The Effects of *Lactobacillus rhamnosus* GR-1 on Cytokines/Chemokines and Prostaglandins in Human Amnion Cells

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

Department of Physiology
University of Toronto

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Department of Physiology
University of Toronto

2011

**Abstract**

The incidence of preterm labor has risen over recent decades and preventative treatments are ineffective. Associated with a 40% increased risk of preterm birth, bacterial vaginosis is characterized by a decrease in lactobacilli and an increase in pathogenic bacteria in the vaginal microbiota. Ascent of bacterial products to the intrauterine environment stimulates cytokine and prostaglandin secretion from invading immune cells and gestational tissues. Probiotic lactobacilli modulate the immune responses in mouse macrophages and human placental trophoblast cells. The focus of this thesis was to determine the influence of *Lactobacillus rhamnosus* GR-1 (GR-1) on cytokines and prostaglandins which are part of the activated pathway in infection and/or inflammation-mediated preterm labour. Pro-inflammatory cytokines were decreased by GR-1. The release of several chemokines and prostaglandin E$_2$ were elevated by GR-1. It is possible that GR-1 may enhance the host defense barriers of the amnion to pathogenic bacteria.
“When is it that nature does anything in vain?”
Paraphrased from Sir Isaac Newton
Dedicated to those who guided me with their years of wisdom, intellectual creativity and passion for academia; to those who insisted on lunch breaks and offered constant encouragement and reassurance during my midnight trips to the laboratory; to wiped tears, shared happiness and laughter; and to my family whose love and support has made me capable of overcoming challenges and of celebrating successes.
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<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>Contraction Associated Protein</td>
</tr>
<tr>
<td>CCB</td>
<td>Calcium Channel Blocker</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-Releasing Hormone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco Modified Eagle Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immuno-Assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid Dehydrogenase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic Acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-Associated Molecular Pattern</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium Sulphate</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin Light Chain Kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa, and Sharpe media</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline + Tween</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGDH</td>
<td>15-Hydroxyprostaglandin Dehydrogenase</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E Synthase</td>
</tr>
<tr>
<td>PGHS</td>
<td>Prostaglandin endoperoxidase H Synthase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PROM</td>
<td>Premature Rupture Of the Membranes</td>
</tr>
<tr>
<td>ra</td>
<td>receptor antagonist</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Cell Expressed and Secreted</td>
</tr>
<tr>
<td>RIPA</td>
<td>RadioImmunoPrecipitation Assay</td>
</tr>
<tr>
<td>ROD</td>
<td>Relative Optical Density</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
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Chapter 1

General Introduction
Chapter 1
General Introduction

Preterm birth accounts for up to 8% and 13% of all births in Canada and the United States of America respectively (Goldenberg et al., 2008; Public Health Agency of Canada, 2008). Thirty percent of all preterm births are associated with infection and/or inflammation. Despite ongoing studies of the mechanisms underlying infection and/or inflammation-mediated preterm birth, no effective preventative treatment exists. Preterm birth results in neonatal morbidities that last into adulthood, cost an estimated $26 billion in medical care annually in the United States of America and place an emotional and psychological burden on families that is immeasurable by any monetary means. Probiotics are increasingly being investigated as preventative therapy due to their ability to modulate immune responses across many tissues and systems. However, the effects of probiotics during pregnancy are largely uninvestigated. Therefore, the focus of this thesis was to determine the possibility of *Lactobacillus rhamnosus* GR-1 as a potential preventative treatment by measuring its effects on cytokines, chemokines and prostaglandins that are known to be elevated in association with preterm delivery in human amnion cells.

1.1 Human Parturition

Human parturition occurs as the result of a series of feed-forward paracrine, autocrine and endocrine events resulting in myometrial contractions, remodeling of the cervix and weakening and rupture of the fetal membranes, ultimately leading to expulsion of the fetus from the uterus (Challie et al., 2002). During pregnancy, the uterus and cervix work together to promote a quiescent state in order to maintain gestation. Throughout gestation, the myometrium transitions through several phases: quiescence, activation, stimulation and expulsion (Challis et al, 2002). During these phases, multiple factors are recruited and interact to promote synchronous uterine activity. These factors include uterine stretch due to fetal growth and biochemical factors associated with an increase in the activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis that occurs in late gestation.

A quiescent state is maintained for approximately 95% of gestation by high levels of circulating progesterone, locally produced prostacyclin, relaxin, nitric oxide and parathyroid hormone-related protein that maintain myometrial inactivity (Challis et al., 2002). Many of these agents
inhibit muscle contractions by activating downstream pathways that increase cytosolic cAMP and/or cGMP. Consequently, increases in cAMP and/or cGMP cause intracellular calcium to be sequestered and the inactivation of myosin light chain kinase (MLCK). Without the activity of MLCK, myosin is not phosphorylated and the actin-myosin cross-bridges in the myometrial smooth muscle remain in the relaxed state (Challis et al., 2000). Progesterone inhibits the actions of estrogen by preventing expression of the estrogen receptor, (ER)-α to also maintain quiescence (Mesiano et al., 2002).

As gestation progresses the uterine myometrium transitions to an active state. Throughout this transition, uterine stretch caused by the growing fetus leads to up-regulation of contraction-associated protein (CAP) genes (Challis et al, 2002). As a result, the myometrium becomes capable of responding to uterotonins, or stimulants of myometrial contractility.

Uterotonins are molecules that alter the myometrial phenotype to an active state making it capable of producing synchronous contractions. Oxytocin, prostaglandins and corticotropin-releasing hormone (CRH) promote myometrial contractility and their concentrations rise throughout gestation. As gestation progresses, the HPA axis of the fetus becomes increasingly active resulting in increased adrenal cortisol production. Cortisol promotes the activity of estrogen and prostaglandins while decreasing the sensitivity of the myometrium to progesterone. The final phase of expulsion occurs as a result of powerful myometrial co-ordinated contractions that are capable of expelling the fetus and placenta from the uterus.

As the myometrium progresses through different phenotypic states, other gestation tissues, namely the cervix and the fetal membranes, undergo alterations in preparation for labour. During pregnancy the cervix remains closed while the fetal membranes maintain the volume of the amniotic fluid and provide mechanical strength necessary in pregnancy. As gestation progresses, prostaglandins increase metalloproteinase (MMP) expression in the amnion. MMPs degrade collagen molecules that constitute a large proportion of the extracellular matrix of both the cervix and the fetal membranes. This results in remodeling of the cervix and weakening of the fetal membranes (Menon et al., 2004; Yoshida et al., 2002).

Therefore, parturition results from the interaction of two interdependent pathways. The first is mechanical stretch of the uterus and the other is biochemical factors and endocrine activities
associated with increased activation of the HPA axis of the fetus. In cases of membrane rupture or infection, the amnion contributes a large pool of prostaglandins that may result in premature myometrial activation, cervical ripening and membrane weakening.

1.1.1  Anatomy

1.1.1.1  Uterine Myometrium

The uterine myometrium is the strong, densely packed smooth muscle layer of the uterus that gives much of the bulk to this organ. In the non-pregnant uterus, this layer contains blood and lymphatic vessels as well as cholinergic, sympathetic and peptidergic nerve innervations. The myocytes of the uterus, like other smooth muscle cells, are highly plastic and are capable of transitioning through several phenotypes as gestation progresses. These include the proliferative, synthetic and contractile stages leading to preparation for expulsion of the fetus during parturition (Shynlova et al., 2009). Throughout pregnancy the control of the myometrial phenotype switches from a global autonomic control system with the loss of cholinergic, peptidergic and sympathetic nerves from the myometrium to one of humoral or local control by prostaglandins, oxytocin and steroid hormones (Riemer et al., 1998).

The first phase of uterine growth during pregnancy is due to myometrial hyperplasia and is under the control of estrogen and progesterone. Estrogen and progesterone simultaneously hyperpolarize myometrial cells making them less responsive to contractile agonists (Riemer et al., 1998; Shynlova et al., 2004; Shynlova et al., 2005). Myometrial quiescence is partially maintained by high levels of oxytocinase and 15-hydroxyprostaglandin dehydrogenase (PGDH) expression. These proteins metabolize oxytocin and prostaglandins, respectively (Mitchell et al., 1995). Studies in pregnant rats have characterized the phenotypic changes that occur across pregnancy (Shynlova et al., 2009). As uterine tension increases, stretch and progesterone act in concert to induce growth and remodeling resulting in hypertrophy and matrix changes conducive to the contractile phase of myometrial activity (Shynlova et al., 2009). The switch to the contractile phenotype is characterized by increased expression of basement membrane proteins such as fibronectin, laminin β2 and collagen IV within myometrial cells. Near the end of gestation, uterine growth ceases while fetal growth continues, resulting in biomechanical distension of the uterus. This distension is necessary, but not sufficient to cause up-regulation of CAP gene expression as demonstrated by the lack of up-regulation of these genes in the absence
of progesterone withdrawal (Shynlova et al., 2009). Therefore, the phenotype of the uterus is dependent upon both the tension of the myometrium and the endocrine environment that is altered throughout pregnancy and at the onset of parturition.

1.1.1.2 Fetal Membranes/Amnion

The fetal membranes consist of the chorion and the amnion. The fetal membranes derive from fetal origins and thus have the same chromosomal sex of the fetus. The amnion begins to form eight days after conception from the ectodermal cell nest in the dorsal aspects of the zygote (Calvin et al., 2007). The amniotic cavity rapidly increases in size as amniotic fluid begins to surround the developing fetus. By 10-12 weeks of gestation, the amnion comes into contact with the chorion forming the chorioamnion membrane. As the amniotic cavity further expands, the fetal membranes contact the decidua along the uterine walls essentially sealing the endometrial cavity by 16 weeks gestation (Calvin et al., 2007). Between the two fetal membranes, a layer of phospholipids running parallel to the two tissues provides a surfactant-like effect to prevent shearing of the membranes during movement. By the end of gestation, the fetal membranes encompass an area of 1000-1200 cm², resistant to the increased pressure placed upon them by increased volumes of amniotic fluid and by fetal movements that become more vigorous as gestation progresses (Bryant-Greenwood, 1998; Myatt et al., 2010).

The amnion has no blood supply or nerve innervation and as such relies on amniotic fluid and the chorion for nutrient supply and waste transport (Parry et al., 1998). It is a largely an acellular tissue in comparison to the chorion and provides the tensile strength and elasticity required to withhold the amniotic fluid (Bryant-Greenwood, 1998). The amnion consists of two major portions. The first is the placental amnion, defined as the portion of the amnion in direct contact with the chorion of the placental chorion plate and the second portion consists of the remaining amnion, termed the membranous amnion (Calvin et al., 2007).

The amnion consists of five layers starting from the visceral side or that in contact with the amniotic fluid, fetus, and umbilical cord to the outer layer, or that in contact with the chorion: 1) Epithelium, 2) Basement Membrane, 3) Compact Layer, 4) Fibroblast Layer, and 5) Spongy Layer or Zona spongiosa (Figure 1.1, page 8; Bourne, 1962; Calvin et al., 2007; Parry et al., 1998).
The epithelial layer of the amnion consists of cuboidal cells that are typically mononuclear with dense granular cytoplasm with many vacuoles and few small mitochondria (Bourne, 1962). The cells have a slight convex shape on the most apical surface in contact with the amniotic fluid and the edges form a brush border of microvilli (Bourne, 1962). Many vacuoles are found in amnion epithelial cells that are connected by fine channels close to adjacent cell membranes (Bourne, 1962). These tunnels and channels form an intricate network that allows amnion epithelial cells to communicate with the extracellular matrix (Bourne, 1962). Additionally, both murine and human amnion epithelial cells contain tight junctions (Kobayashi et al., 2009; Kobayashi et al., 2010a). Amnion epithelial cells produce MMP-1, -2, -9, and collagens I, III, and IV, as well as the non-collagenase proteins laminin, fibronectin and nidogen that compose the other amnion layers (Aplin et al., 1985; Bourne, 1962; Parry et al., 1998). Epithelial cells attach to a basement membrane composed of a network of collagen III, IV and V that act as a scaffold for the assembly of laminin, fibronectin, heparin sulphate proteoglycan and nidogen (Bryant-Greenwood, 1998; Parry et al., 1998). The epithelial cells attach to the basement membrane through digitations of blunt processes from both the reticular network of the second layer, and the first layer cells (Bourne, 1962).

The third layer of the amnion is the compact layer, a dense acellular network of reticular fibres. It is composed of fibronectin, collagen I, III, and IV with low levels of collagens V, VI, and VII (Bryant-Greenwood, 1998; Parry et al., 1998). Collagens I and II form large parallel bundles that sustain mechanical integrity and give the amnion much of its tensile strength. The compact layer is relatively permeable to chemotaxis of macrophages and rarely has antigen presenting cells present unless they are actively phagocytic (Bourne, 1962).

The thickest layer of the amnion, the fibroblast layer is composed of the second major cell type constituting the amnion, the mesenchymal cells. This layer is relatively weak compared to the compact layer and is composed of a loose network of non-collagenous glycoproteins, reticulin, collagens I, III, and IV, lamminin, nidogen, and fibronectin and scattered mesenchymal cells (Bourne, 1962; Parry et al., 1998). This layer contains Hofbauer cells, macrophages of placental origin where they exist as residential cells capable of phagocytosis (Bourne, 1962).

The outer most layer of the amnion, the spongy layer is in direct contact with the chorion. It is composed of loose bundles of reticulin composed of collagens I, III, and IV as well as hydrated
proteoglycans covered by mucin (Bourne, 1962; Parry et al., 1998). This layer is prone to edema as proteoglycans absorb water causing increases in the thickness of the amnion (Bourne, 1962). The presence of mucin and the hydrophilic characteristics of proteoglycans allow the amnion to move along the chorion while absorbing physical stress (Bryant-Greenwood, 1998). This layer also contains resident Hofbauer cells (Bourne, 1962).

The amnion is responsive to many physiologic factors including cytokines, growth factors, bacterial endotoxins, and glucocorticoids. It is equipped with receptors necessary to respond to these factors including cytokine receptors such as CXCR2, bacterial endotoxins, namely toll-like receptor (TLR) 2, TLR4, the family of prostaglandin receptors EP1, EP1, EP3, EP4, and F, as well as the glucocorticoid receptor (Kallapur et al., 2009; Sun et al., 1996; Unlugedik et al., 2010). Progesterone receptors are absent or expressed in very low levels in the amnion as mRNA for both progesterone receptor (PR)-A and PR-B are undetectable (Merlino et al., 2009). The amnion expresses natural antimicrobials and innate immune molecules that have antibacterial, anti-viral, and anti-fungal activity. These include human α-defensins 1, 2 and 3, human β-defensins 1, 2 and 3 as well as elafin and secretory leukocyte protease inhibitor (King et al., 2007). As important components of the innate immune system, these molecules are part of the immune defense against uterine infection during menstruation, pregnancy and labour (King et al., 2007). The amnion produces pro-inflammatory and anti-inflammatory cytokines as well as chemokines and steroids. However, a major product of the amnion is prostaglandin, in particular prostaglandin (PG) E₂ and to a lesser degree, PGF₂α.

The amnion is in an ideal anatomical position to respond to many different molecules allowing for signaling between the maternal and fetal compartments during pregnancy. Direct contact between the amnion and the amniotic fluid provides not only a pathway for molecules secreted from the fetus, but also foreign bacterial products during microbial invasion of the amniotic cavity to influence the release of cytokines, chemokines and prostaglandins from the epithelial layer of the amnion. In addition, the amnion relies on the chorion for nutrients and waste elimination. Consequently, ascending bacteria and bacterial products may influence the mesenchymal cells of the amnion in close proximity to the chorion. Therefore, an intimate relationship between the amnion, fetal environment and chorion exists which differentially dictate the response and role of the amnion throughout gestation and parturition.
Figure 1.1: The layers of the fetal membranes: the amnion and chorion

The inner layer of the fetal membranes, the amnion is an avascular tissue composed of five layers: 1) Epithelium, 2) Basement membrane, 3) Compact Layer, 4) Fibroblast Layer, 5) Spongy Layer of Zona Spongiosa.

[The image has been modified from American Journal of Obstetrics and Gynecology, Vol 79. Bourne GL. Microscopic anatomy of human amnion and chorion, 1070-1073. Copyright Elsevier (1960) and appears here with the permission of the journal.]
1.1.2 Mechanisms of Human Parturition

1.1.2.1 Uterine Stretch

Stretch induces contractions in many muscle systems including the myocardium and the myometrium (Riemer et al., 1998). Ultimately, the ability of the uterine myometrium to contract is regulated by the availability of intracellular calcium. The myometrium consists of smooth muscle cells that contain actin-myosin bridges. As intracellular calcium levels increase, MLCK is activated resulting in increased adenosine triphosphatase activity of the myosin head which becomes phosphorylated and the muscle contracts. Agents that decrease intracellular calcium levels and/or increase cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) levels that sequester intracellular calcium, result in relaxation of the uterine myometrium. In contrast, agents that increase intracellular calcium result in uterine myometrial contraction.

As gestation progresses, the growth of the fetus causes biomechanical distension of the uterus leading to both hypertrophy and hyperplasia of the myometrial smooth muscle cells in the presence of progesterone (Challis et al., 2005). Throughout gestation, circulating progesterone maintains uterine quiescence through nuclear PR-B expressed on both the decidua and the nuclei of myometrial cells (Merlino et al., 2007). Progesterone maintains quiescence by restricting transcription of CAPs genes which translate into proteins involved in myometrial stimulation and include the oxytocin receptor, progesterone receptors, the gap junction protein Cx43, increasing expression of PGDH, an enzyme responsible for degradation of PGE\textsubscript{2} in the chorion and myometrium and inducing phosphokinase A signaling that promotes smooth muscle relaxation by inhibiting phospholipase C activity (Merlino et al., 2007). Unlike other mammalian species, circulating progesterone levels in the human do not decrease and estrogen levels do not increase abruptly near the end of gestation (Challis et al., 2001). However, a functional withdrawal of progesterone accompanied by functional estrogen activation occurs at term. This results in a conversion of the myometrial phenotype from quiescence to activation. Throughout gestation, PR-A expression increases. PR-A resides in the nucleus of myometrial cells and thus is ideally located to inhibit the expression of PR-B (Merlino et al., 2007). As a result, the effects of PR-B that maintain uterine quiescence until late gestation are withdrawn, including the inhibitory effects on estrogen receptor (ER)-\(\alpha\) expression (Merlino et al., 2007; Mesiano et al., 2002).
Circulating estrogen subsequently increases uterine contractility through the up-regulation of CAP genes as the inhibitory actions of progesterone are functionally withdrawn (Kamel, 2010; Mesiano et al., 2002). Estrogen, in conjunction with mechanical distension works in concert to activate the myometrium, thus allowing for high amplitude, high-frequency contractions to occur in the next phenotypic state of the myometrium.

Up-regulation of CAP genes results in increased expression of three major groups of proteins necessary for activation and subsequent contraction of the myometrium: 1) Ion channels, 2) Gap junction proteins, and 3) Contractile agonist receptors (Challis et al., 2000; Challis et al., 2002). Ion channels determine the resting membrane potential of myocytes and thus determine the contractile response to contractile agonists. Gap junction proteins, specifically C43, synchronize longitudinal co-ordinated contractions over the entire uterine myometrium during labor (Merlino et al., 2007; Riemer et al., 1998). Contractile agonist receptors, including the oxytocin receptor as well as the prostaglandin receptors F, EP1 and EP3 increase the sensitivity of the myometrium to oxytocin and prostaglandins that act as uterotonins during myometrial activation. Stretch also causes increases in myometrial prostaglandin H synthase (PGHS)-2 expression (Riemer et al., 1998). Experiments in unilateral pregnant rats demonstrate the inability of experimentally induced stretch to increase the expression of CAP genes in the non-gravid horn, unlike results in the gravid horn (Ou et al., 1998). This suggests that CAP gene expression is dependent on stretch as well as the endocrine environment, which is largely governed by the fetal genes and activation of the HPA axis (Shynlova et al., 2009).

1.1.2.2 Hypothalamic-pituitary-adrenal Axis

A key mechanism for parturition is the endocrine action of increased glucocorticoids and their influence on the gestational tissues. Across many species, including humans, the fetal HPA axis matures near the end of gestation. Glucocorticoids are vital in the maturation of organs necessary for survival outside of the womb and also play a crucial role in determining the length of gestation (Smith et al., 2007). Experiments in sheep have characterized the mechanisms in the fetal brain that account for an increase in HPA activity. Initially, CRH mRNA levels increase in the paraventricular nucleus of the hypothalamus as adrenocorticotropic hormone (ACTH) concentrations in the fetal circulation rise (Challis et al., 2000). The fetal sheep adrenal gland increases expression of enzymes involved in cortisol production as ACTH receptor expression is
up-regulated (Holloway et al., 2000). In this species, the net result of the maturing fetal HPA axis is increased sensitivity of the fetal adrenal gland to ACTH, and greater production of cortisol (Challis et al., 2001). In primates, similar increases in cortisol occur in late gestation. In addition to increased adrenal cortisol output in the human fetus, glucocorticoids influence human gestational tissue (Karalis et al., 1996). In the human placenta, prostaglandins down-regulate the expression of 11β-hydroxysteroid dehydrogenase (HSD) 2 and increase the expression of 11 β-HSD 1 thereby decreasing conversion of cortisol to cortisone (Alfaidy et al., 2001). Similarly, in the fetal membranes cortisone is produced through the activity of 11β-HSD 1 (Sun et al., 2003; Myatt et al., 2010). Near the end of term maternal blood concentrations of CRH increase and CRH-binding protein levels decrease. Altogether, the influence of CRH and glucocorticoids increase near the end of term and activate downstream cascades that lead to parturition onset. This activation includes a feed-forward loop initiated by increased glucocorticoid production and results in increased prostaglandins.

