EFFECTS OF LONG-CHAIN FATTY ACIDS ON PRODUCTION, METABOLISM AND IMMUNITY OF HOLSTEIN COWS

By

CRISTINA CALDARI-TORRES

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009
To my father Pier Luigi Caldari, who has been the driving force behind all my academic achievements
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Lokenga Badinga, for allowing me to be a part of his laboratory during my MS and PhD projects. I am grateful for all his help and support, and for encouraging me to perform well academically. Special thanks to Dr. Carlos Risco and Dr. Nasser Chegini for agreeing to be members of my committee and for helping me understand and expand on the clinical aspects of my research. I greatly appreciate the contributions and insight provided by my committee member Dr. Charles Staples, especially his help in the nutritional aspect of my field trial. I would also like to thank Dr. William Thatcher for always taking time from his busy schedule to meet with me and discuss my research, as well as for his invaluable help with statistical problems. Thanks also go to Dr. Alan Ealy for allowing me the use of his laboratory equipment.

I greatly appreciate the help provided by all the biological scientists who at one time or another served Dr. Badinga’s laboratory or helped me learn how to perform techniques, operate equipment, use computer software, or who actually carried out manual labor during my field trial. These include Werner Collante, Alan Eldred, Max Huisden, Sergei Sennikov, and Joyce Hayen. I would also like to thank Dr. Joel Brendemuhl and Joann Fischer for their advice and help with academic questions. Special thanks to Sabrina Robinson for her help with all kinds of miscellaneous issues.

I owe special thanks to Eric Diepersloot, Grady Byers, David Armstrong, Mary Russell, Jerry Langford, Molly Gleason, James Dunn, Judith Fowler, Patty Best, Sherry Hay, Johnnie Salvino, and all the Dairy Unit employees who helped make my field trial an incredible and invaluable experience. Many thanks are extended to Dan Wang and Jae Shin for all the help provided at the Dairy Unit, and for being efficient and enthusiastic helpers. I have great appreciation for Dr. Flávio Silvestre for performing all the uterine flushings during my trial and
for all the help provided with the uterine cytologies, as well as for the many scientific
discussions regarding immunity of dairy cows. I also extend my appreciation to Dr. Bruno do
Amaral for teaching me absolutely everything I needed to know about performing research at the
Dairy Unit, as well as for always being available to help me in areas extending from statistical
analyses to preparation of laboratory reagents.

Great appreciation goes to my girls, Jessica Belsito, Dr. Jamie Foster, Dr. Kelly Vineyard,
and Jacqueline Wahrmund for their friendship and advice during both good and bad times. I am
very proud of all of you and will miss you immeasurably. Special thanks also go to María
Beatriz Padua, for being my study companion during the difficult Advanced Cell Biology
courses, for sharing her deep knowledge of the field of immunology, and for being a good friend.
I am deeply appreciative of the help provided in the lab and at the Dairy Unit by Milerky
Perdomo, who was very much involved in my field trial and took care of my cows as if they
were her own. Thanks go to IFAS for supporting me during my PhD with the Alumni
Fellowship, and to Virtus Nutrition for the funding provided for the field trial. Most of all, I
would like to thank God for opening my eyes, for helping me hear his guiding voice, and most of
all, for making me a better person.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. 4

LIST OF TABLES .......................................................................................................................... 9

LIST OF FIGURES .......................................................................................................................... 10

LIST OF ABBREVIATIONS .............................................................................................................. 13

ABSTRACT .................................................................................................................................. 17

CHAPTER

1 INTRODUCTION ....................................................................................................................... 19

2 REVIEW OF LITERATURE ........................................................................................................ 22

Overview of the Periparturient Period in Dairy Cows ................................................................. 22
  Metabolic Changes During the Transition To Lactation ............................................................. 22
  Metabolic Disorders .................................................................................................................. 27
  Immunosuppression During the Periparturient Period ............................................................ 35

Overview of the Bovine Immune System .................................................................................... 42
  Innate Immune Function .......................................................................................................... 43
    Physical, chemical and microbiological barriers ................................................................. 44
    Cells of the innate immune system ....................................................................................... 45
    The complement system ....................................................................................................... 48
    The acute phase response .................................................................................................... 52
  Acquired Immune Function ...................................................................................................... 54
    Cell-mediated immune response ......................................................................................... 56
    Humoral immune response .................................................................................................. 59

Long-Chain Fatty Acids .............................................................................................................. 61
  Fatty Acid Nomenclature and Background Information .......................................................... 62
  Long-Chain Fatty Acid Metabolism in Ruminants ................................................................. 65
  Long-Chain Fatty Acid Incorporation in Tissues ..................................................................... 69
  Benefits of Feeding Fats to Dairy Cows .................................................................................. 72

Effect of Supplemental Fat on Feed Intake and Production ....................................................... 74
  Dry Matter Intake ..................................................................................................................... 74
  Body Weight and Body Condition Score ................................................................................ 76
  Milk Yield, Milk Fat, and Milk Protein .................................................................................... 77
  Milk Fatty Acid Profile ............................................................................................................ 79

Effect of Supplemental Fat on Plasma Metabolites and Hormones of Dairy Cows .................... 81
  Non-Esterified Fatty Acids and β-Hydroxybutyric Acid ......................................................... 81
  Glucose ................................................................................................................................... 84

Immunomodulatory Effects of Long-Chain Fatty Acid Supplementation .................................... 85
  Immune Cell Membrane Composition ................................................................................... 85
3 EFFECTS OF MONOUNSATURATED FATTY ACIDS ON CONCANAVALIN A-INDUCED CYTOKINE PRODUCTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS ................................................................. 97

Introduction ........................................................................................................... 97
Materials and Methods .......................................................................................... 99
  Media and Reagents ............................................................................................... 99
  Animals and Blood Sampling .............................................................................. 100
  PBMC Isolation .................................................................................................. 100
  PBMC Treatment ................................................................................................ 101
  Cytokine Assays ................................................................................................ 102
  Statistical Analysis ............................................................................................. 102
Results ..................................................................................................................... 102
  Cytokine Responses of Cultured Bovine PBMCs to ConA, LPS, and PHA ...... 102
  Cytokine Responses of Cultured Bovine PBMCs to Increasing Doses of ConA 103
  Effects of Cis- and Trans-Octadecenoic Acids on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs ......................................................... 103
Discussion ............................................................................................................... 104
Proposed Model ...................................................................................................... 105

4 EFFECTS OF POLYUNSATURATED FATTY ACIDS ON CONCANAVALIN A-INDUCED CYTOKINE PRODUCTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS ...................................................... 116

Introduction ........................................................................................................... 116
Materials and Methods .......................................................................................... 118
  Media and Reagents ............................................................................................... 118
  Animals and Blood Sampling .............................................................................. 118
  PBMC Isolation .................................................................................................. 119
  PBMC Treatment ................................................................................................ 120
  Cytokine Assays ................................................................................................ 120
  Statistical Analysis ............................................................................................. 120
Results ..................................................................................................................... 121
  Effects of CLA on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs ................................................................. 121
  Effects of n-3 FAs on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs ................................................................. 121
Discussion ............................................................................................................... 121
Proposed Model ...................................................................................................... 124
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1 Functional activity and distribution of the major immunoglobulin types.</td>
<td>96</td>
</tr>
<tr>
<td>5-1 Fatty acid profile according to the manufacturers of a highly saturated fat (RBF; Cargill, Minneapolis, MN), a Ca salt lipid enriched in ( \text{trans} ) C18:1 (tFA; Virtus Nutrition LLC, Fairlawn, OH), and a Ca salt lipid enriched in C18:2 (n-6 FA; Virtus Nutrition LLC, Fairlawn, OH) fed during the prepartum and postpartum periods to Holstein cows.</td>
<td>150</td>
</tr>
<tr>
<td>5-2 Ingredient composition of prepartum and postpartum diets.</td>
<td>151</td>
</tr>
<tr>
<td>5-3 Chemical composition of prepartum and postpartum diets.</td>
<td>152</td>
</tr>
<tr>
<td>5-4 Performance of lactating Holstein cows fed a diet containing a highly saturated fat (RBF), a Ca salt lipid enriched in ( \text{trans} ) C18:1 (tFA), or a Ca salt lipid enriched in linoleic acid (n-6 FA).</td>
<td>153</td>
</tr>
<tr>
<td>5-5 Incidence of fever and uterine infections in Holstein cows fed a diet containing a highly saturated fat (RBF) (n=10), a Ca salt lipid enriched in ( \text{trans} ) C18:1 (tFA) (n=10), or a Ca salt lipid enriched in linoleic acid (n-6 FA) (n=8).</td>
<td>154</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Effect of 10 μg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells.</td>
<td>106</td>
</tr>
<tr>
<td>3-2</td>
<td>Effect of 10 μg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells.</td>
<td>107</td>
</tr>
<tr>
<td>3-3</td>
<td>Effect of 10 μg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells.</td>
<td>108</td>
</tr>
<tr>
<td>3-4</td>
<td>Effect of increasing doses of concanavalin A (ConA) on tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells.</td>
<td>109</td>
</tr>
<tr>
<td>3-5</td>
<td>Effect of increasing doses of concanavalin A (ConA) on interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells.</td>
<td>110</td>
</tr>
<tr>
<td>3-6</td>
<td>Effect of increasing doses of concanavalin A (ConA) on interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells.</td>
<td>111</td>
</tr>
<tr>
<td>3-7</td>
<td>Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells.</td>
<td>112</td>
</tr>
<tr>
<td>3-8</td>
<td>Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced interleukin-4 (IL-4) concentration in bovine peripheral blood mononuclear cells.</td>
<td>113</td>
</tr>
<tr>
<td>3-9</td>
<td>Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells.</td>
<td>114</td>
</tr>
<tr>
<td>3-10</td>
<td>Proposed model for trans-vaccenic acid-induced alteration of immune markers in bovine PBMCs.</td>
<td>115</td>
</tr>
<tr>
<td>4-1</td>
<td>Effect of LA and CLA isomers on ConA-stimulated tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells.</td>
<td>126</td>
</tr>
<tr>
<td>4-2</td>
<td>Effect of LA and CLA isomers on ConA-stimulated interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells.</td>
<td>127</td>
</tr>
<tr>
<td>4-3</td>
<td>Effect of LA and CLA isomers on ConA-stimulated interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells.</td>
<td>128</td>
</tr>
</tbody>
</table>
4-4 Effect of n-3 fatty acids on ConA-stimulated tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells. ..................................................129

4-5 Effect of n-3 fatty acids on ConA-stimulated interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells. .................................................................130

4-6 Effect of n-3 fatty acids on ConA-stimulated interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. .................................................................131

4-7 Proposed model for n-3 FA-induced alteration of immune markers in bovine PBMCs. ....132

5-1 Dry matter intake (DMI) as a percentage of body weight (BW) of Holstein cows fed a saturated fat (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. .................................................................155

5-2 Average body weight (BW) of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet ........................................156

5-3 Average body condition score (BCS) of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet ..............157

5-4 Calculated energy balance by week relative to parturition for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet ..................................................................................................................158

5-5 Temporal patterns of milk production by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .............159

5-6 Temporal patterns of 3.5% fat-corrected milk (FCM) yield by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Asterisks indicate statistical differences among means at the indicated week. ........................................................................................................160

5-7 Fat percentage of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .....................161

5-8 Fat yield of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet ......................162

5-9 Protein percentage of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .................163

5-10 Protein yield of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .........................164

5-11 Average feed efficiency as a function of milk yield over intake of periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet ........................................165
5-12 Plasma NEFA concentrations by week relative to calving in periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet .......................................................... 166

5-13 Plasma glucose concentrations by week relative to calving in periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet .......................................................... 167

5-14 Average rectal temperature on d 4, 7 and 12 postpartum of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. .......................................................... 168

5-15 Concentration of plasma interleukin 6 (IL-6), interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) on day 12 ± 2 postpartum from periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. .......................................................... 169

5-16 Concentration of plasma haptoglobin from periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. .......................................................... 170

5-17 Average Metricleck score on d 4, 7, 12 and 30 of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .... 171

5-18 Average neutrophil % in uterine flushings on d 40 ± 3 postpartum of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet .......................................................... 172

5-19 Average accumulated progesterone concentration of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet .......................................................... 173

5-20 Ovulation distribution for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. .......................................................... 174

5-21 Conception rate to first service for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 7)-supplemented diet .......................................................... 175

5-22 Average days open for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 7)-supplemented diet .......................................................... 176

5-23 Proposed model for n-6 FA actions on lipid and glucose metabolism .......................................................... 177
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-Vit D</td>
<td>1,25-dihydrovitamin D</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid-detergent fiber</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APP</td>
<td>Acute phase proteins</td>
</tr>
<tr>
<td>APR</td>
<td>Acute phase response</td>
</tr>
<tr>
<td>ARA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>βHBA</td>
<td>β-hydroxybutyrate</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BCS</td>
<td>Body condition score</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>c9,t11 CLA</td>
<td>Cis-9, trans-11 CLA</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated linoleic acid</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
<td>Calcium salts</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>DGLA</td>
<td>dihomo-(\gamma)-linolenic acid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMI</td>
<td>Dry matter intake</td>
</tr>
<tr>
<td>EB</td>
<td>Energy balance</td>
</tr>
<tr>
<td>EFA</td>
<td>Essential fatty acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GLA</td>
<td>(\gamma)-linolenic acid</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>H(2)O(2)</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>Interferon (\gamma)</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long-chain fatty acid</td>
</tr>
<tr>
<td>LDA</td>
<td>Left displacement of the abomasum</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LNA</td>
<td>Linoleic acid</td>
</tr>
</tbody>
</table>
LOX  Lipoxygenase
LPS  Lipopolysaccharide
LT   Leukotriene
MBL  Mannose-binding lectin
MCFA Medium-chain fatty acid
MHC  Major histocompatibility complex
MUFA Monounsaturated fatty acid
NDF  Neutral-detergent fiber
NEB  Negative energy balance
NEFA Non-esterified fatty acid
NK   Natural killer cell
NO   Nitric oxide
O₂   Superoxide anion
OR   Odds ratio
P4   Progesterone
PAMP Pathogen-associated molecular pattern
PBMC Peripheral blood mononuclear cell
PDV  Portal-drained viscera
PG   Prostaglandin
PGFM 12,13-dihydro-15-keto-prostaglandin F₂α
PGI₂ Prostacyclin
PHA  Phytohemagglutinin A
PMN  Polymorphonuclear granulocytes
PPAR Peroxisome proliferator activated receptor
PTH  Parathyroid hormone
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCC</td>
<td>Somatic cell count</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-chain fatty acid</td>
</tr>
<tr>
<td>t10,c12 CLA</td>
<td>Trans-10, cis-12 CLA</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>Thymus dependent</td>
</tr>
<tr>
<td>tFA</td>
<td>Trans fatty acid</td>
</tr>
<tr>
<td>TI</td>
<td>Thymus independent</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>VA</td>
<td>Vaccenic acid</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acid</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
</tr>
</tbody>
</table>
A series of \textit{in vitro} experiments were conducted to examine the effects of long-chain fatty acids (LCFAs) on mitogen-stimulated cytokine production by bovine peripheral blood mononuclear cells (PBMCs). Additionally, an \textit{in vivo} experiment testing the effect of dietary n-6 and \textit{trans} fatty acids (tFAs) on production, metabolism and uterine health of periparturient Holstein cows was conducted.

Bovine PBMCs co-treated with \textit{trans}-monounsaturated fatty acids (FAs) produced more concanavalin A (ConA)-stimulated pro-inflammatory cytokines than did cells treated with \textit{cis}-monounsaturated FAs. These data further support that dietary tFAs induce systemic inflammation in mammalian species. Addition of the \textit{cis}-9, \textit{trans}-11(c9,t11) and \textit{trans}-10, \textit{cis}-12 (t10,c12) conjugated linoleic acid (CLA) isomers to the culture media did not affect ConA-stimulated cytokine production by bovine PBMCs. Eicosapentaenoic acid (EPA) and linolenic acid (LNA) attenuated ConA-stimulated cytokine production by bovine PBMCs, with LNA suppressing cytokine production to a lesser extent than EPA.

Multiparous dairy cows were fed one of three dietary treatments from approximately 29 d prior to estimated calving date through 49 d postpartum. The control diet contained a 90\% saturated fat supplement, at 1.5\% of dietary dry matter (DM). The second and third dietary
treatments contained calcium salts of tFAs (61% trans C18:1 isomers, tFA) and calcium salts of safflower oil (55% linoleic acid, LA), respectively, at 1.8% of dietary DM. Feeding supplemental LA to multiparous Holstein cows resulted in an earlier return to positive energy balance than those fed the saturated FA or tFA supplements. This improvement in energy status was primarily due to a decreased concentration in milk fat. Plasma concentrations of non-esterified fatty acids were lower and that of glucose were greater in cows allocated to the LA dietary treatment, indicating an improved metabolic status. These animals also demonstrated better uterine health on d 7 postpartum and had greater accumulated progesterone concentration in plasma by day 50 postpartum than animals in the other two treatment groups. Studies with a larger number of animals are warranted to fully document the effect of periparturient fat supplementation on uterine health and reproductive efficiency in dairy cows.
In dairy cows, the onset of lactation causes an abrupt shift in nutritional requirements in order to support milk production (Butler, 2000), resulting in a rapid increase in energy requirements and changes in the metabolic and endocrine status of the animal (Grummer, 1995). A reduction of feed intake occurring during the final weeks of pregnancy when nutrient demands for fetal growth and initiation of milk synthesis are increasing result in higher energy requirements that can be met by dietary energy intake (Bell, 1995). This creates a state of negative energy balance (NEB) during the transition period that can lead to a suppression of the immune system, as well as impaired reproductive function (Kehrli et al., 2006; Butler, 2000). The transition period, which extends from 3 weeks before calving to 3 weeks after calving, is the most critical period of lactation (Grummer, 1995). A NEB during the periparturient period results in a lack of expendable energy for the cells that make up the immune system, therefore resulting in impaired immune function in these animals. In addition, this NEB can lead to metabolic disorders, such as ketosis and fatty liver, which may have a detrimental effect on the cells of the immune system, further predisposing dairy cows to immune diseases during the transition to lactation.

The immunological dysfunction experienced by modern dairy cows from approximately 3 wks before calving to 3 wks after calving, may have practical implications for health and reproductive management. Dairy cows are prone to uterine infections during this transition to lactation and cows that are unable to clear this uterine contamination may eventually have reduced reproductive performance due to delayed calving to conception interval (Le Blanc et al., 2002; Gilbert et al., 2005). Uterine infections can be classified as puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis based on the severity and time of
occurrence of the disease (Sheldon et al., 2006). The diagnosis of subclinical endometritis, which is characterized by the presence of >18% neutrophils in uterine cytology samples collected 20 to 30 days postpartum or > 10% neutrophils at 34 to 47 days postpartum, has been associated with decreased subsequent reproductive performance because of factors such as increased days open and decreased first-service conception risk (Gilbert et al., 2005). Uterine infections may negatively affect fertility through several mechanisms, all caused by the absorption of bacterial endotoxins into the general circulation through the uterine lumen. These mechanisms include the endotoxin-mediated disruption of hypothalamic-pituitary gonadal axis (resulting in a delayed ovulation), the endotoxin-induced production of prostaglandins, prostacyclins and thromboxanes (which also result in a prolonged anovulation period), and the endotoxin-induced production of cortisol which suppresses LH, and inhibits ovulation (Peter et al., 1989; Peter et al., 1990).

Fat feeding has become common practice in the dairy industry due to its ability to increase the energy density of the diet, therefore improving energy balance of dairy cows. In addition, inclusion of fat supplements into dairy rations reduces the incidence of metabolic disorders in dairy cattle (Grummer and Carroll, 1991) through positive effect on metabolic efficiency (Kronfeld et al., 1980). Because addition of lipids to dairy rations improve metabolic status of lactating cows, it is possible that the increased energy balance and reduced incidence of metabolic disorders, will contribute to an improvement of the immunosuppression seen during the periparturient period. Development of new feeding strategies in which dietary fats influence the immune responses could lead to attenuation of the immunosuppressive effect caused by parturition, through improving immune functions involved in defense against pathogenic
organisms (Lessard et al., 2004). The improvement in energy balance and immune responses could, in turn, improve reproductive performance of early postpartum dairy cows.

The goal of this research project was to examine the physiological effects of supplemental long-chain fatty acids on metabolism, health and reproduction of Holstein cows. This dissertation begins with an overview of the periparturient period in dairy cows, including the metabolic disorders and immunosuppression that accompany this period (Chapter 2). The literature review (Chapter 2) will also include information on the bovine immune system, as well as background information on fatty acids, and the effects of supplemental fat on production, plasma metabolites, hormones, immunomodulation and uterine health in dairy cows. Experiments described in Chapters 3 and 4 were designed to elucidate the effects of monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) on mitogen-stimulated cytokine production by cultured bovine peripheral blood mononuclear cells. Chapter 5 presents the in vivo trial conducted in order to study production, metabolic, and uterine health responses of periparturient Holstein cows to dietary trans and n-6 fatty acids. The final chapter is a general discussion of the major findings of this research project (Chapter 6).
Chapter 2
Review of Literature

Overview of the Periparturient Period in Dairy Cows

The Holstein is a breed of dairy cow that was developed in Europe, specifically in North Holland and Friesland, two northern provinces of the Netherlands. The Holstein cow is the world’s highest producing dairy animal, producing an average of 11,500 kg of milk per 305-day lactation, as reported by the USDA for the month of April of 2009 (USDA Agricultural Research Service website). The selection for higher producing dairy cows has led to such a high level of milk production that modern dairy cows are mostly unable to meet their nutrient demand through feed intake alone, leading to nutrient and energy deficits after parturition. This means that during the periparturient period, these animals experience complex homeorhetic changes in order to cope with the staggering metabolic challenge imposed by the initiation of lactation. During the transition period, which spans from three weeks before to three weeks after parturition (Grummer, 1995), dairy cows experience most of the infectious and metabolic diseases that in turn affect their well-being and profitability (Drackley, 1999; Goff and Horst, 1997).

Metabolic Changes During the Transition To Lactation

The major challenge faced by dairy cows during the transition period is an increase in nutrient requirements for milk production, which is not able to be met through feed intake alone because the rate of dry matter intake (DMI) increase is slower than the rate of milk energy output. As parturition approaches, there is a progressive decrease in DMI, with a decrease of about 30% in the last 3 weeks of gestation and almost 90% of that decrease occurring during the 5 to 7 days before calving (Ingvartsen and Andersen, 2000; Hayirli et al., 2002). Theories that have been proposed to explain the drastic decrease in feed intake in dairy cows include a decrease in rumen volume and the hormonal actions that accompany the periparturient period.
There is significant growth of the fetus in the last 60 days of gestation, leading to a considerable decrease in ruminal capacity which subsequently limits the amount of feed that the cow can consume. Still, researchers agree that this is not enough to explain the extensive reduction in DMI that occurs before parturition (Stanley et al., 1993). The hormonal changes that come into play during the periparturient period can also help clarify this reduction in feed intake. These physiological changes alter the animal’s metabolism drastically, and are driven by the rapidly increasing demands of the fetus, the development of the mammary glands, including synthesis of milk, and the approach of parturition. As calving draws near, blood concentrations of progesterone (P4) start to decrease and those of estrogen stay elevated or increase even further (Grummer, 1995). High concentration of estrogen is believed to be one of the major factors contributing to the decrease in DMI around calving (Grummer, 1993). Growth hormone (GH) is also increased close to parturition (Grum et al., 1996), leading to an increase in responsiveness of adipose tissue to lipolytic signals such as norepinephrine. This is important since during the final 30 days of gestation, energy and protein deposition in the uterus and fetus leads to an increased nutrient requirement, which occur at the same time as the cow is having a decline in nutrient intake (Bell et al., 1995), resulting in a NEB. Daily demands for fetal and placental growth in the last 3 weeks of gestation were calculated to be 360 g of metabolizable protein and 3 to 5 Mcal of Net Energy (Bell, 1995). The increase in GH and increase in responsiveness of adipose tissue to lipolytic signals lead to mobilization of adipose tissue, which provides energy in the form of non-esterified fatty acids (NEFAs) (Grummer, 1995). This energy helps compensate for the NEB caused by reduced DMI, fetal growth, calving (stress), and lactogenesis.

As lactation is initiated, the cow’s demands for glucose are increased. Glucose is necessary for milk lactose synthesis, therefore the initiation of milk synthesis and subsequent
increase in milk production results in an increase in glucose demand by the mammary gland. The problem faced by the dairy cow is that glucose demand cannot be met by feed intake since the decrease in DMI that accompanies the transition period results in little glucose being absorbed directly from the digestive tract. Two important homeorhetic adaptations that help satisfy the animal’s glucose demands are an increase in hepatic gluconeogenesis (Reynolds et al., 2003) and a switch to a “glucose sparing mechanism”, where glucose oxidation is decreased in peripheral tissues in order to direct glucose to the mammary gland for lactose synthesis (Bennink et al., 1972). The major substrates for hepatic gluconeogenesis in ruminants are propionate from ruminal fermentation, lactate from Cori cycling, amino acids from skeletal muscle breakdown or net portal-drained visceral absorption, and glycerol from adipose tissue lipolysis (Seal and Reynolds, 1993). Since propionate concentrations are limited due to the decrease in feed intake, cows rely on the breakdown of skeletal muscle and adipose tissue to supply amino acids and glycerol for gluconeogenesis. Low insulin concentrations, combined with high GH levels, allow for extensive mobilization of long-chain fatty acids from adipose tissue, and therefore an increase in circulating NEFAs. Circulating NEFA concentrations become elevated in response to increased energy needs that occur at the same time that feed intake is inadequate (Overton and Waldron, 2004). These breakdown products of adipose tissue become an important source of energy for the cow during this period, particularly for major energy requiring organs such as skeletal tissue, which decrease their reliance on glucose as fuel during early lactation (Overton and Waldron, 2004). Increased concentrations of circulating NEFAs lead to an increase in hepatic NEFA uptake (Pullen et al., 1989; Reynolds et al., 2003). In the liver these NEFAs will be: 1) completely oxidized to carbon dioxide, which can be used as an energy source by the liver, 2) partially oxidized to ketone bodies, which can serve as fuel for other organs, or 3) be
reconverted to triglycerides which can either be stored in the liver or exported out of the liver as very-low density lipoproteins (VLDL). It is believed that the ruminant’s liver has an inherently low capacity to synthesize and export VLDL, but a similar capacity to reconvert NEFAs back to triglyceride (Kleppe et al., 1988). In addition, hepatic triglyceride production is increased at the time of calving (Grum et al., 1996). This combination of factors can lead to the accumulation of lipids in the liver if NEFA uptake by the liver becomes excessive.

Management factors can also result in an increase in circulating NEFAs. Dry-off cows are fed a high forage ration that is higher in neutral detergent fiber and is less energy dense than lactation cows, which are fed high energy diets. Cows fed high forage diets have changes in their ruminal bacterial population. The decrease in readily fermentable starches results in their microbes shifting away from lactate producers and into cellulytic and methane–producing bacteria (Goff and Horst, 1997). The lower energy density of the early dry period diet also results in a reduction in papillae length and in volatile fatty acid (VFA) absorptive capacity by rumen mucosa, with as much as 50% of the absorptive area possibly being lost in the first 7 weeks of the dry period (Dirksen et al., 1985). An abrupt switch to the high energy lactation diet can result in ruminal acidosis in the animal, since the lactate producers in the rumen respond rapidly to the high starch diet and produce high quantities of lactate, but the bacteria that convert lactate require 3 to 4 weeks to reach concentrations that prevent lactate from accumulating in the rumen (Goff and Horst, 1997). In addition to the lactate accumulation, the poorly developed rumen epithelia of the unadapted cow cannot absorb VFAs rapidly enough to prevent accumulation of organic acids in the rumen. This results in a lowering of ruminal pH to the point where the protozoa and bacteria that inhabit it are either killed or inactivated (Goff and Horst, 1997). As the rumen flora die, they release lactic acid, endotoxins and histamine, which can be
absorbed systemically and result in clinical laminitis (Radostits et al., 1994). Also, if the amount of organic acids absorbed into the bloodstream exceeds the amount that can be metabolized by the liver and other tissues, metabolic acidosis will result (Goff and Horst, 1997). In animals that have only a mild accumulation of acids in the rumen, DMI reduction can occur (Owens et al., 1998). It can be speculated that animals that suffer from laminitis will have an even more pronounced decrease in feed intake, because they will limit their movement towards the area where the feed is in order to prevent the pain associated with walking. This reduction in feed intake close to parturition in animals that are suffering from the metabolic changes mentioned before will also result in an increase in NEFAs.

A disruption of mineral balance, primarily calcium (Ca) balance, around parturition can also have profound effects on the periparturient period. The onset of lactation places a large demand on calcium homeostasis mechanisms, so that most cows develop some level of hypocalcemia soon after parturition (Horst et al., 1994). Calcium pools are under strict homeostatic control, endocrinely regulated at the level of intestinal absorption, bone resorption and deposition, renal reabsorption and urinary excretion, milk secretion, fetal deposition, and fecal excretion (Overton and Waldron, 2004). Parathyroid hormone (PTH), calcitonin and 1,25-dihydrovitamin D (1,25-Vit D) are some of the major endocrine factors regulating plasma Ca concentrations. If plasma Ca levels drop too low to support nerve and muscle function, the animal will develop parturient paresis, or milk fever. This hypocalcemic condition can predispose the dairy cow to other periparturient diseases, through exacerbation of the immunosuppression that generally occurs during the periparturient period, as well as loss of muscle tone necessary for expulsion of the placenta and closing of the teat sphincter.
The next section will deal with the metabolic disorders that can occur during the periparturient period, when NEFA uptake by the liver becomes excessive, as well as when there is disruption of the mechanisms involved in calcium homeostasis.

**Metabolic Disorders**

If excessive hepatic lipid infiltration occurs, a condition known as hepatic lipidosis, or fatty liver, may develop. Fatty liver can lead to prolonged recovery from other health disorders as well as increased incidence of health problems (Herdt, 1988), since excessive fat infiltration can impair liver function and maintaining optimal liver function is central to the ability of cows to make a smooth transition into heavy milk production. Excessive fat infiltration in the liver can decrease the liver’s ability to detoxify ammonia to urea (Strang et al., 1998), as well as impair the liver’s ability to detoxify endotoxin (Andersen et al., 1996). High ammonia concentrations can impair gluconeogenesis in the liver, since ammonia decreases the liver’s ability to convert propionate to glucose (Overton et al., 1999), while a decreased ability to detoxify endotoxin can render the cow susceptible to endotoxin shock and death (Andersen et al., 1996). Fatty liver can also precede and induce ketosis (Veenhuizen et al., 1991), a state of elevated ketone bodies in the blood.

