THE EFFECT OF *Lactobacillus rhamnosus* GR-1 SUPERNATANT ON CYTOKINE PRODUCTION AND PROSTAGLANDINS IN GESTATIONAL TISSUES

by

Maryam Yeganegi

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

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Abstract

Preterm birth remains a major challenge in obstetrics. It complicates up to 13% of all pregnancies and accounts for approximately 80% of neonatal mortality and morbidity. Bacterial Vaginosis (BV) is associated with a 1.4-fold increased risk of preterm birth. Due to ineffectiveness of antibiotics in preventing preterm labour, probiotics have been proposed to serve as an alternative for treatment of BV and prevention of preterm birth. The objectives of this thesis were to determine 1) the effect of *Lactobacillus rhamnosus* GR-1 (*L. rhamnosus* GR-1) supernatant on cytokine profile and prostaglandin (PG)-regulating enzyme expression in lipopolysaccharide (LPS)-stimulated human chorion and placental trophoblast cells from human placentae, 2) the potential signaling pathways through which lactobacilli act and 3) the potential role of immune and placental trophoblast cells in initiating a response to LPS and *L. rhamnosus* GR-1 treatments. Primary cultures of human placental trophoblast cells were pre-treated with lactobacilli supernatant and then with LPS. In addition, immune cells were removed from cell suspensions using a magnetic purification technique to determine
their role in modulating cytokine levels. The expression of pro- and anti-inflammatory cytokines and prostaglandin-regulating enzymes was then determined. We found sex-specific differences in the ability of LPS to increase the output of TNF-α, IL-10, and PTGS2. We also showed that \textit{L. rhamnosus} GR-1 is able to act through the JAK/STAT and MAPK pathways to increase IL-10 and G-CSF, and independently down-regulates PTGS2 and TNF-α and up-regulates PGDH. The increase in G-CSF and PGDH were only observed in women carrying a female fetus. \textit{L. rhamnosus} GR-1 may serve as an alternative to antibiotics in preventing some infection/inflammation-mediated cases of preterm birth.
To laugh often and much;

To win the respect of intelligent people

and the affection of children;

To earn the appreciation of honest critics and endure the betrayal of false friends;

To appreciate beauty, to find the best in others;

To leave the world a bit better, whether by a healthy child,

a garden patch, or a redeemed social condition;

To know even one life has breathed easier because you have lived;

This is to have succeeded.

Ralph Waldo Emerson
To my parents...

For their unconditional love and support...
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This journey of academic and personal growth would have not been possible without the support of many individuals whom I will be indebted to forever. The following acknowledgement cannot fully express the sincere gratitude I owe to all those who have accompanied me in this memorable and fulfilling journey.

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<tbody>
<tr>
<td>11β-HSD-1</td>
<td>11β-Hydroxysteroid Dehydrogenase-1</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>BV</td>
<td>Bacterial Vaginosis</td>
</tr>
<tr>
<td>CAP</td>
<td>Contraction-Associated Protein</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CRH</td>
<td>Corticotropin-Releasing Hormone</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<td>ERK</td>
<td>Extracellular signal-Regulated Kinases</td>
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<td>FACS</td>
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<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GSCFR</td>
<td>Granulocyte-Colony Stimulating Factor Receptor</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus Kinases and Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MACS</td>
<td>Magnetic Cell Sorting</td>
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<tr>
<td>MLCK</td>
<td>Myosin Light-Chain Kinase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa and Sharpe</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-Kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxytocin Receptor</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline Tween</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGDH</td>
<td>15-Hydroxyprostaglandin Dehydrogenase</td>
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<td>PGHS</td>
<td>Prostaglandin H Synthase</td>
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<tr>
<td>PTB</td>
<td>Preterm Birth</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-Endoperoxide Synthase 2</td>
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<tr>
<td>ROD</td>
<td>Relative Optical Density</td>
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<tr>
<td>RTK-MAPK</td>
<td>Receptor-Associated Tyrosine Kinase-Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signalling</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-Alpha</td>
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Chapter One

General Introduction
Chapter 1

1. General Introduction

1.1. Human Parturition

Parturition (L. parturition, childbirth) occurs as a result of series of endocrine, paracrine and autocrine events that lead to dilatation of the cervix, rupture of the membranes and coordinated contractions increasing in strength and ending with the birth of the fetus (Bryant-Greenwood and Millar, 2000). Uterine activation, uterine stimulation and involution are the three phases of parturition that end the uterine quiescence that is maintained throughout pregnancy (Challis et al., 2002). Most of the events leading to labour act in a positive feed-forward manner. Up until labour, the uterus remains relatively inactive through the actions of inhibitors such as progesterone, prostacyclin (PGI2) and relaxin. Increased mechanical activity in conjunction with a change in the fetal endocrine profile are considered to be initiating factors in the activation of labour (Challis et al., 2000) and are thought to be driven by fetal genes (Challis et al., 2002). Mechanical stretch leads to up-regulation of contraction-associated proteins (CAP) including prostaglandin and oxytocin receptors, activating myometrial function in phase 1 of parturition. Prostaglandins (PGs) also lead to rupture of the membranes through stimulation of matrix metalloproteinase (MMP) activity (McLaren et al., 2000) and are known to play a key role in cervical ripening and dilatation (Keirse, 1993; Keirse et al., 1983). High levels of prostaglandins and/or the increased sensitivity of the myometrium to prostaglandins lead to stimulation of the uterus during phase 2 (Challis et al., 2002; Olson, 2003). Another contributing factor to the initiation of labour is activation of the hypothalamic-pituitary-adrenal (HPA) axis in the fetus which leads to an increase in fetal adrenal cortisol production (Pepe and Albrecht, 1995). This results in a decrease in progesterone and an increase in estrogen concentrations (Mason et al., 1989; Anderson et al., 1975). The increase in cortisol and estrogen and fall in progesterone cause an up-regulation of the expression of the CAPs and prostaglandin synthesis, leading to uterine contractions and labour (Challis et al., 2000). Therefore, the initiation of labour could be considered as a
transition in uterine activity caused by two interdependent pathways. One pathway is activated by biomechanical stretch of the uterus and the other pathway requires a series of endocrine and biochemical factors associated with activation of the HPA axis. Trophoblast-derived structures in the placenta, which include the fetal membranes (amnion and chorion) and the decidua have a major impact on the onset of labour through the local production of steroid hormones and prostaglandins. Labour ends with the involution of the uterus after the fetus and the placenta have been delivered, primarily due to the activity of oxytocin (Challis et al., 2002).

1.1.1. **Anatomy**

Both the placenta and myometrium play an important role during pregnancy and parturition. Their structural organization and endocrine characteristics are, therefore, designed for maintenance of pregnancy and coordinated contractions leading to successful delivery of the fetus at the time of labour.

**A. Uterine myometrium**

The uterus has a unique structure and at term undergoes a series of peristaltic movements that lead to dilatation of the cervix and expulsion of the fetus and the placenta. The uterus consists of both longitudinal and circular layers of smooth muscle, which consist of three different contractile proteins: actin, myosin and an intermediate filament that binds actin and myosin. Myosin is composed of two heavy chains and two light chains. With increased in intracellular calcium, the activity of the enzyme, myosin light-chain kinase (MLCK) increases. This enzyme then enhances the phosphorylation of the light chains which promotes the interaction of the heavy chains with actin, leading to contractions (Drover and Casper, 1983). The functional cell type of the uterus that acts to conduct action potentials throughout and generate a contractile force is the myocyte. Between myocytes, lies gap junctions through which an action potential passes. Myocytes also contain calcium-activated potassium channels for repolarization of the membrane after each contraction.
B. Placenta

The placenta is the organ responsible for the exchange of gas and nutrients between the fetus and the mother. It also acts as an endocrine organ and synthesizes a variety of protein hormones that maintain pregnancy through to term as well as initiate parturition. Some of the protein hormones secreted by the placenta are: human chorionic gonadotropin (hCG), human chorionic somatomammotropin (hCS), human placental lactogen (hPL), human chorionic thyrotropin (hCT) and human chorionic corticotripin (hCACTH). The placenta also secretes steroid hormones such as progesterone and estrogen, which help to maintain pregnancy (Skalba et al., 2003; Tuckey, 2005).

The human placenta has a discoid shape with two compartments, the fetal portion and the maternal portion (Figure 1.1, Page 6). It is considered to be hemochorial, meaning that maternal blood is in direct contact with the chorionic villi. The fetal portion of the placenta develops from the chorionic sac and the maternal portion is derived from a layer of endometrium that is functional in pregnant women and is referred to as the decidua. The decidua contains a number of immune cells such as T cells, natural killer cells, macrophages and dendritic cells which are sources of cytokines (Sanguansermsri and Pongcharoen, 2008). Decidua cells also protect the maternal tissue from uncontrolled invasion by extravillous trophoblasts (Gordon et al., 1995). In the human, the placental cell types are formed by the end of the third week and its vasculature is established by the fourth week of pregnancy (Gordon et al., 1995). As the chorionic sac grows, the blood supply to a section of decidua is compressed to produce an avascular area called the smooth chorion. On the other hand, the supply of blood to other regions of decidua increases to form a highly perfused region called villous chorion. By week 22-24, the amniotic sac fuses with smooth chorion to make the amniochorionic membrane (Foidart, 1992). The chorionic villi, which are branched from umbilical arteries invade the decidua during placental formation. Therefore, this part of decidua is eroded and such erosion leads to wedge-shaped areas called placental septa which cause the maternal surface of the placenta to have convex areas called cotyledons. There are many villi that branch through each cotyledon. After about 20 weeks of gestation,
histological changes lead to differentiation of cytotrophoblast and formation of patches of syncytiotrophoblast. The syncytiotrophoblast contacts fetal capillaries to form a vascularized membrane. Syncytiotrophoblast formation contributes to the barrier function of the placenta. However, different bacteria are allowed to pass into the intrauterine environment. The downside of this easy access for bacteria is the development of infections and fetal inflammatory syndrome because of the proximity of the fetal membranes to the cervix through which most of these bacteria ascend (Goldenberg et al., 2008b).
Figure 1.1: Potential sites of bacterial infection within the uterus and routes of infection.