Elevated CRH levels stimulate prostaglandin production by modulating the levels of prostaglandin metabolizing enzymes expressed in the amnion, chorion and placenta trophoblast cells. PGHS-2 expression in the fetal membranes and placenta is increased by CRH (Alvi et al., 1999; Challis et al., 2002). Simultaneously, CRH and cortisol decrease the expression of PGDH in chorionic trophoblast cells. As a result, prostaglandins act on the fetal membranes to increase the activity of 11β–HSD 1 in chorionic trophoblasts and decrease expression of 11β-HSD 2 in placental trophoblasts (Alfaidy et al., 2001; Challis et al., 2001). The activity of 11β-HSD 1 converts inactive cortisone to cortisol whereas activity of 11β-HSD 2 inactivates cortisol to cortisone. Elevated CRH levels promote different effects based on the regionalization of the cortisol receptor subtypes throughout the gestational tissues (Hillhouse et al., 2002). In the fundal region, CRH acts through CRH receptor subtype 1 to increase prostaglandin production through increased PGHS-2 and decreased PGDH (Challis et al., 2000). As a result of varying receptor expression, the fundal region of the uterus relaxes allowing for regionalization of activity at term (Cong et al., 2009; Stevens et al., 1998). As well, the surge of CRH in late pregnancy is associated with increased estrogen resulting in altered progesterone to estrogen ratios indicative of a role for CRH in myometrial activation (Smith et al., 2009). Elevated prostaglandins induce expression of MMP-1, 2, and 9 in the amnion that begin to degrade the collagen present in the epithelium, compact, fibroblast and spongy layers that constitute the
tissue. Therefore, increased glucocorticoid levels result in a positive feed-forward loop between cortisol, CRH and prostaglandins that is necessary for activation of the myometrium, cervical ripening and membrane weakening.

1.2 Preterm Birth

Both autocrine and paracrine signals mediated by uterine stretch and activation of the HPA axis interact to promote phenotypic changes in the gestational tissues leading to the onset of labour at term. However, the phenotypic state of the gestational tissues can be altered earlier in gestation if similar autocrine and paracrine signals are up-regulated. During infection and/or inflammation cytokines and prostaglandins levels become elevated that subsequently activate pathways involved in modifying the phenotype of the myometrium, cervix and fetal membranes. Ultimately, the ability of these signals to modify and activate downstream pathways indicates a potential mechanism leading to preterm delivery and highlights potential molecules to be targeted in preventative treatment of infection and/or inflammation-mediated preterm birth.

1.2.1 Epidemiology

Preterm birth is a major obstetrical concern that occurs in 8% and 13% of all pregnancies in Canada and the United States of America, respectively (Goldenberg et al., 2008; Public Health Agency of Canada, 2008). Defined as labour occurring between 20 and 37 weeks gestation, preterm birth accounts for 75-85% of all neonatal morbidities and mortalities (Challis et al., 2002). Many of these morbidities, including pulmonary disorders, blindness, deafness, cerebral palsy, and neurodevelopmental delays linger into adulthood causing poor health later in life. The cost of caring for preterm infants has a large economic impact on the healthcare system. In 2005, it was estimated that $26.2 billion in the United States of America was spent on medical care for preterm neonates (Institute of Medicine Report, 2006). An impact that cannot be measured through monetary means is the emotional and psychological burden placed upon the families caring for infants born prematurely.
1.2.2 Etiology

The incidence of preterm birth has been on the rise since the 1980s (Challis et al., 2002). Much of this trend is accounted for by increased use of reproductive assisted techniques and the associated rise in multi-fetal pregnancies. Other predisposing factors include advanced or young maternal age, black race, low socioeconomic status of either or both the father and mother, single parenthood, type of employment, smoking, alcohol and substance abuse as well as carrying a male fetus (Goldenberg et al., 2003; Meis et al., 1995; Robinson et al., 2001). Certain aspects of the mother’s medical history also increase the risk of preterm birth. Such medical conditions include periodontal disease, lung disease, chronic hypertension, proteinuria or bacteriuria, a history of a previous preterm delivery, uterine malformation or short cervix. Reproductive tract infection, pelvic infection, vaginal microbiota alterations and infections including bacterial vaginosis, Neisseria gonorrhoeae, Chlamydia trachomatis, group B Streptococcus, Ureaplasma urealyticum, and Trichomonas vaginalis as well as obstetrical complications during pregnancy such as early pregnancy bleeding, placental abruption, placenta praevia, hydramnios, preeclampsia, premature rupture of the membranes (PROM), cervical surgery or a previously induced abortion are all associated with increased preterm birth (Goldenberg et al., 1998; Goldenberg et al., 2003; Meis et al., 1995; Norwitz et al., 1999; Robinson et al., 2001; Romero et al., 2002). Preterm birth can be categorized into three groups: 1) Idiopathic (50%), 2) Indicated, those induced by a physician as more deleterious to prolong to either the health of the fetus or the mother due to medical complications (20-30%), and 3) Infection and/or inflammation-mediated (30%). Early preterm deliveries tend to be associated more so with the presence of histologic chorioamnionitis and inflammation compared to later preterm deliveries (Goldenberg et al., 2000; Goldenberg et al., 2003; Vogel et al., 2005). However, despite ongoing research, the etiology of spontaneous preterm birth remains largely unknown.

1.2.3 Current Diagnostic Strategies and Treatments for Preterm Birth

The diagnosis and prevention of preterm birth remains problematic despite ongoing research. Commonly, pregnant women are informed by their physician of how to recognize signs of labour including contractions, pelvic pressure, vaginal discharge and back pain (Denney et al., 2008). However, only one third of women showing signs of preterm labour give birth within 24–48
hours (Bocking et al., 1999; Katz et al., 1999). Despite ongoing research, the onset of labour is only delayed up to 72 hours using tocolytic therapy. This allows for glucocorticoid treatment to mature fetal lungs; however the other developmental consequences associated with preterm birth cannot be corrected within this relatively short prolongation of gestation. Therefore, it is important to develop effective preventative therapy.

Assessment of a woman’s medical history plays an important role in determining the individual risk she possesses of undergoing preterm delivery. A history of a previous spontaneous preterm birth is one of the highest predictive indicators of an increased risk of preterm birth (Goldenberg et al., 2003). The preterm prediction study by the Maternal Fetal Medicine Network, which included 3000 pregnancies, indicated the three most effective predictive assessments for preterm delivery: 1) A positive cervical or vaginal fluid fetal fibronectin test, 2) A cervical length of less than or equal to 25 mm, and 3) Elevated serum levels of α-fetoprotein, alkaline phosphatase and granulocyte-colony stimulating factor (G-CSF; Goldenberg et al., 2003). As well, amniotic fluid samples with elevated levels of interleukin (IL)-6, IL-8, or Ureaplasma urealyticum are associated with high levels of preterm delivery (Vogel et al., 2005). Within maternal serum or vaginal and cervical fluids many molecules have been identified as potential predictive markers of preterm birth. These include low levels of ferritin, folate, zinc and high levels of C-reactive protein, cytokines, MMPs as well as altered levels of activin, relaxin, collagenase, non-phosphorylated insulin-like growth factor and CRH (Andrews et al., 2003; Norwitz et al., 1999; Vogel et al., 2005). Recently, single nucleotide polymorphisms (SNPs) in the promoter region of cytokines that up-regulate their transcription levels have been correlated with an increased risk of preterm birth (Harper et al., 2011).

It is important for physicians to determine whether or not prolonging gestation may be more detrimental to the fetus in a hostile intrauterine environment exposed to hypoxia or infection and/or to the mother as is the case in preeclampsia. In these cases, preterm delivery may be the most beneficial option compared to allowing gestation to progress. Tocolytics are agents that ameliorate uterine contractions and thus prevent expulsion of the fetus from the uterine environment for 24-72 hours (Norwitz et al., 1999). Ethanol was the first effective tocolytic identified (Fuchs et al., 1981). However, due to severe fetal side effects, its use was abandoned. Four routine tocolytic treatments are used in the United States of America: 1) β-mimetics, 2)
Magnesium sulphate, 3) Non-steroidal anti-inflammatory drugs, and 4) Calcium channel blockers (Giles et al., 2007; Katz et al., 1999; Meis et al., 2003; Vercauteren et al., 2009). However, like early tocolytic drugs, many of these options are associated with adverse side effects, including myocardial ischemia, tachycardia and arrhythmias as well as severe consequences to the health of the fetus including intraventricular hemorrhage and premature closure of the ductus arteriosus (Giles et al., 2007; Katz et al., 1999; Vercauteren et al., 2009). Therefore, the benefits of tocolytic drugs have been questioned and their use abandoned in many cases for the prevention of preterm delivery.

After the diagnosis of a pregnancy at high risk of undergoing preterm delivery, it is essential to provide effective preventative treatment if prolongation of gestation is not deleterious to the health of the fetus or the mother. However, currently no effective preventative treatment exists. A common method once used to prevent preterm birth was the use of antibiotics. Although bacterial vaginosis and Trichomonas vaginalis are largely asymptomatic, these alterations to the healthy vaginal microbiota are associated with an increased risk of preterm birth (Romero et al., 2002). When these conditions are diagnosed, antibiotics such as metronidazole, erythromycin, clindamycin and/or ampicillin, are administered in an attempt to replenish the altered microbiota by eliminating pathogenic bacteria. However, numerous meta-analyses, cohort studies, and randomized trials indicate that antibiotic treatment is ineffective at decreasing the risk of preterm birth (Andrews et al., 2003; Carey et al., 2000; Kenyon et al., 2001; Leitich et al., 2003; McDonald et al., 2007; Okun et al., 2005). In some cases, the use of metronidazole treatment actually increases the risk of preterm birth (Andrews et al., 2003; Okun et al., 2005). Antibiotics may be ineffective at decreasing the risk of preterm birth for several reasons: 1) Bacteria may have already ascended into the upper genital tract, 2) Antibiotics are unable to eradicate biofilms, 3) Antibiotics are unable to inactivate sialidases and, 4) Antibiotics can eradicate bacteria that are necessary for host defense mechanisms (Reid et al., 2003). Also, prolonged use of antibiotics has led to increasingly resistant bacterial strains and promotes an alkaline environment in the vagina that encourages pathogenic bacterial growth (Locksmith et al., 2001).

### 1.3 Infection and Inflammation

Infection and/or inflammation accounts for 30% of preterm births. Inflammation acts as a first line of defense against pathogenic threats by recruiting and activating immune cells while
forming a physical barrier to stop the spread of infection. In many cases, the signs and symptoms of infection and/or inflammation during pregnancy are undetectable unless invasive procedures such as amniocentesis are performed. The prevalence of chorioamnionitis, an inflammation of the chorioamnion membranes has been found in up to 50% of pregnancies ending prematurely. Many of the organisms found in the upper genital tract are of vaginal origin and bacterial vaginosis, an alteration to the vaginal microbiota where lactobacilli levels decrease, increases the risk of preterm delivery by 1.4 to 3.8 fold (Andrews et al., 2000). Treatment of bacterial vaginosis with antibiotics is associated with a high incidence of re-occurrence.

Consequently, current research has shifted to determine the use of candidate probiotic strains of bacteria including lactobacilli, to reinstate a healthy vaginal microbiota. Many *Lactobacillus* strains exert immunomodulatory effects across various systems; however the role *Lactobacillus* strains may exert on the inflammatory processes in the gestational tissues that are associated with infection and/or inflammation preterm delivery remain largely uninvestigated.

### 1.3.1 Vaginal Microbiota

The fetal vagina is a sterile environment which is first colonized by bacteria endogenous to the mother’s vaginal birth canal, the skin of caretakers and the infant’s faeces upon birth (Reid et al., 2011; Spiegel, 1991). It is not until after menarche, when estrogen levels increase that the childhood vaginal microbiota characterized by intestinal and cutaneous bacterial species becomes dominated by *Lactobacillus* species, the hallmark species of a healthy adult vaginal microbiota (Forsum et al., 2005; Spiegel, 1991). Estrogen levels at puberty promotes the production of glycogen in the vaginal epithelial cells necessary for the survival of lactobacilli as it provides a source of glucose through fermentation (Forsum et al., 2005; Spiegel, 1991). The bi-product of fermentative metabolism, lactic acid creates an acidic vaginal environment with a pH of 3.8–4.2 which promotes self-growth of *Lactobacillus* species within a healthy microbiota. Three or four species, including *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* are present in much higher levels in the vaginal microbiota (Antonio et al., 1999; Burton et al., 2002; Forsum et al., 2005; Lamont et al., 2011; Pavlova et al., 2002; Song et al., 1999; Vasquez et al., 2002; Yamamoto et al., 2009). As well, over twenty species of *Lactobacillus*, including *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus oris*, *Lactobacillus ruminis*, *Lactobacillus reuteri*, and
Lactobacillus rhamnosus have been isolated from the vaginal microbiota (Antonio et al., 1999; Forsum et al., 2005; Pavlova et al., 2002). Gram-negative bacteria, including Streptococcus species, Atopobioum vaginae, Prevotella spp. and Gardnerella vaginalis are present in low levels in healthy vaginal microbiota (Yamamoto et al., 2009). Using 16S RNA gene sequencing analysis, Gardnerella vaginalis was identified as a core component in the vaginal microbiota of the entire female population sampled (Hummelen et al., 2010). Therefore, many bacteria that may become pathogenic if levels are not maintained are present in healthy vaginal flora at detectable levels.

The vaginal microbiota provides a barrier to exterior influences and actively participates in adaptive immunity, the portion of the immune system that is specific and non-inherited (Forsum et al., 2005). However, the vaginal microbiota is susceptible to alterations resulting in bacterial vaginosis, Vulvovaginal candidiasis, and Trichomonas vaginalis. Accordingly, women with altered vaginal microbiota are more susceptible to the transmission of sexually transmitted diseases including HIV and gonorrhoea, pelvic inflammatory disease, urinary tract infections, post-operative infections, and upper genital tract infections including chorioamnionitis (Antonio et al., 1999; Deb et al., 2004; Forsum et al., 2005). Pathogenic bacteria are equipped with molecules, including proteases, mucinases, and sialidases that promote self-growth through degradation of the cervicovaginal mucous lining. Beyond the vaginal environment, bacterial collagenases, phospholipases and endotoxins directly or through stimulated production of downstream molecules result in remodeling of the collagen structuring of the fetal membranes and cervix and initiate inflammatory cascades in the gestational tissues of the intrauterine environment (Goldenberg et al., 2000).

1.3.2 Routes of Intrauterine Infection

Microbial invasion of the amniotic cavity is associated with adverse outcomes including PROM and preterm birth. Intrauterine infection may result from four different routes of bacterial access: 1) Retrograde migration from the abdominal cavity through the fallopian tubes, 2) Haematogenous spread from the maternal blood through the placenta, 3) Iatrogenic introduction through invasive procedures, such as amniocentesis, and 4) Ascent from the vagina and cervix through the fetal membranes (Figure 1.2, page 21; Goldenberg et al., 2000). The latter of these, is thought to be the most common route for bacteria to reach the uterine cavity. Evidence for this
includes the fact that histologic chorioamnionitis is more severe at the location of membrane rupture than other sites including the chorionic plate or the site of umbilical cord attachment (Benirschke, 1960; Romero et al., 1989d). As well, microbes found in congenital infections are similar to those isolated from the maternal vagina (Benirschke, 1960). The most common microorganisms found in amniotic fluid samples are *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Bacteroides* spp. and *Mycoplasma hominis* (Goldenberg et al., 2000). The morphotypes of these bacteria are used by the Nugent scoring system to diagnose bacterial vaginosis (Goldenberg et al., 2000; Nugent et al., 1991). During twin gestations, twins are commonly oriented one over the other, such that the membranes of only one twin come in contact with the cervical os, while the other twin is located superior to this twin. Histologic chorioamnionitis is more frequent in the fetal membranes directly over the cervix. Bacteria present in these membranes are commonly of vaginal origin indicative of their ascent from the lower genital tract (Benirschke, 1960). Together, this evidence indicates the most common route of infection of the intrauterine environment is ascending from the lower genital tract.

### 1.3.3 Bacterial Vaginosis

#### 1.3.3.1 Epidemiology

Bacterial vaginosis accounts for approximately 80% of all diagnosed vaginal microbiota alterations and is present in 15% - 20% of pregnant women (Hillier et al., 1995; Imseis et al., 1997; Romero et al., 2004). Bacterial vaginosis is associated with many factors including: single marital status, African American ethnicity, low socioeconomic status, previous delivery of a low-birth-weight infant, douching practices, spermicide and antimicrobial use, smoking, absence of barrier birth control, and sexual activity (Hawes et al., 1996; Hillier et al., 1995; Reid, 2008). Bacterial vaginosis increases the incidence of pelvic inflammatory disease, post-operative infections, post-cesarean endometritis, PROM, histologic chorioamnionitis, and subsequent preterm delivery as the infection may reach the intra-uterine environment (Hawes et al., 1996; Imseis et al., 1997; MacPhee et al., 2010; Nugent et al., 1991; Romero et al., 2004; Soper, 1993; Sweet, 1995).

#### 1.3.3.2 Etiology

Bacterial vaginosis is an alteration to the endogenous vaginal microbiota where levels of *Lactobacillus* species decrease and anaerobic bacteria begin to dominate. It is unknown if the
A decrease of lactobacilli precedes the growth of pathogenic bacteria or overgrowth of harmful bacteria causes a decrease in *Lactobacillus*; however, the total concentration of bacteria in the vagina increases by 100 to 1000 times that of the healthy microbiota (Forsum et al., 2005). Characteristic bacteria that dominate the microbiota in bacterial vaginosis include *Gardnerella vaginalis*, *Bacteriodes* spp., *Mycoplasma hominis*, *Mobiluncus* spp., and *Atopobium vaginae*; many of which reside in low levels in a healthy vaginal microbiota (Falagas et al., 2007; Hummelen et al., 2010; Nugent et al., 1991). The microbial profiles of women with bacterial vaginosis show large inter-patient variability and greater diversity when compared to profiles of women with a healthy vaginal microbiota. In agreement, no singular microbe has been associated with the etiology of bacterial vaginosis and diagnosis is dependent on symptoms and signs as well as a proportional analysis of bacterial species present in characteristically healthy versus altered vaginal microbiota.

### 1.3.4 Current Diagnostic Strategies and Treatments for Bacterial Vaginosis

Currently, the diagnosis of bacterial vaginosis combines both clinical syndrome observation with Nugent scoring according to the following four hallmark indicators: 1) A vaginal pH greater than 4.5, 2) An amine fishy odour when vaginal fluid is mixed with potassium chloride, 3) The presence of clue cells - vaginal epithelial cells covered in *Gardnerella vaginalis* making them appear rough edged under a microscope in a vaginal fluid sample, and 4) A Nugent score greater than 6. Nugent scoring is based on a weighted representation of the bacterial species visualized by gram-staining that summate to a score from 0 to 10. Bacteria scored include: *Lactobacillus* (gram-positive rods), *Gardnerella vaginalis* (small gram-variable rods), *Bacteroides* spp. (small gram-negative rods), and *Mycoplasma* spp. (curved gram-variable rods; Nugent et al., 1991). The values 0-3 represent a normal microbiota, with high proportions of *Lactobacillus* spp. and few of the other morphotypes; 4-6 represents an intermediate microbiota with higher proportions of pathogenic morphotypes, and a score of 7-10 represents bacterial vaginosis, with low lactobacilli morphotypes present. Inaccuracies in diagnosis persist due to the lack of symptoms in up to an estimated 50% of women with bacterial vaginosis and the acuity required to identify associated symptoms (Nugent et al., 1991). Other diagnostic techniques once used for bacterial vaginosis include laboratory cultures positive for *Gardnerella vaginalis* growth, gas chromatography for products of pathogenic vaginal bacteria and a proline amniopeptidase test.
(Nugent et al., 1991). However, finding *Gardnerella vaginalis* is no longer deemed a definitive diagnosis. Gram-staining is the most inexpensive, efficient and reproducible; therefore, gram-staining and Nugent scoring is the method of choice for bacterial vaginosis diagnosis.

As bacterial vaginosis is a disruption to the vaginal eubiosis, treatment has largely been focused on the use of antibiotics, administered both orally and vaginally. Despite evidence of cure after initial treatment, a high reoccurrence rate exists in many patients regardless of the route and/or type of antibiotic administration. Repetitive antibiotic use is avoided as it may encourage the development of resistant bacterial strains. In addition, antibiotic treatment of bacterial vaginosis in pregnant women has not shown any decrease in the incidence of preterm birth.

The association of bacterial vaginosis with preterm delivery has led to the study of adjunctive probiotic and antibacterial treatment, or probiotic supplementation independently. Despite relatively low endogenous levels in the vaginal microbiota, *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 are the most effective at restoring and maintaining a normal vaginal microbiota (Reid et al., 2011). These two strains of lactobacilli persist in the vagina after vaginal insertion and also alter the host immune defenses (Cadieux et al., 2002; Gardiner et al., 2002). *Lactobacillus rhamnosus* GR-1 in particular can populate the vagina, reduce pathogenic bacteria from reaching the vagina from the anal-rectal area, inhibit growth, adhesion and biofilm formation of gram-negative bacteria as well as inhibit the persistence of *Candida albicans* (Cadieux et al., 2002; Reid, et al., 2003; Reid, 2008).

Bacteria associated with bacterial vaginosis are capable of releasing proteases and endotoxins that free arachidonic acid from phospholipid membranes and subsequently increase prostaglandin production in the amnion, chorion and decidua. Increased prostaglandins elevate the risk of preterm labour by inducing collagen remodeling in the fetal membranes. Antibiotic treatment is ineffective at preventing preterm birth. This may be a consequence of the inability of antibiotics to alter downstream pathways that have been activated prior to their administration. There is little research on the role lactobacilli may play at preventing the production of cytokines and prostaglandins in the amnion, a major contributor to the amniotic fluid cytokine pool and prostaglandins. However, due to the known immunoregulatory and other beneficial roles of *Lactobacillus* species, it is possible that these bacteria may act as a probiotic to decrease the risk of preterm birth associated with infection and/or inflammation.
Figure 1.2: Potential sites of bacterial infection amongst the intrauterine tissues.

Potential routes for microbes to gain access to the intrauterine environment: 1) Retrograde migration from the abdominal cavity through the fallopian tubes, 2) Haematogenous spread from the maternal blood through the placenta, 3) Iatrogenic introduction through invasive procedures, such as amniocentesis, and 4) Ascent from the vagina and cervix through the fetal membranes.

1.4 Probiotics

Probiotics are defined as, “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO, 2001). Probiotics have been used therapeutically and have shown beneficial effects across a wide spectrum of medical conditions. These effects include modulation of immunity, lowering cholesterol, treatment of rheumatoid arthritis, prevention of cancer, improvement of lactose intolerance, prevention of diarrhea and constipation, reduction or prevention of acute dermatitis and treatment of urinary tract infections (Kligler et al., 2007; Lee do et al., 2011; Ohara et al., 2010; Ojetti et al., 2010; Reid, 1999; So et al., 2011; Verna et al., 2010). Although the use of lactobacilli as probiotics have shown promise in restoring vaginal microbiota for the treatment of vaginal infections and microbiota alterations during pregnancy, there are currently insufficient studies to assess the impact probiotics have on pregnancies at risk of undergoing preterm birth associated with infection and/or inflammation (Othman et al., 2007).