Ketosis is one of the metabolic disorders that can predispose the animal to other periparturient diseases. Ketone bodies are by-products of the lipid metabolic pathway after fat is converted to energy. They are formed when the liver reaches its maximum capacity to completely oxidize fatty acids through the Krebs cycle or to export them as VLDL, resulting in the condensation of acetyl-CoA units that are not incorporated into the Krebs cycle (Goff and Horst, 1997). The product of this condensation reaction are ketone bodies such as acetone, acetoacetate, and β-hydroxybutyrate (βHBA). High ketone concentrations are normally seen
soon after parturition in dairy cows, since the decreased DMI and high energy demands for lactation lead to an intense mobilization of stored body fat, which can easily overload the liver’s capacity to completely oxidize these circulating NEFAs. One also needs to keep in mind that the ruminant liver is known to have a low capacity to export VLDLs, resulting in a higher probability of acetyl-CoA units condensing into ketones. High ketone concentrations can result in the metabolic disorder known as ketosis, which can impair gluconeogenesis, therefore resulting in hypoglycemia (Goff and Horst, 1997). Ketosis can also affect the level of milk production in dairy cows by causing an additional decrease in DMI, and can further exacerbate the immunosuppression seen in early postpartum dairy cows, predisposing them to other periparturient diseases.

As mentioned before, the initiation of lactation imposes a stringent demand on the mechanisms of calcium homeostasis in dairy cows. In fact, most dairy cows develop some level of hypocalcemia soon after parturition (Goff et al., 1987). When blood Ca concentrations fall to the point where nerve and muscle function cannot be supported, parturient paresis, or milk fever, develops (Goff and Horst, 1997). This condition affects up to 7% of periparturient dairy cows in the United States (Jordan and Fourdraine, 1993), with cows having a high milk output, such as third and greater parity cows, being at a higher risk of developing this metabolic disorder. On the other hand, first lactation heifers rarely develop this metabolic disorder. Parturient paresis can predispose cows to a number of other periparturient diseases such as metritis, ketosis, retained placenta, and displaced abomasum. Calcium is important for intracellular communication and muscle function, which are necessary for optimal function of the immune and musculoskeletal systems, respectively. This immunosuppression that can occur due to the
negative effect of the low Ca concentration on immune cell signaling will be described further in the section “Immunosuppression during the periparturient period”.

Left displacement of the abomasum (LDA) is another metabolic disorder that can occur in early postpartum dairy cows. The abomasum is the final stomach compartment in ruminants and is found in the ventral portion of the abdomen, close to the midline, with the pylorus extending to the right side of the animal, caudal to the omasum. As pregnancy progresses, the growing uterus moves under the rumen, reducing its volume which leads to the abomasum being displaced cranially and towards the left side, with the pylorus still extending across the abdomen towards the right side of the animal (Habel, 1981). When LDA occurs, the pyloric end of the abomasum moves completely towards the left side of the cow, under the rumen. In dairy cows several factors can contribute to LDA. These include a failure of the rumen to take up the void left by the retracting uterus after parturition, stretching of the omentum that attaches to the abomasum allowing for the abomasum to move to the left side of the body cavity and the occurrence of abomasal atony (Goff and Horst, 1997). Decreased plasma Ca concentrations can lead to abomasal atony, with plasma Ca concentrations of 5 mg/100 mL and 7.5 mg/100 mL reducing abomasal motility by 70 and 30%, and force of contractions by 50 and 25%, respectively.

This decrease in muscle motility and force of contraction can also predispose lactating dairy cows to mastitis, an inflammation of the tissue of the udder. Impairment of smooth muscle contraction does not seem to be the only factor involved in the dairy cow’s higher propensity for developing mastitis after freshening. One also needs to keep in mind that the immunosuppression occurring around calving can result in a decrease in neutrophil and lymphocyte function, which can allow intramammary infections that have been kept in check
during the dry period to bloom after overcoming the animal’s weakened immune system. Other factors that allow the entry and colonization of new microorganisms into the mammary gland after parturition include the reduction in lactoferrin concentration as colostrum production starts, as well as the breakdown of the keratin plug, which occurs 7 to 10 days before calving (Smith et al., 1985). Lactoferrin is an anti-microbial protein that is part of the innate immune defense against pathogens. This protein binds iron, which is necessary for bacteria to bind host cell membranes. Lactoferrin is high in colostrum, so as its concentration increases in colostrum, less of it is available in the circulation. The keratin plug which seals the streak canal is formed within the first week after dry-off, and it helps prevent bacterial entry into the udder. Breakdown of this plug close to parturition can allow the entry of new microorganisms into the teat canal, and colonization of these can result in clinical mastitis.

In addition to mastitis periparturient dairy cows are prone to nonspecific bacterial uterine infections, which will be the focus of this dissertation. These uterine infections are referred to as puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis, based on the severity and time of occurrence of the disease. The process of calving results in a breakdown of the physical barriers that prevent the entry of microorganisms into the reproductive tract, mainly the cervix, vagina and vulva. This allows for bacteria from the external environment, including from the animal’s skin and feces, to enter and colonize the uterine lumen. Data from Elliot et al. (1968), Griffin et al. (1974) and Sheldon et al. (2002) show that there is a high proportion of uterine bacterial contamination in cattle in the first 60 days postpartum. Most of these uterine contaminations are resolved with no detrimental effect on the animal, but in those animals that cannot clear the bacterial load, a uterine infection may develop. It appears that there is no specificity in the bacteria involved in uterine contamination, with a wide range of bacteria
being attributed to these uterine infections (Sheldon et al., 2002). The pathogens that are mostly associated with clinical disease during uterine infection are *Arcanobacterium pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum* and *Prevotella melaninogenicus*. These bacteria can act synergistically to enhance the likelihood and severity of the disease. *A. pyogenes* can produce a growth factor for *F. necrophorum*, which in turn produces a leukotoxin, while *P. melaninogenicus* produces a phagocytosis-inhibiting substance (Griffin et al., 1974; Olson et al., 1984; Bonnett et al., 1991). This bacterial contamination can be prevented or cleared by the animal’s innate immune system, which includes physical, physiological, phagocytic and inflammatory defenses. Physical barriers that prevent bacterial contamination include the vulva, vestibule, vagina and cervix, which act to prevent the ascension of bacteria into the reproductive tract. During estrus there is production of large quantities of mucus by the vagina and cervix, which act as a physiological barrier to pathogens coming from the external environment. Neutrophils coming into the uterus from the peripheral circulation are the main phagocytic defense, and serve in the internalization and killing of pathogens that have invaded the uterine lumen, but the reduction in their functional capacity that is seen after parturition (Zerbe et al., 2000) is one of the factors that can predispose animals to uterine infections. Macrophages are also important in this innate immune response, acting in the recognition of bacterial components which leads to the production of inflammatory cytokines that can cause pyrexia, stimulate hepatic secretion of acute phase proteins (APPs), and provide a positive feedback loop to further increase immune cell mobilization (Sheldon and Dobson, 2004). In some cows the innate immune system is unable to combat the bacterial load, leading to the establishment of a uterine infection which can have a negative impact on subsequent fertility through an increase in days open, lower first service conception, increased number of inseminations to conception, and
failure to become pregnant (Gilbert et al., 2005). The level of impact on fertility of uterine infections is dependent on the severity as well as time of occurrence of the disease. Uterine infections can be further characterized based on their histological and clinical aspects, as well as the duration of neutrophil infiltration into the uterine lumen.

Generally metritis is defined as an inflammatory disease that involves all layers of the uterus (endometrium, submucosa, muscularis and serosa), and that is characterized by pyrexia (≥ 39.5°C) up to 10 days postpartum with a fetid purulent vaginal discharge, and which is often associated with delayed involution of the uterus (BonDurant, 1999; Sheldon and Dobson, 2004). Endometritis is a term used to describe a condition where there is a superficial inflammation of the endometrium which extends no deeper that the stratum spongiosum (BonDurant, 1999). In animals suffering from endometritis there is infiltration of inflammatory cells into the surface epithelium, presence of pus in the vagina 21 days or more postpartum, and delayed uterine involution (Sheldon and Noakes, 1998). In 2006, Sheldon and co-workers published a paper with the aim of providing clear clinical definitions of uterine disease that could be adopted by researchers. The purpose of stating definitions for uterine disease that could be adopted by researchers came from the observation that “frequently the definition or characterization of the various manifestations of uterine disease either lack precision or definitions vary among research groups and/or were not validated as to their effect on reproductive performance, making assessment of the effects of treatment difficult” (Sheldon et al., 2006). This dissertation will adopt the definitions for uterine disease presented by Sheldon and co-workers (2006), which include puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis. Puerperal metritis is defined as a condition that results in an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, in conjunction with signs of systemic illness such as
decreased milk yield, dullness or other signs of toxemia, as well as fever >39.5°C, within 21 days after postpartum. Animals categorized as having clinical metritis are not systemically ill, but exhibit an abnormally enlarged uterus and a purulent uterine discharge, within 21 days after parturition. Clinical endometritis is defined as a uterine disease where animals present a purulent (>50% pus) uterine discharge 21 days or more after calving, or a mucuopurulent (approximately 50% pus, 50% mucus) uterine discharge detectable in the vagina after 26 days after parturition. Uterine cytology samples can help determine if an animal is suffering from subclinical endometritis, which is characterized by the presence of >18% neutrophils in uterine cytology samples collected 21-33 days postpartum, or >10% neutrophils at 34-47 days postpartum. The accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix characterizes animals suffering from pyometra.

A survival analysis study by Le Blanc et al. (2002) showed that cows with endometritis were 27% less likely to conceive in a given period, and 1.7-times more likely to be culled than cows without endometritis. In another survival analysis study, the authors concluded that the rate at which subclinical endometritis-negative cows became pregnant was higher than cows diagnosed with subclinical endometritis, with the median days open for endometritis-positive cows being 206 days, compared to 118 days for endometritis-negative cows (Gilbert et al., 2005). This study also reported that first-service conception risk was diminished in cows with endometritis (11 vs 36%), and that these cows required more services per conception than endometritis-negative cows (median 3 vs 2). On the other hand, Miller at al. (1980) reported no significant effect of mild metritis on reproductive performance. There is also a lack of uniformity in reports of the prevalence of uterine infections among dairy herds. A study with 141 Holstein cows from five commercial dairy herds in central New York reported the overall
prevalence of subclinical endometritis to be 53% (Gilbert et al., 2005), which is similar to the prevalence reported by Hammon et al. (2001), who studied 115 Holstein cows and classified 52% of these as suffering from endometritis. Studies by Kasimanickam and co-workers (2004) and LeBlanc and co-workers (2002) have reported a lower prevalence of endometritis in dairy herds (37% and 17%, respectively). The variability in the reported prevalence of this disease can be attributed to the different methods utilized to obtain the cytological samples, as well as the definitions used to characterize these uterine infections.

Several mechanisms of action seem to be related to the reduction in fertility that is attributed to uterine infections. The absorption of bacterial endotoxins through the uterine wall into the circulation seems to be the major culprit resulting in subfertility, through its disruption of the hypothalamic-pituitary-ovarian axis, as well as the stimulation of prostaglandin and cortisol production. These all result in a delay in folliculogenesis, which would explain the increased interval from calving to first detected estrus and first service that has been attributed to uterine infections since the 1970’s (Callahan et al., 1971; Oltenacu et al., 1983; Holt et al., 1989). Endotoxins are bacterial toxins that are released when the bacterial cell wall is damaged (Janeway et al., 2005). Cows with spontaneous uterine infections show increased plasma endotoxins concentrations (Mateus et al., 2003), demonstrating that these bacterial cell wall components can be absorbed by the uterine lumen into the circulation. In 1989, Peters et al. showed that infusing the uterine lumen of heifers with *Escherichia coli* (*E. coli*) endotoxin prevented the pre-ovulatory LH surge and ovulation, causing persistent follicular cysts. This shows the endotoxin-mediated inhibition of pulsatile LH secretion from the pituitary, which suppresses hypothalamic GnRH secretion and reduces pituitary responsiveness to GnRH pulses. Nevertheless, in ewes, endotoxin caused a block on the pre-ovulatory increase in peripheral
plasma estradiol concentration even though there was normal LH pulsatility, suggesting that endotoxin may also have direct effects on the ovary (Battaglia et al., 2000). Another factor that may come into play is the production of prostacyclin (PGI\(_2\)), prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) and thromboxane A\(_2\) (TXA\(_2\)) that has been observed in day 5 postpartum cows that were administered *E. coli* endotoxin intrauterinely (Peter et al., 1990). Also, intravenous administration of *Salmonella typhimurium* or *E. coli* endotoxins to cows resulted in an increase in 12,13-dihydro-15-keto-prostaglandin F\(_{2\alpha}\) (PGFM) and a decrease in P4 concentrations (Frederiksson, 1984; Peter et al., 1990). Concentrations of the PGI\(_2\), PGF\(_{2\alpha}\) and TXA\(_2\) metabolites tend to be high in newly calved cows that show signs of endometritis (Peter et al., 1987). This contributes to a prolonged anovulation period since ovarian activity and the first P4 rise after calving can only resume when prostaglandin concentrations have returned to basal levels (Kindahl et al., 1982). In addition, recently it has been shown that bacterial LPS induces an endocrine switch from PGF\(_{2\alpha}\) to PGE\(_2\) in the bovine endometrium, which can explain the prolonged luteal phase observed in animals with uterine disease (Herath et al., 2009). A third possible mechanism through which subclinical endometritis can result in decreased fertility is through an increase in serum cortisol concentrations. A single treatment of heifers during estrus with LPS raised their serum cortisol concentrations, while multiple infusions suppressed the preovulatory surge of LH, truncating ovulation (Peter et al., 1989).

**Immunosuppression During the Periparturient Period**

Studies dating back to 1970’s have recognized that the periparturient bovine immune system is less capable of battling pathogens and exhibits a wide range of immunological dysfunctions when compared to the immune system of non-pregnant cows (Newbould, 1976; Wells et al., 1977). This immunosuppression predisposes the dairy cow to develop infections
such as metritis and mastitis. This review will focus on how this depression of the immune system can result in uterine infections in postpartum dairy cows.

In a review by Kehrli and coworkers (2006), the authors stated that the immune system has a level of protein and energy requirements for optimal function that cannot be met by the negative energy and protein balances that characterizes the early postpartum period. This results in impaired immune function and can account, in part, for the periparturient immunosuppression seen in dairy cows. In order for neutrophils to combat infection, they need to expend a level of cellular energy. In fact, a component of dietary energy and protein consumption for maintenance is spent on the replenishing of neutrophils by the bone marrow (Kehrli et al., 2006). A study with mastectomized cows showed that these cows recovered from immunosuppression within one week postpartum, compared to intact cows that remained in an immune-depressed state for 2-3 weeks after calving (Goff et al., 2002).

Another factor that can account for the immunosuppression seen during the periparturient period is the development of the various metabolic disorders that can affect dairy cows during the early postpartum period. Metabolic disorders such as milk fever and ketosis can increase the odds of the animal developing other metabolic and immune disorders, including metritis (Curtis et al., 1985; Kaneene and Miller, 1995). Low circulating Ca concentrations, as those seen during parturient paresis, can affect the cells of the immune system, which use cell surface receptors to sense changes in their environment and to recognize stimuli. Usually, binding of an antigen, cytokine, or some other ligand to the cell surface receptor will result in the production of a signal that will be transmitted across the plasma membrane and converted into an intracellular biochemical event. The conversion of a signal from one form to another is known as signal transduction (Janeway et al., 2005). Several enzymes and second messengers are necessary for
intracellular signaling, and their function is to transmit the signal onward from the membrane and amplify it. Calcium is a common second messenger in intracellular signaling. Enzymes such as inositol trisphosphate (IP₃) allow the movement of Ca from the endoplasmic reticulum to the cytosol, which in turn triggers the opening of channels in the plasma membrane, allowing Ca to enter the cell from the external fluid (Janeway et al., 2005). The increased intracellular Ca activates the Ca-binding protein calmodulin, which in turn binds to and regulates the activity of several other proteins and enzymes in the cell, transmitting the signal onward until it finally reaches the nucleus (Janeway et al., 2005). Calcium is indispensable for the proper signaling and functioning of immune cells. Decreased concentrations of Ca would result in a decrease in signal transduction and impaired function of immune cells, making the animal further immunosuppressed. In fact, both classical and recent studies support this notion. In 1976, Swierenga and coworkers experimented with rats injected with sheep red blood cells (RBCs), which results in splenic DNA synthesis and a rise in circulating antibody concentrations. Removing the parathyroid glands from rats, which resulted in acute hypocalcemia 24 hours before injecting the RBCs, significantly blunted the splenic cell proliferative response. When the parathyroid gland was removed after injecting the RBCs, the splenic response was normal. This suggested that the parathyroid hormone and Ca could affect the immune response by affecting lymphocyte proliferation. A recent study (Kimura et al., 2006) used Ca-sensitive dyes to monitor intracellular Ca concentration by flow cytometry in stimulated peripheral blood mononuclear cells (PBMCs) from Jersey cows. Plasma Ca concentrations and intracellular Ca stores reached a nadir at calving and intracellular Ca response to stimuli tended to decrease at calving but greatly increased after calving. These data suggest that the hypocalcemic state commonly observed at parturition in dairy cows decreases the intracellular Ca stores, reducing
the ability of immune cells to respond to activating stimuli. In addition, cows that develop milk fever have higher concentrations of circulating cortisol compared to cows that don’t develop milk fever (Goff et al., 1989). Cortisol is a known immunosuppressant, so high concentrations of this corticosteroid can further aggravate the immunosuppression that occurs during the periparturient period. Increased incidence of uterine prolapse and mastitis are associated with decreased uterine and teat sphincter muscle tone, respectively (Goff and Horst, 1997; Risco et al., 1984). The reduction in teat sphincter contraction can lead to the entry of microorganisms into the teat canal and eventually the mammary gland (Goff and Kimura, 2002). This, in addition to the fact that hypocalcemic cows spend more time lying down than normal cows (therefore the name “downer cows”), increasing their chances of teat end exposure to microorganisms (Goff and Kimura, 2002), is the perfect setup for these animals to develop mastitis. There is also a decline in feed intake and prevention of insulin secretion associated with periparturient paresis, which exacerbates the negative energy balance associated with early lactation (Goff and Horst, 1997), as well as reduces glucose uptake by tissues, resulting in increased lipid mobilization. This increase in adipose tissue breakdown can increase the risk of ketosis in these animals, while the decrease in DMI predisposes them to displacement of the abomasum.

Several studies have reported an association between ketosis and metritis, and data indicates that high plasma concentration of ketone bodies may also result in immunosuppression as well as other metabolic disorders in dairy cows. In 2009, Duffield and co-workers reported that serum BHBA concentration at or above 1,200 μmol/L in the first week postpartum were associated with increased risk of displaced abomasum (odds ratio (OR) = 2.6) and metritis (OR = 3.35). Several studies have focused on determining the specific mechanism of action through
which the elevated concentration of ketones that is associated with ketosis can result in an impaired immune response. These studies have looked at the functional changes in the immune cells of animals that suffer from metabolic disorders of early lactation. As mentioned before, for a cow to develop metritis the functional capacity of its uterine neutrophils are probably downregulated or inefficient (Zerbe et al., 2000). In fact, in a study by Klucinski and coworkers (1988) ketone bodies at concentrations found in subketotic and ketotic cows were associated with an inhibition in the phagocytic activity of polymorphonuclear granulocytes (PMNs) that were extracted from blood and milk. Also, blood PMNs of periparturient dairy cows diagnosed with mastitis or metritis had a lower capacity to produce reactive oxygen species (ROS) compared to PMNs of healthy cows (Cai et al., 1994). Along these same lines, another group showed that incubating PMNs with BHBA concentrations typical of subketotic states reduced the respiratory burst of these leukocytes (Hoeben et al., 1999). Also, in vitro chemotaxis of bovine leukocytes isolated from postpartum cows seems to be negatively influenced by ketone bodies (Suriyasathaporn et al., 1999). This would prevent the white blood cells from reaching the area where the infection is and clearing it. It has also been speculated that high blood ketone concentrations could inhibit the growth and differentiation of bone marrow progenitor cells (Hoeben et al., 1999). Since leukocytes are derived from the bone marrow, this would decrease the number of circulating immune cells. Evidence for this was presented by Wheelan and Clay (1992). In addition, cytokine production by lymphocytes seems to be affected by ketone concentration. High blood ketone concentration resulted in a reduction of mitogen-induced lymphocyte proliferation and secretion of interleukin (IL)-2 (Griebel et al., 1987). Interleukin-2 is crucial for lymphocyte activation and proliferation. A second important cytokine that is diminished during ketosis is the pro-inflammatory cytokine interferon-gamma (IFN-γ)
(Kandefer-Szerszen et al., 1992). This cytokine is produced by T helper 1 cells and drives the activation of monocyte/macrophages, which are also important in innate immunity. In order to save energy and prevent injury to tissues, once a pathogen is phagocytosed, a macrophage will not destroy it until it is activated through the signaling of a T helper 1 cell, with that signal being in the form of IFN-γ. In conclusion, these studies support the notion that a ketotic state in cows results in a decrease in innate immunity through several mechanisms. It seems to start with a decrease in the circulating number of leukocytes due to the inhibitory effect of ketones on progenitor cells in the bone marrow. From there, appears to be decreased chemotaxis which prevents the neutrophils/PMNs from reaching the infected area, and those that do reach have a reduced phagocytic activity, accompanied by a decreased respiratory burst, which is probably due to their diminished capacity to produce ROS. This means that whatever bacteria/pathogens are being ingested by the neutrophils are not being killed. In addition, there is a decrease in production of cytokines which are necessary for the activation of both innate and adaptive immunity. The reduced production of IFN-γ will prevent the activation of macrophages, which are also part of the innate immunity, or the first line of defense in the prevention of infections. Inhibition of IL-2 prevents activation of T lymphocytes, which are crucial for adaptive immunity in case the infection cannot be cleared by the cells of the innate immune response. A reduction in all these factors or a combination of these factors would undoubtedly result in an inefficient capacity of PMNs to resolve uterine infections, leading to metritis.

A study by Lacetera and coworkers (2005) concluded that body condition score (BCS), intensity of lipomobilization, or increase of plasma NEFA concentration affects lymphocyte functions in dairy cows around calving. Overconditioned cows have greater circulating NEFA concentrations postpartum than medium or thin cows due to the increased lipid mobilization in
greater prepartum BCS cows as compared to medium or thin cows (Lacetera et al., 2005). Peripheral blood mononuclear cells isolated from overconditioned cows secreted less immunoglobulin M (IgM) compared to thin cows on days 14 and 35 postpartum. These PBMCs also produced less IFN-γ than thin or medium cows on day 7 before parturition (Lacetera et al., 2005). This author also performed a series of in vitro studies which documented direct negative effects of NEFAs on leukocyte functions in dairy heifers (Lacetera et al., 2004). In that particular study a mixture of fatty acids that reflected the composition of NEFAs in ruminant plasma and at concentrations mimicking those that occur under conditions of intense lipid mobilization resulted in an inhibition of DNA synthesis by PBMCs as well as their ability to secrete IgM and IFN-γ. These studies show that overconditioned cows are at a higher risk of developing diseases associated with immunosuppression due to the intense lipomobilization that occurs during the periparturient period, which is associated with alterations of lymphocyte functions.

It is possible that the association between ketosis and uterine infections is also related to the negative energy balance experienced by the dairy cow during the periparturient period. It is widely known that this negative energy balance contributes to the immunosuppression observed in these animals. Neutrophils are the first line of defense in combating infection, and they require energy expenditure. A great component of the dietary energy and protein consumption for maintenance is used for replenishing neutrophils by the bone marrow (Kehrli et al., 2006). It is fair to assume that the other components of the immune system require energy, too. Ketotic animals are hypoglycemic and hyperketonemic. Could it be possible that the reduced glucose and increased ketone concentrations are also detrimental to immune cells? Studies determining the effects of ketones and glucose on mitogen-induced lymphocyte proliferation of age-matched
heifers suggested that the reduction in blood glucose concentration during clinical ketosis does not inhibit lymphocyte proliferation, and that the presence of ketones may have no effect on the proliferative response (Franklin et al., 1991). This suggests that NEFAs could be a major causative agent in the immunosuppressive effect in transition dairy cows. Of course, many scientists believe that fatty liver is a prelude to ketosis (Kehrli et al., 2006), and increased NEFA concentrations will occur right before fatty liver develops. A suppression of the immune system would allow for bacteria that enter the uterus during the act of parturition when there is an opening of the cervix, to colonize, and therefore for metritis to develop.

**Overview of the Bovine Immune System**

The immune system is one of the most complex and intriguing systems in biology due to its ability to protect the host from foreign invaders while at the same time preventing damage to its own tissues. Due to the complexity of the immune system, only a very brief overview of the bovine immune system with emphasis on the major components of the innate and adaptive immune responses will be presented in this review. The immune system is made up of specialized cells, enzymes and serum proteins which are found throughout the body. Cells of the immune system are derived from pluripotent hematopoietic stem cells found in the bone marrow (Janeway et al., 1995). These pluripotent stem cells divide to produce two types of stem cells: the common lymphoid progenitor and the common myeloid progenitor (Janeway et al., 2005). The common lymphoid progenitor cells give rise to natural killer (NK) cells, and T and B lymphocytes, whereas the common myeloid progenitor cells give rise to erythrocytes and different types of leukocytes such as dendritic cells, monocytes, mast cells and PMNs (Janeway et al., 2005). The innate immune system, also known as the native immune system, is the first line of defense against invading pathogens. It mounts an almost immediate non-specific immune response towards infectious agents that enter the body. This native defense system is composed
of physical and chemical barriers that prevent the entry of microorganisms, the complement system and acute phase response which induce inflammation, and the phagocytic cells which engulf and kill invading bacteria and control viral infections. The adaptive or acquired immune response involves the activation of naïve lymphocytes that recognize foreign antigens with their antigen receptor molecules. These lymphocytes are specific for the antigen they recognize, making the adaptive immune response slower than the innate response, but very specific, flexible and powerful. One of the important features that characterize the acquired immune system is the presence of memory cells which are lymphocytes that have already been exposed to antigen, making them more sensitive and allowing them to react very rapidly when re-exposure to the same antigen occurs. This feature of the adaptive immune system makes up the basis for vaccination. There are two types of acquired immune function: humoral responses which are mediated by antibody-producing B lymphocytes, and cell-mediated responses, which are attributed to T lymphocytes. It is important to keep in mind that even though the discussion of the immune system will be divided into its major components, this is an integrated system where all components work together in order to provide maximum protection for the animal. An important example of this is the stimulation of adaptive immune responses by the innate immune system (Medzhitov et al., 1997). Failure of any of these components to work properly can result in the establishment of infection in the host.

**Innate Immune Function**

The innate immune system is a universal and ancient form of host defense against infection (Janeway and Medzhitov, 2002). It is the front line of host defense and can be found in species that lack the more evolved adaptive immune system, such as invertebrates and plants (Janeway et al., 2005). The native immune system is made up of physical and chemical barriers that prevent the entry of invading microorganisms, as well as protein and cellular-mediated
defense mechanisms such as the complement system, acute phase response, and phagocytic cells which come into effect if the physical and chemical barriers are bypassed. Innate immunity is responsible for the initial inflammation that is associated with infection. Inflammatory processes are triggered by cells of the innate immune system such as macrophages, PMNs, and mast cells. These cells recognize pathogen-associated molecular patterns (PAMPs) on foreign invaders through their pattern recognition receptors (Kindt et al., 2007). Recognition of invading microorganisms by innate immune cells leads to the production of cytokines which help induce inflammation and attract more immune cells into the area, as well as the eventual phagocytosis and destruction of these invaders by cells such as neutrophils and macrophages.

Physical, chemical and microbiological barriers

The epithelial surfaces of the body provide the animal’s first line of defense against invading microorganisms. Mechanical, chemical and microbiological barriers prevent pathogens from crossing epithelia and colonizing tissues. The epithelial cells of the skin, gut, lung, eye and nose are joined by tight junctions that prevent the passage of invad
presence of normal nonpathogenic bacterial flora which competes with invading microorganisms for attachment and nutrients, and which also produce antimicrobial substances (Janeway et al., 2005). If the pathogen is effective in evading these physical, chemical and microbiological barriers, they will be recognized, ingested and killed by phagocytic cells of the innate immune system.

**Cells of the innate immune system**

The cells of the innate immune system include macrophages, PMNs (neutrophils, basophils, and eosinophils), mast cells, dendritic cells (DC) and NK cells. Of special importance in the linking of the innate and adaptive immune responses are the macrophages and dendritic cells, which act as antigen-presenting cells (APCs) for lymphocytes. Once these cells internalize a pathogen, they can present a pathogen-derived antigen on their major histocompatibility complex (MHC), which can then be recognized by the T cell receptor on T lymphocytes specific for this antigen. In this way, cells from the innate immune system can activate an adaptive immune response. B lymphocytes can also act as APCs, which will be discussed later on.

Polymorphonuclear leukocytes received their name due to the presence of a multilobed nucleus and cytoplasmic granules. Eosinophils are important in the control of parasitic infections, and need to be activated by lymphocytes of the adaptive immune response (Janeway et al., 2005). They are phagocytic cells that can migrate from the blood into the affected tissues (Kindt et al., 2007). Basophils are nonphagocytic and have a major role in certain allergic responses through the release of pharmacologically active substances from their cytoplasmic granules (Kindt et al., 2007). Eosinophils and basophils are so named because their granules stain with eosin and basic dyes, respectively. Mast cells are released into the blood as undifferentiated cells; they differentiate once they leave the blood and enter the tissues (Kindt et al., 2007). They are similar to basophils in that they contain cytoplasmic granules that contain
histamine and other pharmacologically important substances (Kindt et al., 2007). These cells play an important role in the development of allergies. As mentioned before dendritic cells are important in innate immunity because they serve as APCs. There are different kinds of dendritic cells, including the Langerhans cells, the interstitial dendritic cell and the monocyte-derived dendritic cells (Kindt et al., 2007). The NK cell is important in innate immunity against viruses and other intracellular pathogens, as well as in the killing of certain tumor cells, and is the only innate immune cell that is derived from the common lymphoid progenitor (Janeway et al., 2005).