Microorganisms causing an inflammatory response could access the uterine environment by 1) ascending through the vagina and the cervix, passing through the fetal membranes; 2) haematogenous means through the placenta; 3) retrograde spread from the fallopian tubes and 4) invasive procedures such as amniocentesis. [The image is taken from “Goldenberg, R. L., J. C. Hauth, and W. W. Andrews."Intrauterine infection and preterm delivery." N.Engl.J.Med. 342.20 (2000): 1500-07” and appear here with the permission of the journal.]
1.1.2. Mechanism of human parturition

A. Uterine stretch

Throughout the first trimester, the uterus undergoes rapid growth due to increased cell number (hyperplasia). This leads to an increase in uterine mass of about 24-fold (Johansson, 1984). This phase is then replaced by hypertrophy, which is a state of cell growth that continues until the end of pregnancy. It has been suggested that stretch imposed by the growing fetus during mid-gestation together with a particular hormonal environment leads to ischemia and hypoxia, which in turn could activate a transient apoptotic pathway (caspase cascade). Activation of this pathway, however, does not accompany any morphological changes associated with apoptosis. In fact, this might be the trigger for a change in morphology, which provides a condition for future extensive uterine growth. There is also an increase in protein and extracellular matrix synthesis (Shynlova et al., 2004). This remodeling of the uterus is maintained by progesterone and is also mediated by an increase in expression of focal adhesion molecules, such as focal adhesion kinase (FAK), which consist of series of integrins at points of contact with extracellular matrix (Hanks and Polte, 1997; Macphee and Lye, 2000). It is proposed by Shynlova and colleagues that an unexpected early change in the myometrial phenotype and activation of transiently apoptotic pathway (caspase cascade) which is medicated by pro-inflammatory cytokines could indeed be responsible for preterm labour (Shynlova et al., 2006).

Through activation of the contractile phase, the uterine myometrium becomes highly active, with coordinated contractions in order to expel the fetus (Shynlova et al., 2006). At this point, a fall in progesterone in most species or its functional withdrawal in humans causes a decrease in the activity of FAK and remodeling is inhibited (Macphee and Lye, 2000). The stretch from the fetus and the presence of estrogen also lead to an increase in the concentration of oxytocin/oxytocin receptor (OTR) (Alexandrova and Soloff, 1980; Carsten, 1974). It is observed that the mRNA expression of OTR increases 300-fold at the end of gestation (Kimura et al., 1996; Fuchs et al., 1984) and this is due to stretch-associated
activation of OTR gene promoter activity. Oxytocin activates myometrial contractility in late gestation through its interaction with OTR (Terzidou et al., 2005). Stretch also activates prostaglandin receptors and the increase in prostaglandin synthesis and activity decreases the threshold of myometrium excitation by allowing for calcium efflux and movement into myometrial cells (Drover and Casper, 1983). Phosphorylation of the light-chain by MLCK, which needs calcium, promotes contraction. The functional withdrawal of progesterone, which is responsible for muscle relaxation by decreasing calcium efflux, helps the uterus to remain in a more contracted state (Drover and Casper, 1983). The unified and coordinated nature of the contractions is mainly due to the presence of gap junctions that conduct the action potential throughout the body of the uterus (Garfield et al., 1980; Garfield et al., 1978). Stretch in phase 1 of parturition helps with the activation of CAPs such as connexin 43, which is a key component of these gap junctions (Challis et al., 2000; Chow and Lye, 1994). In phase 2 of parturition, the above factors stimulate the uterus and in phase 3, oxytocin is mainly responsible for the delivery of the fetus and the placenta (Challis et al., 2000).

B. Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is known to be important in initiation of labour in many species. The paraventricular nucleus in the hypothalamus secretes neuroendocrine factors such as vasopressin and corticotropin-releasing hormone (CRH) that stimulate the anterior lobe of the pituitary gland to produce adrenocorticotropic hormone (ACTH) from proopiomelanocortin (POMC). ACTH in turn acts on the adrenal glands to produce glucocorticoid hormones such as cortisol. Levels of glucocorticoids are in turn regulated through a negative feedback loop system (Challis et al., 2001b).

There are several studies that support the role of HPA axis and the associated endocrine pathways in initiation of labour. It was originally observed that pregnant ewes fed V. californicum gave birth to lambs with hypoplastic pituitary and adrenal glands and experienced a longer gestational length (Liggins and Howie, 1972; Liggins et al., 1973). Other studies have shown that interruptions of the HPA axis through hypophysectomy,
adrenalectomy or hypothalamic paraventricular nuclear lesions all lead to prolonging the length of pregnancy (Challis et al., 2002; Challis et al., 2000; McDonald et al., 1992; Gluckman et al., 1991; McDonald and Nathanielsz, 1991). In contrast, it has been shown that administration of ACTH to sheep fetuses upregulates P450 enzyme 17α-hydroxylase (P450 C17), an enzyme essential for cortisol production, leading eventually to premature birth (McLaren et al., 1996; Thorburn et al., 1991; Challis et al., 2001b).

In sheep, it has also been shown that the concentration of CRH mRNA in the paraventricular nucleus of the fetal hypothalamus is increased even further in late gestation (Challis et al., 2000; Challis et al., 2001). The adrenal glands use the progesterone supplied by the placenta as substrate to make cortisol. Alternatively, the availability of active glucocorticoids depends on the relative expression of enzymes 11β-hydroxysteroid dehydrogenase-1 (11β-HSD-1) and 11β-HSD-2 in the placenta. These two enzymes act in an opposite manner with 11β-HSD-1 having a reductase activity, converting cortisone to cortisol, whereas 11β-HSD-2 exhibits an oxidase activity and converts cortisol to cortisone, hence having a protective role by acting as a barrier to maternal glucocorticoids throughout pregnancy. Therefore, cortisol can be formed from cortisone in amniotic fluid or maternal circulation by the enzyme 11β-HSD-1 of the choriodecidua (Mastorakos and Ilias, 2003; Yang et al., 1995). As well, at labour, prostaglandins down-regulate the expression of 11β-HSD-2 and increase the expression of 1β-HSD-1 exposing the fetus to high levels of glucocorticoids (Sato et al., 2008; Hardy et al., 1999). The increase in fetal cortisol levels throughout gestation helps with the maturation of fetal organs and in particular, the lung (Bolt et al., 2002). The increase in fetal glucocorticoids as a result of activation of the fetal HPA axis also leads to up-regulation of prostaglandin (PGE2 and PGF2α) receptors (Gyomorey et al., 2000). Cortisol also increases the expression of prostaglandin synthase (PGHS), which synthesizes prostaglandin from arachidonic acid, and decreases the expression of 15-hydroxyprostaglandin dehydrogenase (PGDH), which metabolizes prostaglandins, leading to an overall increase in the output of prostaglandins (Challis et al., 2000).
In women, it has been shown that maternal plasma levels of CRH increase during the third trimester up until advanced stages of cervical dilatation (Petraglia et al., 1996; Petraglia et al., 1990; Campbell et al., 1987). As well, maternal pituitary and plasma ACTH increase leading to an increase in plasma cortisol levels. Similarly, there is a rise in CRH expression in the fetal hypothalamus as early as the 12th week of pregnancy (Mastorakos and Ilias, 2003). It is known that CRH stimulates dehydroepiandrosterone sulphate (DHEA-S), the precursor of estrogen. The availability of more estrogen will lead to up-regulation of CAPs (Smith et al., 1998b). The increase in CRH also stimulates MMP-9, which in turn initiates rupture of the membrane at labour (Li and Challis, 2005).

The fetal HPA axis can be prematurely activated by an adverse intrauterine environment caused by different factors such as maternal undernutrition, reduction of uteroplacental blood flow and fetal hypoxaemia, leading to preterm labour (Challis et al., 2002).

1.2. Preterm Birth

1.2.1. Epidemiology

Preterm birth (PTB) remains a major challenge in obstetrics and is defined as a delivery that occurs after 20 and before 37 weeks of gestation (Lopez, 2007). Canadian Perinatal Health Reports indicate that the incidence of preterm birth in Canada was 7.6% in 2003. This incidence is higher in the United States where preterm birth complicates up to 13% of all pregnancies (Goldenberg et al., 2008b). Preterm birth also accounts for approximately 80% of neonatal mortality and morbidity (Mattison et al., 2001), contributing to long-term injuries such as periventricular leukomalacia, cerebral palsy, chronic lung disease, blindness, deafness and neuro-developmental delay (Chan et al., 2001; McCormick, 1985). In addition, preterm birth creates a large financial burden to society. Based on a report funded by the National Institutes of Health, it is estimated that the annual cost associated with preterm birth in the United States was approximately $26.2 billion in 2005, or $51,600 per infant born preterm (Behrman and Butler, 2007). This dollar figure does not take into account the impact
on the family of raising these children and the loss of jobs parents have to face due to increased responsibility or the emotional stress of having a premature infant.

1.2.2. Etiology

The etiology of preterm birth is multifactorial. With the increased use of assisted reproductive technology and greater frequency of multiple gestations, the number of premature deliveries is increasing (Jackson et al., 2004). Preterm birth could also be attributed to other predisposing factors such as extremes of maternal age, poor socioeconomic status, low maternal body-mass index, endocrine changes in the placenta, fetal hypoxia, infection, periodontal disease, short cervical length, in vitro fertilization, hydramnios, choronic hypertension, single marital status, previous history, smoking, male babies and black race (Goldenbe rg et al., 2008b; Goldenberg et al., 2005; Lang et al., 1996; Zeitlin et al., 2002; Ingemarsson, 2003). The causes of preterm birth fall into three categories: 1) 50% being idiopathic- where there is no specific cause identified; 2) 20-40% indicated- where maternal or fetal clinical conditions such as pre-eclampsia or intrauterine growth restriction (IUGR) necessitate delivery (Meis et al., 1995), and 3) 30% infection/inflammation-mediated (Goldenberg et al., 2000; Romero et al., 1991). It is also suggested that most of the spontaneous births that happen in early gestation (20-28 weeks) are associated with histological chorioamnionitis and inflammatory responses associated with inflammation or infection in the amniotic fluid, whereas only 10% of the cases later in gestation (32-36 weeks) are. The late preterm births are mostly due to activation of the HPA axis and are considered stress-induced (Goldenberg et al., 2008b; Gray et al., 1992; Romero et al., 1989c; Romero et al., 2006; Guzick and Winn, 1985).

1.2.3. Current strategies for diagnosis and treatment of preterm birth

Although, prediction of preterm birth has become more accurate, no effective diagnostic marker or preventative measure currently exists for preterm birth (Chandiramani and
Methods of diagnosing preterm birth generally have a low positive predictive value and a high negative predictive value and, therefore, are unable in all cases to differentiate between true and false premature labour. Only one third of women presenting with signs of premature parturition will actually undergo preterm delivery within 24 to 48 hours (Bocking et al., 1999). Physicians need to determine whether it is beneficial to the mother and the fetus to delay the birth if the signs indicate that labour is inevitable or if there is a detrimental underlying cause such as fetal hypoxia and infection that warrants premature delivery (Bocking et al., 1999).