Although the exact mechanism by which probiotics exert their beneficial effects remains unknown, various types of probiotic bacteria are known to benefit their host through competitive exclusion of bacterial adherence, bacteriocin and butyrate production, maintenance of the epithelial barrier, modulation of immune responses through NFkB transcriptional activity modification and immune cell recruitment and activation as well as increased IgA production (Ahn et al., 1990; Falagas et al., 2007; Floch, 2010; Jijon et al., 2004; Lara-Villoslada et al., 2007; Madsen et al., 2001; Moorthy et al., 2009; Reid, 2008; Yan et al., 2002). Pathogenic bacteria possess pathogen-associated molecular patterns (PAMPs) on their cellular membrane that are recognized by the body’s immune system through activation of TLRs. Unlike probiotic bacteria that instead harbor microbe-associated molecular patterns (MAMPs), recognition of PAMPs initiate an immune response to defend against pathogenic bacterial threats (Servin, 2004). Additionally, the signaling pathway activated by TLR9 has been shown to be necessary for immunostimulation by bacterial CpG DNA sequences that play a role in the anti-inflammatory effects exerted by various probiotics (Rachmilewitz et al., 2004). Therefore, an opportunity exists for probiotics and/or their byproducts to be used as treatment for many infectious and autoimmune conditions.
1.4.1 *Lactobacillus* Species as Candidate Probiotics for use During Pregnancy.

The criteria for defining a probiotic candidate has been established including: 1) Of no threat to the host, therefore they are non-carcinogenic, non-pathogenic, and non-invasive, 2) Able to persist and multiply, 3) Resistant to vaginal microbicides, 4) Capable of co-aggregation and forming a normal eubiosis, 5) Capable of adherence to cells, 6) Capable of exclusion or reduction of pathogenic adherence, and 7) Capable of antagonistic growth through the production of acids, hydrogen peroxide and bacteriocins (Reid, 1999). In addition, when identifying a potential probiotic strain of bacteria, it is important to consider the commensal bacterial microbiota as well as the intrinsic resistance of the probiotic to antibiotics (Servin, 2004). In the case of the vaginal microbiota, loss of the endogenous dominant species of a healthy vagina, lactobacilli, is associated with pathologies including bacterial vaginosis. Bacterial vaginosis in pregnant women increases risk of preterm delivery; therefore, a strain of *Lactobacillus* may exert beneficial effects during pregnancy. In particular, *Lactobacillus rhamnosus* GR-1 possesses many of the characteristics required to define it as a probiotic bacteria for use in the urogenital tract.

1.4.1.1 Safety of lactobacilli administration

*Lactobacillus* species are safe for use in humans with low intrinsic immunogenicity as they are neither carcinogenic nor pathogenic (Cadieux et al., 2002; Servin, 2004). In fact, *Lactobacillus rhamnosus* GR-1 injected directly into the bladder of human patients failed to induce an infection (Hagberg et al., 1989). In addition, a meta-analysis of randomized controlled trials involving lactobacilli and bifidobacterium during pregnancy indicated that either bacterium do not alter the incidence of caesarean section, birth weight, malformations, or gestational age at birth in pregnant women (Dugoua et al., 2009). Therefore, the safety of *Lactobacilli rhamnosus* GR-1 in humans is established and has a low or negligible chance of threatening the health of a mother and her fetus throughout pregnancy.

1.4.1.2 Ability of lactobacilli to colonize the vagina

Studies have shown that after instillation, *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 persist in the vaginal environment for 19 days compared to only 5 days for *Lactobacillus rhamnosus* GG (Cadieux et al., 2002). Another study showed *Lactobacillus*
rhamnosus GR-1 present in the vaginal microbiota up to 7 weeks after instillation (Reid et al., 1994). Importantly, *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 are capable of restoring and maintaining a healthy vaginal microbiota (Reid et al., 2001a, Reid et al., 2003).

Passive forces including electrostatic interactions, hydrophobic steric forces and steric hinderence via lipoteichoic acid (LTA) allow *Lactobacillus* to interact with the mucosal layer of urogenital cells (Servin, 2004). *Lactobacillus rhamnosus* strains have a high capacity for adherence to urogenital cells compared to other lactobacilli strains (Reid et al., 1987; Servin, 2004). Since adherence to epithelium plays an important role in initiating the pathogenesis involved with many urogenital tract infections, the ability of lactobacilli to compete, inhibit, and/or prevent colonization of the urogenital tract by pathogenic bacteria is significant (Servin, 2004).

1.4.1.3 Pathogenic bacterial defense mechanisms of lactobacilli

Lactobacilli promote exclusion or reduction of pathogenic bacteria adherence through the production and actions of biosurfactants, hydrogen peroxide, lactic acid and bacteriocins. Together these molecules limit the adherence of pathogenic bacteria to urogenital cells and minimize their ability to colonize and flourish within the vaginal environment.

Biosurfactants are surface-active compounds that help restore homeostasis by exclusion or reduction of pathogenic bacteria (Reid et al., 2011). Biosurfactants produced from vaginal lactobacilli strains are composed of lipids, proteins and carbohydrates that bind to collagen III and VI as well as fibronectin which are major components of the extracellular matrices of the vagina and other intrauterine tissues (Howard et al., 2000; Reid et al., 2011). *Lactobacilli rhamnosus* GR-1 and/or *Lactobacillus reuteri* RC-14 effectively disrupt *Escherichia coli*, *Enterococcus faecalis*, *Gardnerella vaginalis*, *Shigella dysenteria*, *Candida albicans*, *Streptococcus aureus*, and other bacteria biofilms and/or adherence that are associated with bacterial vaginosis, urinary tract infections and yeast vaginitis (Howard et al., 2000; Moorthy et al., 2009; Reid et al., 1995; Reid, 2008; Saunders et al., 2007; Velraeds et al., 1996; Velraeds et al., 1998; Xu et al., 2008).

Hydrogen peroxide is a non-specific oxidizing agent that promotes the production of free radicals and activates epithelial cell responsiveness to inflammatory stimuli (Voltan et al., 2008).
*Gardnerella vaginalis* is a non-catalase producing bacteria and therefore, hydrogen peroxide inhibits its growth (MacPhee et al., 2010). Women with hydrogen peroxide-producing lactobacilli present in their vaginal microbiota have lower incidence of bacterial vaginosis (Eschenbach et al., 1989; Hillier et al., 1992; Hillier et al., 1993). Although *Lactobacillus rhamnosus* GR-1 does not produce much hydrogen peroxide, it produces lactic acid through the fermentation of glycogen from the vaginal epithelium (Saunders et al., 2007). Bacteriocins have a narrow killing spectrum in comparison to hydrogen peroxide and lactic acid as they damage the cytoplasmic membranes of bacteria (Reid et al., 2011; Servin, 2004).

### 1.4.1.4 Immunomodulatory properties of lactobacilli

Various strains of lactobacilli have been shown to modulate the immune response in immune cell lines, gut epithelial and placental trophoblast cells. DC429, a strain of *Lactobacillus rhamnosus* increased chemotaxis of polymorphonuclear immune cells, activated phagocytosis in the recruited cells and increased cytokine production in murine air pouch models (Kotzamanidis et al., 2010). The cytokine profile stimulated by DC429 was characterized by increased production of IFNγ, IL-5, IL-6, and IL-10 in the gut mucosal lining of mice. The authors of this study suggested that DC429 helped to maintain a physiologic state of inflammation crucial to the defense of the gut epithelial layer (Kotzamanidis et al., 2010). Heat-killed *Lactobacillus rhamnosus* GG stimulated IL-4, IL-10 and urocortin release from human placental trophoblast cells (Bloise et al., 2010). Similar to results shown with the supernatant of *Lactobacillus rhamnosus* GR-1, heat-killed *Lactobacillus rhamnosus* GG prevents LPS-stimulated TNFα release from primary placental trophoblast cultures (Bloise et al., 2010; Yeganegi et al., 2010). Additionally, *Lactobacillus rhamnosus* GR-1 supernatant increased IL-10 output in primary cultures of human placental trophoblast cells (Yeganegi et al., 2010). Interestingly, *Lactobacillus rhamnosus* GR-1 supernatant is capable of altering levels of PGDH, PGHS-2 and G-CSF in a sex-dependent manner (Yeganegi et al., 2009; Yeganegi et al., 2011). These results suggest that the supernatant of *Lactobacillus rhamnosus* GR-1 contains metabolites capable of exerting the beneficial effects regardless of the presence of live or heat-killed bacteria. Therefore, *Lactobacillus rhamnosus* strains and/or supernatant metabolites are capable of modifying both immune cell chemotaxis and activity levels as well as the cytokine and prostaglandin metabolizing enzymes in a gestational tissue.
1.4.2 The Potential Mechanisms of Probiotics

The exact mechanism by which lactobacilli exert their beneficial effects on the host remains elusive. However, studies have shown that the supernatant of some Lactobacillus species can be beneficial. After separating the supernatant from Lactobacillus plantarum 10hk2 into protein and polysaccharide portions and subsequent analysis of fractions based on molecular weight, an active protein fraction of 8.7 kDa was identified. This protein fraction was capable of inducing IL-10 production from a murine macrophage cell line and eliminates activation of key molecules in the NFκB-cytokine transcription pathway (Chon et al., 2010). Similarly, a soluble protein factor eluted from a gastrointestinal probiotic reduced Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ) secretion from T84 monolayers (Madsen et al., 2001). The active product in two other lactic acid bacterium family members, Bifidobacterium breve and Streptococcus thermophilus have active enzymes resistant to digestive enzymes with a molecular mass of < 3 kDa in culture medium capable of inhibiting lipopolysaccharide (LPS)-induced TNFα in intestinal cell monolayers. This inhibitory effect was preserved after transepithelial transport across the intestinal cell monolayer (Menard et al., 2004). Additionally, medium collected from cultured Lactobacillus rhamnosus GG was sufficient in preventing cytokine-induced apoptosis in intestinal epithelial cells (Yan et al., 2002). Together, these studies indicate the possibility that probiotic metabolites are sufficient to exert their effects without the presence of live or heat-killed bacteria. Although no such evidence has been found regarding the active molecule in Lactobacillus rhamnosus GR-1, it stands that the culture medium from these bacteria may also be capable of exerting effects independent of the presence of live bacteria.

The experiments in this thesis were designed to further determine the effects of probiotics on gestational tissues during pregnancy. Taken together, the above evidence identifies Lactobacillus rhamnosus GR-1 is a safe candidate for use in pregnancy. Lactobacillus rhamnosus GR-1 displays many anti-pathogenic characteristics that may prevent pathogenic bacteria from dominating the vaginal flora. If access to the gestational tissues is possible, it is more likely that a small protein byproduct or metabolite would be capable of accessing the intrauterine environment than live probiotic bacteria. Currently, however, there is little research focused on the effects of probiotics and their metabolites to access the intrauterine tissues during pregnancy, or on the effects this interaction may have on the other gestational tissues including the amnion.
1.5 Cytokines and Chemokines

Preterm labour associated with infection is accompanied by an inflammatory cascade activated by invasive bacterial products that stimulate increased secretion of cytokines from invading immune cells and gestational tissues (Figure 1.3, page 33). Elevated cytokine levels stimulate prostaglandin production in the amnion, chorion and decidua activating downstream pathways that result in myometrial contractility, membrane rupture and cervical ripening mimicking parturition events. Evidently, cytokines and chemokines play crucial roles in the mechanisms involved in preterm labour associated with infection and/or inflammation. Activated neutrophils are capable of entering into the compact, mesenchymal and spongy layers of the amnion and may enter the amniotic cavity between the amnion epithelial cells while stimulating cytokine and chemokine production in the fetal membranes (Saji et al., 2000). Intra-amniotic infection, microbial invasion of the intrauterine environment and/or chorioamnionitis have been associated with elevated levels of LPS, IL-6 and IL-8 as well as IL-1, TNFα, monocyte chemotactic protein (MCP)-1, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) and G-CSF in amniotic fluid compared to women with uncomplicated pregnancies (Andrews et al., 1995; Coultrip et al., 1994; Esplin et al., 2005; Hillier et al., 1993; Romero et al., 1988b; Romero et al., 1989a; Romero et al., 1989c). In many cases, however, amniotic fluid is sterile despite elevated cytokine levels indicating that the presence of bacteria is not necessary for inflammatory markers to be present (Seong et al., 2006; Smulian et al., 1999). Elevated maternal serum levels of IL-8 and cervical or amniotic fluid levels of IL-6 are indicative of chorioamnionitis and subsequent increased risk of preterm delivery (Andrews et al., 1995; Jacobsson et al., 2005). Cytokine levels are increased in the gestational tissues and bodily fluids indicative of an ongoing inflammatory response.

The amnion is composed of epithelial and mesenchymal cells that work in concert to produce cytokines and chemokines. Many cytokines and chemokines are produced and secreted from primary cultures of human amnion including: IL-2, IL-4, IL-6, IL-8, IL-15, IL-1 receptor antagonist (IL-1ra), MCP-1, macrophage inflammatory protein-1α, RANTES, and TNFα. Production and secretion of cytokines and chemokines from the amnion and chorion decreases when the two portions of the fetal membranes are cultured independently compared to explants of intact membrane (Menon et al., 2004). This indicates that coordinated interaction exists between the amnion and chorion in vivo. It is hypothesized that shuttling of mRNA as well as
bidirectional communication and cooperation occurs between the amnion and chorion (Zaga et al., 2004; Zaga-Clavellina et al., 2007).

During infection, bacterial products such as LTA and LPS activate pathogen recognition receptors that activate the inflammatory cascade. LTA and LPS are wall-component proteins of gram positive and gram-negative bacteria respectively. LPS activates TLR4 by binding to LPS-binding protein. TLR4 co-localizes with CD14 resulting in activation of downstream MAP kinase pathways and transcription of NFκB genes including IL-1β, TNFα, IL-6 and IL-8. The amnion is responsive to both bacterial endotoxin and cytokine stimulation as it expresses many cytokine receptors as well as TLR2 and TLR4. Amnion cells maintained in vitro and as membrane explants, secrete cytokines and chemokines after stimulation with pro-inflammatory molecules to levels similar to those found in amniotic fluid samples from women with pregnancies complicated by infection (Fortunato et al., 1996).

1.5.1 Pro-inflammatory Cytokines

Cytokines are small proteoglycan molecules that are secreted from immune cells and other tissues that modulate the immune response through up-regulation of other cytokines and chemokines and direct activation of immune cells and other aspects of immunity. Pro-inflammatory cytokines are elevated in the amniotic fluid during preterm labour associated with infection and/or inflammation. Pro-inflammatory cytokines induce the production of other cytokines and chemokines, growth promotion and inhibition, angiogenesis, cytotoxicity and other mechanisms to defend against pathogenic threats. The major cascade of pro-inflammatory cytokines involves the first response cytokines, TNFα and IL-1β as well as IL-6. IL-6 has been shown to play a more immunoregulatory role than its counterparts in the triad. In the female reproductive system and throughout pregnancy, TNFα plays a role in follicular development, ovulation, menstruation and endometrial function regulation (Argiles et al., 1997). However, in preterm labour associated with infection and/or inflammation TNFα, IL-1β and IL-6 stimulate prostaglandin production in the amnion. Elevated prostaglandins initiate the onset of parturition and if levels are increased due to infection, pro-inflammatory cytokines may promote early onset of labour.

TNFα and IL-1β are the first cytokines found in serum in response to acute infection (Petersen et al., 2006). In LPS-induced fetal loss in pregnant mice, both TNFα and IL-1β levels are increased
(Silver et al., 1994; Silver et al., 1997). Unlike IL-1β levels that become detectable in the third trimester of pregnancy, TNFα is undetectable in amniotic fluid unless an infection is present (Romero et al., 1989c). The amnion does not produce IL-1β and its mRNA is not localized to the amnion. However, IL-1β mRNA is found in both the chorion and decidua and it may be shuttled and therefore account for secretion found on the fetal side of in vitro membrane explants (Menon et al., 1995; Thiex et al., 2009; Zaga et al., 2004). IL-1β and TNFα both increase the availability of phospholipids, the substrate for prostaglandin production, phospholipase protein levels as well as the expression of PGHS-2, the rate-limiting step in prostaglandin production in the amnion (Blumenstein et al., 2000; Bry et al., 1992; Pollard et al., 1993; Romero et al., 1989b; Romero et al., 1989c). As well, IL-1β and TNFα also decrease PGDH expression in chorion cells resulting in decreased prostaglandin metabolism (Brown et al., 1998; Pomini et al., 1999). Therefore, pro-inflammatory cytokines cause a net increase in prostaglandin production from the gestational tissues (Kent et al., 1993; Pollard et al., 1993).

Levels of IL-6 are increased in vaginal fluid at term and preterm labour, in cervical fluid obtained from women with intrauterine infection and in the amniotic fluid of women with chorioamnionitis (Imseis et al., 1997). Importantly, IL-6 increases amnion prostaglandin production in cultured amnion cells to levels similar to those found in the amniotic fluid of women with intrauterine infections and preterm labour (Kent et al., 1993; Mitchell et al., 1991). IL-6 is part of the pro-inflammatory cytokine triad alongside TNFα and IL-1β. Despite its pro-inflammatory initiation of the acute phase response from the liver in response to acute infection, IL-6 is defined as a pleiotropic immunomodulatory cytokine. In LPS-stimulated IL-6 deficient knock-out mice, levels of both TNFα and neutrophilia were three times higher than in wild-type counterparts (Xing et al., 1998). These effects were abolished with treatment of recombinant IL-6 (Xing et al., 1998). Furthermore, in cancer patients receiving recombinant IL-6 intravenous therapy, serum levels of both IL-1ra and sTNFα receptor, the antagonists to TNFα and IL-1β, were increased (Tilg et al., 1994). IL-6 treatment increases levels of the anti-inflammatory cytokine, IL-10 (Steensberg et al., 2003). Negative feedback is provided by IL-6 against TNFα and IL-1β which may help maintain and facilitate the decrease and resolution of pro-inflammatory immune responses (Steensberg et al., 2003).
1.5.2 CXC and CC Chemokines

Chemokine release is stimulated by pro-inflammatory molecules from immune cells and other immune responsive tissues. Classified by patterns of amino acids in their amino terminus, chemokines are separated into two families: CXC or CC. Chemokines are responsible for immune cell recruitment, chemotaxis, activation and motility. Levels of chemokines that recruit immune cells are elevated at term and prior to the onset of labour. However, the levels of many chemokines increase throughout gestation and in association with infection during pregnancy and many of their specific roles remain unknown.

IL-8 or CXCL8 is a member of the CX chemokines and plays a role in endothelial cell proliferation and survival as well as inducing directional motility and activation of neutrophils and T-lymphocytes (Li et al., 2003). As such, elevated concentrations of IL-8 in amniotic fluid are associated with microbial infection, increased neutrophil count, membrane rupture, and cervical ripening (Cherouny et al., 1993; Hsu et al., 1998). IL-8 is constitutively expressed in the amnion and as mechanical tension increases towards the end of the mid-trimester, the expression of the stretch-responsive IL-8 gene is increased and subsequently levels of IL-8 in the amniotic fluid rise (Kendal-Wright, 2007; Romero et al., 1991). It is thought that increased IL-8 levels associated with intrauterine infection recruit neutrophils into the fetal membranes and placenta (Cherouny et al., 1993). Unlike TNFα, IL-1β, and IL-6, IL-8 does not stimulate amnion prostaglandin production. However when the amnion is stimulated with endotoxin, amnion cells respond with increased IL-8 to levels similar to those found in women with intra-amniotic infection.

The family of CC chemokines includes MCP-1 and RANTES which recruit immune cells that express the receptor, CCR5 including monocytes, basophils and eosinophils. MCP-1 is released from ovarian follicles, endometrium and the choriodecidua as well as the amnion in the third trimester (Arici et al., 1995; Arici et al., 1997; Denison et al., 1998). MCP-1 levels in amniotic fluid are increased during preterm labour with chorioamnionitis and with preterm labour with or without infection (Esplin et al., 2005). RANTES is released by the endometrium, first trimester trophoblast cells and the amnion (Denison et al., 1998; Hornung et al., 1997; Svinarich et al., 1996). RANTES levels in amniotic fluid decrease with gestational age and increase with preterm labour associated with microbial invasion as well as at term (Athayde et al., 1999).
RANTES attracts monocytes and lymphocytes and increases immune cell adhesion. CC chemokines activate immune cells. The important role of CC chemokines is demonstrated by the inability of MCP-1 deficient knock-out mice to resist microbial infection and an inability to clear bacteria (Yadav et al., 2010).

1.5.3 Immune Cell Activating Cytokines

Both IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF) play important roles in the adaptive immune system by participating in the activation of recruited immune cells at the site of infection. IFNγ is an immunomodulatory cytokine crucial in the adaptive immune system for defense against viral and intracellular bacterial infections. IFNγ increases antigen presentation through up-regulation of MHC I and MHC II complexes, release of reactive oxygen species in macrophages and promotes adhesion and binding of leukocytes during migration. IFNγ plays a role in maternal recognition of pregnancy as it is involved in implantation and blastocyst attachment (Murphy et al., 2009). In the decidua, IFNγ receptor expression decreases in both term and preterm placentae (Hanna et al., 2004). IFNγ decreases PGHS-2 expression and consequentially the production of PGE2 in placental explants in vitro (Hanna et al., 2004). It is suggested that IFNγ receptor down-regulation occurs at term. With a loss of IFNγ receptor expression, IFNγ is no longer able to repress PGHS-2 expression in the placenta resulting in an increase in prostaglandin production (Hanna et al., 2004). IFNγ has not been identified in the amniotic fluid; however, IFNγ is expressed in amnion tissue and shows no changes as labour progresses (Veith et al., 1999).