The main phagocytic cells of the innate immune response are the macrophage and neutrophil. Among these two cells only macrophages can act as APCs for cells of the adaptive immune system. Macrophages are mononuclear phagocytes derived from monocytes that circulate in the blood. Once these monocytes leave the circulation and migrate into tissues they become macrophages capable of recognizing PAMPs through the expression of receptors for bacterial components (Janeway et al., 2005). Neutrophils are short-lived cells that are abundant in blood, but not in normal, healthy tissues (Janeway et al., 2005). They are the first cells to migrate from the circulation into infected tissues and can recognize pathogens through pattern recognition receptors (Kindt et al., 2007). Some of the pattern recognition receptors found on the macrophage’s and/or neutrophil’s cell surface include those for bacterial carbohydrates (mannose and glucan receptors), lipids [lipopolysaccharide (LPS) receptor] and other pathogen-derived components [Toll-like receptors (TLRs) and scavenger receptor] (Janeway et al., 2005). These pattern recognition receptors evolved to recognize conserved products of microbial metabolism produced by microbial pathogens, but not the host, allowing the immune system to distinguish infectious nonself from noninfectious self (Janeway and Medzhitov, 2002). Binding of PAMPs to the phagocyte’s pattern recognition receptors results in the phagocytosis and internalization of
the pathogen through intracellular vesicles called phagosomes, which fuse with lysosomes to form phagolysosomes (Janeway et al., 2005). In these phagolysosomes the lysosomal contents are released resulting in the destruction of the pathogen. Phagocytes also produce other toxic products that help kill the engulfed microorganism, including ROS such as nitric oxide (NO), superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) and reactive nitrogen species (RNS) (Janeway et al., 2005). These ROS and RNS are produced by the NADPH phagosome oxidase enzyme complex, which utilizes oxygen provided by a metabolic process known as the respiratory burst for the production of these bactericidal products (Kindt et al., 2007).

Neutrophils are short-lived and die soon after phagocytosis of a pathogen, contributing to the pus that is associated with infections, while macrophages are longer-lived and continue generating new lysosomes (Janeway et al., 2005). Activation of macrophages through their interaction with pathogens allows them to produce cytokines and chemokines which help attract and activate other cells of the immune system as well as plasma proteins. Cytokines are low molecular weight, soluble proteins secreted by most cells of the body, which are especially important in immune cell regulation (Blecha, 1991). They act as chemical messengers to modify the activity of various immune cells. Cytokines can have either pro-inflammatory or anti-inflammatory actions. Pro-inflammatory cytokines increase protein catabolism and induce amino acid uptake by the liver for acute-phase protein production (Johnson, 1997). These cytokines cause inflammation, and are inhibited by anti-inflammatory cytokines. Pro-inflammatory cytokines are associated with cells of the adaptive immune response known as T helper 1 cells, while anti-inflammatory cytokines are associated with T helper 2 cells. Cytokines will be further discussed in the section entitled “Acquired immune function”. Chemokines are chemoattractant cytokines that function in the recruitment of immune cells into the affected tissues by creating a
concentration gradient which can be detected and followed by cells found in the neighboring areas. Activation of macrophages and subsequent cytokine production leads to a state of inflammation in the tissue, which helps attract neutrophils and plasma proteins to the site of infection. Inflammation has three essential roles that are important for effective pathogen combat. First, it helps the movement of extra effector molecules and cells to the site of infection to help augment the disposal of the invading microorganisms by the macrophages (Janeway et al., 2005). Second, it leads to coagulation, which helps contain the infection to prevent spreading of the microorganisms through the bloodstream. Third, it promotes repair of the injured tissue.

Production of the pro-inflammatory cytokine tumor necrosis factor $\alpha$ (TNF-$\alpha$) by activated macrophages causes dilation of local small blood vessels and changes in the endothelial cells of their walls (Janeway et al., 2005). One important change is the expression of adhesion molecules by the cells lining the blood vessels, which promote the binding of circulating leukocytes, leading to their eventual extravasation and movement to the infected tissues. Circulating leukocytes such as neutrophils and monocytes are attracted to the area of inflammation by the chemokines produced by the activated macrophages already present at the infection site. The blood vessels also become more permeable, allowing plasma proteins and fluid to leak into the infected tissue (Janeway et al., 2005). These changes result in the signs associated with inflammation, including heat, swelling, redness and pain.

**The complement system**

Another component of the innate immune system that helps hasten the accumulation of phagocytes at the site of infection is the complement system. The primary effect of the complement reaction is to increase blood flow to the affected area. Its name is derived from the fact that it ‘complements’ the antibacterial activity of antibodies through increasing the
opsonization and killing of bacteria by antibodies (Janeway et al., 2005). Complement is a major effector of the humoral branch of the immune system but can also be activated early in infection in the absence of antibodies. It now appears that this system first evolved as part of the innate immune system, where it still has an important role (Janeway et al., 2005). Therefore it will be discussed as part of the innate immune system for the purpose of this literature review. The complement system is made up of at least 20 serum proteins which act together to attack extracellular forms of pathogens. The plasma proteins that make up this system can act as chemoattractants for other cells of the immune system, as well as bind to the pathogen and opsonize it, or cause direct lysis and destruction of the pathogen. Activation of the complement system can occur spontaneously on certain pathogens or through binding of antibody to the pathogen. The plasma proteins involved in the complement reaction interact with one another creating an active complement triggered-enzyme cascade. This type of cascade consists of the activation of an active complement enzyme generated by cleavage of its zymogen precursor, which can in turn proteolytically cleave its substrate, another complement zymogen, to its active form (Janeway et al., 2005). This cascade keeps going leading to the amplification of the complement response due to the activation of a small number of complement proteins. The complement system has not been thoroughly studied in cattle, although some of the plasma proteins have been isolated from bovine serum (Rainard et al., 2008; Boehmer et al., 2008). Therefore, only a brief overview of this system will be presented in this section, and some of the proteins necessary for complement activation will be omitted.

There are three pathways of complement activation: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway. All three pathways can be activated independent of antibody as part of the innate immune response (spontaneous activation).
early events of all three pathways involve the proteolysis of complement proteins resulting in the formation of an enzyme called the C3 convertase (Janeway et al., 2005). The C3 convertase cleaves complement components C3 into C3a and C3b. The C3 convertase can cleave up to 1000 molecules of C3 to C3b, allowing many of these opsonizing molecules to bind to the surface of bacteria (Janeway et al., 2005). In addition, binding of C3b to the C3 convertase leads to the formation of the C5 convertase. At this point, the main effector functions of complement are generated. The C5 convertase cleaves C5 into C5a and C5b. C3a and C5a act as powerful peptide mediators of inflammation, while C3b binds covalently to the target cell wall to opsonize it. C5b also binds to the pathogen’s cell wall allowing the formation of the membrane-attack complex (MAC) through the binding of subsequent complement components (Kindt et al., 2007). The MAC forms a large channel through the membrane of the target cell, which allows the diffusion of ions and small molecules across the membrane and can result in lysis of the pathogen (Kindt et al., 2007).

The classical pathway is initiated by activation of the C1 complex, which is made up of a single molecule of C1q, bound to two molecules each of C1r and C1s. C1q can bind to antibodies bound to antigen, therefore linking the adaptive and humoral immune response to the complement system (Janeway et al., 2005). It can also bind directly to the surface of pathogens or to C-reactive protein bound to phosphocholine on bacteria leading to the activation of complement in the absence of antibody (Janeway et al., 2005). Once activated, the C1s enzyme cleaves C4 and C2, leading to the formation of the C3 convertase, the complement step where all three pathways converge.

The mannose-binding lectin pathway initiates complement activation in a similar way to the classical pathway. The complement cascade is triggered by an acute phase protein called
mannose-binding lectin (MBL), which gives this pathway its name. This protein is produced by
the liver during the acute phase response, and is found in low concentration in the plasma of
healthy individuals (Janeway et al., 2005). Binding of MBL to pathogen cell surface
carbohydrates leads to association of MBL-associated serine proteases, MASP-1 and MASP-2,
with MBL (Kindt et al., 2007). The complex formed by this association can cleave and activate
C4 and C2 resulting in the formation of the C5 convertase. This means of antibody-independent
C5 convertase formation is an important innate defense mechanism (Kindt et al., 2007).

The alternative pathway also generates active products similar to those of the classical
pathway, but it is only activated in an antibody-independent manner (Kindt et al., 2007). The
lack of antibody requirement means that the alternative pathway is considered strictly a
component of the innate immune system (Kindt et al., 2007). This pathway does not require
pathogen-binding protein for its activation; it is initiated by spontaneous hydrolysis of C3
resulting in the formation of C3(H2O) (Janeway et al., 2005). C3(H2O) binds factor B, enabling
this bound factor to be cleaved by factor D and leading to the formation of a soluble C3
convertase. This fluid phase C3 convertase is present in small quantities, but can cleave many
molecules of C3 into C3a and C3b (Janeway et al., 2005). The alternative pathway of
complement activation can amplify the classical or MBL pathways through formation of this
alternative C3 convertase, which can deposit more opsonizing C3b molecules on the pathogen
(Janeway et al., 2005).

Tissue damage may occur due to the spontaneous activation of complement components
in plasma or the inadvertent binding of activated complement components to host cells (Janeway
et al., 2005). The activity of the complement system can be regulated at different stages through
the action of several proteins. An important passive mechanism of regulation found in all three
pathways is the presence of highly labile components that are spontaneously inactivated if not stabilized by reacting with other components (Kindt et al., 2007). Active regulation occurs through regulatory proteins that may inactivate various complement components. These regulatory mechanisms prevent the inadvertent damage of the host’s tissue by activated complement components.

**The acute phase response**

The acute phase response (APR) is a term used to define the shift in protein synthesis and secretion that occurs in the liver in response to cytokines released by activated phagocytes found at an infection site. Some of the important cytokines produced by activated macrophages are TNF-α, IL-1β, and IL-6. These pro-inflammatory cytokines are termed endogenous pyrogens because they lead to an elevation in body temperature and are derived from an endogenous source, as opposed to exogenous pyrogens, such as bacterial components like LPS. This elevated body temperature can benefit host defense through induction of a more intense adaptive immune response, protection of host cells from the injurious effects of TNF-α, as well as diminishing pathogen growth (Janeway et al., 2005). Tumor necrosis factor α, IL-1β, and IL-6 are some of the major cytokines that act on the liver to drive the APR. These cytokines stimulate the production of APPs by hepatocytes, which can interact with the pathogen’s cell wall due to their broad specificity for PAMPs (Janeway et al., 2005). These APPs increase in cattle in response to stress (Conner et al., 1988), with different stressful situations eliciting a differential extent of the APR. For example, research performed at the University of Florida reported that co-mingling of cattle does not increase plasma APP concentration in cattle to the extent that transportation does, suggesting that transportation is a more stressful event than co-mingling (Arthington et al., 2003). Acute phase proteins can be quantified from frozen serum, making
them a good marker for measuring the intensity of inflammation in several species. Some of the important APPs overviewed in this section are C-reactive protein (CRP), MBL, fibrinogen, and haptoglobin.

C-reactive protein is a pathogen recognition molecule that binds to the phosphocholine portion of LPS that makes up the cell wall of certain bacteria and fungi as well as phosphorylcholine, which is found on the surface of many microbes (Janeway et al., 2005; Kindt et al., 2007). As was mentioned earlier, membrane-bound CRP can lead to the antibody-independent activation of the complement cascade. It can also act as an opsonizing agent, promoting the uptake of microbes by phagocytes. It is a major APP in several species, and although it can be measured in bovine plasma its concentration does not change significantly in response to stress (Conner et al., 1989), suggesting that it is not a major APP in bovids.

Mannose-binding lectin is another APP that can initiate complement activation. It recognizes mannose-containing molecular patterns on microbes, but not on vertebrate cells (Kindt et al., 2007). It is a member of the collectin family, binding to mannose residues on pathogens through globular lectin-domains attached to a collage-like stalk (Janeway et al., 2005).

Fibrinogen is an important factor involved in blood clotting. It is a precursor to fibrin, which is formed through the action of thrombin on fibrinogen when damage to blood vessels occurs (Kindt et al., 2007). Even though it is a widely measured APP in cattle, it seems to have limited value as an indicator or predictor of disease (Carter et al., 2002).

The last APP reviewed in this chapter is haptoglobin. Haptoglobin binds free hemoglobin, sequestering the iron found in it which is necessary for microbial growth (Godson et al., 1996). Its concentration correlates positively with the destruction of erythrocytes. Haptoglobin may be the most studied APP in bovids (Horadagoda et al., 1999), probably because
its concentration increases in response to injury and stress, and particularly infection (Alsemgeest et al., 1995). Compared to other APP which can be detected at baseline concentrations, haptoglobin is usually not detected in unstressed calves (Arthington et al., 2003), but its concentration peaked 3 d following stressful episodes such as weaning or transport (Arthington et al., 2005). Cattle challenged with LPS responded with a peak haptoglobin concentration on d 5, and a return to baseline concentration by d 9 after challenge (Conner et al., 1989). A study by Huzzey and co-workers (2009) reported that cows with plasma haptoglobin amounts greater or equal to 1 g/L on d 3 postpartum were 6.7 times more likely to develop severe or mild metritis, suggesting that high haptoglobin concentration is a positive indicator of this uterine disease.

**Acquired Immune Function**

When the innate immune response is incapable of recognizing and/or combating an invading pathogen, the cells that make up the acquired or adaptive immune response come into play with an array of mechanisms that target the pathogen specifically. Failure of the innate immune response to dispose of a pathogen results in activated APCs reaching draining lymphoid tissues where they can encounter lymphocytes specific for that antigen, which will then be induced to proliferate and differentiate into effector cells. While the innate immune system can only recognize microorganisms that present common surface or internal molecules that have been conserved over the course of evolution, the cells of the adaptive immune system can recognize an almost infinite array of antigens (Janeway et al., 2005). This is possible due to the phenomenon of specificity, as a result of the enormous diversity of the receptor repertoires of lymphocytes (Abbas and Janeway, 2000). This vast receptor repertoire is achieved through the somatic recombination of gene segments during the maturation of these cells (Abbas and Janeway, 2000). The second characteristic that defines the adaptive immune response is
immunological memory, which refers to the ability of the immune system to respond more efficiently to an insult by a previously seen antigen. This is due to the differentiation of a subset of activated proliferating lymphocytes into memory cells.

In order for T lymphocytes to respond to an antigen, it needs to be presented to them by an APC in the form of a MHC-bound antigen-derived peptide. Major histocompatibility complex molecules are polymorphic cell-surface glycoproteins that contain a peptide-binding groove that can bind a wide variety of antigen-derived peptides (Kindt et al., 2007). T cells can only recognize antigen presented by self-MHC molecules (MHC restriction), meaning that T cells undergo dual specificity for both peptide antigens and MHC molecules (Abbas and Janeway, 2000). Peptides from antigen can be presented in two different MHC classes depending on the type of pathogen the antigen was derived from. These two MHC classes are MHC I and MHC II. Antigen-presenting cells, which include macrophages, dendritic cells, and B lymphocytes, are the only cells to express class II MHC molecules on their membrane. Class I MHC molecules, on the other hand, are expressed by almost all nucleated cells of vertebrate species (Kindt et al., 2007). Recognition of an antigen complexed to a MHC molecule by a naïve T lymphocyte leads to the activation and proliferation of this cell into memory T cells and various effector T cells. Different subsets of T lymphocytes recognize different MHC classes. Major histocompatibility complex class I molecules bind peptides from protein antigens located in the cytosol, while MHC class II present peptides from extracellular pathogens and proteins that are internalized into the vesicles of phagocytes. T lymphocytes that recognize peptides bound to class I MHC molecules differentiate into cytotoxic T (T_C) cells that can destroy viral and tumor-infected cells. On the other hand, peptides presented in class II MHC molecules lead to the differentiation of helper T (T_H) cells that can activate other cells of the immune system to create antibodies and kill...
intravesicular pathogens. The adaptive immune response in which antigen-specific T cells play the main role is termed the cell-mediated immune response. This type of immune response cannot be transferred to a naïve recipient through serum antibodies. On the other hand, the humoral immune response, which is antibody mediated, can be transferred to unimmunized recipients through serum antibodies (Janeway et al., 2005). These two types of acquired immune response will be the major focus of this section.

**Cell-mediated immune response**

Cell-mediated immunity is mediated by T lymphocytes. These lymphocytes express a T-cell receptor (TCR) on their membrane, which has the main function of recognizing and binding antigen which is presented in the form of antigen:MHC complex. There are two subpopulations of T cells: T\textsubscript{H} cells, which express cluster of differentiation (CD) 4, and T\textsubscript{C} cells, which express CD8. Clusters of differentiation are groups of monoclonal antibodies that identify the same cell-surface molecule (Janeway et al., 2005). Natural killer cells also form part of the cell-mediated immune response. These cells share some of the features of T cells, and can kill infected cells in a way similar to T\textsubscript{C} cells. They are the first line of defense against viral infection, and are important in the early response while activation, proliferation and differentiation of T\textsubscript{C} occurs (Kindt et al., 2007).

T cells are important in the control of intracellular pathogens, as well as in the activation of B cells. When pathogens such as some bacteria and parasites, as well as all viruses, replicate inside the host’s cells, they cannot be detected by antibodies. In such cases T cells need to mount a cell-mediated reaction which eventually kills the infected cells, preventing replication of the pathogen inside the cell. Cytotoxic T cells recognize virus-infected cells through the display of virus-derived antigen on class I MHC found on the surface of infected cells. It is imperative to destroy the cell harboring the virus since these pathogens utilize the cell’s biosynthetic
machinery to replicate, eventually killing the cell and releasing new viral particles (Janeway et al., 2005). Cytotoxic T cells kill virus-infected cells through the activation of caspases, which in turn activate a cytosolic nuclease in the infected cell leading to the cleavage of host and viral DNA (Janeway et al., 2005). Helper T cells, in contrast, do not kill pathogen-infected cells. These T cells have a role in the activation of other cells of the immune system through the production of cytokines. This subset of T lymphocytes can be further divided into T\textsubscript{H1} cells, T\textsubscript{H2}, and T\textsubscript{H3} cells. This review will focus mostly on T\textsubscript{H1} and T\textsubscript{H2} cells. These CD4 T cells are especially important in the control of intracellular and extracellular bacteria. Once a T\textsubscript{H} cell recognizes an antigen presented on a class II MHC, it becomes activated and begins secreting cytokines that activate B cells, T\textsubscript{C} cells, macrophages and other cells of the immune system. As mentioned before, cytokines are proteins produced by cells that can affect the behavior of other cells. They act through specific cytokine receptors present on the cells that they affect (Janeway et al., 2005). T helper 1, T\textsubscript{H2}, and T\textsubscript{H3} cytokines are produced by T\textsubscript{H1}, T\textsubscript{H2} and T\textsubscript{H3} cells respectively. The main role of T\textsubscript{H1} cells is to activate macrophages through the production of pro-inflammatory cytokines such as TNF-\textalpha, IFN-\gamma, IL-1, IL-2, and IL-12 (Fonfara et al., 2007; Zenclussen et al., 2002). Because of this release of pro-inflammatory proteins, these cells are commonly known as inflammatory CD4 T cells. On the other hand, T\textsubscript{H2} cells secrete anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 (Fonfara et al., 2007). A third subset of CD4 cells is the T\textsubscript{H3} cells which is defined by Janeway et al. (2005) as “unique cells that produce mainly transforming growth factor beta (TGF-\beta) in response to antigen”. The pro-inflammatory cytokines produced by T\textsubscript{H1} cells induce cell-mediated immunity through the production of macrophage-activating effector molecules such as those discussed above. In contrast, production of T\textsubscript{H2} cytokines by T\textsubscript{H2} cells lead to an inhibition of a cell-mediated response and the

Virus-infected cells are induced to undergo apoptosis by cytotoxic CD8 T cells through the Ca-dependent release of specialized lytic granules. These lytic granules contain proteins which are stored in an active form, but prevented from acting until released. Release of these granules occurs only after recognition of an antigen:MHC I complex on the surface of a target cell by the Tc cell. The cytotoxic effector proteins released by Tc cells are perforin, granzymes, and granulysin. These proteins kill virus-infected cells by forming transmembrane pores, acting as serine proteases, and activating apoptosis, respectively (Janeway et al., 2005). The membrane of some activated Tc expresses Fas ligand (FasL), which can also induce apoptosis of the infected cell through interaction with Fas on the target cell (Kindt et al., 2007).

Intracellular bacteria that are phagocytosed by macrophages can evade destruction because the vesicle in which they are found does not fuse with lysosomes, therefore preventing the formation of the phagolysosome. This lack of macrophage activation is important for the prevention of tissue damage and energy expenditure. Activation of TH1 cells by infected macrophages is especially important in controlling macrophage activation through a positive feedback mechanism. Infected macrophages stimulate TH1 cells to produce IFN-γ, leading to the activation of macrophages and inducing the formation of phagolysosomes, as well as other cytokines that stimulate other antimicrobial activities of the phagocyte (Janeway et al., 2005). Another important effect of activated TH1 cells is the induction of macrophage differentiation in the bone marrow, which is accomplished through the release of IL-3 and granulocyte-macrophage-colony stimulating factor (GM-CSF). These fresh macrophages are then recruited
to the site of infection by T\(_\text{H1}\) cells through the action of cytokines and chemokines, such as TNF-\(\alpha\), TNF-\(\beta\) and CCL2. Macrophages that have been chronically infected with intracellular bacteria lose their ability to kill these bacteria. Release of Fas ligand or TNF-\(\beta\) by T\(_\text{H1}\) cells leads to the killing of these macrophages, releasing the bacteria which can then be destroyed by fresh macrophages (Janeway et al., 2005). Another important mechanism mediated by T\(_\text{H1}\) cells in the fight of bacterial infections is the release of IL-2, a cytokine that induces T cell differentiation, increasing the number of effector cells available to fight infection.

**Humoral immune response**

B lymphocytes are important in the protection of extracellular spaces, through the production of antibodies that can bind to the cell surface of pathogenic organisms neutralizing them and preventing the spread of infection. The binding of antibodies to the pathogen’s surface helps opsonize them so that receptors on phagocytic cells can recognize them and ingest them. Alternatively, antibodies bound to the pathogen’s surface help activate the complement reaction. In contrast to T cells, B cells can recognize antigen that is not presented in the form of a peptide:MHC complex. In fact, B cells can bind antigen through their B-cell receptor (BCR) and internalize and process it into peptides that can be presented to T cells through class II MHC. In this way B cells can also act as APCs. The internalization of antigen by B cells is one of the steps required for their activation into antibody-secreting plasma cells. In addition, B cells require accessory signals from antigen-specific armed T\(_\text{H}\) cells or from microbial constituents. Antigens known as thymus-dependent antigens (TD antigens) can only activate B cells if a second signal is given to the B cell through an activated T\(_\text{H}\) cell. An integral part of this second signal is the interaction of CD40 ligand (CD40L) on the T cell and CD40 on the B cell (Janeway et al., 2005). Another important signal delivered by T\(_\text{H}\) cell is in the form of cytokines such as
IL-4, IL-5 and IL-6, which drive B cell proliferation and differentiation into plasma cells. During the differentiation phase, cytokines released by T_H cells can guide the change of antibody isotype in B cells. Thymus-independent (TI) antigens can induce antibody production without the help of T cells. These antigens are usually microbial constituents, such as bacterial polysaccharides, that can deliver the second signal themselves through direct binding to a receptor of the innate immune system found on the surface of the B cell, or through extensive cross-linking of membrane IgM in the case of polymeric antigens (Janeway et al., 2005).

An important characteristic of the humoral response is its difference in kinetics and other characteristics depending on whether it’s a primary or secondary response. A primary humoral response involves the activation of naïve lymphocytes, while the secondary response involves memory lymphocytes. Both primary and secondary responses are characterized by activation of B cells leading to the secretion of antibodies of various isotypes, which differ in their ability to mediate specific effector functions (Kindt et al., 2007). The primary response is composed of a lag phase, which is attributed to the time naïve B cells undergo clonal selection, a time of clonal expansion, and finally differentiation into plasma or memory cells (Kindt et al., 2007). Immunoglobulin M is the first immunoglobulin secreted during the primary response, with an eventual switch to an increasing proportion of IgM. Even though the T-cell dependent primary antibody response begins with the secretion of IgM, additional isotypes are eventually produced, with each isotype being specialized both in its location in the body as well as the functions it can perform (Janeway et al., 2005). Table 1-1 shows the simplified distribution and functional activities of various immunoglobulin isotypes.

The secondary humoral response is characterized by having a more rapid onset and greater magnitude. This is due to the fact that there is already an established population of
memory B cells for the given antigen that are able to respond to exposure to this previously seen antigen, and that are more easily activated. There is also higher affinity and the presence of different isotypes due to the processes of affinity maturation and class switching that occur during the secondary response (Kindt et al., 2007). The presence of higher quantities of antibodies that express higher affinities give the secondary humoral response its characteristic faster onset of reaction and stronger response.

**Long-Chain Fatty Acids**

A fatty acid (FA) is a monocarboxylic acid with a long, unbranched aliphatic tail that can be either saturated or unsaturated. Fatty acids occur as products of the hydrolysis of naturally occurring fats and oils. They contain a methyl group at one end and a carboxylic group at the other end of the chain. Their aliphatic tail can be four to 28 carbons long and is usually unbranched and of even numbered carbons in naturally occurring fatty acids. The length of the aliphatic tail determines if the FA is considered short, medium, long-chained, or very long-chained. Fatty acids can also be classified as saturated or unsaturated based on the absence or presence of double bonds in their chain. Short-chain FAs (SCFAs) are those containing an aliphatic tail of less than six carbons. Medium-chain FAs (MCFAs) contain a six to twelve carbon tail, while long-chain FAs (LCFAs) are fatty acids with aliphatic tails longer than 12 carbons (Frackenfield, 2000). Generally, FAs are not found as free carboxylic acids due to their high affinity for many proteins. In mammals, though, albumin-bound FAs are referred to as free FAs (FFAs) or NEFAs (Gurr et al., 2002). This section will deal primarily with LCFAs. Long-chain FAs are physiologically important because they combine to form triacylglycerols, which provide a long-term energy storage form in adipose tissue of animals (Gurr et al., 2002).
Fatty Acid Nomenclature and Background Information

This section will discuss the numbering system standardized by the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC-IUB, 1977) as well as to give some background information on LCFAs. In order to understand FA nomenclature some background information on saturation and positional and geometric isomerism will be given.

Saturated FAs are those that do not contain any double bonds in their carbon chain, and are therefore saturated with hydrogen. Unsaturated FAs, on the other hand, contain at least one double bond. Fatty acids that contain only one double bond are referred to as monounsaturated FAs (MUFAs), while FAs with more than one double bond in their carbon chain are known as PUFAs (Gurr et al., 2002). Fatty acids are numbered starting at the carbon atom of the carboxyl group, which is designated C-1 (IUPAC-IUB, 1977). The position of unsaturation is determined based on the first of the pair of carbons where the double bond occurs. An older system of identifying carbon atoms in the FA chain using Greek letters is still utilized when naming PUFAs. The main PUFAs discussed in this section are the ω-3 and ω-6, which are PUFAs in which the last double bond is found three and six carbons away, respectively, from the carbon at the methyl end, designated the ω-carbon. Nowadays, these FAs are referred to as n-3, and n-6 FAs, which will be the naming system used in this dissertation. When two FAs with the same chain length contain the same number of double bonds but at different positions, they are said to be positional isomers. Geometric isomerism refers to the configuration of the hydrogen atoms in respect to the double bond. The configuration at the double bond can be either cis or trans. In the cis configuration adjacent hydrogen atoms are found on the same side of the double bond, while in the trans configuration they are found in opposite sides (Gurr et al., 2002).
Both the systematic name and shorthand nomenclature for a FA provide information on the chain length, degree of saturation, and location and configuration of the double bonds that make up the FA. The systematic name for linoleic acid, which is an 18-carbon FA with two cis double bonds at positions 9 and 12, is cis-9, cis-12 octadecadienoic acid. The shorthand nomenclature consists of two numbers separated by a colon. The first number is used to denote the number of carbons in the FAs, and the second number gives the number of double bonds (Gurr et al., 2002). An example of this type of nomenclature for a saturated FA would be 16:0 (palmitic acid).

Additional information can be provided in the shorthand system in order to identify the presence and type of double bond(s), as well as the type of PUFA being described. For example, the MUFA octadecenoic FA vaccenic acid (VA) can be represented as 18:1, trans-11, since it contains its double bond at the 11th carbon. Similarly, the n-6 PUFA linoleic acid (LA) would be represented by 18:2, n-6, since PUFAs are classified based on the position of their double bond in relation to the methyl end.

Saturated FAs are found in meats, poultry, fish, fats, oils, dairy products, and a variety of other foods. Hydrogenation of unsaturated fatty acids can occur during anaerobic fermentation in the rumen or by chemical methods used to form stable, solid fats, resulting in the formation of saturated fatty acids. Monounsaturated fatty acids have a single double bond in the carbon chain that can be in either the cis or trans configuration. The introduction of a cis double bond results in a bend in the carbon chain, while the trans configuration allows the carbon chain to maintain an extended conformation and properties similar to those of similar length saturated FAs (Gurr et al., 2002). As more cis double bond are introduced into the carbon chain, the less flexibility it has, limiting the ability of fatty acids to be closely packed and making them less thermodynamically stable than trans forms (Gurr et al., 2002). This is the reason why oils which
are made up of PUFAs containing cis bonds are liquid at room temperature, while animal-derived fats made up of trans-FAs (tFAs) are solid at room temperature. Most naturally occurring unsaturated FAs are in the cis configuration. The most common tFAs in human and animal diets are octadecenoic FAs with a single double bond (18:1) (Bauman et al., 2004). Most of these tFAs are a product of partially hydrogenated vegetable oils used in cooking and the preparation of processed foods (Bauman et al., 2004). A small amount of dietary tFAs come from ruminant products, as a consequence of biohydrogenation of dietary PUFAs by ruminal bacteria. The most common tFA found in ruminant products is VA, which is an intermediate in the biohydrogenation of LA and LNA which can escape from the rumen and therefore account for about 60-70% of the tFAs in ruminant milk and meat (Emken, 1995).

There are two main types of PUFAs, n-3 and n-6. They are considered essential fatty acids (EFAs) because they cannot be endogenously synthesized by mammals (Sampath and Ntambi, 2005). Essential fatty acids that cannot be endogenously produced by ruminants include LA, which can be obtained from plant oils, α-linolenic acid (LNA; 18:3, n-3), which predominates in forage lipids and in linseed, and eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexanoic acid (DHA; 22:6, n-3), which are primarily found in fish and fish oil (Cheng et al., 2001). Metabolic conversions can occur only within the same PUFA family, meaning that LA can only be converted to other n-6 fatty acids such as γ-linolenic acid (GLA; 18:3), dihomo-γ-linolenic (DGLA; 20:3), AA (20:4), and docosapentaenoic acid (DPA; 22:5), while LNA can be converted to other members of the n-3 family such as EPA and DHA (Bezard et al., 1994). These processes occur by the action of desaturase and elongase enzymes present in the animal. These longer chain PUFAs can also be obtained directly from the diet.
Conjugated linoleic acids (CLAs) are specialized forms of trans fats. These are positional and geometric isomers of linoleic acid, meaning that they are 18-carbon unsaturated FAs that contain two double bonds. These double bonds can be in the cis/cis, trans/trans, cis/trans, or trans/cis configuration (Bauman et al., 2004). Conjugated linoleic acids differ from naturally occurring FAs in that their double bonds are conjugated, meaning that there is no interceding methylene group between the double bonds as with typical PUFAs (Bauman et al., 2004). Conjugated linoleic acids are also a product of ruminal biohydrogenation (Harfoot and Hazlewood, 1997), therefore they can only be obtained from ruminant-derived products. The two most studied isomers of CLA are the cis-9, trans-11 (c9,t11) and the trans-10, cis-12 (t10,c12) isomer. The c9, t11 isomer is the principal dietary form of CLA exhibiting biological activity, but recently biological activity has been proposed for the t10, c12 isomer, too (Dhiman et al., 2005). Of these two isomers, the c9, t11 isomers is the most abundant in products of ruminant origin, constituting 73-94% of total CLA in milk, dairy products, meat and processed meat products (Chin et al., 1993).