Many studies have proposed different tests that could help physicians more accurately identify true preterm labour. Maternal plasma CRH levels are 2 to 3 times higher in women undergoing preterm birth (28-36 weeks) 24 hours after presenting with labour symptoms when compared to those that do not (Bocking et al., 1999). There is evidence that maternal plasma urocortin, which is a member of the CRH family and binds with great affinity to CRH receptors and subsequently increases myometrial activity, are high in women with threatened preterm labour (Florio et al., 2007). In addition, in some studies, 69% of spontaneous preterm births are associated with an increase in inflammatory markers, such as serum TNF-alpha (Vogel et al., 2007). A positive cervical-vaginal fetal fibronectin test (less than the 10th percentile cervical length and a high concentration of alpha-fetoprotein, alkaline phosphatase and granulocyte colony-stimulating factor (G-CSF) in maternal plasma) are all potential predictors of preterm birth (Goldenberg et al., 2003).

Many approaches to prevent preterm birth in women with threatened preterm labour have been attempted. A previously marketed medication for preventing preterm birth was the betamimetic, β2-adrenoceptor (ADRB2) agonist Ritodrine, which enhances uterine relaxation. However, betamimetics are only effective for 48 hours, providing additional time for patient transfer to a tertiary perinatal unit as well as glucocorticoid administration. It has been shown that betamimetics also cause maternal cardiovascular, metabolic and neuromuscular side effects (Lopez, 2007). Oxytocin receptor antagonists such as atosiban and barusiban have also been used to decrease myometrial contractility. However, the
efficacy of these drugs remains to be determined (Lopez, 2007; Phaneuf et al., 1994). Meloxicam, a selective inhibitor of cyclooxygenase-2 (COX2/PTGS2) and indomethacin, an inhibitor of both COX2 and cyclooxygenase-1 (COX1) are other options that suppress myometrial contractility, but both carry maternal and fetal side effects (Rac et al., 2006; Lee et al., 2003). Progesterone and other progestational agents have been shown to reduce preterm birth. However, the safety of such agents remain unclear (Jayasooriya and Lamont, 2009; O'Brien and Lewis, 2009). PGHS-2 inhibitors, which down-regulate prostaglandin synthesis have been shown to be able to reverse CAP gene expression (DeWitt, 1999). Calcium channel antagonists, prostaglandin PTGFR receptor antagonists, and phosphodiesterase (PDE4) inhibitors are other drugs used to prevent preterm birth. However, the efficacy of many of these drugs are still not fully known (Lopez, 2007).

Administration of glucocorticoids is one of the most effective means of improving neonatal outcomes with preterm birth. These steroids increase surfactant production in fetal lungs and reduce the risk of neonatal respiratory syndrome (Katz and Farmer, 1999). The benefits of a single dose of glucocorticoids have led in some cases to the use of repeated courses of this treatment. Although the long-term outcome of multiple uses of glucocorticoids has not yet been reported, several studies have indicated an increased risk of poor neuronal development, lower birth weight and potential for adult onset of diseases (Sloboda et al., 2000; Barker, 1995). A Canadian study revealed that 61% of women not in true labour received multiple courses of betametasone whereas only 25% of those in preterm labour received optimal treatment as a result of administration of these glucocorticoids (Skoll et al., 2002). A recent study concluded that the use of multiple courses of antenatal corticosteroids did not improve preterm-birth outcomes, and was associated with a decreased weight, length, and head circumference of the fetus at birth (Murphy et al., 2008).

One strategy that has been proposed to prevent infection-mediated preterm birth is administration of antibiotics. Since many conditions of infection or inflammation such as BV are asymptomatic during pregnancy in approximately 50% of patients, these conditions often go undetected (Sobel, 1997; Eschenbach et al., 1988). When diagnosed, antibiotics may be
prescribed to correct the flora and potentially prevent preterm birth. However, this approach has been shown to be unsuccessful at delaying parturition (Leitich et al., 2003; McDonald et al., 2007; Okun et al., 2005). Prolonged use of antibiotics is associated with the production of resistant bacterial strains as well as creation of an alkaline environment that promotes growth of highly pathogenic bacteria. Moreover, antibiotics are not always able to eliminate the biofilms created by the pathogenic bacteria or reverse their sialidase activity (Reid and Bocking, 2003). Evidence from seventeen different trials indicates that treatment of women with BV with antibiotics does not reduce the risk of preterm birth. In fact, in some studies, the use of metronidazole actually increases the risk of preterm birth (Shennan et al., 2006).

Despite efforts by many investigators to find diagnostic and preventative measures for preterm birth, current medical interventions remain generally unsatisfactory. This has provided the major rationale for this thesis.

1.3. Infection and Inflammation

It is well established that infection or inflammation contributes to up to 30% of preterm births (Goldenberg et al., 2000; Romero et al., 1991). Early preterm births are generally associated with histological chorioamnionitis, in keeping with most preterm births at early gestation being caused by an infection or inflammation (Lahra and Jeffery, 2004). There is also evidence that the fetus initiates an inflammatory response as a result of intra-amniotic inflammation, which is linked to the onset of preterm labour. This can further lead to long-term injuries and developmental handicap (Goldenberg et al., 2008b; Gomez et al., 1998).

Intrauterine colonization with bacteria is often asymptomatic and even after labour, only histologic findings or cultures will show a presence of an intrauterine infection such as chorioamnionitis. Patients often do not exhibit fever, abdominal pain or peripheral-blood leukocytosis and there are no fetal symptoms such as tachycardia. Therefore, it is a major challenge to diagnose women with intrauterine bacterial colonization.
1.3.1. Characteristics of the vaginal microbiota

The normal vaginal ecosystem is a complex microenvironment. Colonization begins at birth and continues throughout life. The presence of microorganisms, such as acidophilic lactobacilli as well as their metabolic products helps maintain the pH of the vaginal tract (3.8-4.2) and provides the host with effective immunity. Any alteration in the normal balance of the flora by pathogenic bacteria or following antimicrobial treatment can lead to infection (Jarvis, 1996).

The most common and predominant components of a normal healthy vaginal flora are lactobacilli. Six species of Lactobacillus acidophilus (L. acidophilus (A1), Lactobacillus crispatus (A2), Lactobacillus amylovorus (A3), Lactobacillus gallinarum (A4), Lactobacillus gasseri (B1), and Lactobacillus johnsonii (B2)) are obligatory homofermentative and part of the rRNA group L. acidophilus (Vasquez et al., 2002; Fujisawa et al., 1992). Other species of lactobacilli have been also identified. Some examples are Lactobacillus rhamnosus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus jensenii, Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus vaginalis, with Lactobacillus iners being the most common (Saunders et al., 2007; Vasquez et al., 2002; Song et al., 1999; Zhong et al., 1998; Redondo-Lopez et al., 1990).

The lower genital tract is a suitable environment for pathogenic bacteria to initiate an infection and cause inflammatory diseases such as BV, vulvovaginal candidiasis, or trichomoniasis (Vasquez et al., 2002). Some potential pathogenic urogenital species associated with preterm labour with intact membranes are Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, peptostreptococci, and Bacteroides species (Goldenberg et al., 2000). Neisseria gonorrhoeae and Chlamydia trachomatis, are species that have been associated with preterm birth and lower birth weight after membrane rupture (Holst et al., 1994). Other pathogenic species found in the urogenital tract are, Mobiluncus sp., Morganella morganii, Escherichia coli, Prevotella sp., Streptococcus acidominimnus, Streptococcus morbilllorun, Atopobium vaginae and Staphylococcus epidermis. Many of
these pathogens produce proteases, sialidases, and mucinases, which are able to break down the barrier of cervicovaginal mucous. As well, they contain collagenases that may help degrade the fetal membranes (McGregor et al., 1986; McGregor, 1988). Some strains produce phospholipase A2 which releases the arachidonic acid from the phospholipid of the fetal membranes and promotes prostaglandin synthesis (McGregor et al., 1992a). As well, lipopolysaccharides, which are a major component of gram-negative bacteria stimulate pro-inflammatory cytokine production and initiate infection/inflammation-mediated preterm birth (Bejar et al., 1981; Bennett et al., 1990).

1.3.2. Routes of Infection

Microorganisms involved in an inflammatory response can access the uterine environment through four routes (Figure 1.1, Page 6): 1) by ascending through the vagina and the cervix; 2) by haematogenous means through the placenta; 3) by retrograde spread from the fallopian tubes and 4) through invasive procedures such as amniocentesis (Goldenberg et al., 2008b). The most common route for pathogenic bacteria to access the amniotic cavity is thought to be through ascending colonization. The evidence for this comes from a study where the same species of bacteria was found in amniotic fluid and the cervicovaginal fluid of 609 women undergoing early cesarean sections before rupture of membranes (Goldenberg et al., 2000). Bacterial sialidases facilitate the attachment of the pathogenic species and break down the mucin to allow their ascent. Proteolytic enzymes then act on fetal membranes and may cause premature cervical ripening and weakening of the membranes, predisposing to premature birth. Disorders such as an incompetent cervix could further expose the chorioamniotic membranes to the microorganisms present in the vaginal flora as the short distance may facilitate their ascent. A short cervix has been associated with many markers of chorioamnionitis (Klein and Gibbs, 2004; Romero et al., 2002; Guzman et al., 1999; Howe et al., 1999; McGregor and French, 1997). It is also thought that microorganisms are able to cross the intact membrane and enter the amniotic cavity (Romero et al., 1989c), in which case the invasion of microorganisms can further initiate a fetal inflammatory response. Studies have shown the presence of microorganisms in 23% of umbilical cord blood cultures.
from infants born at less than 32 weeks of gestation (Goldenberg et al., 2008a). The fetal inflammatory response is in turn associated with the activation of preterm labour (Gomez et al., 1998).

1.3.3. Bacterial Vaginosis (BV)

Bacterial Vaginosis (BV), which is defined as an alteration in the endogenous vaginal microbiota (Eschenbach et al., 1988), is associated with a 40% increase in the risk of PTB and is present in up to 20% of pregnant women (Ugwumadu, 2002; McGregor and French, 2000; Hillier et al., 1995; Goldenberg et al., 1996). BV is 3-4 times more prevalent in African Americans (Reid and Bocking, 2003) and this might contribute to the higher incidence of preterm birth that is observed in these women compared to other ethnic groups (Goldenberg et al., 2005). Bacterial vaginosis is also associated with other infective complications such as post-surgical sepsis and a higher chance of acquiring a sexually transmitted disease including HIV (Devillard et al., 2005; Sobel, 2000; Gray et al., 1997).