Similarly, GM-CSF promotes proliferation of monocyte, neutrophils and eosinophils, GM-CSF also activates phagocytosis, chemotaxis and adhesion as well as promoting cell growth and survival (Gasson, 1991). During early pregnancy, GM-CSF is involved in murine normal placental growth and differentiation as well as embryonic implantation and cytotrophoblast differentiation (Bry et al., 1997; Garcia-Lloret et al., 1994; Robertson et al., 1992). Transgenic mice with overexpression of GM-CSF show high levels of macrophage proliferation and activation (Metcalf et al., 1988). Levels of GM-CSF increase throughout gestation and women with PROM show higher levels compared to those with intact membranes during pregnancy (Bry et al., 1997). GM-CSF is produced in the amnion, trophoblasts and decidua. Both TNFα and IL-1β stimulate GM-CSF release from amnion cells (Bry et al., 1995). Expression of GM-CSF in
the amnion and levels of GM-CSF in the amniotic fluid are elevated during chorioamnionitis and intrauterine infection respectively (Keelan et al., 2003; Stallmach et al., 1995). It is suggested that GM-CSF may amplify the immune response in the amnion while increasing host resistance to infection.

The interaction between cytokines and chemokines plays a crucial role in the immune response against pathogenic threats. However, during pregnancy an imbalance of TH$_1$ to TH$_2$ cytokines results in increased risk of preterm delivery through subsequent increases in prostaglandin production. Infection of the amniotic cavity and the accompanying inflammatory processes are harmful to the fetus because it may lead to Fetal Inflammatory Response Syndrome associated with cerebral white matter damage and development of cerebral palsy (Yoon et al., 2003). Although the fetal membranes act as a barrier to cytokines produced in the decidua, both the chorion and the amnion are responsive to bacterial endotoxin and cytokine stimulation (Kent et al., 1994; Menon et al., 2004). Therefore, the contribution of the amnion to the cytokine pool in response to bacterial endotoxin and cytokines in infection and inflammation may play a major role in determining the onset of parturition and outcome of labour. By controlling the level of cytokines and chemokine stimulation during pregnancy, it may be possible to eliminate activation of downstream pathways leading to preterm delivery. Therefore, this has led to the investigation of the ability of *Lactobacillus rhamnosus* GR-1 to modulate the cytokine and chemokine production from the amnion.
Figure 1.3: Proposed pathway of infection and/or inflammation-mediated preterm birth

1.6 Prostaglandins

The family of biologically active lipid molecules consists of 20 carbon fatty acids that categorized into leukotrienes, lipoxins, and prostaglandins (Olson, 2003). Prostaglandins are produced by all cells of the body and act as local hormones initiating downstream cascades in a paracrine manner. Prostaglandin production and metabolizing pathways are well characterized and many of the enzymes involved are mediated by cytokines, bacterial products and endotoxins, growth factors and glucocorticoids that increase prostaglandin production in states of infection and/or inflammation. The biosynthesis of prostaglandins begins with the cleavage of the lipid bilayer membrane either directly by members of the phospholipase A2 family or indirectly through the actions of phospholipase C (Olson, 2003). As a result, arachidonic acid is freed from the phospholipid membrane into the cytosol where it is readily converted through varying enzymatic pathways to form lipoxins, leukotrienes, or prostaglandins. In early gestation, metabolism leading to lipoxygenase products is favoured; however, as term approaches metabolism is shifted towards production of prostaglandins dominating prior to the onset of labour (Figure 1.4, page 38; Mitchell et al., 1995).

1.6.1 Prostaglandin Production

1.6.1.1 Phospholipases

Prostaglandin production begins with the cleavage of arachidonic acid from phospholipid membranes by phospholipase enzymes. Amnion cell membranes are rich in arachidonate, therefore cleavage of amnion cell membranes are ideal residues for prostaglandin substrate (Curbelo et al., 1981). The family of phospholipase A2 enzymes consists of up to 15 isoforms. Type IV, cytosolic phospholipase A2 has been shown to have a prominent role in the amnion; however, many isoforms are also expressed in the fetal membranes (Myatt et al., 2010). Cytosolic phospholipase 2 translocates to the cell membrane in response to agonist stimulation, including the cytokines, IL-1 and IL-6 as well as bacterial endotoxins (Challis et al., 2002). Therefore, much like PGHS, the enzyme involved in the rate-limiting step in prostaglandin production, levels of phospholipase enzymes can be up-regulated in a state of infection and or inflammation in the amnion.
1.6.1.2 Prostaglandin H Synthase

Freed arachidonic acid is converted into PGH$_2$ by two subsequent reactions involving PGHS. The first reaction is a cyclooxygenase reaction that combines two free oxygen molecules with arachidonic acid to form PGG$_2$, a relatively unstable intermediate. A peroxidase reaction follows between PGG$_2$ and PGHS to form PGH$_2$. Two major forms of PGHS, PGHS-1 and PGHS-2, are present in the amnion, similar to most cell types including various gestational tissues throughout pregnancy (Hirst et al., 1995; Rose et al., 1990; Teixeira et al., 1994). PGHS enzymes commonly reside near the membrane and within lipid bodies in various cell types ideally situated for conversion of freed arachidonic acid substrate (Dvorak et al., 1994; Smith et al., 1981). PGHS-1 and PGHS-2 share considerable sequence homology and up to 60-65% cDNA sequence homology despite being the products of two separate genes (Blumenstein et al., 2000; Mitchell et al., 1995; Xu et al., 1995). Both enzymes are homodimers and have equal affinity and capacity to convert arachidonic acid to PGG$_2$ and subsequently PGH$_2$ with equal relative efficiency (Olson, 2003). The major difference in the role of the two enzymes is their differential expression and regulation. PGHS-2 is inducible as its promoter consists of many binding elements that can influence its expression pattern: TATA box, two NFκB, NF-IL6, GRE, CRE, and AP-2, SP-1, and ETS-1 binding sites (Inoue et al., 1995; Tazawa et al., 1994). The expression of PGHS-2 is regulated and altered by cytokines, oxytocin, mechanical stretch, glucocorticoids, bacterial endotoxins, growth factors, activators of protein kinase C and factors that increase intracellular calcium concentrations such as CRH (Bennett et al., 1987; Blumenstein et al., 2000; Economopoulos et al., 1996; Masferrer et al., 1992; Mohan et al., 2007; Molnar et al., 1999; Smieja et al., 1993; Zakar et al., 1994; Zakar et al., 1995). Due to the ability of PGHS-2 mRNA levels, protein expression and activity to be modulated by external stimuli, conversion of arachidonic acid to PGH$_2$ is a likely candidate for the rate-limiting step in prostaglandin production. Amnion PGHS-2 mRNA levels, protein expression and activity levels increase throughout gestation and further at term and with the onset of labour in an NFκB dependent manner (Fuentes et al., 1996; Gibb et al., 1996; Hirst et al., 1995; Johnson et al., 2006; Mijovic et al., 1999; Mitchell et al., 1995; Slater et al., 1998; Teixeira et al., 1994). TNFα, IL-1β and bacterial endotoxin increase PGHS-2 expression levels up to 80-fold compared to a 2 to 3 fold increase seen in PGHS-1 expression (Blumenstein et al., 2000; Mitchell et al., 1993; Pollard et al., 1993). Therefore, by altering PGHS-2, cytokines and bacterial products associated with
infection and/or inflammation during labour have an ability to largely affect amnion prostaglandin production.

1.6.1.3 Prostaglandin Synthases

The last step in prostaglandin production is performed by the prostaglandin synthase enzymes. PGH$_2$ is converted by a member of the prostaglandin synthase enzyme family to either prostaglandin E$_2$, F$_{2\alpha}$, D$_2$ and I$_2$. The major prostaglandin product formed in the amnion is prostaglandin E$_2$ and the minor product PGF$_{2\alpha}$ due to the enzymatic activity of prostaglandin E synthase (PGES) and prostaglandin F$_{2\alpha}$ synthase, respectively. Three isoforms of PGES are expressed in the amnion: microsomal (m)PGES$_1$, mPGES$_2$, and cytosolic (c)PGES also known as mPGES$_3$ (Ackerman et al., 2008). cPGES is constitutively expressed and its activity is thought to be coupled to PGHS-1 (Han et al., 2002; Myatt et al., 2010). cPGES is localized to the cytosol, in particular at lipid bodies of amnion epithelium as well as in fibroblasts whereas mPGES$_1$ is localized only to amnion epithelium membranes and lipid bodies (Meadows et al., 2003). mPGES$_1$ is the most catalytic efficient form of PGES above both other isoforms (Thoren et al., 2003). Expression of mPGES$_1$ is induced by various cytokines such as IL-1β, LPS and other pro-inflammatory stimuli (Forsberg et al., 2000; Jakobsson et al., 1999; Mancini et al., 2001). As well, mPGES$_1$ is coupled to expression of PGHS-2 and is thought to be regulated through NFκB transcriptional activity (Kudo et al., 1999; Thoren et al., 2000). The third isoform of PGES is mPGES$_2$ which is formed as a golgi-membrane bound protein that translocates to the cytosol to perform its enzymatic function (Murakami et al., 2003). mPGES$_2$ is coupled to both PGHS-1 and PGHS-2 and is constitutively expressed (Murakami et al., 2003). No change in either cPGES or mPGES$_2$ is seen in the fetal membranes at term or preterm with or without labour; however mPGES$_1$ can be induced by pro-inflammatory cytokines (Meadows et al., 2003). The exact influence alterations to PGES expression has on labour with and without infection is unknown (Premyslova et al., 2003).

1.6.1.4 15-Hydroxyprostaglandin Dehydrogenase

PGDH is a catabolic enzyme for PGE$_2$ and PGF$_{2\alpha}$ that plays an important role in minimizing the spread of prostaglandins produced in the amnion from reaching the other gestational tissues (Sangha et al., 1994). Unlike the amnion where expression is absent, chorion trophoblast cells express high levels of PGDH (Cheung et al., 1990). This enables the chorion to act as a
metabolic barrier to amnion produced prostaglandins from acting on the other gestational tissues. Chorionic PGDH activity decreases at term and in idiopathic preterm deliveries allowing amnion produced prostaglandins to influence the other intrauterine tissues (Sangha et al., 1994; Van Meir et al., 1996). Cytokines have been shown to decrease the expression of PGDH in the chorion. As well, inconsistencies of PGDH expression throughout the chorion can be induced during infection and inflammation (Cheung et al., 1990; Sangha et al., 1994; Van Meir et al., 1996; Van Meir et al., 1997). Therefore, changes in PGDH throughout the chorion may grant passageway of amnion produced prostaglandins access to the uterine myometrium and cervix ultimately leading to activation and ripening respectively and a subsequent increased risk of preterm delivery (Challis et al., 2002).
Figure 1.4: Effects of stimulants on the prostaglandin production pathway in the amnion.

Molecules that stimulate prostaglandin production are in blue font at the top of the diagram. Red font indicates enzymes involved in prostaglandin production. Hatched lines indicate metabolizing enzymatic activity and solid lines indicate synthesizing enzyme activity. The major product of the amnion is PGE2 with a minor product of PGF2α.
1.6.2 The Role of Amnion Produced Prostaglandins in Preterm Labour

At term, elevated amniotic fluid prostaglandins are paralleled with increased levels of prostaglandin producing enzymes and activity in amnion epithelial and fibroblast cells. Amnion produced prostaglandins play a role in fluid or ion balance as well as mediation of transmembrane ion flow (Challis et al., 2002; Saunders-Kirkwood et al., 1993). Although the amnion, chorion and decidua produce increasing amounts of prostaglandins throughout gestation, only the amnion and chorion show increased prostaglandin production prior to the onset of labour (Olson et al., 1983; Skinner et al., 1985). Increased prostaglandin levels initiate a set of events necessary for parturition onset and successful completion of labor including: myometrial activation, cervical ripening, up-regulation of MMPs leading to membrane rupture, fetal adaptations to labour including decreased breathing and movements, increases in fetal HPA axis activity and changes in uterine and placental blood flow (Kitterman, 1987; Rankin, 1976; Sastry et al., 1997; So, 1993; Thorburn, 1992). Elevated levels of prostaglandins can lead to preterm labour as is demonstrated by administration of prostaglandins in mammalian species that result in induction of labour or abortion at any stage of gestation (Embrey, 1971).

The two cell types of the amnion, epithelial and mesenchymal cells respond differently to stimuli when cultured separately in vitro. It is suggested that the two cell types of the amnion exert paracrine actions on one another to form a regulation loop for prostaglandin production (Whittle et al., 2001). Mesenchymal cells produced greater per cell quantities of prostaglandins; however since epithelial cells outnumber amnion fibroblast cells 10 000:1, the overall contribution of epithelial cells to the prostaglandin pool is greater (Whittle et al., 2000). Many cytokines including IL-1α, IL-1β, TNFα, IL-6, MIP-1α increase prostaglandin production from the amnion (Dudley et al., 1996; Furuta et al., 2000; Mitchell et al., 1991; Romero et al., 1989b; Romero et al., 1989c). Additionally, gram-negative bacteria, LPS and arachidonic acid increase prostaglandin production in the amnion (Reisenberger et al., 1998; Romero et al., 1988a; Romero et al., 1988b). In contrast to the typical anti-inflammatory properties of IL-10 across many tissues, the amnion shows increases prostaglandin production in response to IL-10 treatment (Economopoulos et al., 1996; Gibb et al., 1990; Mitchell et al., 2004; Potestio et al., 1988). As well, glucocorticoids stimulated prostaglandin production in amnion mesenchymal cells contrary to its well-established anti-inflammatory effects in many other tissues (Whittle et al., 2000).
In cultured human placenta trophoblast cells, the supernatant of *Lactobacillus rhamnosus* GR-1 modulates expression levels of enzymes involved in prostaglandin production. Pre-treatment of *Lactobacillus rhamnosus* GR-1 prevents LPS-induced PGHS-2 expression in placentae derived from pregnancies carrying a male fetus (Yeganegi et al., 2009). As well, in placentae derived from pregnancies carrying a female fetus, *Lactobacillus rhamnosus* GR-1 supernatant increases PGDH expression to a higher degree than in placentae derived from pregnancies carrying a male fetus (Yeganegi et al., 2009). Although the exact mechanism for the decreased expression of PGDH is unknown, however these results may give a molecular basis for the higher incidence of preterm labour that occurs in pregnancies carrying a male fetus compared to a female. Comparatively, another strain, *Lactobacillus rhamnosus* GG induces PGHS-2 expression in a time and dose-dependent manner in T84 colon epithelial cells (Korhonen et al., 2004).

Therefore, the effects of *Lactobacillus* species are dependent on the strain as well as the cell type and gender of the host tissue. Many stimuli associated with infection and/or inflammation are capable of stimulating prostaglandin production in the amnion. In addition, molecules that typically decrease prostaglandin production across tissues, such as cortisol, also up-regulate prostaglandin production in the amnion. The amnion has been identified as the main site of prostaglandin production at the end of gestation and the onset of labour (Challis et al., 1997). If the amnion is stimulated early in gestation by molecules associated with infection and/or inflammation, events normally associated with term labour may be activated leading to an increased risk of onset of preterm delivery.
1.7 **Summary**

Bacterial vaginosis, which is associated with increased risk of preterm labour by 40%, is characterized by the colonization of pathogenic bacteria in the vaginal tract and decreased levels of endogenous *Lactobacillus* species (Hillier et al., 1995). Bacterial endotoxins and bacterial products stimulate cytokine, chemokine and prostaglandin production that may activate downstream pathways leading to preterm labour (Figure 1.3, page 33). Interfering with the upstream stimulants of these pathways may allow the prevention of preterm delivery mediated by infection and/or inflammation. The particular interest of this thesis was on the immunomodulatory role of *Lactobacillus* species in the amnion. *Lactobacillus* species have been previously shown to modulate the inflammatory response in LPS-stimulated placental trophoblasts and mouse macrophages and are capable of colonization and restoration of the vaginal microbiota. However, the effect of lactobacilli on the amnion remains unknown. In order to help determine its ability to be a potential preventative treatment, this thesis examined the role of *Lactobacillus rhamnosus* GR-1 in regulating mediators of inflammation and/or infection associated with preterm birth release from the human amnion. In particular, the effects of *Lactobacillus* on cytokines, chemokines, prostaglandins and prostaglandin metabolizing enzymes in human amnion were investigated.
Chapter 2

Rationale, Hypothesis and Specific Aims
Chapter 2

Hypotheses, Rationale and Specific Aims

2.1 Overall Hypothesis

*Lactobacillus rhamnosus* GR-1 will prevent elevation of cytokines, chemokines and prostaglandins associated with infection and/or inflammation-mediated preterm birth from the human amnion.

2.1.1 Rationale

Infection-mediated preterm birth is associated with stimulation of an exaggerated inflammatory response in both the gestational tissues and recruited immune cells. Bacteria and bacterial products stimulate cytokines and chemokines from the gestational tissues. During chorioamnionitis and microbial invasion of the amniotic cavity, the cytokine pool in the amniotic fluid is characterized by increases in pro-inflammatory cytokines and chemokines. As many cytokines produced in the decidua are unable to cross the fetal membranes, the amnion is thought to be the prominent producer of elevated cytokines during an inflammatory response associated with infection. The amnion also produces prostaglandin E\(_2\) (PGE\(_2\)) that may exert paracrine actions on both the myometrium and cervix to cause activation and remodeling respectively. As well, PGE\(_2\) exerts paracrine effects on the fetal membranes resulting in collagen degradation and weakening. Probiotics, namely *Lactobacillus* species have known immunomodulatory roles in murine macrophages, monocytic cell lines and human placental trophoblasts which promote an anti-inflammatory cytokine profile and increased chemokine production. Additionally, *Lactobacillus* species modulate prostaglandin-metabolizing enzymes. Therefore, the effects of *Lactobacillus rhamnosus* GR-1 on cytokines/chemokines and prostaglandin production from the amnion was determined in these experiments.

Although numerous strains of lactobacilli have been shown to exert immunomodulatory effects, *Lactobacillus rhamnosus* GR-1 was used to treat amnion cells in the current study. Due to its ability to colonize and replenish the vaginal flora when administered orally and ability to alter the immune response in human placental trophoblasts and other epithelial cell types, *Lactobacillus rhamnosus* GR-1 was assessed in its effects to possibly exert similar effects in
human amnion cells. Although the exact route of orally administered probiotics to the vaginal microbiota is unknown, *Lactobacillus* species have been shown to be bile and acid resistant and have been suggested to migrate from the rectum to the vagina after travelling through the gastrointestinal tract (Reid et al, 2001b).

The intrauterine tissues during healthy pregnancies are sterile; therefore we chose to use treat amnion cells with the supernatant of *Lactobacillus rhamnosus* GR-1 as opposed to live or freeze dried bacteria. It is currently unknown whether endogenous lactobacilli are capable of interacting with the fetal membranes; however, recent research has identified that small protein molecules are capable of exerting the effects of other probiotics, including strains of *Lactobacillus*. It may be that a small molecule is more capable of accessing the amnion compared to live or heat killed bacteria.

During bacterial vaginosis, gram-negative bacteria, such as *Bacteroides* spp. and gram-positive/gram-variable bacteria, such as *Gardnerella vaginalis* and *Mycoplasma* spp. levels are elevated. Therefore, both LTA, a wall-component of gram-positive bacteria and LPS, a wall-component of gram-negative bacteria were used to stimulate amnion cells in this study. Importantly, *Lactobacillus rhamnosus* GR-1 is a gram-positive bacterium; therefore, LTA treatment acts as an internal control to separate the effects of *Lactobacillus rhamnosus* GR-1 metabolites present in the supernatant used from the effects of the characteristic gram-positive bacterial endotoxin, LTA.

2.2 Chapter 3: Effect of *Lactobacillus rhamnosus* GR-1 on cytokines and chemokines in human amnion cells at term

**Hypothesis:**

*Lactobacillus rhamnosus* GR-1 supernatant will ameliorate increases in LTA and/or LPS-stimulated pro-inflammatory cytokines and chemokines in human amnion cells in order to prevent activation of downstream pathways associated with infection and/or inflammation-mediated preterm birth

**Specific Aims:**

1. To determine the optimal dose and length of exposure to LTA and LPS on cytokine and chemokine release in medium collected from cultured human amnion cells at term.
2. To determine the effects of *Lactobacillus rhamnosus* GR-1 on the release of interleukin (IL)-6, IL-8 and tumor necrosis factor α (TNFα) in medium collected from LPS and LTA treated human amnion cells at term.

3. To determine the effects of *Lactobacillus rhamnosus* GR-1 on global cytokine and chemokine release in medium collected from LTA and LPS treated human amnion cells at term.

### 2.3 Chapter 4: Effect of *Lactobacillus rhamnosus* GR-1 on Prostaglandin Output and Prostaglandin Metabolizing Enzymes in Human Amnion Cells at Term

**Hypothesis:**

*Lactobacillus rhamnosus* GR-1 supernatant will ameliorate increases in LTA and/or LPS-stimulated prostaglandin production in human amnion cells in order to prevent propagation of downstream pathways associated with infection and/or inflammation -mediated preterm birth

**Specific Aims:**

1. To determine the effects of *Lactobacillus rhamnosus* GR-1 on prostaglandin E$_2$ output in LPS and LTA treated human amnion cells at term.

2. To determine expression profiles of prostaglandin metabolic and synthetic enzymes (Prostaglandin H Synthase-2, cytosolic Prostaglandin E Synthase, microsomal Prostaglandin E Synthase 1, microsomal Prostaglandin E Synthase 2) that may account for any changes in PGE$_2$ release caused by LTA, LPS and *Lactobacillus rhamnosus* GR-1 in human amnion cells at term.
Chapter 3

Effect of *Lactobacillus rhamnosus* GR-1 on Cytokines and Chemokines in Human Amnion Cells at Term
Chapter 3
Effect of *Lactobacillus rhamnosus* GR-1 on Cytokines and Chemokines in Human Amnion Cells at Term

3.1 Introduction

Approximately half of all preterm births are idiopathic. However, 30% of preterm births are associated with infection and/or an activated inflammatory process (Goldenberg et al., 2008; Hillier et al., 1995). This inflammatory process is characterized by bacterial endotoxin stimulation of amnion cytokine and chemokine production. Despite ongoing research, current efforts at prevention are largely ineffective and consequentially, preterm birth remains a major obstetrical concern.

The presence of bacteria triggers a response by the body’s innate immune system which is characterized by increased levels of cytokines and chemokines. During pregnancy, intrauterine infections are associated with increased levels of cytokines, including IL-1β, TNFα, IL-6, IL-8, RANTES, and MCP-1 in the amniotic fluid (Andrews et al., 1995; Coultrip et al., 1994; Esplin et al., 2005; Hillier et al., 1993; Romero et al., 1989c). Many cytokines produced in the decidua are not capable of passing through the membranes. Therefore, the amnion and chorion play a major role in contributing to the pool of cytokines and chemokines in the amniotic fluid. Elevated levels of cytokines and chemokines recruit immune cells and increase prostaglandin production in the amnion, chorion, and decidua (Challis et al., 1997). The net result is activation of downstream pathways leading to increased metalloproteinase expression and subsequent cervical ripening and membrane weakening as well as myometrial activation.