**Long-Chain Fatty Acid Metabolism in Ruminants**

The splanchnic organs, meaning the liver and portal-drained viscera (PDV), are indispensible in the delivery of dietary nutrients to the rest of the body. The PDV is composed of the stomach, the large and small intestine, omental and mesenteric adipose tissues, and the pancreas (Drackley and Andersen, 2006). This section will review how the splanchnic organs metabolize LCFA and coordinate nutrient use in ruminants. The ruminant animal is unique in that its stomach consists of three fore-stomachs (reticulum, omasum, and rumen) and a true stomach (abomasum). Of special importance to this review is the fact that the rumen contains a large microbial population that can act on LCFAs, effecting a change on the unsaturated fat that enters the rumen. In addition, these microbes can add to the amount of lipid that flows from the
rumen to the abomasum through the addition of microbial phospholipids. Rumen microorganisms change unsaturated FAs to saturated FAs through the addition of hydrogen molecules, a process termed microbial biohydrogenation. This results in ruminant animals absorbing more saturated fat than simple-stomach animals. One important aspect to keep in mind is that because of this relationship between lipids and ruminal bacteria, the feeding of large quantities of unsaturated FAs can be detrimental to rumen bacteria, depressing fiber digestion and lowering ruminal pH. Once lipases produced by ruminal microorganisms hydrolyze complex lipids in the rumen (Harfoot, 1978), a mixture of saturated and unsaturated LCFAs flow into the small intestine, where most LCFA absorption occurs. In addition, LCFAs may also enter the small intestine due to some dietary triacylglycerol (TAG) escaping ruminal degradation, as well as microbial phospholipids, and phospholipids from bile and sloughed intestinal endothelial cells flowing towards the duodenum (Drackley and Andersen, 2006). As mentioned previously, most of the unsaturated LCFAs consumed by ruminants are extensively biohydrogenated by the rumen microbes, resulting in mostly saturated LCFAs reaching the small intestine. However, some products of FA ruminal metabolism may escape this process, leading to the accumulation of different biohydrogenation intermediates into ruminant tissues and milk. The most common unsaturated FAs found in seeds and forages, and therefore the cow’s diet, are the EFAs LA and LNA, respectively. Complete biohydrogenation of these FAs by rumen bacteria results in the production of stearic acid (18:0). Escape of intermediate products leads to a small amount of tFAs accumulating in ruminant tissues and milk. One of the intermediates in the biohydrogenation of both LA and LNA is VA, which explains why this 18-carbon FA is the most abundant tFA in products of ruminant origin (Emken, 1995). Other tFA isomers can also be formed in the rumen through less important PUFA biohydrogenation pathways, contributing to
the large distribution of tFAs that can be found in ruminant fat (Bauman et al., 2004). Alternatively, in vitro studies suggest that biohydrogenation of oleic acid (18:1, cis-9) can also lead to the production of trans 18:1 isomers (Mosley et al., 2002). Conjugated linoleic acid isomers are also found in ruminant fat, with the c9, t11 isomer being the most abundant (75-90% of total CLAs), followed by t7, c9 CLA (10% of total CLAs) (Bauman et al., 2004). The c9, t11 isomer is formed as an intermediate product in the biohydrogenation of LA, but data from several research groups has shown that this isomer is predominantly formed by endogenous synthesis through the action of the Δ9-desaturase enzyme on VA (Griinari et al., 2000; Kay et al., 2004). Similarly, t7, c9 CLA is formed by Δ9-desaturase using rumen-derived trans-7 18:1 as a substrate (Corl et al., 2002). By the time dietary fats reach the main site of LCFA absorption, the jejunum, a mixture of saturated and unsaturated FAs has been formed due to the addition of biliary phospholipids (Noble, 1978). Free LCFAs become emulsified into micelles which are efficiently absorbed into the intestinal epithelial cells, where they are esterified to form acetyl-CoA. Acetyl-CoA molecules are used to form TAG that can be incorporated into TAG-rich lipoproteins that are primarily absorbed into the lymphatic system (Bauman et al., 2004). It has been proposed that this intestinally derived TAG is the primary source of circulating TAG in ruminants (Emery et al., 1992).

Dairy cows experience changes in splanchnic metabolism of NEFAs and TAGs during the periparturient period, as a result of the NEB and hormonal changes related to parturition and the initiation of lactation that characterize this phase. This leads to increased lipolysis of adipose tissue and the resulting NEFAs being released by the PDV constantly being taken up by the liver, with an increase in circulating NEFAs leading to an increased uptake by the liver (Drackley and Andersen, 2006). Once taken up by the liver, NEFAs can either be partially oxidized to acetyl-
CoA through β-oxidation or completely oxidized to CO₂ in the tricarboxylic acid (TCA) cycle. Acetyl-CoA is the substrate for two competing reactions: with oxaloacetate to form citrate or with acetoacetyl-CoA for ketogenesis (Gurr et al., 2002). Which of these reactions predominates is determined on the rate of β-oxidation and the redox state of the mitochondrial matrix, which controls the oxidation of malate to oxaloacetate (Gurr et al., 2002). This lack of oxaloacetate needed to react with acetyl-CoA and allow the entry of citrate into the TCA means that the reaction that predominates is the condensation of acetyl-CoA units to form ketone bodies (acetoacetate, acetone and β-hydroxybutyrate). Ketone bodies serve as important water-soluble fuels that can be used as an alternative to glucose in a “glucose sparing mechanism” that allows the preferential use of glucose by the mammary gland. The rate of β-oxidation is determined by several mechanisms: FA availability and rate of utilization of β-oxidation products (Gurr et al., 2002). The availability of FAs is dependent on the entry of these into the mitochondria, a process regulated by the activity of carnitine palmitoyltransferase 1 (CPT1), an enzyme that has been shown to have increased activity in early lactation cows as compared to mid-lactation cows (Aiello et al., 1984). The rate of utilization of β-oxidation products can lead to specific inhibition of particular enzymes or ‘feedback inhibition’ of the whole sequence (Gurr et al., 2002). Ketogenesis can be regulated by the activity of the mitochondrial enzyme hydroxymethylglutaryl-CoA synthase, whose activity is increased in response to depletion of TCA cycle intermediates, as well as the presence of increased acetyl-CoA from β-oxidation (Drackley and Andersen, 2006). Non-esterified fatty acids that are not oxidized are esterfied to cellular TAG, which may be subsequently exported from the liver as VLDL or deposited in the liver as lipid droplets. Secretion of VLDL-TAG from the liver is inherently low in ruminants, even under intense lipid mobilization periods, leading to hepatic lipid accumulation being
associated with NEB periods in dairy cows. The cow’s physiological state changes the liver’s ability to oxidize or esterify NEFAs, suggesting this area of research may yield data that can help prevent TAG accumulation, and therefore optimize production and health of lactating dairy cows.

**Long-Chain Fatty Acid Incorporation in Tissues**

In order to establish if FAs can have any beneficial effects when supplemented in the diet, it is important to determine if these are incorporated into tissues, and if they are, how long this protective effect can last. There is strong scientific evidence supporting the incorporation of FAs into membrane phospholipids after their supplementation. Numerous human studies have found that administration of n-3 FA-enriched diets can result in the incorporation of these FAs into gut mucosal tissue (Gee et al., 1999; Senkal et al., 2005; Courtney et al., 2007). A study with patients that consumed fish oil prior to surgery for colorectal cancer resulted in the treatment group having EPA incorporated into the colonic mucosal lipid fraction as compared to the control group, which received no fish oil supplementation (Gee et al., 1999). Similarly, patients undergoing gastrointestinal surgery received a PUFA-supplemented liquid orally 5 days preoperatively, which resulted in an increased incorporation of EPA and DHA in gut mucosa, among other tissues (Senkal et al., 2005). Courtney et al. (2007) also concluded that dietary supplementation with EPA significantly increased the amount of this FA in colonic mucosa. In relation to ruminants, it seems that the problem of ruminal biohydrogenation makes it unlikely for unsaturated LCFAs to be incorporated into ruminant’s tissues without being completely or almost completely saturated first. Several studies with beef cattle have determined that this is not the case. Studies with rumen-protected fat supplements show that these products do confer some level of biohydrogenation protection in the rumen. A study by Ashes and co-workers (1992) documented that the feeding of ruminally-protected fish oil resulted in an increase in the
amount of EPA in serum as well as muscle phospholipids in ruminants. In a more recent trial, a University of Georgia group concluded that feeding a rumen-protected CLA supplement increased total CLA isomers in adipose tissue by 22%, compared to feeding corn oil (Gillis et al., 2004). In addition, total CLA and c9, t11 CLA isomers were significantly increased in perianal and subcutaneous adipose depots, suggesting that lipid supplementation of feedlot cattle can increase adipose tissue CLA concentration. In 2003, Burns and co-workers set out to determine the effects of supplementation with a rumen-unprotected source of n-3 FAs on plasma and endometrial FA composition of nonlactating beef cows. Animals fed a fish meal-supplemented diet had higher plasma concentrations of EPA and DHA, as well as caruncular endometrial concentrations of EPA, than animals fed a corn gluten-rich meal (Burns et al., 2003), indicating that feeding of a ruminally-unprotected high n-3 FA diet can alter plasma and endometrial n-3 FA composition in beef cows. Studies performed at the University of Florida have concluded that supplementing dairy cow’s diets with n-3 FAs in either a protected or unprotected form can also increase the amount of EPA and DHA in endometrial tissues (Mattos et al., 2005; Bilby et al., 2006). Feeding periparturient dairy cows a diet supplemented with fish oil increased the concentration of total and individual n-3 FAs in milk fat and caruncular tissue as compared to cows fed an olive oil-supplemented diet (Mattos et al., 2005). Similar results where obtained when feeding early postpartum dairy cows a diet containing calcium salts (CS) of fish oil-enriched lipid. Animals allocated to the CS of fish oil diet had increased concentration of n-3 FAs in endometrial, liver, and mammary tissue, as well as milk fat (Bilby et al., 2006). These data indicate that feeding of protected and unprotected FA sources can result in the incorporation of these FAs into tissues of both beef and dairy cattle.
It is also important to document the rate of clearance of these FAs after tissue incorporation. A study conducted by Cao et al. (2006) with twenty healthy humans who received fish oil (1296 mg EPA + 864 mg DHA/day) supplementation for 8 weeks resulted in a 300% and 42% increase in erythrocyte membrane EPA and DHA, respectively. In plasma phospholipids EPA increased 245%, while DHA increased 73% over baseline. The changes in erythrocyte membrane FA compositions were monitored at two week intervals postsupplementation and the results indicated a steep decrease in both n-3 FAs in the first two weeks after supplementation, with a more gradual decrease following in the next six weeks. After eight weeks, the concentrations of EPA and DHA in erythrocyte membrane were still higher than before starting supplementation with the fish oil, but returned to baseline amount after 16 weeks postsupplementation. In the plasma phospholipid fraction there seemed to be a faster clearance of the n-3 FAs. Eicosapentaenoic acid was down to baseline level after only two weeks postsupplementation. On the other hand, DHA had a more gradual decrease with 54% of the increase in mean DHA concentration being retained two weeks postsupplementation, and 25% six weeks postsupplementation. A study with horses performed at the University of Florida also suggests that the clearance rate for FAs is on the faster side (Vineyard et al., 2007). Horses fed encapsulated fish oil had higher plasma and erythrocyte EPA and DHA than control horses, and 5 weeks postsupplementation plasma and erythrocyte EPA had declined to baseline concentrations. Even though DHA in erythrocytes returned to presupplementation level after 5 weeks, plasma DHA did not reach baseline concentration until 8 weeks postsupplementation. The researchers hypothesized that the disparity seen in clearance rate in the human study and the horse study could be due to the fact that yearling horses were used, and that there probably are differences in erythrocyte turnover or lipid mobilization between growing horses and adult
humans. There is a lack of published data determining the clearance rate of FA from tissues of ruminants after supplementation. It is a well documented fact that during the periparturient period of dairy cows there is an extensive mobilization of adipose tissue in order to supply energy in the form of NEFAs. This suggests that the rate of lipid mobilization, and therefore tissue FA clearance rate, may follow different kinetics in these high-producing animals as compared with other species. It seems fair to assume that lipid turnover is going to occur at a faster rate in dairy cows than what has been documented for humans and horses. This is an area that requires further research in order to determine how long-lasting the effects of FA supplementation would be in dairy cows.

From the results of these studies it is clear that even though the tissue FA clearance rate appears to be different depending on the tissue, animal, and physiological state of the animal being studied, the FA concentrations shortly return to baseline concentration. These data suggest that FA supplementation will lead to the incorporation of FAs into tissues, but that the higher FA amounts will be short-lived, with clearance from tissues occurring shortly and resulting in a return to baseline FA concentrations soon after dietary supplementation is terminated.

**Benefits of Feeding Fats to Dairy Cows**

Fat supplementation has become a common practice in the dairy industry due to the inability of high-producing dairy cows to maintain a positive energy balance during the transition to lactation. Inadequate energy intake prepartum and during early lactation contributes to the NEB associated with the peripartum period of dairy cows, and has been associated with an increased incidence of metabolic disorders (Curtis et al., 1985). This lack of energy also contributes to the reproductive inefficiency which is directly related to the selective breeding of high producing dairy cows that has occurred in the dairy cattle industry. Increased milk production in dairy cattle is associated with a decrease in reproductive performance, specifically
a reduction in occurrence and intensity of estrus as well as embryo survival, due to alterations in metabolic rate associated with lactation and management (Thatcher et al., 2003). Supplemental fat feeding increases energy intake, therefore reducing the severity of the NEB the animal experiences. The increase in energy density achieved through the feeding of supplemental fat may enhance lactation performance (Shaver, 1990) as well as metabolic efficiency of dairy cattle (Kronfeld et al., 1980). Supplementation of the dairy cow diet with fats not only increases the energy content of the diet, but also seems to improve reproductive efficiency through mechanisms not related to energy levels. Besides an improvement in reproductive performance, evidence suggests that feeding of certain fats also seems to reduce the incidence of metabolic disorders in dairy cows (Grummer and Carroll, 1991).

The profile of dietary LCFA can affect hepatic β-oxidation, which in turn can determine the level of hepatic FA infiltration and, therefore, fatty liver and ketosis that can occur in early lactation cows. For example, soybean oil (rich in unsaturated LCFAs) can cause accumulation of TAG in the liver of calves (Graulet et al., 2000). *In vitro* studies with bovine hepatocytes determined that the n-3 FAs EPA and DHA were more readily oxidized than oleic or palmitic acid, which were the best substrates for TAG incorporation (Mashek et al., 2002). In addition PUFAs decreased the esterification of palmitic acid to cellular TAG, while saturated LCFAs increased its β-oxidation (Mashek et al., 2002; Mashek and Grummer, 2003).

Feeding fats to lactating dairy cows may also improve reproductive performance through several mechanisms. It may supply EFAs that are lacking in the general diet (Staples and Thatcher, 2005), and may improve oocyte development and fertilization (Cerri et al., 2004; Bilby et al., 2005). The section entitled “Effect of Supplemental fat on Uterine Health and
Reproductive Hormones of Dairy Cows” will discuss how these fats can improve reproductive efficiency in cattle.

Effect of Supplemental Fat on Feed Intake and Production

Dry Matter Intake

The effect of FA supplementation on the DMI of dairy cows is dependent on the type and amount of FA fed, although the general consensus among dairy cow nutritionists is that a high fat content in the diet will depress intake. Factors that determine if a reduction in DMI will occur due to FA supplementation include the degree of ruminal protection of the FA supplement and the amount of fiber fed in the diet. The depression in DMI due to fat feeding is due, in part, to a reduction of fiber digestion leading to prolonged ruminal fill, and decreased palatability attributed to fat supplements (Allen, 2000). In addition, dietary fat supplementation increases plasma concentration of cholecystokinin (Choi and Palmquist, 1996), which can inhibit reticuloruminal motility (Matzinger et al., 2000) and act as an anorexigen on brain satiety centers.

In a study by Mattos et al. (2004), addition of fish oil (6% C20:5 n-3 and 28% C22:6 n-3) to the diet reduced DMI in the prepartum and postpartum periods by 30.3 and 18.1%, respectively, compared to cows fed an olive oil (61% C18:1 n-9)-rich diet. In this study, prepartum rations contained 2% oil (DM basis) and postpartum diets contained 1.8% oil. This data agree with the reduction in DMI observed in dairy cows fed a diet containing 4.5% oil in the form of unprotected mixture of sunflower and fish oils (Shingfield et al., 2006), as well as for primiparous cows fed diets containing unprotected fish oil at 1-2% of DM basis (AbuGhazaleh et al., 2002). Other studies that have reported a negative effect of fish oil on DMI include those of Donovan et al. (2000) and Whitlock et al. (2002). Ruminal infusion of unprotected fish oil also resulted in a decrease in intake (Castañeda-Gutiérrez et al., 2007). Feeding of protected fats, or
abomasal infusion of fats, seems to have a less pronounced effect on DMI, which could be related to the CS of fatty acids conferring ruminal inertness and minimizing the effects of these fats on fiber digestion. Feeding of CS of tFAs or CLAs had minimal effects on prepartum DM consumption compared to cows fed a control diet (no fat inclusion), but there was a decrease in DMI at four, five and six weeks postpartum for tFA-fed animals and at six weeks postpartum for CLA-fed animals compared to control animals (Selberg et al., 2004). Data agree with other studies that have concluded that abomasal infusion of long-chain FAs or tFAs causes a reduction in DMI (Bremmer et al., 1998; Romo et al., 2000). On the other hand, no effect of lipid supplementation was observed in cows fed a mixture of protected palm and fish oil at 2.7% of dietary DM compared to cows receiving a control (no fat) diet (Allred et al., 2006). Feeding of protected fats up to 5% of dietary DM also had no significant effect on DMI (Moallem et al., 2000; Schroeder et al., 2003). Rodríguez-Sallaberry and co-workers (2007) reported there was no significant difference in pre-or postpartum DMI of heifers or cows fed a CS of tFA (58% C18:1 trans family; 1.8% of DM)-supplemented diet as compared with cows fed a highly saturated fat supplement (RBF; 1.55% of DM). Similarly, do Amaral (2008) found that the pattern of DMI over time did not differ among primiparous and multiparous animals fed sunflower oil (80% C18:1 cis-9), CS of tFAs (61% C18:1 trans family), CS of vegetable oils (36% C16:0 and 29% C18:2), or linseed oil (55% C18:3 and 16% C18:2)-rich diets, when the supplemental fats were fed at 1.35% of dietary DM during the prepartum period and at 1.5% (oil sources) and 1.75% (CS sources) of dietary DM during the postpartum period. The same laboratory reported no significant differences in pre- or postpartum DMI among animals fed a control (no fat), CS of safflower oil (63% C18:2 n-6; 1.5% of DM), or CS of palm oil and fish oil (5.4% C20:5 n-3 and 5.3% C22:6 n-3; 1.5% of DM)-supplemented diet, although there was a
trend \((P = 0.09)\) for lower postpartum intake in cows fed the n-3 supplement compared to cows fed the n-6 supplement (do Amaral., 2008). In a study with 1,500 dairy cows fed CS of FAs at 1.5 \% of DM, mean group DMI was significantly greater for the animals allocated to a safflower oil-supplemented diet than for animals fed a palm oil (46\% C16:0)-rich diet both in the pre- and postpartum periods (Silvestre, 2008). Animals from this study had greater DMI during the breeding period when fed the palm oil-supplemented diet as compared with animals fed a fish oil (5.4\% C20:5 n-3, 5.3\% C22:6 n-3)-supplemented diet. Discrepancies between studies suggest that the effects of feeding protected fats on DMI of dairy cows may depend on animal parity, stage of lactation of the animals, source of the fat, protection of the fat, degree of saturation of the fatty acids, and proportion of fat in the diet.

The reduction in fiber digestibility observed with diets containing a high level of PUFAs is a result of the change in microbial population and therefore, ruminal environment, that is attributed to these diets and that can result in a reduction of DMI (Doreau and Chilliard, 1997). Feeding of protected fats seems to have a less pronounced effect on DMI because the slow release of the unsaturated fatty acids from the calcium salt complex prevents rapid modifications of the ruminal environment, therefore minimizing the effect of these fats on fiber digestion.

**Body Weight and Body Condition Score**

Results from research conducted at the University of Florida on the effects of FA-supplementation on production aspects of dairy cows indicate that there is no significant effect of lipid supplementation on body weight (BW) or BCS of primiparous or multiparous animals in either the pre or postpartum period. In 2004, Mattos and co-workers reported no effect of lipid supplementation on BW of Holstein heifers and cows fed diets containing fish oil or olive oil. Studies utilizing protected fats as sources of FAs show similar results (Selberg et al., 2004; Rodriguez-Sallberry et al., 2007; do Amaral et al., 2008; Silvestre, 2008). There was no effect of
diet on BW or BCS when Holstein cows and heifers were fed a basal (no fat) diet, a CS of CLA-supplemented diet or a CS of tFAs-supplemented diet (Selberg et al., 2004). Consistent with these results, Rodríguez-Sallaberry and co-workers (2007), reported no effect of feeding a tFA-rich diet on BW or BCS of dairy cows and heifers. In 2008, Silvestre reported that frequency distribution of cows among different BCS quartiles was not affected by feeding of safflower oil, palm oil or fish oil-rich diets at parturition and at 43 days postpartum. In addition, do Amaral and others (2008) also reported no effect of lipid supplementation on BW and BCS during the pre and post-partum period of Holstein heifers and cows.

Body weight and BCS were not significantly different among primiparous cows fed a control diet, a diet with 2% (DM basis) added fat from menhaden fish oil, a diet with 2% added fat from extruded soybeans, or a diet with 1% fat from fish oil and 1% fat from extruded soybeans (AbuGhazaleh et al., 2002). Another study utilizing the same dietary treatments concluded that there was no effect of lipid supplementation on BW or BCS of multiparous lactating cows (Whitlock et al., 2002). Feeding lactating cows increasing concentration of menhaden fish oil (0 to 3% of ration DM) had no effect on BW or BCS changes (Donovan et al., 2000). These results suggest that FA supplementation has no effect on BW or BCS of dairy cows or heifers.

**Milk Yield, Milk Fat, and Milk Protein**

Several studies have reported no significant effect of lipid supplementation on milk production and milk protein concentration of lactating cows (AbuGhazaleh et al., 2002; Mattos et al., 2004; Selberg et al., 2004; Rodríguez-Sallaberry et al., 2007; do Amaral et al., 2008). Four primiparous cows (2 Holstein, 2 Brown Swiss), averaging 102 days in milk (DIM), were used in a 4 X 4 Latin square with 3 week periods to determine the effect of feeding fish oil (2% DM basis), extruded soybeans (2% DM basis), or their combination (1% fish oil and 1% extruded
soybean) on fatty acid profiles of milk (AbuGhazaleh et al., 2002). Results from this experiment indicated that milk yield, milk fat and milk protein percentages were similar for all fat supplements and control. Mattos and co-workers (2004) also reported no effect of unprotected lipid supplementation (fish oil or olive oil) on milk production, milk fat and milk protein percentage. Supplementing the diet of Holstein heifers and cows with CS of CLA or CS of tFAs had no effect on average milk production and milk protein percentage (Selberg et al., 2004). There was a significant reduction in mean milk fat concentration during weeks 5, 6 and 7 of lactation in animals fed the CS of CLA-supplemented diet, but feeding CS of tFAs had no significant effect on milk fat concentration (Selberg et al., 2004). These results agree with the report by Rodríguez-Sallaberry et al. (2007), in which there were no differences in milk yield, milk fat or milk protein percentage due to addition of CS of tFA to the diet of primiparous and multiparous Holsteins. In 2008, do Amaral and co-workers also reported that fat source did not influence milk yield or milk protein. Other studies have reported a negative effect of n-3 fat supplementation on milk fat percentage of dairy cows (Donovan et al., 2000; Whitlock et al., 2002). In 2000, Donovan and co-workers reported an increase in milk yield corresponding to an increase in dietary fish oil concentration from 0 to 1% (DM basis), followed by a linear decrease as fish oil concentration increased from 1 to 3%. Milk fat also decreased linearly as dietary fish oil increased, but there was no effect on milk protein concentration. A study conducted at South Dakota State University found that milk production, and milk fat content were lower for lactating cows that consumed either a diet containing 2% fat from fish oil, or a combination of 1% fat from fish oil and 1% fat from extruded soybeans, compared to cows fed a control diet or a diet containing 2% fat from extruded soybeans (Whitlock et al., 2002).
The significant reduction in milk yield reported by Donovan et al. (2000) and Whitlock et al. (2002) might be attributed to the reduction in intake associated with dietary fish oil. In spite of reduced DMI AbuGhazaleh et al. (2002), reported no effect of fat feeding on milk production. Differences in fat effects on milk production may be related to differences in parity in the previous two studies. This could also account for the lack of n-3 FA-supplementation effect on milk fat production in the AbuGhazaleh et al. (2002) study, since data seem to consistently indicate that feeding of an n-3 rich diet results in a reduction in milk fat percentage (Donovan et al., 2000; Whitlock et al., 2002; do Amaral et al., 2008). This can be explained by the observation that there was a positive quadratic relationship between supplementation with n-3 FAs from fish oil and production of trans-octadecenoic FAs (Moate et al., 2006). These tFAs are produced as intermediate products of the ruminal biohydrogenation of unsaturated fats, which can be absorbed from the small intestine and inhibit de novo synthesis of lipid in the mammary gland possibly through a reduction in acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) synthesis (Piperova et al., 2002).

Milk Fatty Acid Profile

Fat supplementation has been shown to differentially change the fatty acid profile of milk, depending on the fat supplement being ingested by the cow. Marketability of milk may be improved due to an increased proportion of total unsaturated FAs (O’Donnell, 1989), including CLA, trans-vaccenic acid, and n-3 FAs. Research has shown that the c9, t11 CLA isomer has anticarcinogenic (Ip et al., 1999) and antiatherosclerotic (Lee et al., 1994) properties. This isomer makes up more than 80% of the CLA isomers found in milk fat (Chin et al., 1992). Trans-vaccenic acid may also have anticarcinogenic properties (Awad et al., 1995) and, since it is an intermediate in the synthesis of CLA, can also serve as a precursor for CLA synthesis in human and animal tissues (Salminen et al., 1998). In addition, n-3 FAs are thought to be heart-
healthy because they can help prevent coronary heart disease (Daviglus et al., 1997). Increasing the proportion of these FAs in milk may improve its consumer appeal, enhancing its salability and therefore benefiting the dairy industry.

Feeding of a diet containing 2% fish oil increased the concentrations of CLAs and TVA in milk fat to 356% and 502%, respectively, of basal amounts when no fish oil was fed (Donovan et al., 2000). Increasing the amount of fish oil in the diet to 3% resulted in no further increase in the concentration of these FAs, but did increase the profile of n-3 FAs in milk from a trace when no fish oil was added to the diet, to over 1 g/100 g of milk fatty acids. Most of the increase in n-3 FAs was due to EPA and DHA, because there was no change in LNA concentration. Similar results were observed when feeding a diet with 2% fat from fish oil, 2% fat from extruded soybeans, or a combination of both. All dietary treatments increased the proportion of n-3 FAs in milk, with no significant difference among fat supplements (Whitlock et al., 2002). Concentrations of TVA and c9, t11 CLA were higher in milk fat from cows fed fish oil than milk fat from cows receiving the extruded soybean-supplemented diet. This is in contrast to the data obtained by AbuGhazaleh and co-workers (2002), who reported that concentrations of cis-9, trans-11 CLA and TVA were increased in milk fat by all fat supplements, with no differences in milk CLA and TVA observed among fat supplements. Feeding fish oil also increased the proportion of individual and total n-3 FAs as well as of total CLAs in milk when compared to cows fed olive oil (Mattos et al., 2004). Selberg et al. (2004) also reported an increase in the milk FA content of CLA (namely the t10, c12 isomer) and trans-C18:1 FAs due to supplementation with CS of CLA and CS of tFAs. In addition, supplementation of CLA decreased short- to medium-chain FA concentrations and increased both LA and LNA amounts in milk fat.
The higher concentration of CLA observed when feeding fish oil can be explained by the modification of ruminal or systemic functions caused by fish oil feeding, which stimulates increased conversion of LA and LNA present from other feeds, to TVA and CLA (Whitlock et al., 2002). Ruminal digesta of cows supplemented with n-3, n-6 or a mixture of both FAs, had higher TVA to CLA ratio compared with milk, indicating that the fat supplements increased milk CLA concentration mainly by increasing ruminal production of TVA. This observation indicates a significant role for mammary Δ⁹-desaturase in milk CLA production (Abughazaleh et al., 2002). The decrease in short- and medium-chain FAs observed with CLA supplementation (Selberg et al., 2004) can be explained by the increased proportion of LCFAs in milk as a result of feeding increasing amounts of these FAs, and by their inhibition of de novo synthesis of short- and medium-chain FAs in the mammary gland (Palmquist et al., 1993).

**Effect of Supplemental Fat on Plasma Metabolites and Hormones of Dairy Cows**

**Non-Esterified Fatty Acids and β-Hydroxybutyric Acid**

Measurement of plasma NEFA concentration in cows is a common practice in the dairy industry due to the association between elevated NEFAs during the NEB period of these animals and the development of fatty liver (Skaar et al., 1989). Plasma NEFA concentration reflect the amount of lipomobilization occurring in the animal. Non-esterified fatty acids are almost always increased due to fatty acid supplementation, although some studies report no change in plasma NEFA concentration when fats are added to the diet (Mattos et al., 2004; do Amaral et al., 2008; Silvestre et al., 2008; Castañeda-Gutiérrez et al., 2009). In addition, the type of fat also affects plasma NEFA concentration, with some fatty acids having a more marked effect on lipid mobilization than others (Selberg et al., 2004; Rodríguez-Sallaberry et al., 2007).