Bacterial vaginosis is often associated with a malodorous vaginal discharge, a vaginal pH of greater than 4.5 and the presence of many pathogenic bacteria, such as Gardnerella vaginalis, Prevotella bivia, E. coli, S. aureus, Mobiluncus sp., Mycoplasma hominis and most commonly Atopobium vaginae (Kurki et al., 1992; Klebanoff et al., 2004; Gleeson et al., 1989; Burton et al., 2005). Ascending microbial colonization leads to the release of bacterial proteolytic enzymes including phopholipase A2 and collagenase. These substances initiate the formation of arachidonic acid and increase levels of prostaglandins, which may eventually lead to uterine contraction and preterm labor (Lamont et al., 1990; Bennett et al., 1987). Microorganisms associated with BV can disrupt biofilms in the vaginal tract and release individual cells that in turn stimulate a local inflammatory response (Costerton et al., 2003). In addition, BV is associated with decreased levels of certain Lactobacillus, crucial to the maintenance of normal vaginal flora (Klebanoff et al., 1991).
Bacterial vaginosis is generally diagnosed using a Gram stain assessment of the vaginal fluid developed by Nugent et al. (Nugent et al., 1991). This method examines four bacterial morphotypes and the gram stained smears are scored on the basis of the number of these organisms. A low score of 0 to 3 is characteristic of a field of view that is dominated by Lactobacillus morphotypes and an intermediate score of 4-6 is a mixture of lactobacilli and other microorganisms. A score of 7 to 10 represents an absence of Lactobacillus and presence of large numbers of gram negative rods and gram positive cones such as Gardnerella vaginalis, Escherichia coli and group B streptococci. A Nugent score of 7-10 is considered diagnostic for BV (Spiegel et al., 1983; Nugent et al., 1991) and has been shown to be associated with premature birth in women with no symptoms (Hauth et al., 2003). Although Nugent scoring is used widely, not all the microorganisms that are characteristic of BV can be identified using this method.

Sialidases and pro-inflammatory cytokines are also elevated in the cervico-vaginal fluid of women with preterm birth (Cauci et al., 2005; Cauci et al., 2002). In women with symptoms of BV, microarrays have shown the expression of a number of inflammatory genes such as TNF-2 allele that increase the risk of preterm delivery (Friel et al., 2006; Am J Obstet Gynecol, 193: 6: Abstract 37). These methods are not optimal for a routine test for BV and the cultivation of the vaginal bacteria is also not plausible due to the anaerobic nature of growth of some bacteria and their nutrient requirements (Burton and Reid, 2002). The combination of PCR and denaturing gradient gel electrophoresis (DGGE) is another approach that is used to identify bacterial species and has been successfully used to characterize the vaginal flora. DGGE is a method through which different PCR products can be separated based on different denaturing characteristics of different sequences of DNA from different bacteria, resulting in a different pattern of bands (Burton and Reid, 2002).

Once BV diagnosed in pregnancy, antibiotics may be prescribed in attempt to prevent preterm birth. However, these antibiotics do not restore the endogenous lactobacilli, which are essential to a healthy flora, and in some cases have increased the incidence of preterm
birth as previously noted (Shennan et al., 2006). The studies described in this thesis were
designed to determine the role of lactobacilli in reducing the risk of preterm birth.

1.4. Probiotics

Probiotics are defined as “live microorganisms which, when administered in adequate
amounts, confer a health benefit on the host.” There are numerous publications that have
shown the beneficial role of probiotics for humans.

Infections such as urinary tract infection, BV, and yeast vaginitis affect an estimated one
billion women each year (Reid et al., 2003b). The role of probiotics in reversing urogenital
infections is of great importance. Oral daily intake of $10^9$ to $10^{10}$ \textit{L. rhamnosus} GR-1 and \textit{L. reuteri} RC-14 provides one way for the lactobacillus to reach the vagina and displace
pathogens (Reid et al., 2001). Once-weekly vaginal administration of a suppository
containing $10^9$ \textit{L. rhamnosus} GR-1 and \textit{L. fermentum} B-54 has been shown to potentially
decrease the recurrence of urinary tract infections while GR-1 and RC-14 have been shown
to treat BV in a pilot study (Anukam et al., 2006; Martinez et al., 2009). Due to their safety
and ability to modulate immunity and maintain the normal flora, probiotics are thought to be
potential candidates for preventing BV in pregnancy and, therefore, reducing the risk of
infection/inflammation-mediated preterm birth (Reid et al., 2003b).

The benefit of probiotics is not limited to women’s health. For example, probiotics have had
a positive role in treating many gastrointestinal diseases. Necrotizing enterocolitis is an
intestinal disorder affecting 10 to 25% of preterm infants. It is characterized by abdominal
distension and leads to septic shock and intestinal necrosis (Glass et al., 1991). The presence
of lactobacilli and bifidobacteria, which colonize in the normal flora of the intestine, in the
breast milk or in formula decreases the incidence of necrotizing enterocolitis. The probiotic
organisms survive intestinal passage and are recovered in the stool (Millar et al., 1993).
\textit{Lactobacillus rhamnosus} GG also decreases the risk of diarrhea in children (Oberhelman et
al., 1999). Organisms such as \textit{L. rhamnosus} GR-1, \textit{L. reuteri} RC-14 can help counter
salmonellae and a variety of other enteropathogens (Gopal et al., 2001; Bernet-Camard et al.,
Probiotics are also able to prevent *C. difficile* infection (Plummer et al., 2004) and inhibit the growth of *Helicobacter pylori*, a gram-negative pathogen responsible for type B gastritis and peptic ulcers (Kabir et al., 1997; Midolo et al., 1995). In addition, lactobacilli and bifidobacteria have been shown to decrease the amount of carcinogenic aflatoxin in the stomach lumen (El-Nezami et al., 2000). Several studies in patients undergoing high risk surgeries have shown that the use of probiotics could reduce infection and organ rejection. Post-operatively, probiotics can also assist in skin and wound healing (Rayes et al., 2002).

Probiotic lactobacilli express fragments in both secreted and cell surface forms that can bind to receptors on pathogenic bacteria. This might be a mechanism whereby lactobacilli prevent pathogenic bacteria from colonizing (Hultberg et al., 2007). Lactobacilli also enhance the protective effects of local immunoglobulin A (IgA) antibodies and modulate other host defenses (Lara-Villoslada et al., 2007). Studies have shown that lactobacilli can enhance immune parameters in the elderly through activation of macrophages and secretion of IgA and neutrophils without any release of inflammatory cytokines (Shu and Gill, 2002; Sheih et al., 2001; Matsuzaki and Chin, 2000). Atopic diseases in children are also reduced in early life if mothers consume *L. rhamnosus* GG in the later part of their pregnancy (Kalliomaki et al., 2001; Kirjavainen et al., 2003).

Probiotics are normal commensals of human microbiota, and their use is generally safe. Special care must be taken, however, when administering probiotics to immune-compromised individuals, where excessive immune system stimulation can be a side effect (Marteau, 2002).

### 1.4.1. Characteristics of lactobacilli

Lactobacilli are the most common probiotic used to restore the normal vaginal microbiota and treat urogenital, respiratory and gastrointestinal infections (Hudault et al., 1997; Reid, 2001; Boris and Barbes, 2000; Reid, 1999; McGroarty, 1993). Different strains of lactobacilli
have probiotic properties such as surface hydrophobicity, self- and co-aggregation, adhesion to vaginal epithelium and production of antimicrobial substances (Ocana and Nader-Macias, 2002; Ocana et al., 1999; Ocana and Nader-Macias, 2001). Several studies have shown that the number of vaginal lactobacilli increase significantly after oral use of *L. rhamnosus* GR-1 and *L. reuteri* RC-14. Many women treated with these lactobacilli maintained a normal Nugent score and had their BV resolved (Reid et al., 2003a; Anukam et al., 2006; Reid et al., 2004). Studies also suggest that *L. rhamnosus* GR-1 is able to colonize the vagina and up-regulate the host’s antimicrobial system after its intravaginal use (Reid et al., 1994b; Cadieux et al., 2002). In animal studies, *L. rhamnosus* GR-1 has been shown to strengthen the immune system by up-regulating the anti-inflammatory cytokines (Kim et al., 2006). Probiotic lactobacilli have also been used as a treatment strategy in humans. In Germany, women presenting with a vaginal pH of greater than 4.7 based on self-assessment, have been treated with lactobacilli and followed throughout pregnancy. Lactobacilli treatment in these women has been shown to decrease early prematurity and restore a normal vaginal flora (Hoyme and Saling, 2004).

The production of lactic acid by lactobacilli helps to maintain a low pH and decrease proliferation of pathogenic bacteria by altering their surface membranes and making them less able to adhere to the genital tract (Reid et al., 2005; Cadieux et al., 2002). The production of antimicrobial compounds such as lactocidin, acidolin, lactacin B, bateriocin, candicin as well as hydrogen peroxide (H$_2$O$_2$) can suppress the growth of microbial organisms or interfere with the signaling pathways through which bacterial endotoxins mediate inflammation (Wilks et al., 2004; Heinemann et al., 2000; Hawes et al., 1996; Velraeds et al., 1996; Reid et al., 1995; Reid et al., 1994a). Although antibiotics are used to eradicate pathogens, they do not replace the vaginal flora with lactobacilli. Thus, probiotic lactobacilli are good candidates for use in combination with antibiotics to prevent infection/inflammation-associated PTB.
1.5. Prostaglandins

It is well known that prostaglandins (PG) have a key role in the initiation and progression of labour. Primarily, they promote uterine contractility and ripening of the cervix by changing the extracellular matrix (Challis et al., 2002; Ellwood et al., 1980). Prostaglandins maintain uterine and placental blood flow (Sastry et al., 1997) and enhance membrane rupture (So, 1993). Prostaglandins are classified as eicosanoids, which are a family of oxygenated fatty acids (Smith et al., 1998a). One of the precursors of eicosanoids is arachidonic acid. The structure of PG is a hairpin with a cyclopentane ring and two side chains. Two of the most important prostaglandins responsible for initiation of labour are PGE$_2$ and PGF$_{2\alpha}$. However, these and other prostaglandins are involved in variety of other functions such as ovulation, menstruation, implantation, differentiation, cancer, allergy and homeostasis (Ushikubi et al., 2000; Matsuoka et al., 2000; Rocca et al., 1999; Murata et al., 1997; Olofsson and Leung, 1996; Lupulescu, 1996; Kelly, 1994; Smith, 1989).