Antibiotics, such as metronidazole and clindamycin have been used to prevent infection and/or inflammation-associated preterm birth. However, in some cases antibiotics may increase the incidence of preterm labour (Andrews et al., 2003; Okun et al., 2005). Probiotics, defined as, “live microorganisms which when administered in adequate amounts confer a health benefit on the host,” are candidates for prevention of preterm labour (FAO and WHO, 2001; Reid, 1999). Bacterial vaginosis is present in approximately 20% of pregnant women and increases the risk of undergoing preterm birth by 40% (Hillier et al., 1995). The hallmark characteristic of bacterial vaginosis is a decline in endogenous *Lactobacillus* species and a rise in pathogenic bacteria.
resulting in an alteration to the vaginal microbiota. Due to the ability of *Lactobacillus rhamnosus* GR-1 to colonize and restore the vaginal microbiota and exert immunomodulatory effects in murine macrophages and placental trophoblasts, probiotics stand as a candidate preventative treatment for infection and/or inflammation-mediated preterm birth (Reid et al., 2003; Yeganegi et al., 2010; Yeganegi et al., 2011).

The effects of *Lactobacillus rhamnosus* GR-1 on the human amnion have not been previously investigated. The amnion is responsive to both bacterial products and cytokines and therefore plays an important role during infection and/or inflammation. Therefore, *Lactobacillus rhamnosus* GR-1 may enable the amnion to resist bacterial endotoxin stimulation of cytokine and chemokine production that occurs in infection and/or inflammation-mediated preterm birth.

### 3.2 Methodology

#### 3.2.1 Placenta Collection

All studies were approved by the Faculty of Medicine, University of Toronto and Mount Sinai Hospital (Toronto, Canada) ethics review boards in accordance with the Canadian Tri-Council Policy Statements on Human Ethics Reviews (institutional review board #04-0018-U). Placentae were collected and informed consent was obtained by the Biobank program of the Research Centre for Woman`s and Infant`s Health at Mount Sinai Hospital (http://biobank.lunenfeld.ca) from women undergoing elective caesarean sections at term (>37 weeks) at Mount Sinai Hospital (Toronto, Canada). Inclusion criteria included: 1) No complications during pregnancy, 2) No abnormal antenatal test results (including laboratory results and ultrasounds), 3) An unremarkable maternal medical history, free of any conditions that may significantly impact placental pathology (i.e. renal disease, diabetes, hypertension, cardiovascular disease), 4) No clinical signs of infection or labour 5) Free from any infections during pregnancy including HIV, Hepatitis B and group B streptococcus. Indications for delivery by caesarean section included: abnormal presentation of the fetus, cephalopelvic disproportion, or previous caesarean sections. Patients were not screened for bacterial vaginosis.

#### 3.2.2 Mixed Amnion Epithelial and Mesenchymal Cell Culture

Amnion membranes were stripped from the underlying choriodedical layer and washed in saline until the majority of blood was removed. The amnion was suspended in forceps and cut in
strips by cutting towards the forceps at 1.5 cm intervals. The amnion was then digested for 45 mins in Dulbecco Modified Eagle Medium (DMEM; GIBCO Invitrogen; Burlington, Ontario, Canada) + 0.1% trypsin (GIBCO Invitrogen) to obtain amnion epithelial cells in a 37°C incubator. Tissue was then filtered using a 0.8 nm gauze filter. Filtered DMEM was treated with filtered newborn calf serum (NCS; Wisent Incorporated; St. Bruno, Quebec, Canada) to inhibit trypsin activity. Tissue was cut into fragments of approximately 1.0 cm in length and further digested for 45 mins in DMEM + 0.1% trypsin; filtered and treated with filtered NCS to inhibit trypsin activity. Epithelial cells from the first and second digestion were pelleted by centrifugation at 1250 g for 5 mins. Excess medium was discarded and epithelial cells were re-suspended in culture medium DMEM-F12 (GIBCO Invitrogen) + 10% fetal bovine serum (FBS; Wisent Incorporated) + 1% antibiotics. The remaining tissue was cut into approximately 0.5 cm pieces and digested for 1 hr in DMEM + 0.1% collagenase (Roche Diagnostics; Mannheim Germany) in order to obtain amnion mesenchymal cells. After digestion, mesenchymal cells were centrifuged for 10 mins at 1500 g. Total mesenchymal cells obtained were re-suspended in culture medium and mixed with total epithelial cells from previous digestions, despite in vivo proportions. Mixed amnion cells were counted using a Trypan Blue exclusion assay to ensure greater than 98% viability. 20 µl of mixed amnion cell culture was diluted in 180 µl of culture medium. 40 µl of diluted cultured cells were mixed with 40 µl Trypan Blue. Cells were then viewed under 40x magnification and counted on a hemocytometre. Blue cells representing cell death were assessed in the viewing field and cultures with dispersed cells displaying greater than 2% cell death were discarded. Mixed amnion epithelial and mesenchymal cells were plated to a density of 0.8x10^6 cells per well in a 24-well culture plate.

3.2.3 Lactobacilli Preparation

*Lactobacillus rhamnosus* GR-1 was grown anaerobically for 48 hours to reach the stationary growth phase in de Man, Rogosa, and Sharpe media (MRS) by colleagues in Dr. S.O. Kim’s laboratory at the University of Western Ontario. The culture medium was then collected and centrifuged at 6000 g for 10 minutes at 4°C. Residual bacteria were removed by filtration of the supernatant through a 0.22 µm pore size filter. Supernatant was aliquoted and frozen at -80°C until used.
3.2.4 Treatment Protocol

Cells were cultured for 48 hr at 37°C in 5% CO₂ and 95% room air and washed with room temperature phosphate buffered saline (PBS) without calcium and magnesium. Culture medium was replaced and cell confluency was reached after an additional 48 hr incubation. Endotoxin dose, time and *Lactobacillus rhamnosus* GR-1 supernatant optimization experiments were performed. Both LTA (Invivogen; San Diego, California, United States of America) and LPS (Sigma-Aldrich; St Louis, Missouri, United States of America) at doses 0.1, 1, 10, 100, and 1000 ng/ml were tested for a time period of 12 hr after starvation in serum-free conditions. Medium was collected for LTA (10 ng/ml) and LPS (100 ng/ml) at time 4 hr, 8 hr, 12 hr and 24 hr post-treatment. The effects of various *Lactobacillus rhamnosus* GR-1 supernatant dilutions, 1:10, 1:20, 1:50, and 1:100 were assessed at 12 hr post-treatment. After conditions were optimized, cell cultures were serum starved for 12 hr. *Lactobacillus rhamnosus* GR-1 supernatant was thawed and diluted in DMEM at a ratio of 1:20 and incubated for 12 hr. Excess supernatant was discarded after each experiment. Fresh batches of supernatant were received every two months to maintain similar levels of enzymatic activity across experiments. Cells were then treated with LTA (10 ng/ml) or LPS (100 ng/ml) for 12 hr in serum-free DMEM and GR-1 treatment was washed off and discontinued prior to the addition of the endotoxins. Aliquots of medium (100 µl) were collected and frozen at -80°C (Figure 3.1, page 60).

3.2.5 Lactate Dehydrogenase Assay

Cultured amnion cells were plated to a density of 0.8x10⁶ cells per well in a 24-well culture plate. After incubation, cells were treated with 1% Triton X (Sangon Limited; Mississauga, Ontario, Canada), control, GR-1 (1:20 dilution), LTA (10 ng/ml), or LPS (100 ng/ml). Medium was collected after a 12 hr incubation period and 100 µl was transferred into an optically clear 96-well plate. Reaction mixture was prepared to a ratio of catalyst solution (BioVision; Mountain View, California, United States of America) 1:4 to dye solution (BioVision) and 100 µl was immediately added to corresponding wells. The plate was incubated in the dark at room temperature for 30 mins and then was read using a spectrophotometer at 495 nm with a reference wavelength of 600 nm. Lactate dehydrogenase (LDH) was measured in collected medium from each treatment group in relation to Triton X treatment groups representing one hundred percent LDH release.
3.2.6  Cytokine and Chemokine Measurement

3.2.6.1  Enzyme-Linked ImmunoSorbent Assay

Levels of IL-1β, IL-4, IL-6, IL-10, TNFα, and GM-CSF were measured in culture media in commercially available Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (GM-CSF from R and D Systems, Burlington, Ontario, Canada; All other cytokines from EBioscience; San Diego, California, United States of America). A 96-well plate was coated with 100 µl/well capture antibody diluted in coating buffer to a dilution of 1:250. The plates were incubated overnight at 4°C and were then washed with 300 µl of wash buffer (PBS+0.5% Tween 20) 5 times for 1 minute incubation periods each. Between each wash, plates were blotted on absorbent paper. Assay diluent diluted at a ratio of 1:1 in Millipore filtered water was added to each well at a volume of 200 µl and incubated at room temperature for 1 hr. After 5 washes, prepared standard sets in 1:2 dilutions were added to the plate at a volume of 100 µl/well. Samples were added in 1:10 dilutions for IL-6 and IL-8 kits and 1:1 dilution for IL-1β, IL-4, GM-CSF and TNFα kits at a volume of 100 µl to each well. Plates were incubated overnight at 4°C after which the plates were washed an additional 5 times. 100 µl to each well of detection antibody diluted to 1:250 in assay diluent was added followed by an incubation of 1 hr at room temperature. Plates were washed 5 times and avidin-horseradish peroxidase diluted to 1:250 in assay diluent was added 100 µl to each well and incubated in the dark at room temperature for 30 mins. Plates were washed 7 times before addition of 100 µl of substrate solution to each well for 15 mins at room temperature. 50 µl of stop solution (H₂SO₄) was added to each well before reading the plate in a spectrophotometer at 450 nm using 570 nm as a reference wavelength. The minimum detection limits for IL-1β, IL-4, IL-6, IL-8, IL-10, GM-CSF, and TNFα kits were 4.0 pg/ml, 2.0 pg/ml, 2.0 pg/ml, 4.0 pg/ml, 4.0 pg/ml, 7.8 pg/ml, and 2.0 pg/ml respectively.

3.2.6.2  Bioplex Pro Human 27-Cytokine/Chemokine Assay

A Bioplex Pro Human 27-Cytokine/Chemokine Assay (BioRad Laboratories Incorporated; Mississauga, Ontario, Canada) was performed to measure the levels of the following cytokines and chemokines with minimum detection limits as indicated: Platelet-Derived Growth Factor (1.33 pg/ml), IL-1ra (2.96 pg/ml), IL-4 (0.21 pg/ml), IL-6 (1.54 pg/ml), IL-8 (1.53 pg/ml), IL-9 (1.58 pg/ml), IL-10 (1.48 pg/ml), IL-12 (2.19 pg/ml), IL-15 (1.75 pg/ml), IL-17 (1.36 pg/ml), Eotaxin (1.51 pg/ml), Fibroblast growth factor (0.66 pg/ml), G-CSF (1.76 pg/ml), GM-CSF (0.84...
pg/ml), IFNγ (1.44 pg/ml), IFN-inducible Protein (2.51 pg/ml), MCP-1 (1.22 pg/ml), MIP-1α (1.02 pg/ml), MIP-1β (0.92 pg/ml), RANTES (1.20 pg/ml), TNFα (5.72 pg/ml), Vascular Endothelial Growth Factor (1.93 pg/ml), IL-1β (1.92 pg/ml), IL-2 (1.1 pg/ml), IL-5 (1.79 pg/ml), IL-7 (1.67 pg/ml), and IL-13 (2.2 pg/ml). Samples were prepared in a total volume of 100 µl with 0.5% bovine serum albumin (BSA; Sigma-Aldrich). After addition of BSA, samples were vortexed and centrifuged at 1000 g for 10 mins and stored on ice. Stock standard solution was reconstituted and 96 µl were added to 183 µl culture medium to a total volume of 275 µl. 128 µl of each the reconstituted standard were mixed with 72 µl of standard diluent to prepare standard 1. Serial dilutions of 1:2 were prepared to create standards 2-8. Next, 5 µl of beads were added to each well in a 50 µl of assay buffer. Excess fluid was aspirated and the plate was washed 2 times with wash buffer. Standards and samples were mixed on a vortex shaker, added to wells at a volume of 50 µl and incubated on a shaker covered with foil at room temperature at 1000 g for 30 mins. The plate was washed 3 times and 25 µl of detection antibody, prepared to a 1:10 dilution in detection antibody diluent, were added to each well. The plate was then incubated on a shaker covered with foil at room temperature at 1000 g for 30 mins. The plate was washed 3 times and 50 µl streptavidin-PE, prepared to a 1:100 dilution in assay buffer, were added to each well. The plate was incubated on a shaker with foil for 10 mins, washed 3 times and 125 µl of assay buffer were added to each well. The plate was shaken at 1 100 rpm for 30 secs before it was read in a Bio-Plex reader according to manufacturer`s software.

3.2.7 Statistical Analysis

Data are presented as mean values ± standard error of the mean (SEM) where each n represents individual amnion cultures from placentas received from patients meeting inclusion and exclusion criteria as state previously. Statistical significance between groups was determined using a One-way Anova statistical test if data was normally distributed followed by Student-Newman-Keuls post-hoc analysis or using a non-parametric Kruskal-Wallis statistical analysis if data did not have normality or equal variation using STAT32 version 2.0 statistical software. Statistical significance was accepted at p<0.05.

3.3 Results

There were no significant differences in measured LDH levels between Lactobacillus rhamnosus GR-1 (1:20) dilution, LTA (10 ng/ml) and LPS (100 ng/ml) and control (DMEM only; Figure
3.2, page 61). Triton X treatment, representing maximal cell death confirmed the validity of the LDH assay (n=5). Therefore, treatments do not cause significant amounts of amnion cell death.

After 12 hr stimulation, all subsequent treatments were performed in serum-free DMEM. Previous studies have shown that the presence of serum in medium increased basal levels of prostaglandin output in cultured human amnion cells (Whittle et al., 1994). Thereby eliminating the presence of serum, any confounding stimulants of either cytokines or prostaglandins was prevented.

Throughout experiments in this study, amnion cells were treated with an LTA dose of 10 ng/ml or an LPS dose of 100 ng/ml and medium was collected after 12 hours incubation with or without pre-treatment of Lactobacillus rhamnosus GR-1 supernatant diluted to a ratio of 1:20 with DMEM. These values were chosen based on IL-6 and IL-8 time course and dose optimization studies. LPS increased levels of IL-6 to maximal stimulation at 100 ng/ml where IL-8 peaked at a dose of 10 ng/ml. The concentration of IL-8 was significantly increased by an LPS dose of 100 ng/ml compared to control (Figure 3.3, page 62). Therefore, an LPS dose of 100 ng/ml was used for subsequent experiments. IL-6 concentrations showed two peaks at an LTA dose of 10 ng/ml and 1000 ng/ml. However, in contrast, IL-8 concentrations appeared to peak at an LTA dose of 10 ng/ml which then decreased with higher doses of endotoxin (Figure 3.3, page 63). Therefore, based on preliminary data, a dose of 10 ng/ml was used for LTA treatment.

To assess time course response, medium was collected and analyzed 4, 8, 12 and 24 hours after LTA (10 ng/ml) or LPS (100 ng/ml) treatment (Figure 3.4, page 63). At 12 hr, both IL-6 and IL-8 showed significant increases compared to 4 hr control by LPS stimulation. LTA also showed increased IL-6 and IL-8 at 12 hr compared to 4 hr and 8 hr control. Although LTA significantly increased IL-6 at 24 hr and LPS increased IL-8 at 24 hr compared to 4 hr and 8 hr controls, the 24 hr control levels of both cytokines appear to be elevated above the other control groups. Therefore, a time point of 12 hr was chosen due to the elevated basal cytokine concentrations observed at 24 hr and to the significantly increase in both IL-6 and IL-8 that was stimulated by LTA and LPS. Additionally, numerous cytokines and chemokines showed peak stimulation at 12 hrs as assessed by Bioplex analysis including: MCP-1, RANTES, and IFNγ (data not shown).
Based on analysis of medium collected from amnion cells treated with various dilutions of *Lactobacillus rhamnosus* GR-1 supernatant, the greatest differences in IL-6 concentration compared to control were observed at a *Lactobacillus rhamnosus* GR-1 supernatant dilution of 1:20 (Figure 3.5, page 64). *Lactobacillus rhamnosus* GR-1 significantly increased IL-8 concentration compared to control, 1:50 and 1:100 treatments. Therefore, we chose a 1:20 dilution for all experiments. The same dilution of supernatant, 1:20 was used in previous studies in studies on primary cultures of placental trophoblast and mouse macrophages (Kim et al., 2006; Yeganegi et al., 2009; Yeganegi et al., 2010; Yeganegi et al., 2011). The lack of response for a 1:10 dilution make indicate a possible dose-dependency or threshold level required for the unknown metabolite or metabolites in *Lactobacillus rhamnosus* GR-1 supernatant to exert its effects on the unknown receptor present on the amnion. In addition, the bell curve shape obtained suggests that the unknown signalling molecule present in *Lactobacillus rhamnosus* GR-1 supernatant requires optimal dosage to exert its effects on target tissues. Competition may arise if the concentration of this molecule is too high or homodimerization may occur preventing interaction with the appropriate receptors. However, due to the unknown identity of the molecule responsible for signalling in probiotic strains, these suggestions are merely speculation.

Two different methods were used to analyze cytokines and chemokines in these studies. To ensure results were comparable between both methodologies, the medium collected from both a pregnancy carrying a male fetus and one carrying a female fetus whose levels, as previously measured by ELISA, where closest to the preliminary mean were assessed once again in ELISA and simultaneously in a Bioplex assay for IL-6 (Figure 3.6, page 65). Measured IL-6 levels were highly correlated between the two methodologies ($R^2$ value = 0.8728).

Both LTA (10 ng/ml) and LPS (100 ng/ml) caused statistically significant increases in TNFα concentrations in cultured amnion cell medium compared to control (Figure 3.7, page 66). There was no significant change in TNFα with treatment of GR-1 (1:20) compared to control. The increase in TNFα with either LPS or LTA treatment was attenuated when cultured amnion cells were pre-treated with GR-1 in GR-1+LTA and GR-1+LPS treatment groups. No significant changes were observed with other treatment groups. IL-1β (N=8), IL-10 (N=8) and IL-4 (N=6) levels were undetectable across all treatment groups with lowest detectable limits (data not shown).
Both LTA (10 ng/ml) and LPS (100 ng/ml) caused significant increases in IL-6 concentrations in cultured amnion cell medium compared to control (Figure 3.8, page 67). GR-1 (1:20) caused a significant increase in IL-6 concentration compared to control. GR-1+LTA and GR-1+LPS treatments caused a significant increase in IL-6 concentration compared to control. No other significant changes were observed across all other treatment groups.

Both LTA (10 ng/ml) and LPS (100 ng/ml) caused significant increases in IL-8 concentrations in cultured amnion cell medium compared to control (Figure 3.9A, page 68). GR-1 (1:20) caused a significant increase compared to control. GR-1+LTA and GR-1+LPS treatments caused a significant increase compared to control group. RANTES concentrations were increased by GR-1 compared to control (Figure 3.9B, page 68). As well, GR-1+LTA significantly increased RANTES compared to control. GR-1+ LPS treatment significantly increased RANTES compared to control. No other significant changes were observed between treatment groups. MCP-1 was increased by GR-1 compared to control (Figure 3.9C, page 68). GR-1+LTA significantly increased MCP-1 compared to control, LTA and LPS alone. As well, GR-1+LPS treatment showed significant increases of MCP-1 compared to control, LTA and LPS alone. No other significant changes were observed between treatment groups.

There was no significant change in IFNγ concentrations in medium collected from cultured human amnion cells with either LTA (10 ng/ml) or LPS (100 ng/ml; Figure 3.10A, page 69). GR-1 (1:20) significantly increased IFNγ concentration compared to control, LTA and LPS alone. GR-1+LTA significantly increased IFNγ concentration compared to control, LTA and LPS alone. GR-1+LPS significantly increased IFNγ concentration compared to control, LTA and LPS alone. Neither GR-1 nor LTA and LPS caused significant increases in GM-CSF concentration compared to control (Figure 3.10B, page 69). GR-1+LTA significantly increased GM-CSF concentration compared to control, GR-1 and LTA alone. There was no significant change in GM-CSF concentration with either LPS or GR-1+LPS treatments.

All data were additionally analyzed according to the gender of the fetus carried in the pregnancy from which tissue was received. For each cytokine measured, the numbers for tissues derived from a pregnancy carrying a female fetus versus a male fetus were as follows: TNFα (2 female: 3 male), IL-6 (4 female: 9 female), IL-8 (5 female: 6 female), RANTES (3 female: 5 male), MCP-1 (3 female: 5 male), IFNγ (4 female: 6 male), and GM-CSF (3 female: 5 male). No obvious
trends were observed based on the gender of the fetus carried in pregnancies from which amnion tissue was derived (data not shown).
3.4 Comment

The current study focused on the effects of *Lactobacillus rhamnosus* GR-1 on LTA and LPS stimulated human amnion cells. In accordance with previous results, we have shown that *Lactobacillus rhamnosus* GR-1 attenuated LTA and LPS-stimulated TNFα release from the amnion similar to experiments performed on human placental trophoblasts and murine macrophages (Yeganegi et al., 2010; Kim et al., 2006). Additionally, we demonstrated the ability of *Lactobacillus rhamnosus* GR-1 to increase chemokine production, namely IL-8, MCP-1 and RANTES as well as cytokines capable of activating recruited immune cells, IFNγ and GM-CSF. Together, these results suggest that *Lactobacillus rhamnosus* GR-1 promotes a strong chemotactic response in the amnion while minimizing the potential for an exaggerated pro-inflammatory immune response to enable the amnion to defend against bacterial endotoxin entrance into the fetal environment.