Since condensation of Acetyl-CoA units to form ketone bodies seems to be the reaction that predominates during periods of NEB in lactating dairy cows, it would be expected that
increased NEFA concentration in blood would be accompanied by an increase in circulating β-HBA. Results from fat-feeding studies indicate that β-HBA does not always follow the same pattern of increase or decrease as NEFAs. In fact, it appears that changes in β-HBA concentrations are independent of changes in NEFA concentrations, and that the type of FA being fed dictates the fate of circulating NEFAs and their eventual conversion to ketone bodies.

Multiparous cows fed a diet rich in CS of CLAs had greater plasma NEFA concentration during the first wk postpartum than cows fed a control (no fat) diet, which was accompanied by an increase in plasma β-HBA concentration (Selberg et al., 2004). Rodríguez-Sallaberry and others (2007) reported no effect of feeding rFAs on plasma β-HBA concentration of primiparous or multiparous dairy cows, although multiparous cows had increased plasma NEFA concentration due to rFA supplementation. Similarly, dairy cows fed linseed oil had greater concentration of NEFAs in plasma at wks 2 and 5 postpartum, compared to cows fed CS of vegetable oil (do Amaral, 2008). Results from the previous two studies indicate fat sources enriched in different fatty acids have differential effects on concentration of NEFAs in plasma of dairy cows. Moallem and co-workers (1997) fed CS of FAs to high producing dairy cows and found that the feeding of extra lipogenic nutrients increased plasma NEFA. In a study with multiparous cows fed a glucogenic, a lipogenic diet or glucogenic and lipogenic diet from wk 3 before the expected calving date until wk 9 postpartum, cows fed the lipogenic or mixed diets diet had higher plasma NEFA and β-HBA concentrations than cows fed the glucogenic diet (van Knegsel et al., 2007). However, there was no effect of diet on plasma NEFA concentration in cows fed diets containing either fish oil or olive oil, but concentration of β-HBA tended ($P = 0.08$) to be greater in cows fed the fish oil-rich diet (Mattos et al., 2004). Do Amaral and co-workers (2008) reported no treatment effect on plasma NEFA or β-HBA concentrations for cows
fed CS of n-3 or n-6 FAs. Similarly, feeding a transition diet rich in either CS of palm oil or safflower oil at 1.5% of DM did not affect mean plasma NEFA or β-HBA concentrations in primiparous and multiparous dairy cows (Silvestre et al., 2008). A study conducted at Cornell University reported that feeding prilled FAs or CS of n-6 FAs had no effect on plasma NEFA concentration of multiparous Holstein cows, although all supplements tended to decrease β-HBA concentration during the postpartum period (Castañeda-Gutiérrez et al., 2009).

The increase in circulating NEFA amount due to FA supplementation can be explained by a release of FAs after lipoprotein lipase hydrolysis of triglyceride contained within circulating lipoprotein (Grummer and Carroll, 1991). These hydrolyzed triglycerides may not be completely removed from the circulation by tissues. Mammary tissue is able to take up NEFAs from blood, but the net uptake (arterio-venous difference) is negligible (Annison, 1983). In addition, increased plasma NEFA concentration could occur due to increased release of FAs from adipose tissues, decreased NEFA clearance by tissues, or a combination of both (Grummer and Carroll, 1991).

An increase in circulating NEFA concentration accompanied by increased β-HBA concentration can be explained by the increase in ketone bodies that occurs when the liver reaches its maximum capacity to completely oxidize FAs through the Krebs cycle or to export them as very low density lipoprotein (VLDL) (Goff and Horst, 1997). Increased NEFA concentration without an accompanying increase in β-HBA concentration, suggests that the dietary FAs may enhance FA β-oxidation in the liver, therefore completely oxidizing fats to CO₂, and preventing the formation of ketone bodies.
Glucose

Plasma glucose concentration is not usually affected by fat supplementation (Staples et al., 1998). Trans FA supplementation did not affect plasma glucose concentration in multiparous Holstein cows, compared to cows fed a diet rich in saturated fats (Rodríguez-Sallaberry et al., 2007). Similarly, Moallem et al. (2007) reported no dietary effect on plasma glucose concentration pre- and post-partum for cows supplemented with saturated or unsaturated fats. Concentration of glucose in plasma were unaffected by dietary treatment in multiparous cows fed CS of FAs (Selberg et al., 2004). In addition, supplementing multiparous Holstein cows with prilled FAs (1.9% of DM), or CS of n-6 FAs (2.24% of DM) did not affect circulating glucose concentration compared to cows that received a control (no supplement) diet (Castañeda-Gutiérrez et al., 2009). Silvestre and co-workers (2008) reported that feeding transition diets rich in palm oil or safflower oil had no effect on plasma glucose concentration. Another study showed that glucose concentration in plasma were not different among cows fed a control, an n-6, or an n-3-rich diet (do Amaral et al., 2008). However, Mattos et al. (2004) found that feeding fish oil reduced plasma concentration of glucose. The effect of this fat on glucose concentration was suggested to be a result of the reduction in DMI that was associated with the fish oil-rich diet, or possibly the inhibition of gluconeogenic enzymes by components of the fish oil (Mattos et al., 2004).

It is evident that fat feeding does not routinely increase plasma glucose concentration. It is possible that fat supplementation may result in a sparing of glucose from oxidation, and if tissue clearance of glucose is decreased because of this glucose sparing effect of fat, then stable blood glucose concentration seen during fat feeding may indicate decreased hepatic gluconeogenesis (Grummer and Carroll, 1991).
**Immunomodulatory Effects of Long-Chain Fatty Acid Supplementation**

Dietary FAs can affect immune function through modulation of innate immune responses such as phagocytosis and oxidative burst in neutrophils and macrophages, as well as effects on the adaptive immune response, which include eicosanoids and cytokine production, lymphoproliferative effects, and changes in humoral responses. These immunomodulatory effects are achieved through the incorporation of these FAs into the immune cell membrane, resulting in a change in the FA membrane profile of these cells. This allows the cell to respond differently to external stimuli. This section will deal with the effects of dietary fatty acids on immune cell membrane composition, and adaptive immune responses including eicosanoids and cytokine production, and lymphocyte proliferation.

**Immune Cell Membrane Composition**

Peripheral blood mononuclear cells are a subset of immune cells that include T and B lymphocytes and monocytes. The fatty acid profile of human PBMCs is approximately 20% arachidonic acid (20:4 n-6; ARA), 2% EPA, and 0.8% EPA (Miles et al., 2004). Supplementation with dietary n-3 or n-6 FAs has been shown to alter the fatty acid profile of human PBMCs, with an increase in n-3 FAs occurring at the expense of n-6 FAs, specifically ARA (Calder, 2007). In a study by Yaqoob and colleagues (2000), supplementation of healthy humans with 2.1 g EPA + 1.1 g DHA/d from fish oil for 12 wk resulted in a 4-fold increase of PBMC EPA, accompanied by a significant increase in DHA. Arachidonic acid was also increased, but to a lesser extent than the n-3 FAs. These data agree with an increase in the proportion of ARA in PBMCs from healthy subjects fed ARA-containing capsules for 12 wk (Thies et al., 2001). However, there was no effect of supplementation with sunflower oil (rich in LA) on PUFA immune cell membrane composition of healthy humans (Yaqoob et al., 2000). This might be explained by the already high LA content of human diets, which means a
significant dietary intervention is needed to alter the PBMC membrane PUFA content. Studies with animals agree with the results from human studies. Supplementing rats with fish oil or linseed oil increased the EPA and decreased the ARA content of PBMCs, with fish oil eliciting a stronger response than linseed oil (Brouard and Pascaud, 1990). Additionally, feeding growing pigs a diet containing 5% sunflower oil for 40 d resulted in higher ARA and lower n-3 FAs in PBMCs compared to pigs fed a diet containing 5% fish oil (Thies et al., 1999).

This change in PBMC FA membrane composition has major effects on the production of eicosanoids by immune cells. Eicosanoids are inflammatory molecules produced from the oxygenation of twenty-carbon EFAs. The type of PUFA used as a precursor for eicosanoids synthesis will dictate the biological activity of the end product, with eicosanoids derived from n-3 FAs being less biologically active than those derived from n-6 FAs (Calder et al., 2002). As a result, the n-3:n-6 ratio found in the PBMC membrane will determine the potency of the response that can be mounted by these cells. The mechanism of action through which these FAs can affect eicosanoids production will be reviewed in the next section.

**Eicosanoid Production**

Eicosanoids can be divided into four families: prostaglandins, prostacyclins, thromboxanes, and leukotrienes. It is well established that eicosanoids are derived from ARA, and that n-3 PUFAs can inhibit the metabolism of ARA and yield less biologically active eicosanoids (Calder et al., 2002). This means that inflammation and immunity can be modulated through nutritional supplementation, therefore avoiding the use of pharmacological products. Because of the medicinal effects fatty acids can exert on human health, these can be considered as neutraceutical products. Most of the research on the neutraceutical effects of FAs has been conducted with n-3 FAs, because these have been shown to consistently reduce the production of
inflammatory mediators such as PGE$_2$ (Endres et al., 1989; Trebble et al., 2003), LTB$_4$ (Lee et al., 1985; Sperling et al., 1993) and LTE$_4$ (Von Schacky et al., 1993) by inflammatory cells.

As mentioned before, supplementation with n-3 PUFAs can lead to the incorporation of these FAs into the cell’s membrane, leading to n-3 FAs competing with ARA for metabolization by the cyclooxygenase (COX) or lipoxygenase (LOX) enzymes (Strasser et al., 1985). This results in the production of less biologically active prostanoids and leukotrienes, which are less inflammatory than their ARA-derived counterparts. In the production of inflammatory mediators such as the 2-series prostaglandins (PGF$_{2\alpha}$, PGE$_2$) and the 4-series leukotrienes (LTB$_4$, LTC$_4$, LTD$_4$, LTE$_4$), the n-6 fatty acid ARA is used as a substrate after being from the phospholipid membrane by the action of the enzyme PLA$_2$. Once ARA is cleaved, it can be metabolized via the COX or LOX pathway to yield either the 2-series PGs or the 4-series LTs. When the n-3 content of the lipid membrane is increased, EPA competes with ARA for either cleaving by PLA$_2$ or metabolization by the COX and/or LOX enzymes (Strasser et al., 1985; Caldari-Torres et al., 2006). The action of the COX and LOX enzymes on EPA results in 3-series PGs and 5-series LTs which are less biologically active than the ARA-derived inflammatory mediators. In fact, LTB$_5$ is 10- to 100-fold less potent as a neutrophil chemotactic agent than LTB$_4$ (Goldman et al., 1983), while PGE$_3$ is less potent than PGE$_2$ at inducing COX-2 gene expression in fibroblasts and IL-6 production in macrophages (Bagga et al., 2003).

These data suggest that, in addition to directly reducing the amount of eicosanoids produced by inflammatory cells, n-3 FAs can also indirectly reduce the amount of inflammation through the reduction in COX-2 gene expression and IL-6 production that the less biologically active eicosanoids products exert, compared to the n-6-derived eicosanoids (Bagga et al., 2003).
Cytokine Production

Fatty acids are known to modulate immune responses through changes in the pattern of cytokine production by immune cells. Most studies have focused on the effects of CLAs, and the n-3 and n-6 PUFAs, while only a few studies have been conducted to investigate the role of tFAs on cytokine production.

Besides the direct inhibition on the production of inflammatory eicosanoids, n-3 FAs can also exert their effects by suppressing the production of pro-inflammatory cytokines, namely IL-1, IL-2, IL-6, TNF-α, IFN-γ. A study dating back to 1989 showed that feeding humans fish oil, rich in EPA and DHA, for 6 wk suppressed IL-1 production by PBMCs (Endres et al., 1989). Meydani et al. (1993) conducted a study where they fed a group of volunteers a low fat diet that included oily fish daily (providing 1.2 g EPA + DHA/d) and found that this group had a significantly reduced production of TNF-α, IL-1 and IL-6 compared to the control group, which received a low fat, low fish diet. More recent studies in mice have demonstrated that feeding fish oil reduces _ex vivo_ production of TNF-α, IL-1β and IL-6 by their macrophages and monocytes (Calder 1996, 1997, 1998). Other researchers that measured the effect of n-3 fatty acids on IL-2 production found that high levels of these PUFAs resulted in a significant reduction in IL-2 and IFN-γ production and expression of the IL-2 receptor (Gallai et al. 1993; Calder 1996). Studies with murine macrophages and splenocytes indicate that in these cells n-3 PUFAs decreases the expression of the IFN-γ receptor (Feng et al., 1999). Very few studies have been conducted with LNA, the major n-3 PUFA found in flaxseed. DeLucca and coworkers (1999) concluded that not only did LNA inhibit the production of TNF-α and IL-1 by human monocytes _in vitro_, but it also exerted the same effect _ex vivo_ when fed to healthy volunteers at levels ≥ 2.4 g/d. Recent data from Perunicic-Pekovic et al. (2007) are in agreement with
previous reports that supplementation with n-3 FAs results in a significant decrease in the inflammatory markers IL-6 and TNF-α, but they also give us some insight about the possible mechanism of action through which this suppression in the production of the pro-inflammatory cytokines occurs. In their paper about the immunomodulatory effects of n-3 fatty acids, Chapkin and colleagues (2007) reported that feeding n-3 FAs to mice alters the balance between CD4+ T-helper (TH1 and TH2) subsets by directly suppressing TH1 cell development (i.e., clonal expansion). This is an interesting claim that sheds some insight into the possible mechanism of action through which these FAs could be down-regulating the production of the TH1 cytokines.

Results from studies with CLAs have not been as consistent as those for the n-3 FAs. Three trials where mice where fed CLA isomeric blends demonstrated a significant increase in mitogen-induced IL-2 production (Wong et al., 1997; Hayek et al., 1999; Yang & Cook, 2003), while two other mice studies found no effect (Kelley et al., 2002; Yamasaki et al., 2003). Kelley et al. (2002) demonstrated that feeding the animals the purified c9,t11 or t10,c12 isomers had no effect on ConA-stimulated IL-2 production. Other researchers have focused on the effects of CLA on TNF-α either in vivo or in in vitro systems. A study with weaned pigs showed that animals fed the CLA mixture had lower production of IL-1β, IL-6 and TNF-α in response to an LPS challenge, as compared to control animals (Changhua et al., 2005). Also, CLA enhanced the expression of IL-10 in the spleen and thymus of these animals. Furthermore, experiments with the PBMCs isolated from CLA-fed pigs revealed that the inhibitory actions of CLA on the Th1 cytokines were attributed mainly to the t10,c12 isomer, and that the anti-inflammatory properties of CLA were mediated, at least in part, through a PPARγ-dependent mechanism. In murine macrophage cells (RAW 24.7) treated with IFN-γ, the various CLA isomers decreased the production of different mediators of inflammation, including TNF-α, at both the mRNA and
protein level (Yu et al., 2002). In contrast, two different studies with mice reported that one or both CLA isomers significantly increased mitogen-stimulated TNF-α production (Yamasaki et al., 2003; Kelley et al., 2002).

Given these contradicting results it is hard to conclude what the overall effects of CLA on cytokine production are. Perhaps more research is necessary in order to determine the concentration or proportion of each isomer that needs to be administered in order to consistently achieve the anti-inflammatory properties seen on some of these animal and cell culture models.

Although very little research has been done on the effects of tFAs on immune and inflammatory responses, data from human studies indicate that these FAs increase inflammatory responses through induction of pro-inflammatory cytokines. Han et al. (2002) recently reported that giving healthy men and women (over 50 years of age) butter (rich in saturated fatty acid), stick margarine (rich in tFAs), or soybean oil for 32 days did not change IL-2 or PGE₂ production, but that IL-6 and TNF-α production were significantly higher after consumption of the stick margarine compared with the soybean oil diet. An epidemiologic study by López-García et al. (2005) also found significantly higher IL-6 concentration in women in the highest quintile of tFA intake as compared to women in the lowest quintile. High intake of tFA has been associated with the activation of the systemic inflammatory responses, including substantially increased concentrations of IL-6, TNF-α, TNF-α receptor, and monocyte chemoattractant protein-1 (Mozaffarian et al., 2004a). Regardless of the lack of research in this area, it appears that tFAs consistently increase pro-inflammatory cytokine production, which might explain the detrimental effects on human health attributed to these fats.
Lymphocyte Proliferation

When lymphocytes encounter a specific antigen they are induced to proliferate in order to mount a strong immune response against the invading pathogen. Measuring lymphocyte proliferation is a good way to assess the overall function of these immune cells, as well as the strength of the response being elicited. Usually, lymphocytes are stimulated to proliferate through the use of mitogenic compounds, such as concanavalin A (ConA), phytohemagglutinin A (PHA), and LPS, which stimulate specific lymphocyte subsets. Several studies have reported the effects of n-3 and n-6 FAs, as well as CLAs on lymphocyte proliferation in several species, including bovids (Lessard et al., 2004; Thanasak et al., 2005).

The majority of studies with n-6 FAs have demonstrated a decreased lymphoproliferative response after supplementation of subjects with the fatty acids (Calder et al., 1991; Calder and Newsholme, 1992; Joulain et al., 1995). A study analyzing the effects of LA and LNA on bovine PBMCs in vitro, found that there was significant inhibition of the mitogen-stimulated proliferative responses of lymphocytes when PBMCs were incubated with high concentrations of LA (125 and 250 μM) (Thanasak et al., 2005). However, increasing amounts of LNA had no effect on lymphocyte proliferation. In another study, cows fed diets enriched in n-6 PUFAs during the transition period had a lower lymphocyte response to ConA when the PBMCs were incubated with autologous serum, as compared to cows supplemented with saturated or n-3 PUFAs (Lessard et al., 2004).

Most CLA studies indicate that these FAs improve lymphocyte responses to mitogens. Chew et al. (1997) found that incubation of porcine blood lymphocytes with CLA resulted in a stimulation of mitogen-induced lymphocyte proliferation, lymphocyte cytotoxic activity and macrophage bactericidal activity. Studies with weaned pigs showed that supplementing the
animals’ diet with CLA also improved lymphocyte proliferation and increased CD8+ lymphocyte population. A study by Nugent et al. (2005) with healthy volunteers found that in PBMCs isolated from subjects that had received a 80:20 \((c9,t11: t10,c12)\) CLA isomer blend, PHA-induced lymphocyte proliferation was significantly enhanced as compared to PBMCs from subjects allocated to a 50:50 isomer blend or to a LA treatment. However, Tricon et al. (2004) reported that both CLA isomers decreased mitogen-induced T lymphocyte activation in a dose-dependent manner.

Studies with porcine and human PBMCs indicate that while n-6 PUFAs decrease lymphocyte proliferation, CLAs enhance lymphoproliferative responses in these species. If these data can be repeated in immunocompromised subjects, it could have practical applications through the use of CLAs as nutraceutical products capable of improving immune responses in these subjects.

**Effect of Supplemental Fat on Uterine Health and Reproductive Hormones of Dairy Cows**

Fat supplementation is a common practice in the dairy industry because supplemental fat increases the energy content of the diet and has positive effects on metabolism and production of dairy cows. In addition, FAs can increase plasma P4 concentration of dairy cows, which can translate into a better reproductive efficiency. Since it has been proposed that FAs can also improve the immune status of dairy cows, there has been intense focus on the effects of FAs on uterine health. This section will review the effects of supplemental fat on uterine health and plasma P4 concentration.

**Plasma Progesterone Concentration**

Feeding of FAs can improve the reproductive efficiency of dairy cows through an increase in plasma P4 concentration, and a decrease in P4 clearance (Staples and Thatcher, 2005). It appears that the increase in P4 concentration is due to the increase in blood cholesterol.
concentration attributed to fat feeding (Grummer and Carroll, 1991). Increased P4 concentration could also occur due to an increased size of the corpus luteum (CL) in the ovary. The cells that make up the CL are steroidogenic and are responsible for P4 synthesis, therefore increased CL cell mass means there are more cells available to produce P4. Lactating dairy cows fed fat supplements tended to have a larger dominant follicle, which translates into a larger CL, since the CL arises from the ovulated dominant follicle. In fact, larger ovulating dominant follicles in dairy cows resulted in larger CLs, which were associated with greater circulating concentration of P4 (Sartori et al., 2002). Several studies have reported an increased dominant follicle or CL size due to fat feeding (Lucy et al., 1993; Oldick et al., 1997; García-Bojalil et al., 1998; Robinson et al., 2002). In addition to directly affecting plasma P4 concentration through an increase in precursor and steroidogenic tissue, fat supplementation can also decrease P4 clearance rate. In a study by Hawkins et al. (1995), plasma P4 concentrations were elevated in beef heifers fed CS of palm oil from 100 d prepartum until the third day of the estrus cycle postpartum. After all the animals were ovariectomized, blood samples from the animals fed the fat supplement showed a slower clearance rate for P4. In addition, an in vitro study showed that P4 half-life was increased in liver slices incubated with LA (Sangritavong et al., 2002).

In summary, lipid supplementation has been shown to increase plasma P4 concentration in cattle. The mechanism of action through which fats can increase P4 include increased concentration of the P4 precursor (cholesterol), increased CL size, and decreased plasma P4 clearance.

Uterine Health

Dairy cows are prone to nonspecific uterine infections, due to the breakdown of the physical barriers that prevent the entry of microorganisms into the reproductive tract that occurs during the process of calving, combined with the depression in immune function associated with
the periparturient period. Cows that are unable to clear this uterine contamination may develop uterine infections, including endometritis (Dhaliwal et al., 2001). Endometritis can reduce the reproductive performance of livestock by significantly delaying the calving to conception interval (Le Blanc et al., 2002; Gilbert et al., 2005). Recent research has shown that fatty acids may modulate immune responses in several species including cows, pigs, mice, and humans. However, the effects of individual FAs on immune function in cattle have not been fully documented. It is important to understand how different FAs modulate immune responses in dairy cows, in order to determine which fat isomers may be efficacious in improving the immunological dysfunction that occurs during the periparturient period and that may lead to the development of uterine infections.

Fatty acids can affect the function of cells of the innate and adaptive immune system. A reduction in immune function in these cells can lead to a decreased immune response and subsequent establishment of an infection. For example, the eicosanoids PGF$_{2\alpha}$ has been shown to enhance immune function \textit{in vitro} by increasing chemoattraction of PMNs, as well as enhancing phagocytosis (Hoedemaker et al., 1992). Additionally, a study with neutrophils isolated from ovariectomized mares also showed an increased bactericidal activity of these phagocytic cells when they were incubated in the presence of PGF$_{2\alpha}$ (Watson, 1988). Studies with bovids have reported a reduction in neutrophil function as well as in PGF$_{2\alpha}$ metabolite (PGFM) concentration during the early postpartum period due to n-3 FA feeding (Mattos et al., 2004; Petit et al., 2004; Thatcher et al., 2006). The suppression in neutrophil function is likely an effect of the reduced PGF$_{2\alpha}$ concentration associated with n-3 FAs. In fact, postpartum concentrations of PGFM are inversely related to the emergence of uterine infections (Seals et al., 2002). These effects of n-3 FAs on cells of the innate immune system explain why these FAs are
considered immunosuppressive (Calder, 1997; Pizato et al., 2006; Calder, 2007). However, do Amaral et al. (2008) reported that primiparous dairy cows fed a diet rich in n-3 FAs had lower neutrophil counts in uterine flushings collected at 40 ± 2 days postpartum compared to those animals fed a diet rich in saturated and n-6 FAs, suggesting that the animals fed the n-3 supplement had healthier uterine environments.

In 2006, Thatcher and co-workers reported greater plasma PGFM concentration and fewer health problems in the first 10 d postpartum in cows fed an LA-rich diet at 2% of dietary DM from 4 wk prepartum to 14 wk postpartum. The authors stated that the increased LA in tissues likely enhanced the immunocompetence of the animals due to increased PG production. On the other hand, do Amaral et al. (2008) reported no effect of fat supplementation on the incidence of metritis as measured by vaginoscopy scores. Similarly, Silvestre and co-workers (2008) found that the frequency distribution of cows among cervical discharge categories examined at d 8 postpartum was not affected by fat feeding.

Inconsistencies in the data suggest that more studies are necessary in order to fully elucidate the effects of fat supplementation on uterine health of dairy cows. Specifically, it is important to determine if the feeding of diets that can increase production of inflammatory molecules can help confer some sort of immune protection which can decrease the prevalence of uterine infections during the early postpartum period.
<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Functional Activity</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>Activates complement efficiently</td>
<td>Blood (mainly) and lymph</td>
</tr>
<tr>
<td>IgG</td>
<td>Activates complement, opsonizes pathogens, neutralization of viruses, toxins and bacteria</td>
<td>Blood and extracellular fluids</td>
</tr>
<tr>
<td>IgA</td>
<td>Neutralization of toxins and viruses</td>
<td>Epithelium</td>
</tr>
<tr>
<td>IgE</td>
<td>Local defense reactions, trigger mast cells to produce chemical mediators that induce coughing, sneezing, and vomiting</td>
<td>Bound to mast cells, low levels in blood and extracellular fluid</td>
</tr>
</tbody>
</table>
CHAPTER 3
EFFECTS OF MONOUNSATURATED FATTY ACIDS ON CONCANAVALIN A-INDUCED CYTOKINE PRODUCTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS

Introduction

Modern dairy cows experience varying degrees of immunological dysfunction from approximately 3 weeks before calving to 3 weeks after calving, which may have practical implications for health and reproductive management. During this periparturient period, dairy cows experience a depression in immune functions such as depressed lymphocyte response to mitogens and reduced production of antibodies (Kehrli et al., 1989). Dairy cows are prone to nonspecific uterine infections, due to the breakdown of the physical barriers that prevent the entry of microorganisms into the reproductive tract that occurs during the process of calving. Cows that are unable to clear this uterine contamination may develop uterine infections, including endometritis (Dhaliwal et al., 2001). Endometritis can reduce the reproductive performance of livestock by significantly increasing the calving to conception interval (Le Blanc et al., 2002; Gilbert et al., 2005). Cells of the innate and adaptive immune system work in concert to clear infections and restore homeostasis to the animal. These cells communicate and enhance immune responses through the production of cytokines, which are low molecular weight, soluble proteins (Blecha, 1991) that act as chemical messengers to modify the activity of various immune cells. Cytokines can have either pro-inflammatory or anti-inflammatory actions. Tumor necrosis factor-α and IFN-γ are classical pro-inflammatory cytokines, while IL-4 is considered an anti-inflammatory cytokine (Janeway et al., 2005). The production of appropriate amounts of pro-inflammatory cytokines is beneficial in response to infection, but inappropriate amounts or overproduction of these cytokines can lead to pathological responses (Grimble, 1998).
Recent research has shown that fatty acids may modulate immune responses in several species including cows, pigs, mice, and humans. However, the effects of individual FAs on immune function in cattle have not been fully documented. It is important to understand how different FAs modulate immune responses in dairy cows, in order to determine which fat isomers may be efficacious in improving the immunological dysfunction that is associated with the periparturient period.

Although very little research has been done on the effects of tFAs on immune and inflammatory responses, data from human studies indicate that these FAs increase inflammatory responses through induction of pro-inflammatory cytokines. Han et al. (2002) reported that giving healthy men and women (over 50 years of age) butter (rich in saturated fatty acid), stick margarine (rich in tFAs), or soybean oil (rich in unsaturated FAs) for 32 d did not change their lymphocyte proliferation, or IL-2 and PGE$_2$ production, but that IL-6 and TNF-α concentrations were higher after consumption of the stick margarine compared with the soybean oil diet. A recent epidemiologic study by López-García et al. (2005) also reported higher IL-6 concentration in women in the highest quintile of tFA intake as compared to women in the lowest quintile. Other biomarkers of inflammation, such as CRP, soluble TNF receptor 2 (sTNFR-2), E-selectin, and soluble cell adhesion molecules (sICAM-1 and sVCAM-1) were also found to be higher in women in the highest quintile of tFA intake as compared to women in the lowest quintile. Epidemiological and intervention studies have shown that high intake of tFA may increase the risk of developing heart disease, most likely by adversely altering the blood lipoprotein profile (Willet et al., 1993; Lichtenstein et al., 1999). A meta-analysis by Mozaffarian and co-workers (2006) indicated that a 2% increase in the energy intake from tFA was associated with a 23% increase in the incidence of coronary heart disease. In addition to the detrimental effects of tFA
on the lipid profile, high intake of tFA has been associated with the activation of the systemic inflammatory responses, including substantially increased concentrations of IL-6, TNF-α, TNF-α receptor, and monocyte chemoattractant protein-1 (Mozaffarian et al., 2004a). Unlike human studies, many of the reported detrimental effects of tFA in animals are thought to result from essential FA deficiency rather than a specific effect of trans isomers, because they can be prevented by increasing essential FA availability (Beare-Rodgers, 1988; Mahfouz and Kummerow, 1999). Therefore, it is difficult to ascertain whether some of the effects associated with supplemental tFA are due to tFA or to essential FA deficiencies.

The objective of this study was to determine the effects of monounsaturated cis- and trans-octadecenoic FAs on mitogen-induced cytokine production by bovine PBMCs. Based on results from human and animal studies, we hypothesized that supplemental tFAs would enhance ConA-stimulated proinflammatory cytokine production.

**Materials and Methods**

**Media and Reagents**

The medium 199, Dulbecco’s phosphate buffered saline (DPBS), red blood cell lysing buffer, horse serum, penicillin, streptomycin, β-mercaptoethanol, Trypan blue dye, fatty acid-free bovine serum albumin (BSA), ConA, LPS, and PHA were from Sigma Aldrich (St. Louis, MO). Fico/Lite™ LymphoH was from Atlanta Biologicals (Lowrenceville, GA). *Cis*-9 octadecenoic acid (*cis*-oleic acid), *trans*-9 octadecenoic acid (*trans*-oleic acid), *trans*-11 octadecenoic acid (*trans*-vaccenic acid), and *cis*-11 octadecenoic acid (*cis*-vaccenic acid) were purchased from MP Biomedicals, LLC (Solon, OH). Heparinized Vacutainers were purchased from Becton Dickinson (Franklin Lakes, NJ). Polystyrene tissue culture 6-well plates were from Corning Inc. (Corning, NY). ELISA kits for bovine TNF-α and IL-4 were from Endogen (Rockford, IL), whereas the kit for bovine IFN-γ ELISA was from Mabtech (Mariemont, OH).
Animals and Blood Sampling

Single blood samples (~30 mL) were collected by puncture of coccygeal vessel from clinically healthy Holstein cows (n = 6) averaging 235 days in milk. Experimental cows produced an average of 28.8 kg of milk on the day of blood sampling. The cows were fed the same diet consisting of 53% (dry matter basis) forage and 47% (dry matter basis) concentrate.