Prostaglandins act through G protein coupled excitatory (FP, EP$_3$, EP$_1$) as well as relaxant (EP$_2$, EP$_4$) receptors. These receptors consist of three subunits: α, β, and γ (Hamm, 1998). Upon binding of PG to its receptors, $G_\alpha$ subunit dissociates from $G_{\beta\gamma}$, both of which act as effectors in signal transduction (Clapham and Neer, 1997; Coleman et al., 1994). Stimulation of EP$_2$ and EP$_4$ increases cAMP concentration via activation of adenylate cyclase (Wright et al., 2001; Regan et al., 1994; Bastien et al., 1994). EP$_2$ and EP$_4$ can also couple to eNOS (Abran et al., 1997) or activate T-cell factor signaling (Fujino et al., 2002) rather than adenylate cyclase. EP$_1$ binding leads to inositol phosphate production through the activation of phospholipase C and an increase in intracellular Ca$^{++}$ concentration (Narumiya et al., 1999). Binding with EP$_3$ usually leads to a reduction in cAMP concentrations by inhibiting adenylate cyclase (Negishi et al., 1988), but activates signaling pathways such as Ca$^{++}$ release, the small G protein Rho and protein kinase C (Aoki et al., 1999; Audoly et al., 1999; Asboth et al., 1996; An et al., 1994). Prostaglandins are thought to activate intracrine signaling pathways. The presence of PG transporters in many diverse tissues (Kanai et al., 1995; Lu et al., 1996) supports the transfer of PGs into the nucleus and there is evidence for
the existence of Prostaglandin-Endoperoxide Synthase 2 (PTGS2) in the nuclear region of a human colon cancer cell line, HCA-7 (Coffey et al., 1997). The nuclear envelope also contains EP₂ and EP₃ receptors (Bhattacharya et al., 1999). In addition, phospholipase A2 has been localized in the nucleus (Neitcheva and Peeva, 1995).

1.5.1. Prostaglandins biosynthesis

Prostaglandins are not stored inside the cell, but are secreted as they are generated (Gibb, 1998). Phospholipids lead to arachidonic acid production via the action of phospholipase A/C/D. With the addition of oxygen, arachidonic acid is converted to prostaglandin G₂ (PGG₂) through the action of prostaglandin H synthase 1/2, also known as COX 1/2 or PTGS 1/2. PGG₂ is then reduced to PGH₂, which is the precursor for all the prostaglandins (PGE₂, PGF₂α, thromboxane (TXb) and prostacyclin (PGI)) synthesis (Smith et al., 1998a; Gibb, 1998). The major site of prostaglandin synthesis is the human amnion (epithelial cells and subepithelial mesenchymal layer) (Duchesne et al., 1978). It has also been shown that there is an increase in PGHS2 mRNA in amnion (Mitchell et al., 1978; Gibb and Sun, 1996) and PG output is increased in the amniotic fluid prior to onset of labour (Lee et al., 2008). Prostaglandin is also synthesized minimally by the chorion and decidua. PGHS2 mRNA and protein have been reported to increase (Dong et al., 1996), decrease (Zuo et al., 1994) or remain unchanged (Moore et al., 1999) in myometrium at the onset of labour.

Glucocorticoids are also thought to increase prostaglandins by modulating progesterone and estrogen levels. It has been shown that the increase in fetal glucocorticoids during pregnancy initiates a decrease in progesterone and an increase in estrogen in sheep (Challis et al., 2002). Although in the human, plasma progesterone does not decrease close to parturition, it is suggested that the change in oestrogen:progesterone ratio might increase prostaglandin production (Gibb, 1998). In cultured human amnion cells, glucocorticoids increase PTGS2 expression and prostaglandin synthesis (DeWitt and Meade, 1993; Potestio et al., 1988; Mitchell et al., 1988). In addition, other factors such as epidermal growth factor, transforming growth factor-α, transforming growth factor-β, bacterial endotoxins, oxytocin
and CRH are thought to enhance synthesis of prostaglandins by increasing cAMP concentration (Anteby et al., 1997; Warrick et al., 1985). It has been suggested that cytokines such as IL-1β play a key role in up-regulating cytosolic phospholipase A (cPLA) and PGHS-2 in cultured amnion epithelial cells and contribute to the synthesis of prostaglandins (Gibb, 1998; Mitchell et al., 1993). Figure 1.2 is a schematic representation of the factors that regulate prostaglandin synthesis (Page 25).
Figure 1.2: Regulatory factors involved in stimulation and inhibition of prostaglandin synthesis.

1.5.2. Prostaglandin metabolism

15-Hydroxyprostaglandin Dehydrogenase (PGDH) is the enzyme responsible for prostaglandin metabolism. Its expression is down-regulated in choriodecidual tissue of women giving birth preterm and hence is thought to be a key factor in regulating prostaglandins at the time of parturition (Challis et al., 1999). The main action of this enzyme is to oxidize the 15-hydroxyl group of PGs to 15-keto metabolites, which will have a lower biological activity (Nakano et al., 1969). There are two types of enzymes, type I NAD⁺-dependent hydroxyprostaglandin dehydrogenase (type I PGDH) (Nakano et al., 1969) and NADP-dependent PGDH (type II PGDH) (Okita and Okita, 1996) that are capable of this oxidation reaction. All the major prostaglandins are substrates for PGDH and this enzyme has been localized to many different tissues such as placenta, kidney and lung (Hansen et al., 1999; Schlegel et al., 1974; Jarabak, 1972).

The chorion is an important site of PG metabolism due to high PGDH activity (Skinner and Challis, 1985). The PGDH expression and activity is decreased by pro-inflammatory cytokine addition such as IL-1β and TNF-α in fetal membranes and cultured chorion trophoblast cells (Mitchell et al., 2000; Challis et al., 1999). This effect is reversed by adding anti-inflammatory IL-10 (Pomini et al., 1999). Progestins which are produced from conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) stimulate PGDH to keep levels of prostaglandins low throughout pregnancy and maintain quiescence of the uterus. PGDH is thought to be a protective barrier preventing the transfer of prostaglandins that are produced in the amnion to the underlying decidua or myometrium without being metabolized (Challis et al., 2002). Glucocorticoids, on the other hand, have been shown to inhibit PGDH expression. This is in line with the evidence that there is a higher concentration of glucocorticoids around birth, which in turn correlates with the observation that there is also less expression of PGDH at the time of parturition. Glucocorticoids are produced by the local conversion of cortisone to cortisol via the action of 11β-HSD-1 enzyme in chorion or arise from the amniotic fluid, since there is an increase in
fetal glucocorticoids at birth (Challis et al., 2002). It is postulated that at term, high cortisol levels displace progesterone from glucocorticoid receptors leading to a decrease in PGDH transcription (Challis et al., 1999). Figure 1.3 (Page 28) indicates the factors that regulate prostaglandin metabolism. Other factors, such as bacterial endotoxins, fatty acids, vitamins and thyroid hormones are also thought to play a role in prostaglandin metabolism (Challis et al., 2002). The effect of lactobacilli on prostaglandin production and metabolism in the human placenta is a major component of this thesis.
Figure 1.3: Regulatory factors involved in stimulation and inhibition of prostaglandin metabolism.

1.6. Cytokines

The tissues of the human reproductive tract synthesize a variety of inflammatory mediators, including cytokines and chemokines which are important in processes such as implantation, placental growth and the maintenance of pregnancy. Regulatory peptide cytokines, once defined as mediators of interactions between the immune cells such as macrophages, are now considered to play crucial roles in a variety of cell processes such as proliferation, differentiation and cell death (Bowen et al., 2002). Cytokine production is therefore regulated to ensure homeostasis and an effective defense against pathogens. Any failure to maintain homeostasis between the anti- and pro-inflammatory cytokines will lead to inflammatory conditions (Bowen et al., 2002). Evidence suggests that these molecules possess an important immunoregulatory role especially at the maternal/fetal interface (Haynes et al., 1993).

Several studies have shown that increased intrauterine pro-inflammatory cytokine production is associated with both term and preterm labor (Dudley, 1997; Gomez et al., 1997; Keelan et al., 2003) as they facilitate cervical ripening, and membrane rupture (Challis et al., 2000). There is an increased concentration of Interleukin (IL)-6 in the umbilical vein of infants born to mothers presenting with chorioamnionitis (Chaiworapongsa et al., 2002). These mothers also experience elevated levels of pro-inflammatory cytokines such as IL-6 in their amniotic fluid (Holst et al., 2005). The cervico-vaginal fluid from pregnancies complicated by BV contains higher than normal levels of pro-inflammatory cytokines such as IL-1β and IL-8 (Cauci et al., 2002; Hedges et al., 2006). The levels of intrauterine cytokines, in particular IL-1β, IL-6, IL-8 and Tumor necrosis factor-alpha (TNF-α) are increased in maternal serum, amniotic and cervico-vaginal fluid of women with threatened preterm labour, particularly in those women with intraamniotic infection (Hitti et al., 2001; El-Bastawissi et al., 2000; Jun et al., 2000; Coultrip et al., 1994; von et al., 2000).

Anti-inflammatory cytokines such as IL-4 and IL-10 play a key role in pregnancy maintenance. It is known that IL-10 and IL-4 suppress the harmful maternal immune response in vivo and protect the allogenic human fetus against maternal immune attack during pregnancy (de Moraes-Pinto et al., 1997; Roth et al., 1996). It has also been shown
that anti-inflammatory cytokines are able to inhibit the production of pro-inflammatory cytokines as well as prostaglandins (Bowen et al., 2002; Keelan et al., 2003). Moreover, low levels of G-CSF have been associated with an increased incidence and severity of infection in preterm infants (Chirico et al., 1997). Other cytokines such as leukemia inhibitory factor (LIF), macrophage inhibitory protein-1 (MIP-1), IL-15, macrophage chemotactic protein (MCP), macrophage inhibitory factor (MIF), bone morphogenic protein (BMP) and transforming growth factor-beta (TGF-β) have also been detected at the onset of labour (Bowen et al., 2002; Keelan et al., 2003).

Gestational tissues are important sources of cytokines (Bowen et al., 2002). Amnion, chorion, decidual and endothelial cells are all sources of pro-inflammatory cytokines (Keelan et al., 1999; Steinborn et al., 1999). Anti-inflammatory cytokines IL-10 and IL-4 are produced by fetal membranes, trophoblast and decidual cells (de Moraes-Pinto et al., 1997; Keelan et al., 2003). All these cytokines work in harmony to maintain uterine quiescence throughout pregnancy and promote parturition at term. However, infective agents can infiltrate the decidua and chorion, inducing an inflammatory response accompanied by leukocyte infiltration, release of cytokines and an increase in prostaglandin levels via an increase in the expression of the enzymes of the prostaglandin biosynthetic pathway. Production of prostaglandins in amnion (Bry and Hallman, 1993; Romero et al., 1989a), chorion (Lundin-Schiller and Mitchell, 1990), decidua (Mitchell et al., 1990), and myometrium (Pollard and Mitchell, 1996; Hertelendy et al., 1993) is enhanced by IL-1β and TNF-α stimulation while PG production is inhibited by IL-10 within the human chorion, decidual and placental cells in vitro (Barsig et al., 1995; Fortunato et al., 1997; Trautman et al., 1996).