TNFα is a member of the pro-inflammatory triad alongside IL-1β and IL-6. As expected in accordance with previous literature, IL-1β was not found above detectable limits in our culture system. However, *Lactobacillus rhamnosus* GR-1 attenuated both LTA and LPS stimulated TNFα production and increased IL-6 production to a similar level of LTA and LPS alone. TNFα is only detectable in the amniotic fluid in the presence of an infection and/or inflammatory response and is therefore indicative of an underlying infection (Romero et al., 1989c). The influence of TNFα on the gestational tissues results in the activation of downstream pathways leading to infection and/or inflammation-mediated preterm delivery. Alongside IL-1β, TNFα elevates prostaglandin production in the gestational tissues leading to metalloproteinase stimulation and subsequent cervical ripening and fetal weakening as collagen is degraded as well as myometrial activation (Blumenstein et al., 2000; Bry et al., 1992; Pollard et al., 1993; Romero et al., 1989b; Romero et al., 1989c). These events normally occur in parturition at term and early activation by uterotonins, such as prostaglandins can lead to preterm delivery. By decreasing the release of LTA and LPS-stimulated TNFα from the human amnion, *Lactobacillus rhamnosus* GR-1 may significantly interfere with activation of infection and/or inflammation-mediated processes associated with preterm labour in the amnion.

The amnion responds to *Lactobacillus rhamnosus* GR-1 with increased release of IL-6, IFNγ and GM-CSF prior to LTA and LPS stimulation. IL-6, IFNγ, and GM-CSF all mediate the extent of
the pro-inflammatory response initiated by TNFα and IL-1β. These cytokines increase the production of the antagonists to TNFα and IL-1β, namely the soluble TNFα receptor and IL-1 receptor antagonist (Aderka et al., 1989; Schindler et al., 1990; Tilg et al., 1994; Xing et al., 1998). The soluble TNFα receptor binds and neutralizes TNFα. Similarly, IL-1 receptor antagonist binds to IL-1 receptor preventing attachment of its agonist, IL-1β. Therefore, Lactobacillus rhamnosus GR-1 may indirectly attenuate the effects of TNFα and IL-1β by increasing cytokines that may stimulate the production of pro-inflammatory cytokine antagonists.

The fetal membranes are situated between the amniotic fluid and the maternal gestational tissue. As such, they act as a defensive barrier for pathogens, bacterial products and endotoxin from reaching the fetal environment. We have demonstrated that Lactobacillus rhamnosus GR-1 stimulates a cytokine profile in the human amnion that may be capable of recruiting and activating immune cells to the fetal membranes. Immune cells of monocyte and neutrophil origin are localized to both the amnion and the chorion and neutrophilia in these tissues increases at term (Witkin et al., 2011). Our studies show that Lactobacillus rhamnosus GR-1 increases chemokines of both the CXC and CC family, namely IL-8 and MCP-1 and RANTES. IL-8 is responsible for the recruitment and activation of neutrophils whereas MCP-1 and RANTES play a role in the chemotaxis of monocytes. In addition, Lactobacillus rhamnosus GR-1 enhanced MCP-1 production when challenged with LTA and LPS compared to endotoxin stimulation alone. Therefore, Lactobacillus rhamnosus GR-1 may permit chemotaxis of immune cells, especially monocytes to the amnion to allow for increased defense against bacterial endotoxin.

The activation state of monocytes/macrophages is dependent on the immunological signals present in the microenvironment to which they are recruited. Although classically defined as taking either an M1 or an M2 phenotypic state of activation, recent research has shown that the activity of the macrophage is much more continuous. The M1 phenotype is characterized by strong pro-inflammatory properties that encourage potent bacterial death and clearance (Mehta et al., 2004; Sierra-Filardi et al., 2011). In comparison, the M2 phenotype encourages attenuated pro-inflammatory responses and elevated anti-inflammatory responses (Mehta et al., 2004). In this study Lactobacillus rhamnosus GR-1 increased the production of IFNγ and when pre-treated prior to LTA and LPS stimulation, increased both IFNγ and GM-CSF release from amnion cells.
In conjunction with TNFα, IFNγ and GM-CSF promote the activation of macrophages to an M1 phenotype. However, *Lactobacillus rhamnosus* GR-1 attenuated LTA and LPS-stimulated TNFα production in amnion cells. When stimulated with IFNγ in the absence of TNFα, immune cells are activated to a phenotype characterized by its ability to phagocytose and produce antimicrobial products with a less severe pro-inflammatory response (Mosser et al., 2008). Therefore, *Lactobacillus rhamnosus* GR-1 promotes the amnion to produce cytokines capable of activating immune cells to defend against pathogenic threats while minimizing the associated pro-inflammatory response that may be deleterious to the prolongation of gestation.

In summary, *Lactobacillus rhamnosus* GR-1 increases the ability of the amnion to defend against bacteria and bacterial products while minimizing the extent of the accompanying pro-inflammatory response. However, caution must be taken in interpretation of these results for an exaggerated immune cell response may also be deleterious to the prolongation of gestation if not restricted. Chorioamnionitis is associated with preterm delivery that is characterized by increased neutrophilia in the membranes. Therefore, further studies are necessary to determine whether or not the regulated activation of monocytes/macrophages provided by cytokine and chemokines up-regulated by *Lactobacillus rhamnosus* GR-1 in the amnion is conducive to defense against pathogens as well as the prolongation of gestation. In addition, IL-6 has been shown to stimulate prostaglandin production in the amnion (Kent et al., 1993; Mitchell et al., 1991). IL-6 is also elevated in conjunction with chorioamnionitis and at term in amniotic fluid (Imseis et al., 1997). As mentioned previously, prostaglandin elevation is associated with infection and/or inflammation-mediated preterm birth. *Lactobacillus rhamnosus* GR-1 increases IL-6 to a level comparable to LTA and LPS stimulation alone. Therefore, further experiments are necessary to determine if a controlled immune cell response and IL-6 stimulation of prostaglandin production is deleterious.
Primary amnion cultures were grown for 48 hours and then washed. After another 48 hour when cells reached confluency, cultures were serum starved for 12 hours. Cells were pre-treated with *Lactobacillus rhamnosus* GR-1 supernatant (GR-1) for 12 hours. After 12 hours of LTA or LPS treatment, medium was collected.
Lactate dehydrogenase measured in the media of cultured human amnion cells treated with control (DMEM), *Lactobacillus rhamnosus* GR-1 supernatant (GR-1, 1:20 dilution), lipoteichoic acid (LTA, 10 ng/ml), and lipopolysaccharide (LPS, 100 ng/ml). Results are expressed as mean values ± SEM relative to LDH release by Triton X-treated cell medium. Statistical significance between groups is denoted with a different letter as assessed by One-way Anova followed by a Student-Newman-Keuls post-hoc analysis (N=5, p<0.05).

**Figure 3.2: The effect of treatments on amnion cell toxicity**
Figure 3.3: Lipoteichoic and lipopolysaccharide dose optimization

(A) IL-6 concentration (pg/ml; N=4, NS) and (B) IL-8 concentration (pg/ml; N=7, p<0.05) in media from amnion cell cultures after treatment with control (DMEM), LTA or LPS doses of 0.1, 1, 10, 100, and 1000 ng/ml for 12 hours. Results are expressed as mean values ± SEM. Statistical significance within groups is denoted by different letters as assessed by Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis. Circled points show results at dosages chosen for subsequent experiments.
Figure 3.4: Time course responses of Interleukin-6 and Interleukin-8

Histogram displaying (A) IL-6 concentration (pg/ml) and (B) IL-8 (pg/ml) in media from amnion cell cultures after treatment with control (DMEM), LTA (10 ng/ml) or LPS (100 ng/ml) for periods of 4, 8, 12, and 24 hours. Results are expressed as mean values ± SEM. Significance was determined using a Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis. * denotes significance to 4h control, + denotes significance to 8h control, ** denotes significance to 12h control, ^ denotes significance to 4h LTA (N=6, p<0.05).
Figure 3.5: *Lactobacillus rhamnosus* GR-1 dilution optimization

(A) IL-6 concentration (pg/ml) and (B) IL-8 concentration (pg/ml) in media from amnion cell cultures after treatment with control (DMEM), *Lactobacillus rhamnosus* GR-1 supernatant (GR-1; 1:10, 1:20, 1:50, 1:100 dilutions). Results are expressed as mean values ± SEM. Statistical significance within groups is denoted with a different letter as assessed by Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis (N=3, p<0.05).
Figure 3.6: Correlation between ELISA and Bioplex assay methodologies

Correlation between ELISA measurements and Bioplex measurements for Interleukin-6 in human amnion cultures. (n=2, $R^2=0.8728$).
Figure 3.7: The effects of *Lactobacillus rhamnosus* GR-1 on Tumor Necrosis Factor - α concentration in human amnion cells

Histogram showing TNFα (pg/ml) concentrations in media from human amnion cell cultures. Control (DMEM), GR-1 (1:20), LTA (10 ng/ml), LPS (100 ng/ml). Results are expressed as mean values ± SEM. Statistical significance between groups is denoted with a different letter as assessed by One-way Anova followed by a Student-Newman-Keuls post-hoc analysis (N=5, p<0.05).
Figure 3.8: The effects of *Lactobacillus rhamnosus* GR-1 on Interleukin-6 concentration in human amnion cells

Histogram showing IL-6 (pg/ml) concentrations in media from human amnion cell cultures. Control (DMEM), GR-1 (1:20), LTA (10 ng/ml), LPS (100 ng/ml). Results are expressed as mean values ± SEM. Statistical significance between groups is denoted with a different letter as assessed by One-way Anova followed by a Student-Newman-Keuls post-hoc analysis (N=13, p<0.05).
Figure 3.9: The effect of *Lactobacillus rhamnosus* GR-1 on chemokine concentrations in human amnion cells

Histograms showing (A) IL-8 (pg/ml) (B) RANTES (pg/ml) and (C) MCP-1 (pg/ml) concentrations in media from human amnion cell cultures. Control (DMEM), GR-1 (1:20), LTA (10 ng/ml), LPS (100 ng/ml). Results are expressed as mean values ± SEM. Statistical significance between groups is denoted with a different letter as assessed by One-way Anova followed by a Student-Newman-Keuls post-hoc analysis (N=11 for IL-8, N=8 for RANTES, N=8 for MCP-1, p<0.05).
Figure 3.10: The effects of *Lactobacillus rhamnosus* GR-1 on immune cell activating cytokines

Histograms showing (A) IFNγ (pg/ml) and (B) GM-CSF (pg/ml) concentrations in media from human amnion cell cultures. Control (DMEM), GR-1 (1:20), LTA (10 ng/ml), LPS (100 ng/ml). Results are expressed as mean values ± SEM. Statistical significance between groups is denoted with a different letter as assessed by One-way Anova followed by a Student-Newman-Keuls post-hoc analysis (N=10, for IFNγ, N=8 for GM-CSF, p<0.05).
Chapter 4

Effect of Lactobacillus rhamnosus GR-1 on Prostaglandin Output and Prostaglandin Metabolizing Enzymes in Human Amnion Cells at Term
Chapter 4

Effect of *Lactobacillus rhamnosus* GR-1 on Prostaglandin Output and Prostaglandin Metabolizing Enzymes in Human Amnion Cells at Term

4.1 Introduction

Preterm delivery is a major obstetrical concern as it accounts for up to 80% of all neonate morbidities and mortalities. Its incidence has been rising over the past few decades and over 50% of all preterm births remain idiopathic despite advances in understanding its multifactorial etiology. It is estimated that approximately 30% of all pre-term births are due to an underlying infection and/or inflammation; however, the prevention of preterm birth by antibiotic treatment is largely ineffective and in some cases increase its risk (Andrews et al., 2003; Okun et al., 2005).

Bacterial vaginosis is an alteration to the endogenous vaginal microbiota where *Lactobacillus* species decrease and pathogenic anaerobes increase and is known to increase the risk of preterm birth by 40% (Hillier et al., 1995). Intrauterine infection can result from bacteria and/or bacterial products gaining access to the intrauterine environment by ascent from the vagina through the cervix (Goldenberg et al., 2000). The majority of microbes found in amniotic fluid samples associated with intrauterine infections are of vaginal origin, leading us to study the effect of lactobacilli on the amnion (Benirschke, 1960).

Infectious and/or inflammatory processes present in the intrauterine tissues are known to activate a downstream cascade increasing prostaglandin production from the amnion, chorion and decidua. In a state of infection, the chorionic 15-hydroxyprostaglandin dehydrogenase (PGDH) metabolic barrier to prostaglandins produced in the amnion is decreased due to bacterial endotoxin and cytokine release (Cheung et al., 1990; Sangha et al., 1994; Van Meir et al., 1996). Bacterial endotoxin, bacterial products, cytokines, and chemokines increase net prostaglandin output by up-regulating both phospholipase and prostaglandin H synthase (PGHS)-2 expression in amnion cells (Kent et al., 1993; Mitchell et al., 1991; Pollard et al., 1993; Romero et al., 1988a; Romero et al., 1989a). A net increase of prostaglandin production accompanies infection and/or inflammation due to decreased metabolism and increased production. Prostaglandins
activate downstream pathways that result in a myometrial activation, cervical ripening and membrane weakening.

Although numerous investigators have studied the role of antibiotics in preventing preterm birth, their use has been shown to be largely ineffective and in some cases increases the risk of preterm labour (Andrews et al., 2003; Okun et al., 2005). Probiotics are defined as, “live microorganisms which when administered at adequate amounts confer a health benefit on the host” and have been identified as a potential treatment alternative (FAO and WHO, 2001; Reid, 1999). *Lactobacillus rhamnosus* GR-1 is capable of colonizing and restoring the microbiota of the vagina and modulation of prostaglandin production enzymes in the gestational tissues (Reid et al., 2003; Yeganegi et al., 2009).

In placental trophoblasts, *Lactobacillus rhamnosus* GR-1 increased expression of the prostaglandin metabolizing enzyme, PGDH in pregnancies carrying a female but not male fetus (Yeganegi et al., 2009). Also, *Lactobacillus rhamnosus* GR-1 reduced the LPS-induced increase in PGHS-2 in placental trophoblast cells from pregnancies carrying a male but not female fetus (Yeganegi et al., 2009). One of the major products of the amnion is prostaglandins. In a state of infection and inflammation, where metabolic barriers become less effective, the amnion contribution to prostaglandin elevation may be detrimental to the maintenance of gestation. Therefore, we hypothesized that *Lactobacillus rhamnosus* GR-1 will prevent alterations to prostaglandin production and key prostaglandin metabolizing enzymes in amnion cells in response to bacterial endotoxin stimulation.

### 4.2 Methodology

#### 4.2.1 Placenta Collection

Placenta collection was performed as described in Chapter 3 (Page 48)

#### 4.2.2 Amnion Mixed Epithelial and Mesenchymal Cell Culture

Amnion mixed epithelial and mesenchymal cell cultures were performed as described in Chapter 3 (Page 48-49). Cells were plated to a density of $8 \times 10^6$ cells per well in 60 mm culture dishes. Protein was collected from cell lysates in RIPA lysis buffer + protease inhibitors (Roche Diagnostics) and frozen at -80°C.
4.2.3 Lactobacilli Preparation

*Lactobacillus rhamnosus* GR-1 was grown as described in Chapter 3 (Page 49).

4.2.4 Treatment Protocol

Treatment was performed as described in Chapter 3 (Page 50; Figure 4.1, page 83).

4.2.5 Lactate Dehydrogenase Assay

LDH assay was performed as described in Chapter 3 (Page 50).

4.2.6 Prostaglandin E$_2$ Measurement

4.2.6.1 Enzyme Immuno-Assay

Levels of PGE$_2$ were measured using commercially available quantitative enzyme immuno-assay (EIA) kits (Cayman Chemical; Ann Arbor, Michigan, United States of America). EIA buffer was added to non-specific binding wells and maximum binding wells to a volume of 100 µl and 50 µl respectively. Standards 2-8 were prepared in 1:2 dilutions in EIA buffer and standard 1 of 1000 pg/ml, and 50 µl of each were added to wells. Samples were added in a dilution of 1:10 in EIA buffer to a final volume of 50 µl to wells. Prostaglandin E2 AchE Tracer was reconstituted in 6 ml EIA buffer and 50 µl were added to all wells except Total Activity and Blank wells. Prostaglandin E2 Monoclonal Antibody was reconstituted in 6ml EIA Buffer and 50 µl were added to each well except Total Activity, Non-Specific Binding wells, and blank wells. Plates were incubated overnight at 4°C. Plates were washed 5 times in 300 µl of Wash Buffer. Ellman’s reagent colour developing solution was reconstituted in 20 ml of Millipore-filtered water and 200 µl were added to each well to develop colour reaction. Prior to incubation at room temperature in the dark, 5 µl of Prostaglandin E$_2$ Acetylcholine Esterase Tracer was added to the Total Activity well. The plate was read when the 405 nm absorbance of the Maximum Binding wells minus the 450 nm absorbance of the Blank wells was greater than 0.3, a time ranging from 60 to 90 minutes in a spectrophotometer (Tecan Infinite M200). The minimum detection limit for PGE$_2$ was 80% B/Bo of 15 pg/ml.
4.2.7  Prostaglandin Metabolizing Enzyme Expression Measurement

4.2.7.1  Western Blot Analysis

Protein samples in RIPA were thawed and immediately mixed on a vortex shaker before incubation for 20 mins on ice. This was repeated 3 times followed by centrifugation at 4°C for 30 mins at 1000 g. Supernatant was then collected. Protein concentration was calculated using a Bio-Rad Protein Assay with BSA serial dilutions as a standard. Samples were frozen at -80°C until further analysis. Thirty micrograms of protein extract were mixed with specific volumes of RIPA lysis buffer and 5μl of loading dye to a total volume of 25 μl.

Protein samples were run on a 10% sodium dodecyl sulphate poly-acrylamide gel electrophoresis alongside a Pageruler pre-stained protein ladder (Fermentas; Burlington, Ontario, Canada). After electrophoresis, proteins were transferred to a nitrocellulose membrane at 100 V for 90 mins. Protein transfer was visually assessed using Ponceau S dye followed by 3 washes of 5 mins each in PBS with 0.1 Tween 20 (PBST, Sigma-Aldrich). Membranes were blocked overnight at 4°C in a blocking solution consisting of 5% non-fat skim milk powder (BioShop Canada Incorporated; Burlington, Ontario, Canada) dissolved in PBST.

Membranes were incubated separately on a rocking platform in primary antibody diluted in blocking solution for 1 hr at room temperature at the following dilutions: β-actin monoclonal antibody 1:1000 (Sigma-Aldrich), TLR4 monoclonal antibody 1:250 (Cayman Chemicals), PGHS-2 monoclonal antibody 1:500 (Sigma-Aldrich), PGDH monoclonal antibody 1:500 (Sigma-Aldrich), cPGES polyclonal antibody 1:500 (Cayman Chemicals), mPGES₁ polyclonal antibody 1:500 (Cayman Chemicals), and mPGES₂ polyclonal antibody 1: 500 (Cayman Chemicals). Membranes were washed 5 times for 5 mins in PBST. Secondary anti-mouse IgG (anti-PGHS-2, anti-β-actin; GE Healthcare; Little Chalfont, Buckinghamshire, United Kingdom) and anti-rabbit IgG (anti-cPGES, anti-mPGES₁, anti-mPGES₂; GE Healthcare), horseradish peroxidase linked whole antibody 1:4000 dilution incubation on a rocking platform was performed for 1 hr at room temperature at a dilution of 1:4000 for 1 hr. Membranes were washed 6 times for 5 mins in PBST. Membranes were incubated in enhanced chemiluminescence (ECL; Perkin Elmer, Waltham Massachusetts, United State of America) substrate for 1 min or in ECL Advance (Perkin Elmer) for 2 mins and were exposed to Kodak X-Omat Blue XB film for 30 secs, 1 mins, 5 mins, 10 mins and 15 mins. Optical densities were
measured using Scion Imaging Software. Optical densities of proteins were divided by optical
density of β-actin to standardize for protein loading. These relative densities were then used for
statistical analysis using Scion Image computer analysis software version Beta 4.0.2.

4.2.8  Placental Trophoblast Cell Culture

Placental trophoblast cells were isolated based on previously established primary culture
protocols (Sun et al., 1997). Briefly, placental tissue was separated from fetal membranes and
washed in saline. Approximately 60 g of villous placental tissue was cut and digested in a 37°C
incubator with 0.125% trypsin and 0.02% deoxyribonuclease-I (Sigma-Aldrich) in DMEM for
30 mins. Supernatant was then removed and discarded as tissue was incubated in 0.125% trypsin
in a 37°C incubator two more times for 30 mins. Supernatant was collected after the second and
third digestion and centrifuged at 1700g for 10 mins and resuspended in DMEM. The pooled
supernatant was then filtered through a 200 µm pore size nylon gauze filter. Cells were then
purified using a Percoll gradient (35, 40, 65, 70 increments) and centrifuged for 20 mins at
2500g. Placental trophoblasts were removed from the appropriate Percoll band and resuspended
in culture medium. Cells were plated to a density of 10^6 in 60 mm culture dishes.

4.2.8.1  Treatment

Cells were incubated for 72 hr in 37°C 5% CO₂ and 95% air in order to form a syncytium after
which cells were serum starved for 12 hr. Cells were then treated with Lactobacillus rhamnosus
GR-1 supernatant for 12 hours prior to LPS (100 ng/ml) incubation for 8 hr. Protein was then
collected from cell lysates in RIPA + protease incubator and frozen until use at -80°C.

4.2.8.2  Western Blot Analysis

Proteins were extracted and run on a Western Blot Analysis as previously described (Chapter 4,
page 73). 25 µg of protein was loaded as previously described (Chapter 4, page 73). Primary
antibodies were used to the following dilutions: β-actin monoclonal antibody 1:1000 and PGHS-
2 monoclonal antibody 1:500 (Sigma-Aldrich). Secondary antibodies were used to the following
dilutions: anti-mouse IgG (anti-PGHS-2, anti-β-actin) horseradish peroxidase linked whole
antibody 1:4000 dilution.
4.2.9 Statistical Analysis

PGE\textsubscript{2} data are presented as average values ± SEM. Protein data are presented as average values ± SEM relative to control. Statistical significance between groups was determined using a Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis using STAT32 version 2.0 statistical software. A non-parametric Kruskal-Wallis statistical analysis was performed based on the lack of normality and equal variation in the data of Chapter 4 as is required for parametric One-way Anova statistical analysis. Statistical significance was accepted at p<0.05.