PBMC Isolation

Peripheral blood mononuclear cells were isolated in the following manner. Three tubes of blood were collected from each cow by puncture of coccygeal vessel using heparinized tubes. The tubes were inverted several times to thoroughly mix blood with heparin. The blood was transported to the laboratory at ambient temperature and the tubes were inverted ten times every ten minutes until arrival at the laboratory. Once in the laboratory the tubes were centrifuged at $600 \times g$ for 30 min at room temperature to obtain the buffy coat. The buffy coat was removed from each tube with sterile transfer pipettes and transferred to tubes containing 2 mL of medium 199, and then thoroughly by pipetting up and down several times. This cell suspension was then transferred on top of 2 mL of Fico/Lite LymphoH as slowly as possible to prevent mixing. The cell suspension/Fico/Lite LymphoH solution was centrifuged at $600 \times g$ for 30 min at room temperature. Mononuclear cells were collected from the Fico/Lite interface using sterile transfer pipettes and transferred to pre-labeled tubes containing 2 mL of red blood cell lysing buffer. Exactly 20 seconds after transferring, the solution was neutralized with 8 mL DPBS (1X). The solution was then centrifuged at $600 \times g$ for 15 min at room temperature. The supernatant was removed by aspiration with a sterile glass pipettes attached to a vacuum and the pellet (mononuclear cells) was resuspended in 2 mL of medium 199 by pipetting up and down ten times with a sterile transfer pipette. The solution was then centrifuged at $600 \times g$ for 3 min at room temperature. The supernatant was once again removed and the pellet was resuspended in
medium 199 modified with 5% (v/v) horse serum, 500 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM glutamine, $10^{-5}$ M β-mercaptoethanol. The PBMCs were counted using the Trypan blue dye exclusion method. The cell suspension was adjusted to $2 \times 10^6$ cells/mL and plated in 1 mL of modified medium 199 in each well of a 6-well plate.

**PBMC Treatment**

In preliminary studies, cytokine production was evaluated by specific bovine ELISAs in supernatants collected from control cultures (unstimulated) or those stimulated with ConA (10 μg/mL), LPS (10 μg/mL) or PHA (10 μg/mL) for 48 h. To determine which mitogen would elicit the strongest cytokine response by bovine PBMCs, we examined the effects of incubating bovine PBMCs with ConA, LPS, or PHA on cytokine production. Concanavalin A and PHA are lectin proteins derived from plants which induce proliferation of T cells. Lipopolysaccharide is a component of the outer-membrane of gram-negative bacteria, and acts as a B cell mitogen. Additionally, *in vitro* cytokine concentration was examined following incubation with increasing concentrations of ConA (0, 5, 19, 15, or 20 μg/mL) in the culture medium. No dose response studies were conducted with LPS or PHA, as these mitogens used at 10 mg/μL had negligible effects on PBMC cytokine production. To examine fatty acid regulation of cytokine production, PBMCs were incubated with ConA alone (10 μg/mL) or with a combination of ConA and 100 μM each of cis-oleic, trans-oleic, cis-vaccenic or trans-vaccenic acid for 48 h. Before being added to the cell cultures, fatty acids were complexed with BSA at a molar ratio of 2:1 by incubating at 37°C for 24 h. After 48 h of fatty acid/mitogen incubation, culture plates were centrifuged at 600 g for 3 min and cell-conditioned media was transferred to pre-labeled tubes and stored at -20°C until subsequent analysis.
Cytokine Assays

Concentrations of TNF-α, IFN-γ and IL-4 in the culture media were analyzed by ELISA using commercially available kits. The least detectable concentrations were 40, 15 and 15 pg/mL for TNF-α, IFN-γ and IL-4, respectively.

Statistical Analysis

Cytokine responses to mitogens and FA were analyzed using the GLM procedure of SAS (SAS 9.2, 2008, Cary, NC). Each treatment effect was estimated in duplicate wells. For preliminary experiments, the statistical model included the fixed effect of treatment (n = 4), and the random effects of cow (n = 3), and cow by treatment interaction. The effect of treatment was tested using cow by treatment interaction as error term. For FA experiments, the statistical model included the fixed effects of treatment (n = 5), experiment (n = 2), treatment by experiment interaction, and the random effects of cow nested within experiment and cow nested within experiment by treatment interaction. Three different cows were used for each experiment. The interaction of cow nested within experiment by treatment was used as the error term for all upstream effects. Treatment effects were further analyzed using preplanned orthogonal contrasts. Data are presented as least squares means and statistical differences were declared at $P < 0.05$.

Results

Cytokine Responses of Cultured Bovine PBMCs to ConA, LPS, and PHA

On average, mitogen-stimulated PBMCs produced higher concentrations of TNF-α, IL-4 and IFN-γ ($P = 0.001, P = 0.03, P = 0.03$, respectively) compared to control-treated cells (Figures 3-1, 3-2, 3-3). Concanavalin A and PHA treated cells produced more TNF-α, IL-4, and IFN-γ than LPS-treated cells ($P = 0.0003, P = 0.01, P = 0.006$, respectively). Bovine PBMCs
incubated with ConA or PHA had similar amounts of IL-4 \((P = 0.32)\) and IFN-\(\gamma\) \((P = 0.06)\) (Figure 3-2 and 3-3), but TNF-\(\alpha\) concentrations were higher for ConA-treated cells \((P = 0.0006;\) Figure 3-1). Based on the stronger cytokine response attributed to ConA-stimulated bovine PBMCs, compared with LPS and PHA, we selected this mitogen for use in all remaining \textit{in vitro} experiments.

**Cytokine Responses of Cultured Bovine PBMCs to Increasing Doses of ConA**

Incubating bovine PBMCs with ConA increased TNF-\(\alpha\), IL-4 and IFN-\(\gamma\) \((P < 0.0001)\) concentrations compared to control cells (Figure 3-4, 3-5, 3-6). There was an increase in TNF-\(\alpha\) and IL-4 concentrations as the dose of ConA increased from 0 to 15 \(\mu\)g/mL. Interestingly, IFN-\(\gamma\) responses were numerically higher with 10 \(\mu\)g/mL than 15 and 20 \(\mu\)g/mL. Based on these results, we determined that 10 \(\mu\)g/mL would be the optimal dose of ConA for achieving the strongest cytokine response in cultured bovine PBMCs across cytokines.

**Effects of Cis- and Trans-Octadecenoic Acids on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs**

Effects of \textit{cis}- and \textit{trans}-oleic, and \textit{cis}- and \textit{trans}-vaccenic acids on ConA-stimulated cytokine concentrations are depicted in Figures 3-7, 3-8 and 3-9. ConcanavalinA-stimulated production of TNF-\(\alpha\) and IFN-\(\gamma\) was greater \((P = 0.05, P = 0.02,\) respectively\) for PBMCs co-incubated with \textit{trans}-vaccenic than \textit{cis}-vaccenic acid (Figure 3-7 and 3-9). In addition, cells treated with \textit{trans}-oleic acid had greater ConA-stimulated TNF-\(\alpha\) concentration \((P = 0.01)\) than cells treated with \textit{cis}-oleic acid (Figure 3-7). Co-incubation of PBMCs with the octadecenoic FAs with the double bond at the eleventh position resulted in higher IFN-\(\gamma\) concentration than co-incubation with the octadecenoic FAs with the double bond at the ninth position \((P = 0.009,\) Figure 3-9). There was no FA effect on IL-4 response to ConA in cultured PBMC (Figure 3-8).
Discussion

Concanavalin A, but not LPS, stimulated cytokine production in all cultures. Phytohaemagglutinin A stimulated TNF-α production to a lesser extent than did ConA, but stimulated IL-4 and IFN-γ similarly to ConA. Results are consistent with previous reports (Skopets et al., 1992; Olsen and Stevens, 1993; Lessard et al., 2003; Heaney et al., 2005), and indicate that the bovine PBMCs used in this study likely were highly enriched in T lymphocytes, which are known to be activated by ConA and PHA (Soder and Holden, 1999). Specific mechanisms by which mitogens stimulate cytokine production in PBMCs are complex and were not examined in this study. The observation that ConA-induced IFN-γ mRNA expression was partially inhibited by genistein, a global protein tyrosine kinase (PTK) inhibitor, and PD-98059, an extracellular signal-regulated kinase (ERK) inhibitor, indicated that ConA may also up-regulate cytokine expression, in part, through activation of NF-κB signaling downstream from ERK (Kobayashi et al., 2005).

Although there is strong epidemiological evidence demonstrating the negative effects of tFAs on human health (Willet et al., 1993; Lichtenstein et al., 1999; Mozaffarian et al., 2004b; Mozaffarian et al., 2006), the extent and manner in which individual tFAs affect cytokine production in cattle remain largely unknown. In the present study, trans-vaccenic acid (C18:1 trans-11) greatly enhanced IFN-γ response to ConA in cultured bovine PBMC. The concentration of TNF-α, another pro-inflammatory cytokine, tended to be higher in cultures co-treated with trans-oleic or trans-vaccenic acids than those treated with ConA alone. Results are consistent with previous studies (Gallai et al., 1993; Mozaffarian et al., 2004b), and lend further support to the recognition that dietary tFAs not only alter the blood lipid profile (Katan et al.,
1995), but also induce systemic inflammation in mammalian species (Mozaffarian et al., 2004a; Mozaffarian et al., 2004b; López-García et al., 2005).

The biochemical mechanisms underlying the stimulatory effects of tFAs on pro-inflammatory cytokine production by isolated PBMC are unknown. Available data indicate that tFAs are readily incorporated into cell membranes (Calder et al., 1994; Kummerow et al., 1999), which have several pathways associated with TNF activation (Madge and Pober, 2001). Consequently, as suggested by Grimble and Tappia (1995), tFAs may alter pro-inflammatory cytokine production via effects on lymphocyte membrane phospholipids and signaling pathways.

In summary, results from these experiments with bovine PBMCs suggest that tFAs stimulate production of pro-inflammatory cytokines in cattle, which may induce systemic inflammation in a manner similar to what has been shown in other species. The physiological significance of tFA-stimulated IFN-γ production by cultured PBMC is yet to be elucidated.

**Proposed Model**

Results of these studies provide evidence that supplemental trans-vaccenic acid increases TNF-α and IFN-γ concentrations, but has minimal effect on IL-4 concentration in cultured bovine PBMCs. Whereas the physiological significance of this finding is yet to be elucidated, these data indicate that supplemental trans-vaccenic acid may affect bovine immune function through stimulation of pro-inflammatory cytokine production (Figure 3-10).
Figure 3-1. Effect of 10 μg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (Mitogens), $P = 0.0014$. Contrast 2: (LPS) vs. (ConA) + (PHA), $P = 0.0003$. Contrast 3: (ConA) vs. (PHA), $P = 0.0006$. 
Figure 3-2. Effect of 10 μg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (Mitogens), P = 0.03. Contrast 2: (LPS) vs. (ConA) + (PHA), P = 0.01. Contrast 3: (ConA) vs. (PHA), P = 0.32.
Figure 3-3. Effect of 10 µg/mL of concanavalin A (ConA), lipopolysaccharide (LPS), or phytohemagglutinin A (PHA) on interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (Mitogens), $P = 0.03$. Contrast 2: (LPS) vs. (ConA) + (PHA), $P = 0.006$. Contrast 3: (ConA) vs. (PHA), $P = 0.06$. 
Figure 3-4. Effect of increasing doses of concanavalin A (ConA) on tumor necrosis factor $\alpha$ (TNF-$\alpha$) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (ConA), $P = 0.13$. Contrast 2: (5 $\mu$g/mL) vs. (10 $\mu$g/mL) + (15 $\mu$g/mL) + (20 $\mu$g/mL), $P = 0.24$. Contrast 3: (10 $\mu$g/mL) vs. (15 $\mu$g/mL) + (20 $\mu$g/mL), $P = 0.59$. Contrast 4: (15 $\mu$g/mL) vs. (20 $\mu$g/mL), $P = 0.89$. 
Figure 3-5. Effect of increasing doses of concanavalin A (ConA) on interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (ConA), $P = 0.005$. Contrast 2: (5 $\mu$g/mL) vs. (10 $\mu$g/mL) + (15 $\mu$g/mL) + (20 $\mu$g/mL), $P = 0.007$. Contrast 3: (10 $\mu$g/mL) vs. (15 $\mu$g/mL) + (20 $\mu$g/mL), $P = 0.22$. Contrast 4: (15 $\mu$g/mL) vs. (20 $\mu$g/mL), $P = 0.83$. 
Figure 3-6. Effect of increasing doses of concanavalin A (ConA) on interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (Control) vs. (ConA), $P = 0.18$. Contrast 2: (5 μg/mL) vs. (10 μg/mL) + (15 μg/mL) + (20 μg/mL), $P = 0.47$. Contrast 3: (10 μg/mL) vs. (15 μg/mL) + (20 μg/mL), $P = 0.25$. Contrast 4: (15 μg/mL) vs. (20 μg/mL), $P = 0.90$. 
Figure 3-7. Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced tumor necrosis factor $\alpha$ (TNF-$\alpha$) concentration in bovine peripheral blood mononuclear cells. Data represents least square means $\pm$ SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.47$. Contrast 2: (cis-9) + (trans-9) vs. (cis-11) + (trans-11), $P = 0.48$. Contrast 3: (cis-9) vs. (trans-9), $P = 0.01$. Contrast 4: (cis-11) vs. (trans-11), $P = 0.05$. There was no treatment by experiment interaction ($P = 0.15$).
Figure 3-8. Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced interleukin-4 (IL-4) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.89$. Contrast 2: (cis-9) + (trans-9) vs. (cis-11) + (trans-11), $P = 0.89$. Contrast 3: (cis-9) vs. (trans-9), $P = 0.42$. Contrast 4: (cis-11) vs. (trans-11), $P = 0.16$. There was no treatment by experiment interaction ($P = 0.95$).
Figure 3-9. Effect of cis- and trans-octadecenoic fatty acids (FAs) on ConA-induced interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.37$. Contrast 2: (cis-9) + (trans-9) vs. (cis-11) + (trans-11), $P = 0.009$. Contrast 3: (cis-9) vs. (trans-9), $P = 0.47$. Contrast 4: (cis-11) vs. (trans-11), $P = 0.02$. There was no treatment by experiment interaction ($P = 0.93$).
Figure 3-10. Proposed model for *trans*-vaccenic acid-induced alteration of immune markers in bovine PBMCs.
CHAPTER 4
EFFECTS OF POLYUNSATURATED FATTY ACIDS ON CONCANAVALIN A-INDUCED CYTOKINE PRODUCTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS

Introduction

Recent research has shown that FAs may modulate immune responses in several species including cows, pigs, mice, and humans. Addition of FAs to dairy cow diets has become common practice in an effort to increase the energy density of the diet, and prevent the state of NEB that usually accompanies the periparturient period of these animals. Development of new feeding strategies in which dietary fats influence the immune responses through the modulation of pro-inflammatory factors could contribute to attenuation of the immunosuppressive effect caused by parturition, through improving immune functions involved in defense against pathogenic organisms (Lessard et al., 2004). It is important to understand how different FAs modulate immune responses in dairy cows, in order to determine which fat isomers may be efficacious in improving the immunological dysfunction that is associated with the periparturient period.

Supplemental n-3 PUFAs have been reported to have a beneficial effect on inflammatory symptoms in diseases such as atherosclerosis, rheumatoid arthritis and inflammatory bowel disease (Alexander, 1998; Calder and Zurier, 2001; Yaqoob and Calder, 2003). In both human and animal models, dietary n-3 PUFAs decreased ex vivo concentrations of classical pro-inflammatory cytokines, namely TNF-α, IL-1β and IL-6 (Meydani et al., 1991; Renier et al., 1993; Caughey et al., 1996). However many of these studies used fish oil, which contains various blends of EPA and DHA. The use of such mixtures of long chain n-3 PUFAs has limited the understanding of individual effects of EPA and DHA and has prevented a direct comparison of the relative potencies of these FAs (Weldon et al., 2007). Some studies have focused on the effects of PUFAs on immunologic responses in cattle (Lessard et al., 2004; Thanasak et al.,
One study analyzed the effects of LA and LNA on bovine PBMCs in vitro, and reported inhibition of the mitogen-stimulated proliferative responses of lymphocytes when PBMCs were incubated with high concentrations of LA (125 and 250 μM); (Thanasak et al., 2005). However, increasing amounts of LNA had no effect on lymphocyte proliferation. Linoleic acid (250 μM) decreased ConA-induced PGE₂ response, and increasing amounts of LNA, but not LA, led to a decrease in leukotriene B₄ (LTB₄) concentration. Prostaglandin E₂ and LTB₄ are two potent inflammatory eicosanoids derived from ARA. In another study, dairy cows fed diets enriched in n-6 PUFAs during the transition period had a lower lymphocyte response to ConA when the PBMCs were incubated with autologous serum, as compared to cows supplemented with saturated or n-3 PUFAs (Lessard et al., 2004).

Conjugated linoleic acid, a generic term referring to positional and geometrical isomers of LA, has been shown to decrease PGE₂, TNF-α, IL-1β, IL-6, and NO concentrations in rat macrophage cells (Yu et al., 2002). Bassaganya-Riera et al. (2004) found that in mice fed a CLA-supplemented diet, TNF-α expression in the colon was repressed, and suggested that CLA may work through a PPARγ-dependent mechanism. Other mice and rat studies support the finding that CLA inhibits LPS-stimulated TNF-α production in vivo and in vitro, and that this fatty acid decreases the production of the proinflammatory cytokines IL-1β and IL-6 (Yang et al., 2003; Yu et al., 2002). Lai and others (2005) reported that dietary CLA improved lymphocyte proliferation in weaned pigs, while it decreased leukocyte production and expression of IL-1β, IL-6 and TNF-α, with the inhibitory effects of CLA being attributed mainly to the t₁₀,c₁₂ isomer. In a double blind, randomized, reference-controlled human study (Song et al., 2005), CLA supplementation decreased plasma concentrations of the pro-inflammatory cytokines, TNF-α and IL-1β, but increased the concentration of the anti-inflammatory cytokine,
In contrast with aforementioned studies, Kelley and co-workers (2001) found no evidence for CLA modulation of lymphocyte proliferation, serum antibody titers, \textit{ex-vivo} LPS-stimulated secretion of PGE$_2$, LTB$_4$, IL-1$\beta$, TNF-$\alpha$ or PHA-induced IL-2 production in humans. These studies collectively indicate that the net immunomodulatory effect of CLA may vary with the physiological status of experimental subjects.

The objective of this study was to examine the effects of n-3 FAs and CLA isomers on ConA-induced cytokine production by bovine PBMCs. We hypothesized that supplemental n-3 FAs and CLA isomers would decrease the pro-inflammatory response to ConA.

\textbf{Materials and Methods}

\textbf{Media and Reagents}

The medium 199, Dulbecco’s phosphate buffered saline (DPBS), red blood cell lysing buffer, horse serum, penicillin, streptomycin, $\beta$-mercaptoethanol, Trypan blue dye, fatty acid-free bovine serum albumin (BSA), and ConA were from Sigma Aldrich (St. Louis, MO). Fico/Lite$^{TM}$ LymphoH was from Atlanta Biologicals (Lowrenceville, GA). Linoleic acid, LNA, EPA, DHA, $t_{10}, c_{12}$ CLA (purity $\geq 98\%)$ and $c_{9}, t_{11}$ CLA (purity $\geq 96\%)$ were from Cayman Chemicals (Ann Arbor, MI). Heparinized Vacutainers were purchased from Becton Dickinson (Franklin Lakes, NJ). Polystyrene tissue culture 6-well plates were from Corning Inc. (Corning, NY). ELISA kits for bovine TNF-$\alpha$ and IL-4 were from Endogen (Rockford, IL), whereas the kit for bovine IFN-$\gamma$ ELISA was from Mabtech (Mariemont, OH).

\textbf{Animals and Blood Sampling}

Single blood samples (~30 mL) were collected by puncture of coccygeal vessel from clinically healthy Holstein cows ($n = 6$) averaging 235 days in milk. Experimental cows produced an average of 28.8 kg of milk on the day of blood sampling. The cows were fed the
same diet consisting of 53% forage and 47% concentrate (dry matter basis). Corn silage was the major forage component and ground corn was the primary concentrate.

**PBMC Isolation**

Peripheral blood mononuclear cells were isolated in the following manner. Three tubes of blood were collected from each cow by puncture of coccygeal vessel using heparinized tubes. The tubes were inverted several times to thoroughly mix blood with heparin. The blood was transported to the laboratory at ambient temperature and the tubes were inverted ten times every ten minutes until arrival at the laboratory. Once in the laboratory the tubes were centrifuged at 600 × \( g \) for 30 min at room temperature to obtain the buffy coat. The buffy coat was removed from each tube with sterile transfer pipettes and transferred to tubes containing 2 mL of medium 199, and then thoroughly by pipetting up and down several times. This cell suspension was then transferred on top of 2 mL of Fico/Lite LymphoH as slowly as possible to prevent mixing. The cell suspension/Fico/Lite LymphoH solution was centrifuged at 600 × \( g \) for 30 min at room temperature. Mononuclear cells were collected from the Fico/Lite interface using sterile transfer pipettes and transferred to pre-labeled tubes containing 2 mL of red blood cell lysing buffer.

Exactly 20 seconds after transferring, the solution was neutralized with 8 mL DPBS (1X). The solution was then centrifuged at 600 × \( g \) for 15 min at room temperature. The supernatant was removed by aspiration with a sterile glass pipettes attached to a vacuum and the pellet (mononuclear cells) was resuspended in 2 mL of medium 199 by pipetting up and down ten times with a sterile transfer pipette. The solution was then centrifuged at 600 × \( g \) for 3 min at room temperature. The supernatant was once again removed and the pellet was resuspended in medium 199 modified with 5% (v/v) horse serum, 500 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM glutamine, \( 10^{-5} \) M \( \beta \)-mercaptoethanol. The PBMCs were counted using the
Trypan blue dye exclusion method. The cell suspension was adjusted to $2 \times 10^6$ cells/mL and plated in 1 mL of modified medium 199 in each well of a 6-well plate.

**PBMC Treatment**

To examine CLA regulation of cytokine production, PBMCs were incubated with ConA alone (10 μg/mL) or with a combination of ConA and 100 μM each of LA, $\alpha_9,\alpha_{11}$ CLA, or $\alpha_{10},\alpha_{12}$ CLA for 48 h. To examine n-3 FA regulation of cytokine production, PBMCs were incubated with ConA alone (10 μg/mL) or with a combination of ConA and 100 μM each of LNA, EPA, or DHA for 48 h. For both experiments, treatments were run in duplicates. Culture plates were centrifuged at $600 \times g$ for 3 min and cell-conditioned media was collected and stored at -20°C until subsequent analysis.

**Cytokine Assays**

Concentrations of TNF-α, IFN-γ and IL-4 in the culture media were analyzed by ELISA using commercially available bovine kits. Samples were run in duplicates. The least detectable concentrations were 40, 15 and 15 pg/mL for TNF-α, IFN-γ and IL-4, respectively.

**Statistical Analysis**

Cytokine responses to mitogens and FA were analyzed using the GLM procedure of SAS (SAS 9.2, 2008, Cary, NC). Each treatment effect was estimated in duplicate wells. The general statistical model included the fixed effects of treatment (n = 4), experiment (n = 2), treatment by experiment interaction, and the random effects of cow nested within experiment and cow nested within experiment by treatment interaction. Three different cows were used for each experiment. The interaction of cow nested within experiment by treatment was used as the error term for all upstream effects. Treatment effects were further analyzed using orthogonal contrasts. Data are presented as least squares means and statistical differences were declared at $P < 0.05$.  

120
Results

Effects of CLA on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs

There was no difference in ConA-induced TNF-α, IL-4 and IFN-γ concentrations for bovine PBMCs incubated with LA, c9,t11 or t10, c12 CLA (Figure 4-1, 4-2, 4-3).

Effects of n-3 FAs on ConA-Stimulated Cytokine Production by Cultured Bovine PBMCs

Effects of LNA, EPA and DHA on ConA-stimulated cytokine production in PBMC are shown in Figures 4-4, 4-5 and 4-6. On average, n-3 FAs decreased ConA-stimulated cytokine production by cultured bovine PBMCs. Linolenic acid decreased ConA-induced TNF-α ($P = 0.02$) and IL-4 ($P = 0.02$) production to a lesser extent than did EPA. In addition, co-incubation with EPA decreased ConA-stimulated TNF-α ($P < 0.0001$), IL-4 ($P = 0.0002$) or IFN-γ ($P = 0.002$) production to a greater extent than did DHA.

Discussion

The present study examined the short-term effect of LA, two CLA isomers, and three n-3 PUFAs on mitogen-induced cytokine production in cultured bovine PBMCs. Immunomodulatory properties of CLA are complex and may be species and CLA isomer-specific (Hayek et al., 1999; Song et al., 2005; Li et al., 2005; Changhua et al., 2005). Conjugated linoleic acid has been shown to decrease (Yu et al., 2002; Dunn et al., 2004; Changhua et al., 2005; Song et al., 2005) or have minimal effects (Kelley et al., 2001; Ritzenthaler et al., 2005; Raff et al., 2008) on inflammatory markers in mammalian species. In the present study, supplemental CLA had no detectable effects on Con A-induced TNF-α, IFN-γ, and IL-4 concentrations in bovine PBMC-conditioned media. Results are consistent with previous human studies (Kelley et al., 2001; Nugent et al., 2005; Ritzenthaler et al., 2005) which provided no evidence for enhancing effects of CLA on immunity in healthy subjects. In contrast, Changhua et al. (2005) reported that dietary CLA attenuated the production and gene expression
of pro-inflammatory cytokines in weaned pigs challenged with LPS. In an *in vitro* supplementation study, Yu et al. (2002) found that CLA inhibited the production of inflammatory mediator prostaglandin E$_2$, nitric oxide, TNF-α, IL-1β and IL-6 in murine macrophage RAW 264.7 cells. Whereas exact reasons for differential effects of CLA on immunity in various animal species are unknown, it has been hypothesized that dose, duration and isomeric composition of CLA may each impact the ability of exogenous CLA to modulate immunity in mammalian models. Additionally, the lack of CLA effect on cytokine secretion by PBMCs observed in this study may be due to differences in immune cell populations in isolated PBMCs.

Several investigations have demonstrated that n-3 PUFAs can affect the immune function in animals and humans (references reviewed in Calder et al., 2002). However, the majority of studies examining the immunomodulatory effects of long chain n-3 PUFAs have used fish oils, which contain a heterogeneous blend of EPA and DHA (Weldon et al., 2007). The use of such mixtures of long chain n-3 PUFAs has limited the understanding of the individual effects of EPA and DHA and has prevented a direct comparison of the relative potencies of these FAs in animal and human models (Weldon et al., 2007).

Results of this study provide convincing evidence that EPA and LNA, but not DHA, are potent suppressors of TNF-α, IL-4, and IFN-γ production in isolated bovine lymphocytes. Linolenic acid, the precursor of long-chain n-3 PUFAs, decreased ConA-induced cytokine production to a lesser extent than did EPA. These findings are consistent with the rodent reports, which indicated that fish oil feeding decreased *ex vivo* production of TNF-α, IL-1β and IL-6 by inflammatory macrophages and monocytes (references in Calder et al., 2002). Similar to the rodent data, fish oil providing more than 2.4 g of EPA plus DHA per day greatly decreased TNF-
α, IL-1 and IL-6 production by human mononuclear cells (Meydani et al., 1991; Endres et al., 1989; Gallai et al., 1993). In contrast to these observations, other studies have failed to demonstrate an effect of fish oil on cytokine production. For example, Yaqoob and colleagues (2000) detected no effects of 3.2 g of EPA plus DHA per day on NK cell activity, lymphocyte proliferation and IL-2 and IFN-γ production. Similar results were reported by Kew and coworkers (2003) who reported no effects of foods enriched with plant or marine-derived n-3 PUFAs on human immune function. Exact reasons for discrepancies between studies are unclear, but it has been suggested that some of these differences may be related to different experimental conditions and to different subject characteristics, including the gender, age and basal diet.

Specific mechanisms by which CLAs and n-3 PUFAs modulate cytokine production likely are complex and were not examined in this study. However, available data would indicate that CLA may exert immunomodulatory effects through one of three mechanisms (Song et al., 2005). Firstly, by altering the essential fatty acid concentrations in the phospholipids of the plasma membranes of lymphocytes, CLA isomers may alter the cell’s immunological activity via changes in membrane fluidity or through changes in the activity of membrane-bound enzyme systems (Calder and Grimble, 2002). Secondly, CLA isomers reduce production of eicosanoid products such as PGE₂ (Li and Watkins, 1998; Ogborn et al., 2003), which are involved in early inflammatory events. Finally, there is evidence that CLA directly affects gene expression of inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Changhua et al., 2005) and that this effect may be mediated, in part, via activation of the peroxisome proliferator-activated receptor-γ-dependent mechanism (Yu et al., 2002). Although some of the effects of n-3 PUFAs may also be mediated through alteration of the amount and type of eicosanoids produced (Calder et al.,
2002), it is possible that these FAs elicit some of their immunosuppressive effects by eicosanoid-independent mechanisms, including effects on the membrane fluidity, intracellular signaling pathways and transcription factor activity (Calder et al., 1994; Miles and Calder, 1998; Yaqoob, 1998).

Consistent with an earlier human study (Zhao et al., 2007), supplemental LNA significantly decreased TNF-α production in cultured bovine PBMC. The observation that LNA inhibited TNF-α and IFN-γ production to a lesser extent than EPA also agrees with a previous study (Caughey et al., 1996), which reported a 30% reduction in TNF-α and IL-1β production with LNA, compared with 74-80% reduction with EPA. Whether LNA effects on cytokine production by bovine leukocytes require prior conversion of LNA to EPA is yet to be documented.

In conclusion, our findings provide evidence that LNA and EPA attenuate ConA-stimulated cytokine production by bovine lymphocytes and this data could be used to alter the immunocompetence of the periparturient dairy cow. Unlike the pig and mouse models, short term supplementation of CLA failed to alter TNF-α, IFN-γ and IL-4 responses to ConA in bovine PBMCs, indicating that immunomodulatory effects of CLA may vary from one animal species to another. Whether the current findings reflect the physiological effects of CLA in vivo warrants further investigation.