Different signaling pathways are important in the maintenance of pregnancy and in turn are altered in infection/inflammation-mediated preterm birth. The endotoxin, LPS, is a component of the membrane of pathogenic bacteria and it acts as a signaling molecule that elicits a strong immune response in most tissues. LPS mainly acts through a transmembrane lipopolysaccharide receptor, Toll-like receptor 4 (TLR-4), which is a member of a family of
receptors important for the release of antimicrobial peptides, inflammatory cytokines and chemokines that initiate the immune response to gram-negative bacteria. TLRs are found on the surface of many immune cells and the extracellular domains of these proteins are recognized by different classes of pathogens. TLR-2 recognizes gram-positive bacteria while TLR-4 recognizes the lipid A component of LPS from gram-negative bacteria. TLR-3 recognizes double stranded RNA from virus infected cells and TLR-5 recognizes yeast cells. There are several other TLRs that recognize other components and initiate a downstream signalling pathway (Medzhitov and Janeway, Jr., 2000). It has been shown that pretreatment with a TLR-4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokine release, and PG expression in rhesus monkeys (Adams Waldorf et al., 2008). LPS recognition is enhanced by LPS binding protein (LBP) and Cluster of differentiation 14 (CD14), and is mediated by TLR4/MD-2 receptor complex. LPS/TLR4 signaling can be divided into myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways, which mediate the activation of nuclear factor-kappa B (NF-κB), leading to up-regulation of pro-inflammatory cytokines (Lu et al., 2008). Key members of the NF-κB/Rel family of proteins such as p50 and p65 serve as mediators of cytokine-induced up-regulation of PTGS2 enzyme, leading to an increase in prostaglandin levels (Herve et al., 2008; Kniss et al., 2001). The heterodimer of p50 and p65 is normally in the cytosol bound to inhibitory IκB-α. Several cytokines or bacterial endotoxins activate the IκB kinase (IKK) complex, which phosphorylates IκB-α in order to ubiquitinate and degrade it. This will release NF-κB, enabling translocation to the nucleus. Subsequently, gene transcription will be altered by binding to specific NF-κB binding sites in gene promoter regions (Theiss et al., 2009; Lu et al., 2008). Members of the Receptor-associated Tyrosine Kinase-Mitogen-Activated Protein Kinase (RTK-MAPK) pathway, which are p38, c-Jun N-terminal Kinase (JNK) and Extracellular signal-Regulated Kinases (ERK) are important in infection/inflammation-mediated preterm birth. Studies have shown that a p38 MAPK inhibitor suppresses LPS-induced cytokine and PG production as well as the TNF-α to IL-10 ratio in human choriodecidua, suggesting that p38 MAPK plays a role in the cascade by which infection or inflammation causes preterm birth (Shoji et al., 2007). Pro-inflammatory
cytokines such as TNF-α and IL-1β are known to increase the expression of prostaglandins by up-regulating the expression of PTGS2 (Hansen et al., 1999) and down-regulating PGDH (Mitchell et al., 2000). This is achieved by translocation of the NF-kappaB subunit p65/RelA and RNA polymerase II to the PTGS2 promoter in nucleus, which is dependent on p38 MAPK. Pro-inflammatory cytokines stimulate activation of MMPs, predisposing membranes to break down and rupture (Polzin and Brady, 1998). Trophoblast cells also secrete both MMP-2 and MMP-9, and increased expression of MMP9 in these cells has been associated with preterm premature rupture of the membranes (PPROM) and infection-mediated PTB (Li et al., 2007; Xu et al., 2002). Figure 1.4 (Page 33) shows a proposed pathway for infection/inflammation-mediated preterm birth.

Lactobacilli are able to modulate immunity by having an anti-inflammatory effect. In mouse macrophages, addition of *L. rhamnosus* GR-1 supernatant down-regulates pro-inflammatory TNF-α and up-regulates anti-inflammatory IL-10 and G-CSF outputs (Kim et al., 2006). *L. rhamnosus* GG is also able to down-regulate LPS-induced pro-inflammatory cytokines and up-regulate anti-inflammatory cytokines in the rat (Li et al., 2009). Studies suggest that inhibition of the JNK, a member of the RTK-MAPK pathway, is a key mechanism by which *L. rhamnosus* GR-1 suppresses TNF-α production in mouse macrophages (Kim et al., 2006). In this thesis, we have focused primarily on studying the pro-inflammatory cytokine TNF-α, and the anti-inflammatory cytokines IL-10 and G-CSF.
Figure 1.4: The proposed pathway of infection/inflammation-mediated preterm birth.

1.6.1. **Tumor necrosis factor-alpha (TNF-α)**

Tumor necrosis factor-alpha plays a key role in several different reproductive functions including menstruation, implantation, lactation and parturition as well as in a variety of cell functions such as apoptosis, differentiation and survival (Argiles et al., 1997; Haider and Knofler, 2009). Many different factors such as female sex steroid hormones, and lipopolysaccharide influence the expression of the TNF-α gene (Terranova et al., 1995). TNF-α is a member of the TNF superfamily which consists of 18 ligands and 29 receptors. It mainly acts through two receptor types, TNFR1 and TNFR2 (Haider and Knofler, 2009). Upon binding, silencer of death domain (SODD) is released and TNFR-associated death domain protein (TRADD) binds to the intracellular domain as well as the serine/threonine kinase receptor interacting protein-1 (RIP-1), TNFR associated factor (TRAF)-2, and cellular inhibitor of apoptosis proteins cIAP1 and cIAP2. Signalling through TNFR1 may result in formation of complex I which leads to activation of the key members of NF-κB/Rel family (pathway 1) via MAPK. This pathway is mainly responsible for processes such as inflammation, survival and differentiation. On the other hand, TNF-α might also promote cell death through formation of complex II and activation of caspases (pathway 2). Pathway 3 involves the recruitment of apoptosis-signalling kinase (ASK)-1 (pathway 3), which in turn induces the activation of JNK and activating protein-1(AP)-1. This initiates apoptosis or survival. TNFR2-dependent signalling is involved in cell death or survival by activating pathways 1 and 2, respectively, but it also activates phosphatidylinositol-3-kinase (PI3K)/AKT via vascular endothelial growth factor (VEGF) in order to promote proliferation, migration and survival and suppress apoptosis (Haider and Knofler, 2009).

1.6.2. **Interleukin (IL)-10**

Interleukin-10 is an anti-inflammatory cytokine that plays a key role in pregnancy and parturition. It is produced by type 2 T helper cells, B cells, macrophages, and monocytes and inhibits pro-inflammatory cytokines secreted by these cells (Bogdan et al., 1992; Fiorentino et al., 1991). IL-10 is considered to be a product of type 2 CD4+ T (Th2) cells, which inhibits
several macrophage functions (Piccinni, 2002). The IL-10 gene contains five exons that are separated by four introns (Mosser and Zhang, 2008). IL-10 binds to a two-receptor complex, which contains two copies of each IL-10 receptor 1 (IL-10R1) and IL-10R2 (Donnelly et al., 1999). Upon binding to its receptor on lymphocytes or NK cells, intracellular signals from these receptors are carried through the Janus Kinases (JAK) and Signal Transducers and Activators of Transcription (STAT) system. The JAK/STAT pathway consists of at least 4 different kinases and 7 STATs. Phosphorylation of JAK1 and JAK2 leads to recruitment, phosphorylation and homodimerization of STAT-3. Homodimerization of STAT3 leads to its release from the receptor and translocation to the nucleus to result in transcription of the target genes (Mosser and Zhang, 2008). It has been proposed that IL-10 causes a decrease in NF-κB pathway activity by either suppressing the inhibitor of NF-κB (IκB) kinase (IKK) activity (Mosser and Zhang, 2008) or by blocking the NF-κB translocation to the nucleus and its binding to DNA (Schottelius et al., 1999). STAT3 also activates the suppressor of cytokine signaling 3 (SOCS3), which controls and balances the quality and quantity of STAT3 (Poehlmann et al., 2005). STAT3 activating receptors often initiate an anti-inflammatory response. However, some other STAT3 activating receptors such as IL-6R have a pro-inflammatory role instead. Therefore, although STAT3 activation is required, it is not sufficient for the anti-inflammatory properties of IL-10 to be expressed. It is thought that the difference in regulation of SOCS can contribute to the dual properties observed with STAT3 activation and hence a balance between the pro- and anti-inflammatory cytokine signaling activation (Murray, 2007). It is thought that L. rhamnosus GR-1 could potentially act through the IL-10 receptors to down-regulate the pro-inflammatory cytokines (Kim et al., 2006). This potential effect is studied in human placenta in this thesis.

1.6.3. **Granulocyte-colony stimulating factor (G-CSF)**

Granulocyte-colony stimulating factor is the major cytokine regulator of neutrophilic granulocytes (Panopoulos and Watowich, 2008). It is a protective cytokine with anti-inflammatory effects and is considered essential for the clearance of bacterial pathogens. Human G-CSF weighs around 19 kDa and was originally purified from placental-conditioned
medium (Nicola et al., 1979). It is expressed in several tissues such as fibroblasts, endothelial cells, monocytes, macrophages and bone marrow stromal cells (Panopoulos and Watowich, 2008). Studies have shown that G-CSF is important in promoting the survival of the granulocytic lineage cells and proliferation and migration of the neutrophils (Panopoulos and Watowich, 2008) as well as trophoblast cells (Fitzgerald et al., 2008; Fitzgerald et al., 2005). In addition, G-CSF promotes angiogenesis and reduces ischemic damage (Ripa et al., 2006; Lee et al., 2005). Recombinant G-CSF has been widely used for the treatment of neutropenia and several infections in diabetic patients (Panopoulos and Watowich, 2008). G-CSF acts through its main receptor, G-CSFR, a molecule of 34 kDa, which is detected in the placenta, neurons, endothelial cells, and cardiomyocytes and mostly expressed in granulocytic precursors and mature neutrophils (Panopoulos and Watowich, 2008).