4.3 Results

Out of the stimulants tested and at the amounts stated, only IL-1\textbeta (100 ng/ml) produced a significant increases in PGE\textsubscript{2} release compared to control treatment of DMEM alone in human amnion cells (Figure 4.2, page 84). No significant differences in PGE\textsubscript{2} production between LPS of 1000 ng/ml, LPS of 100 ng/ml compared to control were observed. Dexamethasone had no effect on PGE\textsubscript{2} production. Both IL-1\textbeta and dexamethasone, in mesenchymal cells only, at 100 ng/ml have previously been shown to stimulate prostaglandin production in cultured human amnion cells at term (Romero et al., 1989b, Whittle et al., 2000). MRS (1:20 dilution), the medium in which Lactobacillus rhamnosus GR-1 was cultured was tested as a control for supernatant treatment and did not cause a significant change in PGE\textsubscript{2} release.

Placental trophoblast cell cultures were performed to confirm the duplication of previously established results on LPS stimulation of PGHS-2 expression (data not shown). This was performed to not only confirm culture technique but also to confirm the ability of LPS to modify prostaglandin metabolizing and synthesizing enzyme expression. Placental trophoblasts were cultured and then treated according to the following groups: control (DMEM), GR-1 (1:20 dilution), LPS (100 ng/ml), and GR-1+LPS. Final results were similar to findings by Yeganegi et al. 2009. Although significance was not assessed due to an n-value of 2, similar trends were observed as in experiments performed by Yeganegi et al., namely LPS increased PGHS-2 expression that was prevented by pre-treatment with GR-1.

PGE\textsubscript{2} was significantly increased by GR-1 (1:20) alone and when pre-treated prior to endotoxin stimulation with both LTA (10 ng/ml) and LPS (100 ng/ml) compared to control (DMEM)
In addition, GR-1+LTA stimulation caused a significant increase in PGE\textsubscript{2} concentration compared to control and LTA. GR-1+LPS stimulation caused a significant increase in PGE\textsubscript{2} concentration compared to control and LTA as well as a significant decrease to GR-1. However, without pre-treatment with GR-1, neither LTA nor LPS caused significant changes in PGE\textsubscript{2} concentration compared to control.

To determine whether changes in PGE\textsubscript{2} output were due to prostaglandin enzyme expression modification, PGDH, PGHS-2, cPGES, mPGES\textsubscript{1}, and mPGES\textsubscript{2} levels were measured across all treatment groups. PGDH was found at very low levels across all treatment groups as was expected from previously performed experiments indicating little or no PGDH is expressed in the human amnion (N=6; data not shown). No significant differences in PGHS-2, cPGES or mPGES\textsubscript{1} levels were observed between treatment groups: control (DMEM only), GR-1 (1:20 dilution), LTA (10 ng/ml), GR-1+LTA, LPS (100 ng/ml), and GR-1+LPS (Figure 4.4, 4.5, 4.6, pages 86-88). Interestingly, a significant increase in mPGES\textsubscript{2} expression was observed between GR-1 and GR-1+LTA to control treatment groups (Figure 4.7, page 89). No other significant changes were observed between all other treatment groups.

Expression of the different isoforms of PGES, cPGES, mPGES\textsubscript{1}, and mPGES\textsubscript{2} were correlated with PGE\textsubscript{2} release from human amnion cells (Figure 4.8, page 90). An R\textsuperscript{2} —value of 0.6321, 0.6249, and 0.8214 exist for cPGES, mPGES\textsubscript{1}, and mPGES\textsubscript{2} with PGE\textsubscript{2} respectively.

All data were additionally analyzed according to the gender of the fetus carried in the pregnancy from which tissue was received. For PGE\textsubscript{2} and enzymes measured, the numbers for tissues derived from a pregnancy carrying a female fetus versus a male fetus were as follows: PGE\textsubscript{2} (4 female: 8 male), PGHS-2 (2 female: 4 male), cPGES (2 female: 5 male), mPGES\textsubscript{1} (3 female: 5 male), mPGES\textsubscript{2} (3 female: 6 male). No obvious trends were observed based on the gender of the fetus carried in pregnancies from which amnion tissue was derived (data not shown).
4.4 Comment

The focus of this study was to determine the influence of *Lactobacillus rhamnosus* GR-1 supernatant on amnion prostaglandin output and modulation of the enzymes involved in their metabolism. We have shown that *Lactobacillus rhamnosus* GR-1 significantly increased PGE$_2$ release in human amnion cells. This response appears to be slightly attenuated in amnion cells stimulated with either LTA or LPS after *Lactobacillus rhamnosus* GR-1 pre-treatment compared to *Lactobacillus rhamnosus* GR-1 alone. From these results, we suggest that enhanced prostaglandin production caused by *Lactobacillus rhamnosus* GR-1 permits the amnion to: 1) Control the extent of a cytokine-induced immune cell response and 2) Provide a means of pregnancy termination if the threat of bacterial endotoxin exposure becomes detrimental to the health of the fetus.

Surprisingly, in our culture system we were unable to demonstrate enhanced prostaglandin release by LPS stimulation in amnion cells as has been shown previously (Bennett, et al., 1987; Reisenberger et al., 1998; Romero et al., 1988a; Shon et al., 2002). Consequently, we performed several subsequent experiments to verify our results. These included verification of the viability of our cell culture as well as the activity of LPS and expression of its receptor, TLR4. As well, we verified the ability to produce and secrete prostaglandins and performed parallel experiments in a different cell culture, placental trophoblast cells.

Cells release prostaglandins during apoptosis. If a high proportion of cultured cells were in an apoptotic phenotype at the time our samples were taken, a change in prostaglandin production by LPS may be masked by elevated levels at baseline prior to stimulation. However, an LDH assay indicated little cell death compared to those cells treated with Triton X, a compound that solubilizes cell membranes to release into the cell medium a maximal level of LDH, an enzyme regularly bound to the mitochondrial membrane. In this assessment, the proportion of LDH in the medium collected across treatment groups is representative of the proportion of cell death. All treatment groups including LPS at a dose of 100 ng/ml exhibited low LDH levels indicating little cell death. Therefore, the lack of prostaglandin production stimulated by LPS was not due to an elevated baseline release of prostaglandins due to cell death.

One potential cause for the lack of LPS-stimulated prostaglandin release in our cultured human amnion cells may be a lack of biological activity of our LPS. It is well established that LPS
treatment of cultured human amnion cells increases the production of many cytokines. In our system, we demonstrated that in amnion IL-6, IL-8 and TNFα release were stimulated by LPS at a dose similar to previous experiments of 100 ng/ml. Therefore, the activity of LPS used in our experiments shows biological activity capable of stimulating increased release similar to that previously shown by other investigators.

LPS is a bacterial endotoxin that is recognized by cell membrane receptors of the body known as TLRs. The main receptor for LPS is TLR4 that is expressed on many immune cells and tissues, including the amnion. We also determined that TLR4 is expressed in our culture system verifying the presence of the receptor for LPS on amnion cells present in our cell culture system.

Prostaglandin release from the amnion is stimulated by many factors including bacterial products such as endotoxins, pro-inflammatory cytokines as well as glucocorticoids (Pollard et al., 1993; Romero et al., 1988a; Romero et al., 1989b; Whittle et al, 2000; Zaga et al., 2004). Studies have shown that an LPS dose within a range of 10 ng/ml to 1000 ng/ml stimulated prostaglandin production and release from human amnion cells cultured with a similar method, incubation period and treatment time as used in our experiments (Romero et al., 1988a, Thiex et al., 2009). However, here both 100 ng/ml and 1000 ng/ml of LPS were unable to increase prostaglandin production significantly compared to control groups. Similarly, dexamethasone at 100 ng/ml was unable to increase prostaglandin production. It has been previously demonstrated that mesenchymal amnion cells produce greater amounts of PGE₂ compared to epithelial amnion cells (Whittle et al., 2000). Dexamethasone may not have shown stimulation in mixed amnion cell cultures in this study if the proportion of epithelial cells were much greater than mesenchymal cells. The proportion of epithelial and mesenchymal cells obtained from the culture techniques previously described, were not mixed to a ratio similar to that which exists in vivo. Therefore a greater proportion of epithelial cells may account for the lack of dexamethasone stimulation of PGE₂ observed. Previous studies indicate that IL-1β increases prostaglandin production and release in cultured human amnion cells through increases in phospholipase and PGHS-2 expression at a dose of 100 ng/ml (Blumenstein et al., 2000; Bry et al., 1992; Pollard et al., 1993; Romero et al., 1989a; Romero et al., 1989b). Similarly, we showed that IL-1β at a dose of 100 ng/ml increase prostaglandin release from our cultured human amnion cells (Romero et al., 1989b). Therefore, we have shown that in our system, prostaglandin release can be stimulated by exogenous factors as was previously reported.
Placental trophoblasts are another gestational tissue responsive to LPS. Previously, our laboratory has demonstrated the ability of LPS to modulate enzymes involved in prostaglandin metabolism in cultured human placental trophoblast cells (Yeganegi et al., 2009). To verify not only the activity of LPS by attempting to parallel previously established results, we cultured placental trophoblast cells to verify culture and treatment technique. Previously, it had been shown that LPS-stimulated PGHS-2 expression that was minimized by Lactobacillus rhamnosus GR-1 pre-treatment (Yeganegi et al., 2009). These results were duplicated demonstrating that the LPS used in amnion cell experiments is biologically active and verified culture techniques.

In human placental trophoblasts, Lactobacillus rhamnosus GR-1 supernatant prevented changes in PGHS-2 expression caused by LPS in placentae derived from pregnancies carrying a male fetus (Yeganegi et al., 2009). As well, in placentae derived from pregnancies carrying a female fetus, Lactobacillus rhamnosus GR-1 supernatant increases PGDH expression to a higher degree than in placentae derived from pregnancies carrying a male fetus (Yeganegi et al., 2009). In contrast Lactobacillus rhamnosus GR-1 stimulated PGE2 release from amnion cells in this study. In the amnion Lactobacillus rhamnosus GR-1 significantly increased mPGES2 expression alone and when pre-treated prior to LTA stimulation. Together, with the trends observed in the expression profiles of cPGES and mPGES1, the changes observed in mPGES2 may partly account for the effects of Lactobacillus rhamnosus GR-1 on human amnion prostaglandin production. Placental trophoblasts and the amnion have diverse roles throughout pregnancy and are situated at anatomically distinct locations. Therefore it is not surprising that Lactobacillus rhamnosus GR-1 exerts different effects on prostaglandin production and prostaglandin metabolizing and synthesizing enzymes in placental trophoblasts and the amnion based on the role of each tissue in gestation.

The amnion is the most biologically active producer of prostaglandins amongst the gestational tissues at the onset of labour (Kinoshitak, 1984). Not surprisingly, numerous stimulants of prostaglandin release from human amnion cells have been identified. These include but are not limited to 1) bacterial products and endotoxins including phospholipases, LTA and LPS, 2) Pro-inflammatory cytokines such as IL-1α, IL-1β, TNFα, and IL-6, 3) Chemokines, such as MIP-1α, 4) Anti-inflammatory cytokines, including IL-10 and IL-1ra, and 5) Glucocorticoids in mesenchymal cells (Dudley et al., 1996; Furuta et al., 2000; Mitchell et al., 1991; Reisenberger et al., 1998; Romero et al., 1989b; Romero et al., 1989c). Three of these stimulants stand out as
having paradoxically stimulatory roles in the amnion compared to their well-established anti-inflammatory roles in other tissues: IL-1ra, IL-10, glucocorticoids (Economopoulos et al., 1996; Gibb et al., 1990; Mitchell et al., 2004; Potestio et al., 1988). We have demonstrated that *Lactobacillus rhamnosus* GR-1 can be added to this list of prostaglandin stimulants in cultured human amnion cells.

The amnion increases prostaglandin production in response to stimulants, such as glucocorticoids that normally prevent their production in other tissues. Therefore, one might speculate that prostaglandins may play an important function in the role of the amnion, possibly to enhance the barrier provided by the membranes against pathogenic bacteria and their products from reaching the amniotic fluid. It is well-established that elevated levels of prostaglandins stimulate increased expression of metalloproteinases that cause weakening of the fetal membranes and cervical ripening ultimately leading to preterm delivery. Therefore, uncontrolled elevated levels of prostaglandins stimulated by bacterial endotoxins and cytokines may lead to an elevated risk of preterm birth. However, low or absent prostaglandin levels have been associated with exaggerated immune responses due to the immunomodulatory role this family of eicosanoids exert (Goodwin et al., 1983).

Arachidonic acid metabolites, including prostaglandins have significant roles in suppressing or terminating immune responses (Chizzolini et al., 2009). An established function of PGE$_2$ in particular, is modulation of cytokine production in macrophages (Nataraj et al., 2001). PGE$_2$ acts to shift the activated macrophage phenotype from a strong pro-inflammatory response to a more anti-inflammatory response (Harris et al., 2002). This shift in phenotype is characterized by prevention of LPS stimulated release of type 1 immune response cytokines such as TNF$\alpha$ and IL-12 while elevating the release of type two response cytokines such as IL-4 and IL-10 (Betz et al., 1991, Katamura et al., 1995, Scales et al., 1989, Shinomiya et al., 2001, van der Pouw Kraan et al., 1995). A similar role of PGE$_2$ on the amnion or any other tissue has not been shown, but this effect is modulated through EP4 (Nataraj et al., 2001). EP4 is expressed on the amnion; therefore a similar modulation of cytokine response may occur due to the actions of PGE$_2$ in the amnion. It is important to consider that a metabolic barrier in the chorion expressing high levels of PGDH may be maintained allowing the deleterious effects of increased prostaglandin production to be minimized amongst the other gestational tissues while providing the immunoregulatory effects at the amnion. Therefore, *Lactobacillus rhamnosus* GR-1 stimulation of prostaglandins in the
amnion tissue may aid in maintaining the anti-microbial defenses properties inherent to this layer of the fetal membrane.

The amnion is the layer of tissue in closest proximity to the fetal environment. Therefore, the ability of the amnion to defend against pathogenic threats may be the deciding factor if bacteria and bacterial endotoxin reach the fetal environment causing developmental damage. The amnion possesses inherent anti-microbial, anti-bacterial and anti-viral properties that prevent infection during various obstetrical events. Although elevated levels can lead to activation of downstream pathways associated with preterm labor, the production of prostaglandins may be crucial in the defense provided by the amnion against pathogenic threats. Prostaglandins modulate the immune response by minimizing pro-inflammatory cytokines from recruited immune cells. Therefore, increased prostaglandin release caused by \textit{Lactobacillus rhamnosus} GR-1 may contribute to the overall ability of the amnion to defend against infections by controlling the extent of the inflammatory response if levels of prostaglandins are also controlled and maintained. Conversely, increased prostaglandin production by \textit{Lactobacillus rhamnosus} GR-1 may provide the amnion with the ability to terminate the pregnancy through increased prostaglandin production in order to protect the fetus from exposure to bacterial endotoxin given that the threat of deleterious effects associated with infection may be too great to allow for prolonged gestation. Further experiments are required to differentiate between these potential effects of \textit{Lactobacillus rhamnosus} GR-1 on amnion production of prostaglandins.
Primary amnion cultures were grown for 48 hours and then washed. After another 48 hour when cells reached confluency, cultures were serum starved for 12 hours. Cells were pre-treated with *Lactobacillus rhamnosus* GR-1 supernatant (GR-1) for 12 hours. After 12 hours of LTA or LPS treatment, protein from cell lysates was collected.
Figure 4.2: The effect of IL-1β, dexamethasone (Dex), lipopolysaccharide (LPS) and de Man, Rogosa, and Sharpe (MRS) on prostaglandin production in human amnion cells

Histogram showing PGE$_2$ output in media from human amnion cell cultures by various treatments. Control (DMEM), IL-1β (100 ng/ml), Dexamethasone (100 ng/ml), Lipopolysaccharide (100 and 1000 ng/ml), and de Man, Rogosa, and Sharpe (1:20 dilution). Results are mean values ± SEM. Statistical significance within groups is denoted with a different letter as assessed by Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis (N=4, p<0.05).
Figure 4.3: The effect of *Lactobacillus rhamnosus* GR-1 on Prostaglandin E₂ concentration in medium from cultured human amnion cells

Histogram showing PGE₂ in media collected from human amnion cell cultures. Control (DMEM), LTA (10 ng/ml), LPS (100 ng/ml), GR-1 (1:20). Results are expressed as mean values ± SEM. Statistical significance within groups is denoted with a different letter as assessed by Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis (N=12, p<0.05).
Figure 4.4: The effects of *Lactobacillus rhamnosus* GR-1 on PGHS-2 expression in human amnion cells

Histogram showing (A) PGHS-2 expression fold increase relative to control in human amnion cell cultures. Control (DMEM), LTA (10 ng/ml), LPS (100 ng/ml), GR-1 (1:20). Results are expressed as mean values ± SEM. Statistical significance assessed using a Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis (B) Relative Optical Density (ROD) of PGHS-2/β-actin western blot (N=6, NS).
Figure 4.5: The effect of *Lactobacillus rhamnosus* GR-1 on cytosolic PGES expression in human amnion cells

Histogram showing (A) cPGES expression fold increase relative to control in human amnion cell cultures. Control (DMEM), LTA (10 ng/ml), LPS (100 ng/ml), GR-1 (1:20). Results are expressed as mean values ± SEM. Statistical significance assessed using a Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis. (B) ROD of cPGES/β-actin western blot (N=7, NS).
Figure 4.6: The effect of *Lactobacillus rhamnosus* GR-1 on microsomal PGES$_1$ expression in human amnion cells

Histogram showing (A) mPGES$_1$ expression fold increase relative to control in human amnion cell cultures. Control (DMEM), LTA (10 ng/ml), LPS (100 ng/ml), GR-1 (1:20). Results are expressed as mean values ± SEM. Statistical significance assessed using a Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis. (B) ROD of mPGES$_1$/ β-actin (N=8, NS).
Figure 4.7: The effects of *Lactobacillus rhamnosus* GR-1 on microsomal PGES₂ expression in human amnion cells

Histogram showing (A) mPGES₂ expression fold increase relative to control in human amnion cell cultures. Control (DMEM), LTA (10 ng/ml), LPS (100 ng/ml), GR-1 (1:20). Results are expressed as mean values ± SEM. Statistical significance within groups denoted with a different letter as assessed by Kruskal-Wallis statistical test based on ranks followed by a Dunn’s post-hoc analysis. (B) ROD of mPGES₂/β-actin western blot (N=9, p<0.05).
Figure 4.8: Correlation between PGES expression and PGE2 production in human amnion cells

Correlation plot between (A) cPGES, (B) mPGES$_1$ and (C) mPGES$_2$ expression and PGE2 production in human amnion cell cultures ($n=4$, $R^2=0.6321$ for cPGES, $R^2=0.6249$ for mPGES$_1$, and $R^2=0.8214$ for mPGES$_2$).
Chapter 5

Overall Conclusions and Future Directions
5.1 Overall Conclusions

Infection and/or inflammation-mediated preterm birth is characterized by increased pro-inflammatory actions amongst the gestational tissues. In the amnion specifically this includes induction of a milieu of pro-inflammatory markers that are increased in the amniotic fluid during intrauterine infection. In vitro studies have shown that many of these markers are stimulated by LPS, a gram-negative bacterial endotoxin. This work has described the effects of Lactobacillus rhamnosus GR-1 on LTA and LPS treated amnion cells and the modulation of cytokines, chemokines, prostaglandins and prostaglandin metabolizing and synthesizing enzymes. The effects of Lactobacillus rhamnosus GR-1 can be grouped accordingly as molecules that may cause: 1) Increases in TNFα and IL-1β antagonists, 2) Recruitment of immune cells, 3) Modulation of immune cell activity and, 4) Stimulation of PGE2.

5.1.1 Potential Increases in TNFα and IL-1β Antagonists

LPS induces TNFα from the amnion and IL-1β from intact fetal membranes. Both TNFα and IL-1β are strong TH1 pro-inflammatory cytokines that alongside LPS and other bacterial products, induce transcription of other cytokines through NκFB control as well as increased prostaglandin production through modification of prostaglandin metabolizing enzymes. We have shown that IL-1β is not detectable from our culture system as it was not detected above lowest acceptable limit of the assay. However, similar to previous results shown in placental trophoblast cells and murine macrophages, Lactobacillus rhamnosus GR-1 minimizes LTA and LPS stimulated TNFα release from our human amnion culture system. Release of TNFα may also be decreased by the effects that other cytokines stimulated by Lactobacillus rhamnosus GR-1 cause. Our studies demonstrated that IL-6, IFNγ, and GM-CSF release from the amnion is increased by Lactobacillus rhamnosus GR-1. Each of these cytokines has been shown across various systems to increase the production of the soluble TNFα receptor and IL-1 receptor antagonist, the antagonists to TNFα and IL-1β respectively (Aderka et al., 1989; Schindler et al., 1990; Tilg et al., 1994; Xing et al., 1998). Essentially, IL-6, IFNγ and GM-CSF together provide the potential to minimize TNFα and IL-1β stimulation of prostaglandins in the amnion. Therefore, by up-regulating IL-6, IFNγ and GM-CSF, Lactobacillus rhamnosus GR-1 in addition to the direct effects observed by supernatant alone prevents the actions of pro-inflammatory cytokines.
responsible for initiation of downstream pathways leading to infection and/or inflammation-mediated preterm birth.

*Lactobacillus rhamnosus* GR-1 increased IFNγ release from amnion cells compared to control and endotoxin stimulation. In placental trophoblast cells, IFNγ reduced PGHS-2 expression and prostaglandin production (Hanna et al., 2004). At term, this protective mechanism is lost as IFNγ receptor expression decreases. The result is a net increase in prostaglandin production in placental trophoblasts at term (Hanna et al., 2004). It is unknown if similar effects on expression of amnion PGHS-2 are caused by IFNγ. However, an increase in IFNγ caused by *Lactobacillus rhamnosus* GR-1 release form the amnion may act in a paracrine manner to help maintain low expression levels of PGHS-2 in placental trophoblasts to help minimize prostaglandin production. Consequentially, the cytokines elevated by *Lactobacillus rhamnosus* GR-1 may be able to interact with the other gestational tissues to promote additional beneficial effects beyond the amnion.

### 5.1.2 Recruitment of Immune Cells

The chemokines MCP-1, RANTES, and IL-8 are involved in chemotaxis of both neutrophils and macrophages. *Lactobacillus rhamnosus* GR-1 increases production of these chemokines compared to control and endotoxin treatments. Consequentially, increased chemotaxis may occur in the amnion with the influence of *Lactobacillus rhamnosus* GR-1. Histological analysis of the amnion shows the presence of immune cells of Hofbauer cells of placental origin in the compact layer and as residential cells amongst the mesenchymal cells (Bourne, 1962). Therefore, infiltration and activation of immune cells can occur in the amnion.