**Proposed Model**

Results of these studies provide evidence that supplemental n-3 PUFAs attenuate cytokine concentrations in cultured bovine PBMCs, with EPA having a stronger immunomodulatory effect than LNA. Whereas the physiological significance of this finding is yet to be elucidated, these data indicate that supplemental LNA and EPA may affect bovine immune function through a decrease in cytokine concentrations (Figure 4-7). Docosahexaenoic acid and CLAs appear to
have negligible effects on cytokine concentrations in cultured bovine PBMCs. Whether these results translate to an *in vivo* setting is yet to be determined.
Figure 4-1. Effect of LA and CLA isomers on ConA-stimulated tumor necrosis factor $\alpha$ (TNF-$\alpha$) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.90$. Contrast 2: (ConA + LA) vs. (ConA + CLAs), $P = 0.40$. Contrast 3: (ConA + c9,t11) vs. (ConA + t10,c12), $P = 0.75$. There was no treatment by experiment interaction ($P = 0.50$).
Figure 4-2. Effect of LA and CLA isomers on ConA-stimulated interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.18$. Contrast 2: (ConA + LA) vs. (ConA + CLAs), $P = 0.60$. Contrast 3: (ConA + c9,t11) vs. (ConA + t10,c12), $P = 0.79$. There was no treatment by experiment interaction ($P = 0.81$).
Figure 4-3. Effect of LA and CLA isomers on ConA-stimulated interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.23$. Contrast 2: (ConA + LA) vs. (ConA + CLAs), $P = 0.10$. Contrast 3: (ConA + c9,t11) vs. (ConA + t10,c12), $P = 0.33$. Treatment by experiment interaction was significant ($P = 0.02$).
Figure 4-4. Effect of n-3 fatty acids on ConA-stimulated tumor necrosis factor α (TNF-α) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.0001$. Contrast 2: (ConA + LNA) vs. (ConA + EPA), $P = 0.02$. Contrast 3: (ConA + EPA) vs. (ConA + DHA), $P < 0.0001$. There was no treatment by experiment interaction ($P = 0.83$).
Figure 4-5. Effect of n-3 fatty acids on ConA-stimulated interleukin 4 (IL-4) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.002$. Contrast 2: (ConA + LNA) vs. (ConA + EPA), $P = 0.02$. Contrast 3: (ConA + EPA) vs. (ConA + DHA), $P = 0.0002$. There was no treatment by experiment interaction ($P = 0.21$).
Figure 4-6. Effect of n-3 fatty acids on ConA-stimulated interferon γ (IFN-γ) concentration in bovine peripheral blood mononuclear cells. Data represents least square means ± SEM. Treatment means were separated using orthogonal contrasts. Contrast 1: (ConA) vs. (FAs), $P = 0.07$. Contrast 2: (ConA + LNA) vs. (ConA + EPA), $P = 0.12$. Contrast 3: (ConA + EPA) vs. (ConA + DHA), $P = 0.002$. There was no treatment by experiment interaction ($P = 0.81$).
Figure 4-7. Proposed model for n-3 FA-induced alteration of immune markers in bovine PBMCs.
CHAPTER 5
PRODUCTION, METABOLIC AND UTERINE HEALTH RESPONSES OF PERIPARTURIENT HOLSTEIN COWS TO DIETARY TRANS AND N-6 FATTY ACIDS

Introduction

Early postpartum dairy cows can suffer from numerous metabolic disorders which are related to the negative energy state of the transition period. During the transition period, which spans from three weeks before to three weeks after parturition (Grummer, 1995), dairy cows experience most of the infectious diseases and metabolic disorders that in turn affect their well-being and profitability (Drackley, 1999; Goff and Horst, 1997). The major challenge faced by dairy cows during the transition period is an increase in nutrient requirements due to increasing milk production, which are not able to be met through feed intake. As lactation is initiated, the cow’s demand for glucose increases. Glucose is necessary for milk lactose synthesis, therefore the initiation of milk synthesis and subsequent increase in milk production results in an increase in glucose demand by the mammary gland. The problem faced by the dairy cow is that glucose demand cannot be met by feed intake since the decrease in DMI that accompanies the transition period results in little glucose being absorbed directly from the digestive tract. During this time, cows rely on the breakdown of skeletal muscle and adipose tissue to supply amino acids and glycerol for gluconeogenesis. Low insulin concentration, combined with high GH levels, allow for extensive mobilization of long-chain fatty acids from adipose tissue, and therefore an increase in circulating NEFAs. Circulating NEFA concentrations become elevated in response to increased energy needs that occur at the same time that feed intake is inadequate (Overton and Waldron, 2004). These breakdown products of adipose tissue become an important source of energy for the cow during this period, particularly for major energy-requiring organs such as skeletal tissue, which decrease their reliance on glucose as fuel during early lactation (Overton
High circulating NEFA concentrations can lead to several metabolic disorders, including ketosis and fatty liver. They also seem to contribute to the suppression of the immune system that is seen during the transition to lactation. This immunosuppression can lead to the subsequent establishment of uterine infections during the early postpartum period. It is known that high concentration of ketone bodies, as those seen during ketotic states in cattle, may result in a suppression of the immune system (Klucinski et al., 1998; Duffield et al., 2009). Similarly, NEFAs also seem to have direct negative effects on leukocyte function (Lacetera et al., 2005). In addition, a low concentration of circulating Ca, which is typical of animals suffering from parturient paresis, leads to decreased signal transduction and impairs the function of immune cells (Kimura et al., 2006). Milk fever can also increase serum cortisol concentration, which is a known immunosuppressant (Goff et al., 1989). Fat feeding can help alleviate periparturient immunosuppression by enhancing increasing the energy density of the diet, and reducing the incidence of metabolic disorders in dairy cattle (Grummer and Carroll, 1991).

Modern dairy cows experience varying degrees of immunological dysfunction from approximately 3 weeks before calving to 3 weeks after calving, which may have practical implications for health and reproductive management. During this periparturient period, these animals experience a depression in immune functions such as lymphocyte response to mitogens and production of antibodies (Kehrli et al., 1989). Dairy cows are prone to nonspecific uterine infections, due to the breakdown of the physical barriers that prevent the entry of microorganisms into the reproductive tract that occurs during the process of calving. Cows that are unable to clear this uterine contamination may develop uterine infections, including endometritis (Dhaliwal et al., 2001). Endometritis can reduce the reproductive performance of
livestock by significantly delaying the calving to conception interval (Le Blanc et al., 2002; Gilbert et al., 2005). A survival analysis study by Le Blanc et al. (2002) showed that cows with endometritis were 27% less likely to conceive in a given period, and 1.7-times more likely to be culled than cows without endometritis. In another survival analysis study, the authors concluded that the rate at which subclinical endometritis-negative cows became pregnant was higher than cows diagnosed with subclinical endometritis, with the median days open for endometritis-positive cows being 206 days, compared to 118 days for endometritis-negative cows (Gilbert et al., 2005). This study also reported that first-service conception risk was diminished in cows with endometritis (11 vs 36%), and that these cows required more services per conception than endometritis-negative cows (median 3 vs 2).

Previous studies from our laboratory have shown that the addition of tFAs to the dairy cow ration may reduce the prevalence of uterine infections in these animals (Rodríguez-Sallaberry et al., 2006). This could, in turn, improve their subsequent reproductive performance by preventing the hypothalamic-pituitary-gonadal disruption that is attributed to the endotoxins associated with high uterine bacterial loads. The objective of this study was to examine the effects of feeding tFAs and n-6 FAs on production, metabolic and uterine health responses of periparturient Holstein cows. We hypothesized that peripartum supplementation with trans and n-6 FAs would improve the metabolic status of the dairy cow, ultimately improving uterine health and reproductive performance of the early postpartum dairy cow.

**Materials and Methods**

**Materials**

Fat supplements were provided by Cargill (Minneapolis, MN) and Virtus Nutrition™ (Fairlawn, OH). The control diet was supplemented with a highly saturated rumen bypass fat (RBF; Cargill, Minneapolis, MN, 90% saturated FAs). Dietary treatments were CS of trans-
C18:1 FA mix (EnerG-TR; Virtus Nutrition™, Fairlawn, OH, 57% *trans*-octadecenoic FAs) and CS of n-6 FA mix (Prequel-21, Virtus Nutrition™, Fairlawn, OH, 55% LA). Blood collection tubes (10 mL) containing sodium heparin as anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ), needles (20GA 1”), borosilicate glass disposable culture tubes (12 x 75 mm), 50 mL conical tubes, flat bottom 96-well plates, and serum filters were purchased from Fisher Scientific (Pittsburg, PA). Polypropylene milk sample vials (2 mL) were from Capitol Vial, Inc (Auburn, AL). NEFA-HR(2) and Glucose C2 Autokit test kits were purchased from Wako Chemical USA (Richmond, VA).

**Cows and Diets**

Forty one multiparous (n=29) and primiparous (n=12) Holstein cows were utilized in a completely randomized design to determine the effects of feeding calcium salts of *trans*-C18:1 or n-6 FAs on uterine health, production, metabolic and reproductive responses during the transition period. Due to the final small number of primiparous animals, these were dropped from statistical analyses. A total of twenty-nine multiparous cows were used for statistical analyses. Animals that were diagnosed with any condition that required administering antibiotics either pre- or postpartum were removed from the trial. The experiment was conducted at the University of Florida Dairy Unit (Hague, FL) during the months of February through August, 2008. All experimental animals were managed according to the guidelines approved by the University of Florida Animal Care and Use Committee.

Three dietary treatments were initiated 28-35 d prior to estimated calving date and continued through 49 d postpartum. The control diet contained a highly saturated fat supplement (RBF; Cargill, Minneapolis, MN, 90% saturated FAs) at 1.5% of dietary DM. The second experimental diet contained CS of primarily *trans*-C18:1 FAs (*tFA; EnerG-TR, Virtus Nutrition™, Fairlawn, OH, 61% *trans* C18:1 isomers) at 1.8% of dietary DM. The third dietary
treatment contained primarily CS of LA (n-6 FA; Prequel-21, Virtus Nutrition™, Fairlawn, OH, 55% LA) at 1.8% of dietary DM. Diets were formulated to be isolipidic. The fatty acid profile of each fat supplement is presented in Table 5-1. Diets were formulated for intakes of approximately 150 to 200 g/d prepartum and 250 to 300 g/d postpartum of supplemental lipid. Fat supplements were mixed with the concentrates and offered as a part of the total mixed ration (TMR) to experimental animals.

Preparum cows were housed in pens with a sod base equipped with fans, sprinklers, and Calan gates (American Calan Inc., Northwood, NH). Postpartum cows were housed in a free-stall, sand-bedded barn equipped with fans, sprinklers, and Calan gates. Intake of DM was measured daily using the Calan gate system (American Calan Inc., Northwood, NH). All experimental cows were offered ad libitum amounts of TMR to allow for 5 to 10% refusals. Corn silage was the major forage component and ground corn was the primary concentrate. Dry matter of corn silage was determined weekly by drying samples for 1 h using a Koster® (Koster Crop Tester, Inc., Strongville, OH) and the rations were adjusted accordingly to maintain a constant forage:concentrate ratio on a DM basis. Samples of forage, dried at 55°C and ground to pass a 2-mm scree of a Wiley Mill (C.W. Brabender® Instruments, Inc.), and concentrate mixes were collected weekly, composited monthly, and analyzed by wet chemistry for fat (acid hydrolysis), crude protein (CP), acid-detergent fiber (ADF), neutral-detergent fiber (NDF), and minerals (Dairy One, Ithaca, NY). Detailed ingredient and chemical composition of the experimental diets are listed in Tables 5-2 and 5-3, respectively.

Postpartum cows were milked twice a day and milk weights were recorded at each milking. For each experimental cow, samples of milk from consecutive morning (700 h) and evening (1900 h) milkings were collected the same day during each week postpartum and
analyzed for fat, protein, and somatic cell count (SCC) by Southeast Milk Lab (Belleview, FL) using a Bently 2000 NIR analyzer. Daily values were calculated by averaging morning and evening milk values. Daily concentrations of fat and protein were calculated after adjusting for milk production during those 2 milkings. Body weights were measured and BCS assigned weekly by the same individual.

Prepartum energy balance was calculated using the following equation:

\[
\text{Energy balance (Mcal/d)} = \text{net energy of intake} - \left( \text{net energy of maintenance} + \text{net energy of pregnancy} \right).
\]

Net energy of intake was calculated by multiplying the weekly average DMI (kg) by the calculated energy value of the diet (Mcal/kg) (NRC, 2001). Energy requirement for body maintenance was computed using the following equation (NRC, 2001):

\[
\text{Net energy of maintenance (Mcal)} = 0.08 \times \text{BW}^{0.75}
\]

Pregnancy requirements were estimated using the following equation (NRC, 2001):

\[
\text{Net energy of pregnancy (Mcal)} = \left[ (0.00318 \times \text{days pregnant} - 0.0352) \times \left( \frac{\text{calf BW}}{45} \right) \right] / 0.218
\]

Postpartum energy balance was estimated using the following equation (NRC, 2001):

\[
\text{Energy balance (Mcal/d)} = \text{net energy intake} - \left( \text{net energy maintenance} + \text{net energy of lactation} \right)
\]

Milk energy was estimated by the following equation:

\[
\text{Net energy of lactation (Mcal/d)} = [(0.0929 \times \% \text{ fat}) + (0.0547 \times \% \text{ protein}) + 0.192] \times \text{milk weight (kg)}
\]

**Collection of Blood Samples**

Blood (~20 mL) was collected M, W, and F at 0800 h from d 7 before calculated calving date until parturition and from d 11 until d 49 postpartum. Between the day of parturition (d 0)
and d 7 postpartum, blood samples were collected 2 times per day at 0800 and 1730 h. Between d 8 and d 10 postpartum blood was collected once a day at 0800 h. Blood was collected by puncture of a coccygeal vessel into evacuated heparinized blood tubes. Samples were immediately placed in ice until plasma was separated by centrifugation at 2619 × g at 4°C for 30 min (RC-3B refrigerated centrifuge, H 600A rotor, Sorvall Instruments, Wilmington, DE). Plasma was separated and stored at -20°C for subsequent metabolite, hormone, and cytokine analysis.

**Metabolite, APP and Cytokine Assays**

Plasma concentrations of NEFA and glucose were measured in samples collected on wk -1, 1, 2, 3, 4, 5, 6, and 7 relative to the day of parturition. Plasma concentration of NEFA and glucose were measured with the NEFA-HR(2), and Glucose C2 Autokit test kits, respectively. Samples collected on -3, 1, 3, 6, 9, 12, 15, 18, and 21 d, relative to the day of parturition were analyzed for concentrations of the APP haptoglobin, by measuring haptoglobin/hemoglobin complexing (Makimura and Suzuki, 1982). Plasma concentrations of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ were measured in samples collected on day 12 ± 2 postpartum, because uterine infections normally occur during the first 2 wks postpartum. Cytokine concentrations were measured by Thermo Fisher Scientific (Woburn, MA) using custom protein arrays. Concentrations of P4 were determined in every plasma sample collected after d 12 postpartum using Coat-A-Count Kit (DPC® Diagnostic Products Inc., Los Angeles, CA) solid phase ¹²⁵I RIA. The sensitivity of the assay was 0.1 ng/mL and the intra- and interassay coefficients of variation were 5.1 and 7.4%, respectively.

**Uterine Health**

Cows were evaluated for cervical discharge on days 4, 7, 12 and 30 postpartum using a Metricheck® (Metricheck, Simcro, New Zealand) and the scoring system implemented by
Sheldon et al. (2006). The vulva was cleaned using povidone-iodine scrub and a 10% chlorhexidine diacetate solution (Nolvasan® Disinfectant, Wyeth, Fort Dodge, IA) and dried off with a clean paper towel, until there was no remaining debris in the perivulvar area. The Metricheck was inserted into the cranial portion of the vagina, close to the cervix. The floor of the vagina was scraped, using a posterior sweeping motion, and the discharge was collected in a 50 mL conical tube, and assigned a score of 0 (clear or translucent mucus), 1 (mucus containing flecks of white or off-white pus), 2 (discharge containing ≤ 50% white or off-white mucopurulent material, or 3 (discharge containing ≥ 50% purulent material usually white or yellow but occasionally sanguineous) according to Sheldon et al. (2006).

An assessment of uterine cytology was conducted for each animal at 40 ± 3 DIM (Sheldon et al., 2006). Cows were flushed using a 53.3 silicon Foley catheter. The vulva was cleaned with a 10% chlorhexidine diacetate solution and dried with a paper towel. The catheter was introduced through the cervix into the previously pregnant uterine horn. The air balloon was placed approximately 1 cm past the bifurcation of the uterine horn and inflated to a volume consistent with the size of the uterine horn. Sterile saline (20 mL of 0.9%) was infused into the uterine horn and aspirated back using a syringe with a Foley connector. The aspirated solution was placed into a sterile 50-mL conical tube and vortexed. A 10 µL sample of the solution was smeared onto a glass slide and allow to air dry. The smear was stained using the Protocol Hema 3 (Fisher diagnostics, Middletown, VA) stain method. Slides were examined at a magnification of 40x with oil immersion and 100 total cells (including endothelial cells) were counted. Percent of neutrophils were calculated as follows:

\[
\text{\% neutrophils} = \frac{\text{total number of neutrophils}}{100}
\]
Statistical Analyses

Milk production and intake responses were reduced to weekly means before statistical analysis. Milk composition was analyzed by Least-Square analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS software package (SAS 9.2, Cary, NC, 2008). All other dependent responses were evaluated using the MIXED procedure for repeated measures. Fixed effects included treatment, wk relative to calving (for production responses), and treatment by wk interaction. The variance for cow nested within treatment was used as random error term to test the main effect of treatment. Differential temporal responses to dietary treatments were further examined using the SLICE option of the MIXED procedure. When SLICE option detected treatment differences at point in time the PDIF option was used to separate means. Mean treatment and time (week or day relative to calving) effects are reported as least square means. Furthermore, orthogonal contrasts were used to detect treatment differences. Data that did not have a normal distribution (uterine cytology data) were analyzed by the GLIMMIX procedure using Gaussian distribution. Binomial data also were analyzed using the GLIMMIX procedure and Binomial distribution. Treatment and effects were further examined utilizing the PDIF option of the MIXED or GLIMMIX procedures. Differences were considered to be significant at $P \leq 0.05$ for all analyses.

Results

Production Responses

Pattern of DMI as a % of BW over time did not differ among the dietary treatment groups (Figure 5-1). Prepartum DMI decreased from $1.7 \pm 0.09\%$ of BW on wk -3 before calving to $0.90 \pm 0.09\%$ of BW on day of calving, and subsequently increased to approximately $3.1 \pm 0.09\%$ of BW at wk 7 of lactation (wk, $P < 0.0001$). Cows fed the $\alpha$-FA-enriched diet consumed less DM as a % of BW ($P = 0.001$) than cows fed the control (RBF) diet on day of calving (0.47
± .15% vs. 1.2 ± .15%) and on wk 7 postpartum (2.8 ± .15% vs. 3.4 ± .15%) (Figure 5-1). Body weight did not differ among dietary treatments (Figure 5-2). There was a reduction ($P < 0.0001$) in BW from wk -1 before calving (750 ± 14.0 kg) to wk 5 after calving (628 ± 14.0 kg). Animals from all three dietary treatments had comparable BCS and experienced similar patterns of change throughout the experiment (Figure 5-3). The main loss of BCS occurred between wk -3 prepartum (3.52 ± 0.1) and wk 5 postpartum (3.13 ± 0.1) ($P < 0.0001$).

Patterns of change in energy balance (EB; Mcal/d) were similar for animals in all three experimental diets (Figure 5-4). On average, EB decreased from $4.82 ± 1.0$ Mcal/d at wk -3 prepartum to -8.86 ± 1.0 Mcal/d at wk 1 postpartum, and then increased to -0.96 ± 1.0 Mcal/d by wk 7 postpartum (wk, $P < 0.0001$). Animals fed the n-6 FA-enriched diet had higher ($P = 0.03$) EB on wk 6 (1.1 ± 1.9 Mcal/d) than animals fed the RBF or tFA-enriched diets (-4.01 ± 1.6, -5.6 ± 1.7 Mcal/d, respectively). In addition, n-6 FA-supplemented cows had a higher ($P = 0.03$) EB on wk 7 (2.4 ± 1.9 Mcal/d) than cows fed the tFA-enriched diet (-0.9 ± 1.6 Mcal/d).

Milk production averaged $35.2 ± 1.9$ kg/d across lactation and did not differ among experimental diets (Table 5.4). Milk production increased ($P < 0.0001$) throughout the entire postpartum experimental period (23.8 ± 1.2 kg/d on wk 1 to 40.9 ± 1.2 kg/d on wk 7 postpartum). Although fat source did not influence total milk production over time (Figure 5-5), cows allocated to the n-6 FA supplement produced less ($P = 0.02$, orthogonal contrast) 3.5% FCM ($29.8 ± 1.9$ kg/d) than cows fed the tFA (35.0 ± 1.9 kg/d) or RBF diets (36.8 ± 1.9 kg/d) (Figure 5-6).

There was a reduction ($P < 0.001$) in milk fat concentration from wk 1 (4.8 ± 0.2%) to wk 7 (2.6 ± 0.2%) postpartum (Figure 5-7). Cows fed the n-6 FA diet had less ($P = 0.0004$, orthogonal contrast) mean milk fat concentration (2.8 ± 0.2%) than cows fed the RBF or tFA
diets (3.6 ± 0.2, and 3.8 ± 0.2%, respectively). In addition, cows fed the n-6 FA-rich diet produced less ($P = 0.0018$, orthogonal contrast) milk fat (0.92 ± 0.08 kg/d) than cows fed the tFA or RBF diets (1.3 ± 0.07, 1.2 ± 0.07 kg/d, respectively) (Table 5-4). Concentration (2.9 ± 0.07 %) and yield of milk protein (1.0 ± 0.05 kg/d) were unchanged by dietary treatment (Table 5-4, Figure 5-9 and 5-10). Mean SCC did not differ between dietary treatments (Table 5-4).

Animals in the n-6 FA treatment had lower average feed efficiency (1.75 ± 0.11 kg 3.5% FCM/kg DMI) compared to animals in the control or tFA treatment groups (2.08 ± 0.09 kg 3.5% FCM/kg DMI, 2.18 ± 0.10 kg 3.5% FCM/kg DMI, respectively) (Figure 5-11). Average feed efficiency for all treatment groups decreased ($P = 0.0007$) from wk 1 to wk 7 postpartum.

**Metabolic Responses**

All dietary treatment groups had similar temporal patterns of plasma NEFA concentrations, which were low during the prepartum period, dramatically increased during wk 1 after calving, then gradually declined over time (Figure 5-12). During the postpartum period, NEFA concentrations peaked on wk 1, then declined to a level close to prepartum concentrations by wk 7. Cows in the n-6 FA dietary treatment had lower ($P = 0.05$, orthogonal contrast) plasma NEFA concentrations (0.41 ± 0.12 μEq/L) than cows fed the RBF or tFA diets (0.73 ± 0.11, 0.75 ± 0.11 μEq/L, respectively).

Cows fed the n-6 FA-enriched diet had higher ($P = 0.02$, orthogonal contrast) plasma glucose concentrations (57.8 ± 2.7 mg/dL) than cows fed the RBF (46.8 ± 2.4 mg/dL) or tFA (53.0 ± 2.4 mg/dL) diets (Figure 5-13). Pattern of plasma glucose concentration over time did not differ among dietary treatments. Plasma glucose concentration decreased ($P < 0.0001$) from wk 1 prepartum (58.8 ± 2.5 mg/dL) to wk 1 postpartum (47.2 ± 2.3 mg/dL), and then continually increased until wk 7 postpartum (55.1 ± 2.3 mg/dL).
Rectal Temperature

There was a diet by day postpartum interaction ($P = 0.05$), with animals fed the n-6 FA-enriched diet having an increase in rectal temperature from d 4 (38.6 ± 0.13 ºC) to d 7 (39.1± 0.13 ºC) postpartum (Figure 5-14). Cows allocated to the RBF diet tended ($P = 0.06$) to have a higher rectal temperature on d 4 postpartum (38.9 ± 0.07 ºC) than cows allocated to the n-6 FA diet (38.6 ± 0.13 ºC). Dietary treatment did not affect the incidence of fever (temp > 39.5ºC) (Table 5-5).

Plasma Cytokines and Haptoglobin

Concentrations of the proinflammatory cytokines IL-6, TNF-α and IFN-γ in plasma on d 12 ± 2 postpartum did not differ among dietary treatments (Figure 5-15). Additionally, concentrations of haptoglobin in plasma were not affected by day postpartum. Cows fed the tFA-rich diet tended ($P = 0.06$, orthogonal contrast) to have higher concentration of plasma haptoglobin (0.12 ± 0.01 arbitrary units) than cows fed the n-6 FA-rich diet (0.07 ± 0.02 arbitrary units) (Figure 5-16).

Uterine Health

Metricheck scores (0-3 scale) increased ($P = 0.04$) from d 4 (1.6 ± 0.2) to d 12 (2.1 ± 0.2) postpartum, but subsequently decreased ($P < 0.0001$) to 0.5 ± 0.2 on d 30 postpartum (Figure 5-17). Cows allocated to the n-6 FA diet had lower Metricheck scores on d 7 postpartum (0.9 ± 0.3) than cows fed the RBF (2.5 ± 0.3) or tFA (2.0 ± 0.3) diets ($P = 0.0005$, $P = 0.01$, respectively). Animals fed the tFA or n-6 FA-enriched diets had numerically lower neutrophil % in uterine flushings (1.3% and 2.1%, respectively) compared to cows fed the control (RBF) diet (11.9%), but due to the small number of animals this difference was not statistically significant (Figure 5-18). There was no effect of fat supplementation on the proportion of animals with subclinical endometritis (>10% neutrophils in uterine cytology from uterine flushing collected at
34-47 days postpartum). In addition, the incidence of other uterine infections (puerperal metritis, clinical metritis, clinical endometritis) did not differ among dietary treatments (Table 5-5).

**Reproductive Responses**

Cows fed the n-6 FA-rich diet had increased accumulated P4 by day 50 postpartum than cows fed the control (RBF) or tFA-enriched diets ($P = 0.02$; Figure 5-19). There was no effect of dietary treatment on ovulation distribution of Holstein cows (Figure 5-20), which was determined when animals exhibited a plasma P4 concentration $\geq 1$ ng/mL on two or more consecutive days. There was no dietary effect on conception rate to first service (Figure 5-21). Animals in the n-6 FA treatment tended ($P = 0.20$, orthogonal contrast) to spend less days open (88 d) than animals in the control (RBF) or tFA treatments (122 d) (Figure 5-22).

**Discussion**

During the experimental period BW and BCS responses were similar between dietary groups. This agrees with data from several studies involving the feeding of different types of fat on BW and BCS of dairy cows (Donovan et al., 2000; AbuGhazaleh et al., 2002; Whitlock et al., 2002; Mattos et al., 2004; Selberg et al., 2004). Both BW and BCS decreased from wk 1 to wk 5 postpartum, reflecting the intense lipomobilization that occurs in early postpartum dairy cows. On average, cows fed the RBF diet had higher DMI as a % of BW than cows fed the tFA supplement. These results are not consistent with those previously reported by our laboratory (Rodríguez-Sallaberry et al., 2007), where there was no effect of feeding a diet supplemented with tFAs on DMI of primiparous or multiparous Holstein cows. The difference in these results could be due to the length of postpartum feeding of the diets, since Rodríguez-Sallaberry and co-workers (2007) fed the experimental diets for 21 d postpartum. Postpartum fat feeding in this study extended to 49 d postpartum, with a significant effect of tFA feeding on DMI occurring on wk 7 postpartum. In support of our results, do Amaral (2008) reported that DMI as a % of BW
appeared to increase for a longer period of time for animals fed a diet high in n-6 FA compared to cows fed CS of tFAs.

Cows consuming the n-6 FA supplement had less fat in milk than cows fed the RBF or tFA supplements. However, milk production and milk protein content did not differ among dietary groups. Consequently, yield of 3.5% FCM differed among treatments, with n-6 FA-fed cows producing less 3.5% FCM than cows fed the RBF or tFA diets. Selberg et al. (2004) reported a reduction in mean milk fat concentration during wk 5, 6 and 7 of lactation in animals fed CS of CLA. Since CLAs are isomers of LA and can be derived from this FA through the action of microbial biohydrogenation in the rumen, it is conceivable that the reduction in milk fat in n-6 FA-fed animals was due to an increase in CLA concentration in mammary tissues. Specifically, the t10,c12 isomer of CLA has been shown to be a potent suppressor of milk fat synthesis and secretion (Baumgard et al., 2000).

Animals fed the n-6 FA-enriched diet had higher EB on wk 6 than animals fed the RBF and tFA-rich diets. The higher EB in n-6 FA cows in wks 6 and 7 postpartum are probably associated with the reduction in milk fat concentration and yield, which translates into less energy in the form of fat being allocated towards milk production. Additionally, n-6 FA-fed cows exhibited lower 3.5% FCM production as a function of DMI (calculated feed efficiency), which could also account for their higher EB on wks 6 and 7 postpartum. In the present study, plasma NEFA concentrations were lower for n-6 FA fed cows, which reflects less lipid mobilization occurring in these animals. This is consistent with the higher EB observed in these animals, since n-6 FA fed cows achieved a positive EB by wk 6 postpartum while RBF and tFA animals remained in a negative EB. As animals enter a positive EB state, fat mobilization
diminishes, which is observed as a reduction in circulating NEFAs. In addition, plasma glucose concentration was also higher for n-6 FA-fed cows.

In addition to an improvement in metabolic responses, n-6 FA-feeding also improved uterine health and reproductive responses of dairy cows. Cows allocated to the n-6 FA treatment had lower cervical discharge (Metricheck) scores on d 7 postpartum than cows in the other two treatment groups, indicating that these animals exhibited better uterine health by d 7 postpartum. Cows fed the n-6 FA-enriched diet had an increase in rectal temperature from d 4 to d 12 postpartum, compared to the cows in the other two treatment groups. This observation could be the result of an enhanced immune response in these animals, which would account for an improvement in uterine infection clearance. Although plasma PGF$_2\alpha$ was not measured in this study, it is well documented that inclusion of n-6 FAs in the dairy cow’s diet increases the concentration of this reproductive hormone (Thatcher et al., 2006), which may also have a positive effect on uterine health (Seals et al., 2002). This improvement in uterine health appears to have a positive effect on reproductive responses, which is reflected by the higher accumulated P4 concentration by d 50 postpartum that is observed in the n-6 FA treatment group. Although it has been shown that fat feeding increases plasma P4 concentration through increased cholesterol concentration (Grummer and Carroll, 1991), increased CL size (Lucy et al., 1993; Oldick et al., 1997; García-Bojalil et al., 1998; Robinson et al., 2002), and decreased plasma P4 clearance (Hawkins et al., 1995; Sangritavong et al., 2002), no studies have reported a significant increase in plasma P4 due to n-6 FA compared to other fat sources. This increase in plasma P4 for the n-6 treatment group could be a result of improved uterine health, which is supported by the fact that uterine infections can reduce reproductive performance through a disruption of the hypothalamic-pituitary-gonadal axis (Callahan et al., 1971; Oltenacu et al., 1983; Holt et al.,
1989). Furthermore, animals with uterine infection show higher concentrations of PG metabolites (Peter et al., 1987), which contribute to a prolonged anovulation period since ovarian activity cannot resume until PG concentrations have returned to basal levels (Kindahl et al., 1982). Additionally, cows fed the n-6 FA-enriched diet tended to spend less days open than animals fed the control (RBF) or rFA-enriched diets.