Infection or inflammation up-regulates the production of several factors such as IL-1β, TNF-α and lipopolysaccharide (LPS) (Demetri and Griffin, 1991), which induce the secretion of G-CSF via post-transcriptional and transcriptional mechanisms (Falkenburg et al., 1991). The promoter of G-CSF contains binding sites for transcription factors NF-κB p65 and NF-IL6/C/EBPβ, which are involved in the inflammatory response (Dunn et al., 1994). It is thought that the MAPK pathway is involved in G-CSF production (Pradervand et al., 2006). Moreover, it has been shown that IL-17, which is synthesized by a specialized class of activated CD4+ T cells (Th17) (Park et al., 2005), is a major regulator of G-CSF production. Circulating G-CSF stimulates neutrophil production in bone marrow and its mobilization to the peripheral circulation. Upon binding with its receptor, G-CSF then activates the JAK/STAT pathway. *L. rhamnosus* GR-1 activation of STAT-3 is mediated through production of G-CSF (Kim et al., 2006). Incubation of JEG-3 human trophoblastic cell line with G-CSF also leads to activation of the JAK/STAT pathway (Marino and Roguin, 2008). Both JAK1 and JAK2 are activated by the G-CSFR. The cytosolic domain of the receptor near the membrane is known to couple with the JAK kinase and its role is to help with G-CSF mediated proliferation. The cytosolic domain that is furthest from the membrane regulates gene expression, neutrophil marker protein induction and granulocytic
differentiation (Fukunaga et al., 1993). Furthermore, different signal transduction molecules are activated downstream of the JAKs, such as STAT 1, 3, and 5, the Ras/Mek/Erk1/2 pathway, the Src-related kinases, Lyn and Hck, the serine/threonine kinase Akt, and the Syk tyrosine kinase. These pathways all lead to granulocytic proliferation and differentiation (Avalos, 1996).

1.7. The immune system in pregnancy

Since the fetal extravillous trophoblast (EVT) cells invade the uterus and the uterine spiral arteries, the syncytiotrophoblast cells that line the chorionic villi are in contact with the maternal blood. At this interface, there are a large number of immune cells labeled by different Cluster of differentiation (CD) antigens. CDs are surface molecules that are recognized by monoclonal antibodies and are considered to be markers for identification of different cell types. Some examples of these are CD34 (marker for stem cell), CD20 (marker for B lymphocyte), CD3 (marker for pan T cells), CD45 (marker for all leukocyte group: granulocyte, monocyte, T lymphocyte, T helper cell, cytotoxic T cell, B lymphocyte and thrombocyte), CD4 (marker for helper T cells), CD8 (marker for cytotoxic T cells), CD56/16 (marker for natural killer cells) and CD68 (marker for monocyte/macrophage) (Helige et al., 2008). These tissues of fetal origin could be the target of an allograft rejection and therefore, must protect themselves against a possible maternal immune response (Riley, 2008). Yet, throughout pregnancy, the uterus has a unique and privileged environment that can house the fetus without rejecting the fetal allograft. This is because EVT's express human leukocyte antigen (HLA)-C, HLA-G, HLA-E and HLA-F, which are part of the major histocompatibility complex (MHC)-I. MHC-I are polymorphic proteins that bind pathogen-derived peptides and present them to CD8+(cytotoxic) T cells. However, paternal HLA-C on invading trophoblast cells makes these cells more prone to allograft rejection. This problem is solved by the presence of MHC-I specific inhibitory receptors, which engage with MHC-I molecules and prevent natural killer (NK) cell-mediated lysis by suppressing NK signalling pathways (Riley, 2008).
The decidua contains the highest number of immune cells, including NK cells, macrophages, dendritic cells and T cells compared to the rest of intrauterine tissues (Piccinni, 2002). T lymphocytes (CD4+ T cells) can be divided into two types: 1) Type 1 CD4+ cells (Th1) which carry cell-mediated immunity and also mainly produce IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFN-γ, and TNF-α. These cytokines initiate a strong response against infections; 2) Type 2 CD4+ cells (Th2) which carry humoral immunity, and stimulate the release of cytokines such as IL-4, IL-5, IL-10, IL-13, and granulocyte macrophage colony stimulating factor (GM-CSF). IL-4 mainly promotes IgE and IgG1 antibody production; IL-5 promotes growth of eosinophil colony forming units and IL-13 and IL-10 inhibit macrophage functions (Piccinni, 2002; Challis et al., 2009). Throughout pregnancy, hormones such as progesterone and estrogen have a positive effect on Th2-type cytokines by down-regulating the pro-inflammatory cytokines from macrophages and up-regulating anti-inflammatory cytokines. Urocortins, which are part of the corticotrophin-releasing factor (CRF) family, promote the Th2 response and the production of anti-inflammatory cytokines (Challis et al., 2009). A Th2 response throughout pregnancy can minimize allograft rejection and promote survival of the fetus. This is due to the fact that the Th2 cytokine IL-4 promotes T-cell production of leukemia inhibitory factor (LIF), which is essential for implantation and development of the fetus throughout pregnancy. At birth, there is an increase in production of IFN-γ which shifts the equilibrium toward Th1. Newborn infants who are exposed to a Th2 environment due to variety of factors, such as the mode of delivery, develop more IgE synthesis and allergic reactions such as atopic dermatitis. It has been shown that probiotics, especially strains of *Lactobacillus* and *Bifidobacterium*, are capable of stimulating lymphocytes and monocytes to produce more Th1 cytokines. This is associated with a decrease in incidence of atopic dermatitis (Moore et al., 2006; Vaarala, 2003). Lactobacilli in breast milk increases the number of IgM-, IgA-, and IgG-secreting cells in infants, suggesting that these organisms can improve gut immunity (Rinne et al., 2005). Infection and inflammation can shift the equilibrium to a more Th1 state prematurely and trigger a strong immune response which could lead to problems such as spontaneous abortion, premature birth, preeclampsia and intrauterine growth restriction (Orsi and Tribe, 2008). Recent studies have shown that IL-17-
producing T-helper cells (Th17), also promote an inflammatory state. Bacterial endotoxins lead to an increase in production of IL-17 from Th17 cells, but this can be inhibited by the use of bifidobacteria (Vojdani and Lambert, 2009; Tanabe et al., 2008). Our studies have specifically examined the effect of lactobacilli on pro- and anti-inflammatory cytokine production by human placenta trophoblast cells.

1.8. Fetal sex regulation of gestational diseases

Preterm birth is more frequent in women carrying a male fetus (Ingemarsson, 2003; Zeitlin et al., 2002), and male fetuses are more likely to face intrauterine or neonatal death as well as other complications (Smith, 2000). Male infants are also at greater risk of developing motor and neurological impairments (Smith, 2000). It has recently been shown that diabetic mothers who are carrying a male fetus experience a greater incidence of stillbirth (Engel et al., 2008).

Different mechanisms have been proposed to explain the higher incidence of PTB in women carrying male fetuses. One of the theories suggests that the higher body weight of male fetuses would trigger preterm birth (Hall and Carr-Hill, 1982; McGregor et al., 1992b). Others suggest a high susceptibility to certain complications during pregnancy such as hypertension, preeclampsia and infection (MacGillivray and Davey, 1985; Campbell et al., 1983; Elsmen et al., 2006). It is known, however, that the response to bacterial pathogens is sex-dependent with females being more effective in mounting an immune response and having a better prognosis in septic shock than men in both the neonatal and adult periods (Imahara et al., 2005; Schroder et al., 1998). Studies also suggest that there is a higher concentration of IL-6, IL-1β and chemokine IP-10 in LPS-challenged macrophages from male mice and this could be due to a higher expression of TLR-4 and its co-receptor, CD14 compared to macrophages of female mice. Such differences could account for the higher incidence of sepsis in male premature neonates (Marriott et al., 2006). In mothers with an inflammatory response due to a condition like asthma, there is an increase in Th2 cytokines compared to Th1 cytokines in the placenta of female fetuses, with perhaps a shift to an anti-inflammatory state (Clifton, 2005). Some studies suggest that this gender difference is due to
sex-linked biochemical processes, such as preterm labour being induced by estrogen production from androgen precursors or by IL-1 that could be different among the two fetal sexes (Cooperstock and Campbell, 1996). Female babies born prematurely have also been shown to have a greater activity of 11β-HSD2, the enzyme that converts cortisol to inactive cortisone. This could account for the improved outcomes with premature female infants compared to males (Stark et al., 2009).

1.9. Summary

Bacterial vaginosis, which is associated with a 1.4-fold increase in risk of preterm birth, is characterized by colonization of pathogenic bacteria in the vaginal tract and absence of a crucial component of the normal flora, lactobacilli. Endotoxins released from pathogenic bacteria lead to an increase in the output of the pro-inflammatory cytokines and prostaglandins, which are required for initiation of contraction and preterm birth. Probiotic lactobacilli have been shown to reverse bacterial vaginosis in non-pregnant women and to increase anti-inflammatory cytokines. However, their effectiveness in treating BV in pregnant women and preventing preterm birth remains to be established. Therefore, this thesis examined the role of lactobacilli on the mediators of infection/inflammation-mediated preterm birth. In particular, the effect of lactobacilli on the output of cytokines and expression of prostaglandin-regulating enzymes in LPS-stimulated placental and chorion trophoblast cells was investigated. Moreover, comparisons were made between the response from placentae of female and male fetuses in order to provide an explanation for the higher incidence of preterm birth observed with male fetuses.
Chapter Two

Rationale, Hypothesis and Specific Aims
Chapter 2

2. Rationale, Hypotheses and Specific Aims

2.1. Rationale and hypothesis

Preterm birth (PTB) remains a major challenge in obstetrics. It complicates up to 13% of all pregnancies and accounts for approximately 80% of neonatal mortality and morbidity. Bacterial vaginosis (BV) is associated with a 1.4-fold increased risk of preterm birth. Studies have also shown that there is a higher incidence of spontaneous preterm birth and poorer neonatal outcome in pregnancies with a male fetus. Due to ineffectiveness of antibiotics and other treatments, probiotics may serve as a better alternative to prevent PTB. Studies suggest that *L. rhamnosus* GR-1 is able to resolve some episodes of BV in non-pregnant women as well as up-regulate anti-inflammatory and down-regulate pro-inflammatory cytokines. The objective of the work described in this thesis was to assess the effect of lactobacilli on intrauterine tissues and define the signaling mechanisms by which it has its effects. The long term objective of this work is to develop new therapeutic approaches to prevent preterm birth. We hypothesized that probiotic lactobacilli will help to maintain a healthy pregnancy by interfering with the cascade leading to infection/inflammation-mediated preterm labour and this effect is more predominant in women carrying female fetuses. Manipulation of *L. rhamnosus* GR-1 levels could, therefore, represent an alternative treatment modality to prevent preterm birth.

Microorganisms access the intrauterine environment through ascending infection as well as by haematogenous passage through the placenta. Therefore, we studied the chorion, since it is the barrier to an ascending infection and the major site of PGDH activity. We also studied the placenta since it is where blood borne factors pass from the mother to fetus. We investigated the output of cytokines, since they have been associated with infection of the fetal membranes. We also assessed the expression of prostaglandin-regulating enzymes, since they play a key role in uterine contractility and labour. In order to elucidate the mechanism of action of lactobacilli, we focused on different signaling pathways such as JAK/STAT and MAPK.
The syncytiotrophoblast cells that line the chorionic villi are in contact with the maternal blood which contains a large number of immune cells that are capable of initiating an inflammatory response. Therefore, the source of the cytokines could be the trophoblast cells or the immune cells of the maternal blood. Magnetic pulling was used to separate the two cell types and investigate the role of each cell type in initiation of the inflammatory cascade.