### 5.1.3 Modulation of Immune Cell Activity

Macrophages act as a primary defense against invading pathogenic threats, including bacteria. The microenvironment to which immune cells are recruited dictate their phenotype and activity. Bacterial toxins, cytokines, and chemokines all influence the activity of immune cells. The M1 macrophage phenotype is induced by TNFα, IFNγ, GM-CSF and LPS resulting in macrophages that exhibit strong pro-inflammatory properties and encourage potent bacterial death (Mehta et al., 2004; Sierra-Filardi et al., 2011). In contrast, the M2 macrophage phenotype is stimulated by
IL-4 and IL-13 resulting in macrophages that encourage attenuated pro-inflammatory responses and elevated anti-inflammatory responses (Mehta et al., 2004). Recently, macrophage activation has been described as more fluid than simply having two polar phenotypes. Studies have shown that dependent on the cytokines, endotoxins and other stimulants present in the microenvironment, macrophages can display characteristics along a wide spectrum between the M1 and M2 phenotypes (Mosser et al., 2008).

*Lactobacillus rhamnosus* GR-1 increased IFNγ release from the amnion and *Lactobacillus rhamnosus* GR-1 prior to LTA or LPS treatment increases GM-CSF compared to control treatments. Therefore, *Lactobacillus rhamnosus* GR-1 increased two of the cytokines involved in activation of monocytes/macrophages to a highly active pro-inflammatory state capable of defending against intercellular pathogens. However, studies have shown that optimal activation of an M1 phenotype is dependent on the activities of TNFα and IFNγ. Monocytes/macrophages influenced by IFNγ without the presence of TNFα show a phenotype capable of phagocytosis and production of anti-microbial molecules with a less severe pro-inflammatory phenotype compared to those activated by both IFNγ and TNFα (Mosser et al., 2008). *Lactobacillus rhamnosus* GR-1 attenuated LTA and LPS-stimulated TNFα and increased IFNγ release from the amnion. Therefore, by the effect of *Lactobacillus rhamnosus* GR-1 in the amnion, it is expected that immune cells are recruited and activated to a phenotype capable of bacteria and bacterial product defense accompanied by a less severe pro-inflammatory responses.

Other studies have demonstrated that prostaglandins and other lipid mediators also influence the activity of recruited immune cells. *Lactobacillus rhamnosus* GR-1 increased the release of PGE2 from amnion cells. Amongst all prostaglandins, PGE2 shows the greatest effect on modulation of macrophage activity (Chizzolini et al., 2009). The role of PGE2 on macrophages is described as immunosuppressive. In particular, PGE2 modifies the activity of macrophages towards a M2 phenotype as strong pro-inflammatory responses are minimized as anti-inflammatory responses dominate (Harris et al., 2002). This shift in phenotype is characterized by prevention of LPS stimulated release of type 1 immune response cytokines such as TNFα and IL-12 while elevating the release of type two response cytokines such as IL-4 and IL-10 (Betz et al., 1991, Katamura et al., 1995, Scales et al., 1989, Shinomiya et al., 2001, van der Pouw Kraan et al., 1995). Other
studies demonstrate an immunomodulatory role for *Lactobacillus rhamnosus* GR-1 in mouse macrophages. In particular, LPS-treated mouse macrophages pretreated with supernatant show increased levels of G-CSF production, an anti-inflammatory cytokine (Kim et al., 2006). G-CSF is also increased by *Lactobacillus rhamnosus* GR-1 supernatant in a fetal sex-dependent manner in LPS-stimulated placental trophoblast cells (Yeganegi et al., 2011). Therefore, *Lactobacillus rhamnosus* GR-1 increases many molecules involved in the activation of immune cells that appear to promote defense against bacteria and their products while minimizing pro-inflammatory responses.

Therefore, *Lactobacillus rhamnosus* GR-1 increased chemokines that may recruit immune cells while also increasing chemokines, cytokines and prostaglandins that influence the activity of activated macrophages. The recruitment and activated phenotype of immune cells is difficult to interpret from the given data and future experiments are necessary to determine this. However, these results suggest it may be possible that *Lactobacillus rhamnosus* GR-1 causes immune cells to be recruited and activated to a state capable of defending against pathogens while the pro-inflammatory actions that accompany this response are minimized.

### 5.1.4 Stimulation of Prostaglandin E₂

The supernatant of *Lactobacillus rhamnosus* GR-1 increases cytokines and immune cell activity that have the potential to minimize LPS and pro-inflammatory prostaglandin stimulation. In parallel, *Lactobacillus rhamnosus* GR-1 directly enhances prostaglandin production in amnion cells. This is similar to the effects of *Lactobacillus rhamnosus* GG demonstrated to increase PGHS-2 expression in T84 colon epithelial cells (Korhonen et al., 2004). As well, *Lactobacillus rhamnosus* GR-1 increased IL-6 in amnion cells. IL-6 has been shown to stimulate prostaglandin production in the amnion (Kent et al., 1993; Mitchell et al., 1991). These results suggest that prostaglandins are stimulated in a more controlled manner, independent of LPS and pro-inflammatory stimulation in the presence of *Lactobacillus rhamnosus* GR-1. Amnion produced prostaglandins may exert stimulatory actions on the myometrium, and cause structural changes to the fetal membranes and cervix in conjunction with preterm birth when produced in uncontrolled levels. *Lactobacillus rhamnosus* GR-1 attenuates LPS stimulation of prostaglandins perhaps minimizing the harmful effects of deregulation caused in infection and/or inflammation-
mediated preterm birth. However, prostaglandins may be produced both directly and indirectly by actions exerted by *Lactobacillus rhamnosus* GR-1 in the amnion.

Various physiological stimuli have been shown to increase prostaglandin production in the amnion including glucocorticoids and IL-10 (Economopoulos et al., 1996; Gibb et al., 1990; Mitchell et al., 2004; Potestio et al., 1988). Both glucocorticoids and IL-10 exert typically anti-inflammatory actions in a wide variety of other systems. This indicates that the role of amnion prostaglandins may be crucial to amnion function. The amnion displays anti-bacterial, anti-fungal, and anti-viral properties. These include the expression of natural antimicrobials and innate immune molecules including human α-defensins 1, 2 and 3, human β-defensins 1, 2 and 3 as well as elafin and secretory leukocyte protease inhibitor (King et al., 2007). The innate expression of such a variety of components of the innate immune system suggests a major role of the amnion is to prevent uterine infection during pregnancy and labour (King et al., 2007). Prostaglandins are released from macrophages, NK cells, mast cells, basophils and eosinophils as part of the immediate innate immune response. Prostaglandins act to perpetuate the inflammatory response by modulating endothelial cells to allow for entrance of additional immune cells including leukocytes and are capable of degrading membrane phospholipids of pathogenic bacteria. Therefore a basal level of prostaglandin production stimulated by various physiological stimuli may help maintain the important anti-microbial role innate to the amnion. Consequentially, glucocorticoids, IL-10 and *Lactobacillus rhamnosus* GR-1 stimulation of prostaglandins may act to enhance the protective mechanism of the amnion to bacterial and endotoxin exposure to the fetal environment.

During infection and/or inflammation-mediated preterm birth, bacteria, bacterial products and pro-inflammatory cytokines, TNFα and IL-1β stimulate amnion prostaglandin production. As the chorionic PGDH barrier is decreased by similar stimulants, elevated prostaglandin levels lead to myometrial activation and increased metalloproteinase expression and subsequent cervical remodeling and membrane weakening. We suggest that *Lactobacillus rhamnosus* GR-1 prevents this uncontrolled pathway of prostaglandin production in the amnion and replaces it with a more controlled probiotic and immunomodulatory cytokine induced pathway. This pathway is possibly characterized by direct stimulation of prostaglandin production by *Lactobacillus rhamnosus* GR-
1 metabolites or through indirect consequences of *Lactobacillus rhamnosus* GR-1 induced IL-6 production (Figure 5.1, page 101). The abundance of stimulants of prostaglandin production in the amnion including the paradoxical actions of IL-10, glucocorticoids and *Lactobacillus rhamnosus* GR-1, suggest that prostaglandins play an essential role in the innate immune functions of the amnion.

### 5.1.5 Additional Effects of Prostaglandins on the Amnion

The amnion is the closest layer of tissue to the fetal environment and as such is an important barrier for entrance into the fetal environment. Part of the barrier function the amnion plays is dictated by the permeability of its epithelial layer dictated by the sealing of the intercellular space by part of the intercellular complex, tight junctions. In states of infection, bacteria, bacterial products, cytokines and prostaglandins have been shown to alter tight junction integrity, structure, and function in various different epithelial layers (Bruewer et al., 2003; Lecuit et al., 2000; McNamara et al., 2001; Nusrat et al., 2001; Prasad et al., 2005; Talavera et al., 2004; Tanaka et al., 2008). Disruption to the epithelial barrier is a common step in the inflammatory response and causes initiation of a mucosal immune response that can then stimulate a stronger pro-inflammatory immune response that may harm instead of protect the host.

Commensual strains of bacteria in the gastrointestinal tract, including lactobacilli can prevent changes in tight junction function and structure induced by pathogenic bacteria. *Lactobacillus rhamnosus* GG, a strain that adheres to the intestinal cell wall, prevents alterations to tight junction structure and function caused by *Escherichia coli* O157:H7 in MDCK-I cell line (Johnson-Henry et al., 2008). *Lactobacillus rhamnosus* GG is capable of preventing alterations caused by *Escherichia coli* and thus may prevent increased permeability in the gut thereby preventing entrance of the bacteria into the interstitial space. *Lactobacillus plantarum* can prevent similar changes caused by *Escherichia coli* 0124:NM to both tight junction structure and function in intestinal epithelial cells (Qin et al., 2009). Recently tight junctions have been identified in both murine and human amnion (Kobayashi et al., 2009; Kobayashi et al., 2010a). Inflammatory markers such as TNFα, LPS, and PGE₂ have been shown to disrupt the amniotic barrier through induction of apoptosis and alterations to tight junction structure (Kobayashi et al., 2010b). Therefore, *Lactobacillus rhamnosus* GR-1 may also exert protective effects on human
amnion tight junction structure and function to help maintain their innate role in a state of infection and/or inflammation-mediated preterm birth.

*Lactobacillus rhamnosus* GR-1 administered both orally and through vaginal instillation has been shown to colonize and replenish the vaginal microbiota. As well, when injected directly into the bladder of patients, this strain of lactobacilli does not result in any pathogenic responses. Therefore, the use of therapeutic *Lactobacillus rhamnosus* GR-1 will most likely be safe for use in pregnant women. In addition, *in vitro* studies have shown that both primary amnion and placental trophoblast cultures, *Lactobacillus rhamnosus* GR-1 attenuated release of the pro-inflammatory cytokine, TNFα demonstrating a beneficial immunomodulatory role when the tissues are challenged with either LTA or LPS. This prevention of pro-inflammatory cytokine release may act in ameliorating the activation of downstream pathways associated with infection and/or inflammation-mediated preterm delivery. Additionally, in the amnion, *Lactobacillus rhamnosus* GR-1 increases the defensive capabilities of the fetal membranes by recruiting and activating immune cells capable of defending against pathogenic threats at the fetal environment interface. Therefore, given the well-established safety of lactobacilli, the ability to colonize the vagina and the beneficial immunomodulatory effects on the gestational tissues, the results of this thesis have further demonstrated the potential for probiotic use in pregnancy to prevent infection and/or inflammation-mediated preterm birth.

### 5.1.6 *Lactobacillus rhamnosus* GR-1 Supernatant Metabolite(s)

Although the exact signaling mechanism and molecule or molecules responsible for the effects caused by the supernatant collected from *Lactobacillus rhamnosus* GR-1 is unknown, studies on other probiotics have been performed. Previously, probiotic supernatant was shown to exert pro-inflammatory actions through polysaccharide molecules and anti-inflammatory molecules extracted from collected media (Chon et al., 2010). As well, probiotics have been shown to signal through the MAPK and JAK/STAT pathways as JNK and JAK/STAT molecules are phosphorylated through unknown metabolites present in the supernatant of various probiotics (Kim et al., 2008; Yeganegi et al., 2010). Interestingly, the current study demonstrates a threshold dilution of *Lactobacillus rhamnosus* GR-1 to increase IL-6 and IL-8 concentrations in human amnion cells of 1:20 compared to 1:50 and 1:100 where no stimulation was shown.
Therefore, the metabolite or metabolites present in the *Lactobacillus rhamnosus* GR-1 supernatant may only exert its effects if it is present above a certain threshold at optimal concentrations. As suggested by previously performed experiments, the unknown metabolite may interact with TLR2 or TLR4, or unknown receptors. This may occur either through antagonistic activity to the ligands of a given receptor by either blocking the ligands interaction with the receptor of by leading to downstream signaling that may interfere with those activated by pathogenic ligands such as LTA or LPS or by activating downstream signaling independent of TLR2 and TLR4 that leads to an anti-inflammatory response. In addition, the current study demonstrated that a 1:10 dilution of *Lactobacillus rhamnosus* GR-1 supernatant does not exert the same effects on IL-6 and IL-8 as 1:20. This may suggest that homodimerization may occur between the metabolite or competition between various metabolites may occur to block the ability to activate associated receptors. Therefore, the effects exerted by probiotics are dependent on both the strain of bacteria and the tissue and cell type of the host as well as the concentration of metabolites produced by the bacteria.

5.1.7 *Lactobacillus rhamnosus* GR-1 and Prevention of Preterm Birth Associated with Infection and/or Inflammation

When considering the use of *Lactobacillus rhamnosus* GR-1 as a preventative treatment, the effect of its supernatant on the individual gestational tissue must be considered. In placental trophoblasts, *Lactobacillus rhamnosus* GR-1 exerts anti-inflammatory effects characterized by increased IL-10 and G-CSF and decreased LPS-stimulated TNFα release (Yeganegi et al., 2009; Yeganegi et al., 2010; Yeganegi et al., 2011). This prevention of LPS-stimulated TNFα release is paralleled in the amnion; however it is accompanied by a strong chemotactic response capable of modulating immune cell activity and increasing prostaglandin production. Although further studies are required to determine if the *in vivo* effects *Lactobacillus rhamnosus* GR-1 on immune cell recruitment and activation to the amnion are beneficial or detrimental to the prolongation of gestation, the effect of this probiotic on up-regulation of PGE₂ must be taken into consideration when determining the route of administration for preventative treatment for infection and/or inflammation mediated preterm birth. As preventative therapy, the most user-friendly method would be oral administration possibly as part of a daily supplement for pregnancy women. Orally administrated probiotics have been shown capable of reaching the vaginal flora and
despite the sterility of the amnion in healthy pregnancies, bacteria or their metabolites may be capable of reaching the fetal membranes in vivo. Therefore, the dose and the potential interactions with the inner layer of the fetal membranes, the amnion must be taken into consideration in a more representative model than in vitro cell cultures to determine the best method of preventative treatment for pregnancy women from undergoing infection and/or inflammation mediated preterm birth.

5.1.8 Limitations

Several limitations are of importance in regards to the experiments and conclusions presented in this study. Many experiments performed on the amnion are done on either intact membranes together with the chorion or on dispersed cells separated into epithelial and mesenchymal cell types. As the response of the amnion varies dependent on the presence of absence of not only the other tissue of the membranes, the chorion as well as the partner cell type, epithelial or mesenchymal cells the results of this study are limited in their in vivo representation. In addition, cultured cells were not mixed in proportion to in vivo amnion tissue thus over or under representation of either cell type may be possible.

When intact membranes are tested in a transwell system, shuttling of cytokine mRNA is thought to occur between the amnion and chorion. Due to the lack of the chorion in the system used in this study, both IL1β and IL-10 were not detected from mixed amnion cell cultures. IL-1β is a strong pro-inflammatory cytokine that is thought to play a role in initiation of infection and/or inflammation mediated preterm birth whereas IL-10 is an anti-inflammatory cytokine that counteracts many pro-inflammatory actions and is compatible with the TH2 pregnancy cytokine profile. Therefore, the effects of Lactobacillus rhamnosus GR-1 on IL-1β and IL-10 from intact membranes cannot be determined using the system of mixed amnion cell cultures used in this study.
Figure 5.1: The effects of *Lactobacillus rhamnosus* GR-1 on human amnion cells. (A) Untreated state of infection and/or inflammation. TNFα levels increase PGE₂ production leading to collagen degradation, membrane weakening and entrance of bacterial endotoxin to the amniotic fluid and the onset of preterm labour. (B) Effect of *Lactobacillus rhamnosus* GR-1 treatment on immune cells. Treatment prior to LTA or LPS stimulation results in attenuation of TNFα and increase chemotaxis and activation of immune cells to a phenotype capable of defending against bacteria and bacterial products while minimizing pro-inflammatory responses. (C) Effect of *Lactobacillus rhamnosus* GR-1 on prostaglandins. Increased PGE₂ production by *Lactobacillus rhamnosus* GR-1 may act as a protective defense against pathogens reaching the fetal environment by terminating pregnancy if bacterial threats become detrimental to the health of the fetus.
5.2 Future Directions

Currently, there is no effective treatment to prevent preterm delivery that occurs in approximately 10% of all pregnancies. Consequentially, preterm neonates account for 80% of all neonate morbidities and mortalities. The ultimate goal of the research presented in this thesis was to aid in developing an effective preventative treatment for infection and/or inflammation mediated preterm birth. The effects of *Lactobacillus rhamnosus* GR-1 on cytokine, chemokine and prostaglandin production in the amnion described in this thesis hopefully bring researchers one step closer to developing a treatment to prevent preterm birth and its associated consequences.

The future directions of the current study include further investigation into the effects of *Lactobacillus rhamnosus* GR-1 on products of the human amnion. Arachidonic acid is metabolized into several different end products including leukotrienes, lipoxins and eicosanoids. Both leukotrienes and lipoxins influence inflammation and immune cell responses. Another molecular target that may be of interest is the metalloproteinases and TIMPs that are differentially expressed under LPS stimulation. These enzymes are responsible for degradation or prevention of degradation of collagen during membrane weakening and cervical remodeling. They are up-regulated by prostaglandins and are part of the downstream pathways associated with infection and/or inflammation-mediated preterm labour. Therefore, the effect of *Lactobacillus rhamnosus* GR-1 on leukotrienes, lipoxins, MMPs and TIMPs may further illustrate the effects of probiotic treatment on the immune response from the amnion.

The cytokine and chemokine profile produced in response to *Lactobacillus rhamnosus* GR-1 described in this thesis suggests an effect on the recruitment and activation of immune cells. However, many of the cytokines and chemokines mentioned in this study have well-defined roles amongst the other gestational tissues that have not yet been established in the amnion. Therefore, before the conclusion of immune cell recruitment and activation can be confirmed, determining the precise effects of cytokines and chemokines on the amnion is crucial. Of particular interest are the effects of IFNγ on PGHS-2 expression and prostaglandin production as well as GM-CSF and IL-6 on production of sTNFα receptor and IL-1ra in the amnion. In
addition, it will be important to determine if immune cell recruitment can occur in vivo and if this reaction provides additional defenses or is deleterious to the prolongation of gestation.

In order to better understand the mechanism of action of the metabolites of *Lactobacillus rhamnosus* GR-1, studies identifying the downstream signaling associated with probiotic treatment should be determined. Previously, *Lactobacillus rhamnosus* GR-1 was shown to modify the phosphorylation state of enzymes involved in MAP kinase and NFκB signaling pathways. Probiotic enhancement of G-CSF in mouse macrophages is caused by a JNK-dependent mechanism (Kim et al., 2006). Determining the effects of *Lactobacillus rhamnosus* GR-1 on common pathways known to modulate cytokine transcription and translation will provide insight into the signaling pathway.

By determining the effects of *Lactobacillus rhamnosus* GR-1 on the type of molecules produced in the amnion that are associated with infection and/or inflammation-mediated preterm birth, a better understanding of the effects of probiotic treatment on this tissue should be determined. Since *Lactobacillus rhamnosus* GR-1 may cause both recruitment and activation of immune cells and increased prostaglandin production from the amnion the use of its supernatant on pregnancy may be two-fold. Firstly, by administering lactobacilli in a route that minimizes contact and interaction with the amnion, pro-inflammatory responses in placental trophoblasts and amnion may be attenuated and therefore prevent infection and/or inflammation preterm labour. Secondly, it may be possible that administration of *Lactobacillus rhamnosus* GR-1 in a route that allows for direct interaction of the supernatant with the amnion may be used to promote protection from bacterial endotoxin entrance to the fetal environment for use in idiopathic or induced labour where prolongation of gestation is deemed deleterious to the health of the fetus or mother. However, these options may be dependent on the dose effects of *Lactobacillus rhamnosus* GR-1 on the amnion which were not assessed in this study. Therefore, various routes of supernatant administration including oral, vaginal or directly into the cervix may result in different interaction of supernatant with the amnion and consequently different effects on infection and/or inflammation-mediated preterm birth.
The goal of the research presented in this thesis was to aid in the development of an effective preventative treatment for infection and/or inflammation-mediated preterm birth. Hopefully, the presented work has not only answered apparent questions but also opened routes and areas of interest for future research. By answering these questions, an effective treatment will help prevent the devastating consequences of preterm birth and lead to greater overall health and success of high-risk pregnancies.
References


cytoplasmic lipid bodies in human eosinophils and 3T3 fibroblasts. International Archives of Allergy and Immunology. 105, 245-250.


Gardiner, G. E., Heinemann, C., Bruce, A. W., Beuerman, D., & Reid, G. (2002). Persistence of Lactobacillus fermentum RC-14 and Lactobacillus rhamnosus GR-1 but not L. rhamnosus GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clinical and Diagnostic Laboratory Immunology. 9, 92-96.


Reid, G., Beuerman, D., Heinemann, C., & Bruce, A. W. (2001a). Probiotic Lactobacillus dose required to restore and maintain a normal vaginal flora. FEMS Immunology and Medical Microbiology. 32, 37-41.

Reid, G., Bruce, A.W., Fraser, N., Heinemann, C., Owen, J., Henning, B. (2001b). Oral probiotics can resolve urogenital infections. FEMS Immunology and Medical Microbiology. 30, 49-52.


and immunoreactive PGHS-1 and PGHS-2 levels in human amnion throughout gestation, at term, and during labor. Journal of Clinical Endocrinology and Metabolism. 78, 1396-1402.


resistance to nonoxynol-9 and vancomycin for urogenital isolates of lactobacilli. FEMS Microbiology Letters. 73, 101-104.