Taken together, these results indicate that animals fed the n-6 FA supplement had improved metabolic state and returned to positive EB faster than cows on RBF or rFA supplements. It has been proposed that the immunosuppression seen during the periparturient period can be related, in part, to the NEB which results in a lack of energy for the immune system to function properly (Kehrli et al., 2006). A faster return to positive EB in n-6 FA-supplemented cows would mean these animals have more energy that can be expended on processes of the immune system, including the clearance of uterine infections. This improvement in uterine health was accompanied by an increase in plasma P4 concentration, which translated into less days open for these cows. In addition, NEB is associated with extended interval to first ovulation (Beam and Butler, 1999) through inhibition of LH pulse frequency and low concentrations of blood glucose, insulin, and insulin-like growth factor (Butler, 2000). An improvement in EB in postpartum dairy cows could directly improve reproductive efficiency through a reduction in the interval to first ovulation. In conclusion, feeding n-6 FAs to early postpartum Holstein cows improved their metabolic status, which might have ultimately improved their uterine health and their reproductive performance.

**Proposed Model**

Feeding supplemental n-6 FAs to periparturient dairy cows improves their EB, which might lead to an attenuation of periparturient immunosuppression by increasing the amount of energy available for immune system. Improved energy status due to n-6 FA feeding can improve
reproductive efficiency directly and indirectly. Results from this study provide evidence that dietary n-6 FA decrease body fat mobilization and increase peripheral glucose concentration in periparturient Holstein cows. The regulating adipose TAG turnover is determined by the inversely regulated actions of lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL), both of which are regulated hormonally (Kern et al., 1987; Slavin et al., 1994). Hepatic gluconeogenesis is controlled not only by substrate availability, but also by the activity and or expression of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose bisphosphatase (FBPase) and glucose 6-phosphatase (G6Pase) (Lindsay, 1978). There is scarcity of data documenting the relationship between dietary fats and adipose TAG turnover and liver gluconeogenesis in cattle. We propose that dietary n-6 FAs will alter pancreatic endocrine secretions, which ultimately induce or activate rate-limiting enzymes related to lipid and glucose metabolism. Alternatively, supplemental n-6 FAs may alter tissue responsiveness to homeostatic and homeorhetic signals controlling lipolysis and glucose synthesis in dairy cattle (Figure 5-23).
Table 5-1. Fatty acid profile according to the manufacturers of a highly saturated fat (RBF; Cargill, Minneapolis, MN), a Ca salt lipid enriched in \textit{trans} C18:1 (tFA; Virtus Nutrition LLC, Fairlawn, OH), and a Ca salt lipid enriched in C18:2 (n-6 FA; Virtus Nutrition LLC, Fairlawn, OH) fed during the prepartum and postpartum periods to Holstein cows.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>RBF</th>
<th>tFA</th>
<th>n-6 FA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/100 g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>...</td>
<td>0.04</td>
<td>...</td>
</tr>
<tr>
<td>C14:0</td>
<td>5.60</td>
<td>0.31</td>
<td>...</td>
</tr>
<tr>
<td>C16:0</td>
<td>37.80</td>
<td>12.21</td>
<td>8.00</td>
</tr>
<tr>
<td>C16:1</td>
<td>...</td>
<td>0.15</td>
<td>...</td>
</tr>
<tr>
<td>C18:0</td>
<td>48.00</td>
<td>6.70</td>
<td>2.00</td>
</tr>
<tr>
<td>C18:1</td>
<td>...</td>
<td>...</td>
<td>13.00</td>
</tr>
<tr>
<td>C18:1, trans 6-8</td>
<td>...</td>
<td>20.62</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, trans-9</td>
<td>...</td>
<td>10.47</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, trans-10</td>
<td>...</td>
<td>10.62</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, trans-11</td>
<td>...</td>
<td>7.05</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, trans-12</td>
<td>...</td>
<td>8.73</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, cis-9</td>
<td>4.80</td>
<td>10.04</td>
<td>...</td>
</tr>
<tr>
<td>C18:1, cis-9, cis-12</td>
<td>...</td>
<td>1.97</td>
<td>...</td>
</tr>
<tr>
<td>C18:2</td>
<td>...</td>
<td>...</td>
<td>55.0</td>
</tr>
<tr>
<td>C18:3</td>
<td>...</td>
<td>...</td>
<td>1.00</td>
</tr>
<tr>
<td>Unknown</td>
<td>3.80</td>
<td>11.09</td>
<td>21.00</td>
</tr>
</tbody>
</table>
Table 5-2. Ingredient composition of prepartum and postpartum diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Prepartum</th>
<th>Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of DM</td>
<td>% of DM</td>
</tr>
<tr>
<td>Corn silage</td>
<td>30.0</td>
<td>38.0</td>
</tr>
<tr>
<td>Bermudagrass silage</td>
<td>35.0</td>
<td>...</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>...</td>
<td>12.0</td>
</tr>
<tr>
<td>Ground corn</td>
<td>11.1</td>
<td>19.8</td>
</tr>
<tr>
<td>Citrus pulp</td>
<td>9.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Fat supplement(^1)</td>
<td>1.5 - 1.8</td>
<td>1.5 - 1.8</td>
</tr>
<tr>
<td>Corn gluten feed</td>
<td>...</td>
<td>5.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>6.7</td>
<td>7.5</td>
</tr>
<tr>
<td>SoyPlus(^2)</td>
<td>...</td>
<td>7.0</td>
</tr>
<tr>
<td>Mineral and vitamin mix(^3)</td>
<td>6.1</td>
<td>...</td>
</tr>
<tr>
<td>Mineral and vitamin mix(^4)</td>
<td>...</td>
<td>3.5</td>
</tr>
<tr>
<td>Trace mineralized salt(^5)</td>
<td>0.1</td>
<td>...</td>
</tr>
</tbody>
</table>

\(^1\) Fat sources were 1.5%, saturated fat (RBF; Cargill, Minneapolis, MN), 1.8% Ca salt lipid enriched in \(trans\) C18:1 (EnerG-TR; Virtus Nutrition™, Fairlawn, OH), and 1.8% Ca salt lipid enriched in linoleic acid (Prequel-21; Virtus Nutrition™, Fairlawn, OH).

\(^2\) West Central Soy, Ralston, IA.

\(^3\) Prepartum mineral and vitamin mix contained 22.8% CP, 22.9% Ca, 0.20% P, 0.2% K, 2.8% Mg, 0.7% Na, 2.4% S, 8% Cl, 147 mg/kg of Mn, 27 mg/kg of Fe, 112 mg/kg of Cu, 95 mg/kg of Zn, 7 mg/kg of Se, 8 mg/kg of I, 11 mg/kg of Co, 268,130 IU of vitamin A/kg, 40,000 IU of vitamin D/kg, and 1129 IU of vitamin E/kg (DM basis).

\(^4\) Postpartum mineral and vitamin mix contained 26.0% CP, 1.4% NDF, 0.3% ADF, 6.4% Ca, 1.2% P, 0.07% K, 4.0% Mg, 12.5% Na, 0.8% S, 3.1% Cl, 854 ppm Mn, 1660 ppm Fe, 251 ppm Cu, 945 ppm Zn, 9.6 added ppm Se, 18.7 ppm I, 24.7 ppm Co, 114,419 IU vitamin A/kg, 1,144,200 IU of vitamin D/kg, 41,191IU of vitamin D/kg and 462 g/tonne Rumensin (Elanco, Greenfield, IN) (DM basis).

\(^5\) Trace mineralized salt contained minimum concentrations of 40% Na, 55% Cl, 0.25% Mn, 0.2% Fe, 0.033% Cu, 0.007% I, 0.005% Zn, and 0.0025% Co (DM basis).
Table 5-3. Chemical composition of prepartum and postpartum diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Prepartum</th>
<th></th>
<th></th>
<th>Postpartum</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>45.0</td>
<td>45.0</td>
<td>45.0</td>
<td>56.0</td>
<td>56.0</td>
<td>56.0</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>14.2</td>
<td>14.4</td>
<td>14.5</td>
<td>17.8</td>
<td>17.4</td>
<td>17.6</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>26.0</td>
<td>25.1</td>
<td>25.4</td>
<td>17.4</td>
<td>16.7</td>
<td>16.6</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>43.0</td>
<td>41.8</td>
<td>42.7</td>
<td>27.9</td>
<td>27.9</td>
<td>28.2</td>
</tr>
<tr>
<td>Lignin, % DM</td>
<td>4.0</td>
<td>3.9</td>
<td>4.0</td>
<td>3.1</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>NFC, % DM</td>
<td>30.6</td>
<td>31.9</td>
<td>31.0</td>
<td>44.0</td>
<td>45.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Lipid, % DM</td>
<td>3.7</td>
<td>4.5</td>
<td>4.5</td>
<td>4.7</td>
<td>5.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>11.5</td>
<td>11.9</td>
<td>12.0</td>
<td>6.9</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>NE\textsubscript{L}, Mcal/kg</td>
<td>1.36</td>
<td>1.30</td>
<td>1.32</td>
<td>1.75</td>
<td>1.68</td>
<td>1.70</td>
</tr>
<tr>
<td>Ca, % DM</td>
<td>1.90</td>
<td>2.16</td>
<td>2.12</td>
<td>0.85</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>P, % DM</td>
<td>0.30</td>
<td>0.32</td>
<td>0.30</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Mg, % DM</td>
<td>0.40</td>
<td>0.42</td>
<td>0.40</td>
<td>0.37</td>
<td>0.30</td>
<td>0.38</td>
</tr>
<tr>
<td>K, % DM</td>
<td>1.83</td>
<td>1.82</td>
<td>1.82</td>
<td>1.35</td>
<td>1.28</td>
<td>1.32</td>
</tr>
<tr>
<td>Na, % DM</td>
<td>0.20</td>
<td>0.20</td>
<td>0.18</td>
<td>0.35</td>
<td>0.38</td>
<td>0.40</td>
</tr>
<tr>
<td>S, % DM</td>
<td>0.33</td>
<td>0.32</td>
<td>0.38</td>
<td>0.20</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>Cl, % DM</td>
<td>1.08</td>
<td>1.10</td>
<td>1.10</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Fe, mg/kg</td>
<td>239</td>
<td>243</td>
<td>270</td>
<td>180</td>
<td>168</td>
<td>163.0</td>
</tr>
<tr>
<td>Zn, mg/kg</td>
<td>41</td>
<td>45</td>
<td>43</td>
<td>76</td>
<td>77</td>
<td>61</td>
</tr>
<tr>
<td>Cu, mg/kg</td>
<td>23</td>
<td>21</td>
<td>23</td>
<td>28</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>Mn, mg/kg</td>
<td>38</td>
<td>37</td>
<td>40</td>
<td>78</td>
<td>80</td>
<td>76</td>
</tr>
<tr>
<td>Mo, mg/kg</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.6</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Table 5-4. Performance of lactating Holstein cows fed a diet containing a highly saturated fat (RBF, n = 10), a Ca salt lipid enriched in trans C18:1 (tFA, n = 10), or a Ca salt lipid enriched in linoleic acid (n-6 FA, n = 9).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatments</th>
<th>P-value</th>
<th>Orthogonal Contrasts²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diet</td>
</tr>
<tr>
<td>DMI (% of BW)</td>
<td>2.39</td>
<td>2.06</td>
<td>2.26</td>
</tr>
<tr>
<td>Milk, kg/d</td>
<td>36.85</td>
<td>34.65</td>
<td>34.10</td>
</tr>
<tr>
<td>3.5% FCM¹, kg</td>
<td>36.78</td>
<td>35.01</td>
<td>29.80</td>
</tr>
<tr>
<td>Milk fat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>3.62</td>
<td>3.78</td>
<td>2.81</td>
</tr>
<tr>
<td>kg/d</td>
<td>1.28</td>
<td>1.21</td>
<td>0.92</td>
</tr>
<tr>
<td>Milk true protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>2.89</td>
<td>2.75</td>
<td>2.92</td>
</tr>
<tr>
<td>kg/d</td>
<td>1.03</td>
<td>0.93</td>
<td>0.96</td>
</tr>
<tr>
<td>SCC (x 1000)</td>
<td>123</td>
<td>206</td>
<td>131</td>
</tr>
</tbody>
</table>

¹ 3.5% fat-corrected milk (FCM) = (0.4324) (kg of milk) + (kg of milk fat) (16.216).
² Orthogonal contrast of means were the following: 1 = (RBF) + (tFA) vs (n-6 FA), 2 = (RBF) vs (tFA).
Table 5-5. Incidence of fever and uterine infections in Holstein cows fed a diet containing a highly saturated fat (RBF, n = 10), a Ca salt lipid enriched in \textit{trans} C18:1 (tFA, n = 10), or a Ca salt lipid enriched in linoleic acid (n-6 FA, n = 9).

<table>
<thead>
<tr>
<th>Variable</th>
<th>RBF</th>
<th>tFA</th>
<th>n-6 FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever(^1), %</td>
<td>20</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Puerperal metritis(^2), %</td>
<td>20</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Clinical metritis(^3), %</td>
<td>90</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td>Clinical endometritis(^4), %</td>
<td>20</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Subclinical endometritis(^5), %</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Animals unable to clear metritis before 21 DIM, %</td>
<td>22.2</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Animals with temperature > 39.5°C.
2 Puerperal metritis is defined as a condition that results in an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, in conjunction with a fever >39.5°C, within 21 days postpartum.
3 Clinical metritis is defined as animals that exhibit a purulent uterine discharge, within 21 days after parturition.
4 Clinical endometritis is defined as a uterine disease where animals present a purulent (>50% pus) uterine discharge 21 days or more after calving, or a mucuopurulent (approximately 50% pus, 50% mucus) uterine discharge detectable in the vagina 26 days after parturition.
5 Subclinical endometritis is defined as animals with the presence of >10% neutrophils in uterine cytology samples collected at 34-47 days postpartum (Sheldon et al., 2006).
Figure 5-1. Dry matter intake (DMI) as a percentage of body weight (BW) of Holstein cows fed a saturated fat (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), $P = 0.77$. Contrast 2: (RBF) vs. (tFA), $P = 0.02$. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week ($P < 0.05$).
Figure 5-2. Average body weight (BW) of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. There was an effect of week ($P < 0.0001$).
Figure 5-3. Average body condition score (BCS) of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. There was an effect of week \( (P < 0.0001) \).
Figure 5-4. Calculated energy balance by week relative to parturition for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week. There was an effect of week (P < 0.0001).
Figure 5-5. Temporal patterns of milk production by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. There was an effect of week (P < 0.0001).
Figure 5-6. Temporal patterns of 3.5% fat-corrected milk (FCM) yield by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), \( P = 0.02 \). Contrast 2: (RBF) vs. (tFA), \( P = 0.51 \). Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week (\( P < 0.05 \)).
Figure 5-7. Fat percentage of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), $P = 0.0004$. Contrast 2: (RBF) vs. (tFA), $P = 0.50$. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week ($P < 0.05$).
Figure 5-8. Fat yield of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), $P = 0.0018$. Contrast 2: (RBF) vs. (tFA), $P = 0.51$. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week ($P < 0.05$).
Figure 5-9. Protein percentage of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. There was an effect of week ($P < 0.0001$).
Figure 5-10. Protein yield of milk produced by Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. There was an effect of week ($P < 0.0001$).
Figure 5-11. Average feed efficiency as a function of milk yield over intake of periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), $P = 0.005$. Contrast 2: (RBF) vs. (tFA), $P = 0.46$. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week ($P < 0.05$).
Figure 5-12. Plasma NEFA concentrations by week relative to calving in periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), P = 0.05. Contrast 2: (RBF) vs. (tFA), P = 0.67. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week (P < 0.05).
Figure 5-13. Plasma glucose concentrations by week relative to calving in periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), P = 0.02. Contrast 2: (RBF) vs. (tFA), P = 0.08. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated week (P < 0.05).
Figure 5-14. Average rectal temperature on d 4, 7 and 12 postpartum of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. Asterisks indicate statistical differences among means at the indicated day ($P < 0.05$). There was a treatment by day interaction ($P = 0.05$).
Figure 5-15. Concentration of plasma interleukin 6 (IL-6), interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) on day 12 ± 2 postpartum from periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. Data represents least square means ± SEM.
Figure 5.16. Concentration of plasma haptoglobin from periparturient Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. Data represents least square means ± SEM.
Figure 5-17. Average Metricheck score on d 4, 7, 12 and 30 of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM. Means were separated using the PDIFF procedure. Cows fed the n-6 FA-enriched diet had lower Metricheck scores on d 7 postpartum compared to cows fed the control (RBF) diet ($P = 0.0005$) or the tFA diet ($P = 0.01$).
Figure 5-18. Average neutrophil % in uterine flushings on d 40 ± 3 postpartum of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 9)-supplemented diet. Data represents least square means ± SEM.
Figure 5-19. Average accumulated progesterone concentration in plasma of Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet. Cows fed the n-6 FA-enriched diet had increased accumulated progesterone by d 50 postpartum than cows fed the control (RBF) or tFA-enriched diets ($P = 0.02$).
Figure 5-20. Ovulation distribution for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 8)-supplemented diet.
Figure 5-21. Conception rate to first service for Holstein cows fed a control (RBF, n = 10), trans-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 7)-supplemented diet.
Figure 5-22. Average days open for Holstein cows fed a control (RBF, n = 10), *trans*-C18:1 (tFA, n = 10), or linoleic acid (n-6 FA, n = 7)-supplemented diet. Treatment effects were separated using orthogonal contrasts. Contrast 1: (RBF + tFA) vs. (n-6 FA), \( P = 0.21 \). Contrast 2: (RBF) vs. (tFA), \( P = 0.56 \). Data represents least square means ± SEM.
Figure 5-23. Proposed model for n-6 FA actions on lipid and glucose metabolism. Picture sources: Pancreas: http://www.dramaout.com/; Liver: http://www.zdsolutions.it; Adipose tissue: http://joe.endocrinology-journals.org; Blood vessel: www.turbosquid.com/
CHAPTER 6
GENERAL DISCUSSION

In dairy cows, a reduction of feed intake occurring during the final weeks of pregnancy when nutrient demands for fetal growth and initiation of milk synthesis are increased result in higher energy requirements that can be met by dietary energy intake (Bell, 1995). The initiation of lactation causes a shift in nutritional requirements in order to support milk production (Butler, 2000), resulting in a rapid increase in energy requirements and changes in the metabolic and endocrine status of the animal (Grummer, 1995). Most cows experience a state of NEB during this transition period that can directly suppress the immune system through a lack of expendable energy, and impair reproductive function (Kehrli et al., 2006; Butler, 2000). In fact, during the periparturient period, dairy cows experience a depression in immune functions such as lymphocyte response to mitogens and production of antibodies (Kherli et al., 1989). A severe NEB during the periparturient period can also lead to metabolic disorders, such as ketosis and fatty liver, which may have detrimental effects on the cells of the immune system, further predisposing dairy cows to immune diseases during the transition to lactation. Uterine infections, in particular, may negatively affect fertility through several mechanisms, all caused by the absorption of bacterial endotoxins into the general circulation through the uterine lumen. Long-chain FA are generally added to dairy rations to increase the energy density of the diet. Supplementation of the diet with FAs reduces the incidence of metabolic disorders in dairy cattle (Grummer and Carroll, 1991) through positive effects on metabolic efficiency (Kronfeld et al., 1980), and enhances reproductive efficiency through energy dependent and independent mechanisms (Lucy et al., 1993; Sartori et al, 2002; Oldick et al., 1997; Sangritavong et al., 2002). Fatty acids have been shown to modulate responses in several species, including humans, pigs, mice, and cows. It is important to determine the effects of feeding lipid supplements on
modulation of immune responses in cattle, since improved immune health can subsequently improve uterine health and, therefore, reproductive efficiency.

We first set out to examine the effects of MUFAs and PUFAs on mitogen-stimulated cytokine responses in cultured bovine PBMCs. Different mitogens were tested in order to select one that would best stimulate bovine PBMC cytokine production. Concanavalin A, but not LPS, stimulated cytokine production in all cultures. Phytohaemagglutinin A stimulated TNF-α production to a lesser extent than did ConA, and there were no detectable differences between IL-4 and IFN-γ responses to ConA and PHA. Results are consistent with previous reports (Skopets et al., 1992; Olsen and Stevens, 1993; Lessard et al., 2003; Heaney et al., 2005), and indicate that the bovine PBMCs used in this study likely were highly enriched in T lymphocytes, which are known to be activated by ConA and PHA (Soder and Holden, 1999).

Addition of trans-vaccenic acid to the culture media greatly enhanced IFN-γ response to ConA in cultured bovine PBMC. The concentration of TNF-α, another pro-inflammatory cytokine, tended to be higher in cultures co-treated with trans-oleic or trans-vaccenic acids than those treated with ConA alone. Results are in agreement with previous reports (Gallai et al., 1993; Mozaffarian et al., 2004b), and lend further support to the recognition that dietary tFAs induce systemic inflammation in mammalian species (Mozaffarian et al., 2004a; Mozaffarian et al., 2004b; López-García et al., 2005).

Results from experiments on the effects of MUFAs on ConA-induced cytokine production by bovine PBMCs indicate that tFAs stimulate production of pro-inflammatory cytokines in cattle, which may induce systemic inflammation in a manner similar to what has been shown in other species. The physiological significance of tFA-stimulated IFN-γ production by cultured PBMC is yet to be elucidated.
Supplemental CLA had no detectable effects on ConA-induced TNF-α, IFN-γ, and IL-4 concentrations in PBMC-conditioned media. This is in agreement with previous human studies (Kelley et al., 2001; Nugent et al., 2005; Ritzenthaler et al., 2005) which provided no evidence for enhancing effects of CLA on immunity in healthy subjects. On the other hand, other studies have reported effects of CLA on production and gene expression of pro-inflammatory cytokines in vivo and in vitro (Yu et al., 2002; Changhua et al., 2005). Unlike the pig and mouse models, short term supplementation of CLA failed to alter TNF-α, IFN-γ and IL-4 responses to ConA in bovine PBMCs, indicating that immunomodulatory effects of CLA may vary from one animal species to another. Whether the current findings reflect the physiological effects of CLA in vivo warrants further investigation. Whereas exact reasons for differential effects of CLA on immunity in various animal species are unknown, it has been hypothesized that dose, duration and isomeric composition of CLA may each impact the ability of exogenous CLA to modulate immunity in mammalian models. Additionally, the lack of CLA effect on cytokine secretion by PBMCs in vitro observed in this study may be due to differences in immune cell populations in isolated PBMCs.

Results from studies with n-3 PUFAs provide convincing evidence that EPA and LNA, but not DHA, are potent suppressors of TNF-α, IL-4, and IFN-γ production in isolated bovine lymphocytes. Linolenic acid, the precursor of long-chain n-3 PUFAs, decreased ConA-induced cytokine production to a lesser extent than did EPA. These findings are consistent with the rodent reports, which indicated that fish oil feeding decreased ex vivo production of TNF-α, IL-1β and IL-6 by inflammatory macrophages and monocytes (references in Calder et al., 2002). On the other hand, other studies have failed to demonstrate an effect of fish oil on cytokine
production (Yaqoob et al., 2000; Kew et al., 2003). Exact reasons for discrepancies between studies are unclear, but it has been suggested that some of these differences may be related to different experimental conditions and to different subject characteristics, including the gender, age and basal diet. Our findings provide strong evidence that LNA and EPA attenuate ConA-stimulated cytokine production by bovine lymphocytes and could alter the immunocompetence of the periparturient dairy cow.

Chapter 6 presents the results for the in vivo trial performed to investigate the effect of dietary trans and n-6 FA on production, metabolic and uterine health responses of periparturient Holstein cows. Cows fed the control (RBF) diet had higher DMI as a % of BW than cows fed the tFA diet. This observation is not consistent with previous data from our laboratory (Rodríguez-Sallaberry et al., 2007), which reported that there was no effect of feeding a diet supplemented with tFAs on DMI of primiparous or multiparous Holstein cows. The difference in these results could be due to the length of postpartum feeding of the diets, since in the present study postpartum diets were fed for 4 wk longer than in the Rodríguez-Sallaberry et al. (2007) study. In support of these results, do Amaral (2008) reported that DMI as a % of BW appeared to increase for a longer period of time for animals fed a diet high in n-6 FA compared to cows fed CS of tFAs.

Cows fed the n-6 FA supplements had less fat in milk than cows fed the RBF or tFA supplements, but there was no effect of dietary treatment on milk yield. Therefore, yield of 3.5% FCM differed among treatments, with n-6 FA-fed cows producing less 3.5% FCM than cows fed the RBF or tFA diets. Selberg et al. (2004) reported a reduction in mean milk fat concentration during wk 5, 6 and 7 of lactation in animals fed CS of CLAs. The reduction in milk fat in n-6 FA-fed animals may be due to an increase in the t10,c12 isomer of CLA in mammary tissues,
since this FA has been shown to be a potent suppressor of milk fat synthesis and secretion (Baumgard et al., 2000). Animals fed the n-6 FA-rich diet had higher EB and returned to positive EB faster than animals fed the RBF or tFA-rich diets. The higher EB in n-6 FA cows in wks 6 and 7 postpartum are probably associated with the reduction in milk fat synthesis, which translates into less energy in the form of fat being allocated towards milk production and therefore being available for other physiological processes. Additionally, n-6 FA-supplemented cows exhibited lower milk production as a function of DMI (calculated feed efficiency), which could also account for their higher EB on wks 6 and 7 postpartum. In the present study, plasma NEFA concentrations were lower for n-6 FA fed cows, which reflects less lipid mobilization occurring in these animals, and plasma glucose concentrations were higher. This is consistent with the higher EB observed in these animals, since n-6 FA fed cows achieved a positive EB by wk 6 postpartum while RBF and tFA animals remained in a negative EB. As animals enter a positive EB state through increasing DMI, fat mobilization diminishes as blood glucose concentrations increase, resulting in a reduction in circulating NEFAs. In addition to an improvement in metabolic responses, n-6 FA-feeding also improved uterine health and reproductive responses of dairy cows. Cows allocated to the n-6 FA treatment had an increase in rectal temperature from 4 to 7 d postpartum compared to animals in the other two dietary treatment groups. This could represent an enhanced immune response, which could have resulted in the lower cervical discharge (Metricheck) scores on d 7 postpartum observed in these animals. Lower Metricheck scores in the n-6 FA treatment group indicate that these animals exhibited better uterine health at d 7 postpartum. This improvement in uterine health was also associated with beneficial effects on reproductive responses, as reflected by the higher accumulated P4 concentration by d 50 postpartum that was observed in the n-6 FA treatment group. This
association is supported by the fact that uterine infections can reduce reproductive performance through a disruption of the hypothalamic-pituitary-gonadal axis by bacterial endotoxins that can cross the uterine wall and enter the bloodstream (Callahan et al., 1971; Oltenacu et al., 1983; Holt et al., 1989). Furthermore, studies have reported that animals with uterine infection had higher levels of PG metabolites (Peter et al., 1987), which contribute to a prolonged anovulation period since ovarian activity cannot resume until PG concentrations have returned to basal levels (Kindahl et al., 1982). Additionally, cows fed the n-6 FA-enriched diet tended to spend less days open and had numerically higher higher conception rates to first service than those receiving the RBF or tFA supplements.

Taken together, these results indicate that animals fed the n-6 FA diet had improved metabolic status and greater glucose concentration than RBF and tFA-fed cows. A faster return to a positive EB could explain the improvement in uterine health seen in n-6 FA-fed cows. It has been proposed that the immunosuppression seen during the periparturient period can be due, in part, to the NEB which results in a lack of energy for the immune system to function properly. A return to positive EB would mean that these animals have more energy that can be expended on processes of the immune system, including the clearance of uterine infections. This improvement in uterine health was associated with an increase in plasma P4 concentration, which tended to translate into less days open for these cows, which was not statistically significant due to the small number of animals in this trial. In addition, NEB is associated with extended interval to first ovulation (Beam and Butler, 1999) through inhibition of LH pulse frequency and low concentrations of blood glucose, insulin, and insulin-like growth factor (Butler, 2000). An improvement in EB in postpartum dairy cows could directly improve reproductive efficiency through a reduction in the interval to first ovulation. In conclusion,
feeding n-6 FAs to early postpartum Holstein cows improved their metabolic status, which might have led to improved uterine health and reproductive performance.

Studies with a larger number of animals are warranted to fully document the effect of periparturient fat supplementation on uterine health and reproductive efficiency in dairy cows.
LIST OF REFERENCES


Do Amaral BC. Effect of supplemental fat source on production, immunity, hepatic gene expression, and metabolism of periparturient dairy cows. Dissertation presented to the Graduate School of the University of Florida, 2008.


Gee JM, Watson M, Matthew JA, Rhodes M, Speakman CJM, Stebbings WSL, Johnson IT. Consumption of fish oil leads to prompt incorporation of eicosapentaenoic acid into colonic mucosa of patients prior to surgery for colorectal cancer, but has no detectable effect on epithelial cytokinetics. J Nutr 1999;129:1862-5.


191


Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KV, Bauman DE. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. J Nutr 2000;130:2285-91.


NRC. Nutrient requirements of dairy cattle. 7th rev. ed. Natl Acad Sci 2001; Washington, DC.


Silvestre FT. Nutraceutical and hormonal regulation of immunity, uterine health, fertility, and milk production of postpartum dairy cows. Dissertation presented to the Graduate School of the University of Florida, 2008.


BIOGRAPHICAL SKETCH

Cristina Francesca Lucia Caldari-Torres was born in San Juan, Puerto Rico, in 1980. She is daughter of Yvette Torres-Rivera and Dr. Pier Luigi Caldari-Collini. She graduated from University High School in 1997, and started her Bachelor of Science that same year at University of Puerto Rico, Río Piedras Campus. She graduated magna cum laude in 2002. After graduation, she worked as a veterinary technician at a small animal clinic and as a working student at a horse farm. In fall 2003, she moved to Gainesville, Florida where she started her Master of Science at the University of Florida, Department of Animal Sciences, under the guidance of Dr. Lokenga Badinga. She graduated with a M.S. in animal sciences in 2005, and that same year was awarded the prestigious Alumni Fellowship to pursue her doctoral degree with Dr. Lokenga Badinga. Her research at the University of Florida has focused on the effects of fatty acids on reproduction, production, metabolism and health of dairy cows. Cristina is an avid horse rider and has worked as barn manager, horse trainer and horse show rider at several hunter/jumper farms throughout her graduate education.