2.2. Specific Aims

Chapter three: The effect of *Lactobacillus rhamnosus* GR-1 supernatant and fetal sex on cytokine profile and PG-regulating enzyme expression in LPS-stimulated human chorion and placental trophoblast cells

We hypothesized that *L. rhamnosus* GR-1 supernatant would down-regulate the pro-inflammatory cytokines and PG synthesizing enzyme, PTGS2, and up-regulate the anti-inflammatory cytokines and the PG metabolizing enzyme, PGDH, in both placental and chorion trophoblast cells and that this would be more evident in placentae of female fetuses. The specific aims were as follows:

1. To investigate the effect of *L. rhamnosus* GR-1 supernatant and fetal sex on pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and anti-inflammatory cytokine IL-10 output in LPS-induced placental and chorion trophoblast cells

2. To assess the effect of *L. rhamnosus* GR-1 supernatant and fetal sex on the PG-synthesizing enzyme, PTGS2 and the PG-metabolizing enzyme, PGDH in LPS-induced placental and chorion trophoblast cells

3. To compare expression of TLR-4 receptor in placentae of male and female fetuses

4. To investigate the effect of *G. vaginalis*, *L. crispatus* and *L. iners* on pro-inflammatory TNF-α and anti-inflammatory IL-10 as well as prostaglandin-regulating enzymes, PTGS2 and PGDH
Chapter four: The potential mechanism of action of *Lactobacillus rhamnosus* GR-1 supernatant on placental trophoblast cells

We hypothesized that JAK/STAT and MAPK pathways play a key role in the mechanism of action of lactobacilli. The specific aims were as follows:

1. To investigate the effect of *L. rhamnosus* GR-1 supernatant on phospho-STAT3 and phospho-p38 expression in placental trophoblast cells
2. To determine the effect of JAK and p38 inhibitors on IL-10 output in placental trophoblast cells
3. To study the effect of JAK, p38, JNK and NF-κB inhibitors on TNF-α output in placental trophoblast cells
4. To determine the effect of TNF-α neutralizing antibody on the expression of PTGS2 in placental trophoblast cells
5. To compare the expression of TLR-2 receptor in placentae of male and female fetuses

Chapter five: The effect of *Lactobacillus rhamnosus* GR-1 supernatant and fetal sex on G-CSF and the potential role of immune and placental trophoblast cells in initiating an immune response.

We hypothesized that *L. rhamnosus* GR-1 supernatant would increase the production of G-CSF in placentae from female fetuses more than in placentae from male fetuses. As well, we hypothesized that the immune and placental trophoblast cells are both necessary for the initiation of an inflammatory cascade and modulating cytokine output. The specific aims were as follows:

1. To investigate the effect of *L. rhamnosus* GR-1 supernatant and fetal sex on anti-inflammatory G-CSF in LPS-induced placental trophoblast cells
2. To determine the effect of JAK and p38 inhibitors on G-CSF output in placental trophoblast cells

3. To determine the effect of G-CSF inhibitor on TNF-α output in placental trophoblast cells

4. To determine the effect of fetal sex on G-CSFR expression in placental trophoblast cells

5. To investigate the effect of *L. rhamnosus* GR-1 supernatant on TNF-α, IL-10 and G-CSF output in pure placental trophoblast cells, CD45+ cells and in combination
Chapter Three

The effect of *Lactobacillus rhamnosus* GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: implications for treatment of bacterial vaginosis and prevention of preterm labour

The contents of this chapter were published in Am J Obstet Gynecol. 2009 May;200(5):532.e1-8 and appear here with the permission of the journal (refer to attached authorization). My role was conducting the experiments, analyzing the results and preparing the manuscript.
Chapter 3

3. Effect of Lactobacillus rhamnosus GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: implications for treatment of bacterial vaginosis and prevention of preterm labour

3.1. Introduction

Preterm birth (PTB) remains a major challenge in obstetrics. It complicates up to 13% of all pregnancies (Goldenberg et al., 2008b) and accounts for approximately 80% of neonatal mortality and morbidity (Mattison et al., 2001). It has been reported that preterm birth is more frequent in women carrying a male fetus (Zeitlin et al., 2002; Ingemarsson, 2003). In addition, male fetuses are more likely to face intrauterine or neonatal death as well as other complications (Smith, 2000).

Preterm birth is multifactorial, but one of the major causes is infection (Goldenberg et al., 2008b; Hillier et al., 1995). Bacterial Vaginosis (BV), an alteration in the endogenous vaginal microbiota associated with decreased levels of certain Lactobacillus (McGregor and French, 2000), is associated with a 40% increase in risk of PTB (Hillier et al., 1995). BV is also associated with the presence of pathogenic bacteria such as Gardnerella vaginalis (Saunders et al., 2007). Two common ways through which such microorganisms could access the uterine environment are by ascending through the vagina and the cervix, passing through the fetal membranes or by haematogenous means through the placenta (Goldenberg et al., 2008b). Therefore, both placental and chorion trophoblast cells were examined in the present study. Previous studies have reported an increase in the release of the pro-inflammatory cytokines associated with infection in humans, animals and in vitro models of infection-mediated PTB (Dudley, 1997; Romero et al., 2004; Mussalli et al., 1999; Elovitz and Mrinalini, 2004). In addition, BV-associated pathogens up-regulate expression of prostaglandins, leading to activation of premature uterine contractions and preterm birth (Challis et al., 2002; Romero et al., 1989b). Sexual dimorphism exists in immune response to bacterial pathogens with women being more effective in mounting an immune response to
pathogens and having a better prognosis in septic shock than men in both neonatal and adult periods (Imahara et al., 2005; Schroder et al., 1998; van Eijk et al., 2007). To date, antibiotics have been the most common treatment for BV and prevention of infection-mediated preterm birth. However, these treatments are largely unsuccessful (Simcox et al., 2007) and in some studies, have been shown to increase the risk of preterm birth (Shennan et al., 2006).

A potentially novel way to protect against infection-mediated preterm birth is to use probiotic lactobacilli (Reid and Bocking, 2003). Probiotics defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Reid and Bocking, 2003) are being studied as a treatment alternative (Reid and Bocking, 2003). Although many strains of lactobacilli such as Lactobacillus crispatus and Lactobacillus iners are thought to be good probiotic candidates (Saunders et al., 2007; Martin et al., 2008), L. rhamnosus GR-1 is the most commonly used probiotic strain mainly due to its ability to colonize and replenish vaginal lactobacilli and modulate immunity (Kim et al., 2006; Reid et al., 2003a; Reid et al., 2005).

At present, the effect of probiotics on intrauterine tissues and the mechanisms underlying the sex difference observed in the incidence of preterm birth or effectiveness of treatments are unexplored. Therefore, we sought to determine possible sexual dimorphisms in the expression of the inflammatory mediators as a result of bacterial endotoxin treatment, as well as the ability of probiotic lactobacilli supernatant to antagonize these effects.

3.2. Materials and Methods

3.2.1. Samples

All studies were approved by the Research Ethics Boards of Mount Sinai Hospital and Faculty of Medicine, University of Toronto in accordance with the Canadian Tri-Council Policy Statements on Human Ethics Reviews (IRB# 04-0018-U). Placental tissues were collected from women undergoing term (> 37 weeks of gestation) elective caesarean section at Mount Sinai Hospital, Toronto. The subjects experienced a healthy pregnancy with no clinical infection and had no signs of labour prior to delivery. Indications for delivery by elective cesarean section included abnormal presentation of the fetus, cephalopelvic
disproportion or previous caesarean sections. Informed consent was obtained before tissue collection.

3.2.2. Placental and chorion trophoblast cell culture

Placental and chorion trophoblast cells were isolated using established primary culture protocols (Sun et al., 1997). Placentae with adherent membranes were collected. Briefly, placental tissue was separated from the membranes and washed with saline. Approximately 60 grams of placental tissue were cut and digested in 37°C water bath using 0.125 % trypsin (Sigma, St Louis, MO) and 0.02% deoxyribonuclease-I (Sigma) in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Inc., Grand Island, NY) three times for 30 minutes each. Supernatant was collected each time, centrifuged at 37°C, 1700 x g for 10 minutes and resuspended. The pooled supernatant was then passed through a 200 μm pore size nylon gauze filter and loaded onto a continuous Percoll (Sigma) gradient (5-70 % at step increments of 5 %), and centrifuged at 2500 x g for 20 minutes to separate different cell types. Cytotrophoblasts between the density markers of 1.049 and 1.062 g/ml were collected and washed using DMEM and centrifuged at 1700 x g for 10 minutes. The precipitate was then diluted using DMEM culture medium containing 10% fetal bovine serum (FBS; Sigma) and 1% antibiotic solution (Sigma). The cells were plated in 24 well plates at a density of 10^6 /well (for ELISA measurements and immunostaining) or in 60 mm dishes at a density of 10^7 /dish (for protein extraction). Cells were then cultured for 72 hours at 37°C in 5 % CO₂ and 95 % O₂. Cells aggregated to form a syncytium. The purity of the cell preparation was assessed by histochemical staining for cytokeratin, an epithelial cell lineage marker (DAKO Corp., Glostrup, Denmark), or vimentin, a mesenchymal cell lineage marker (DAKO Corp.). Cells were counterstained with Carrazzi’s hematoxylin. Immunohistochemical analysis showed approximately 90-95% of placental cells were cytokeratin positive as expected with a minority of vimentin stained fibroblast contamination (Figure 3.1, Page 63). The percentage of purity of each cell type was quantified based upon counting each cell type and expressing the value as a percentage of the total number of cells in each field of view. The percentage of purity for duplicate wells of same treatment, with 5 sample field views per well were then averaged and reported. In the case of chorion culture, the same protocol as described above was applied with minor modifications. Chorion with adherent decidua was peeled away from
the amnion. The decidual tissues were removed using a cell scraper (Sarstedt, Germany). Chorion was then digested three times for 60 min each time with DMEM, containing 0.2% collagenase (Sigma, St Louis, MO) and 0.125 % trypsin (Sigma) without DNase I. Chorionic trophoblast cells do not aggregate to form a syncytium. Instead, they form clumps or remain as single cells.

3.2.3. Treatment of placental and chorion trophoblast cells

Supernatant from *L. rhamnosus* GR-1 culture was prepared as previously described (Kim et al., 2006). Briefly, the bacteria were grown in MRS (de Man, Rogosa and Sharpe) broth to an optical density of 1.5 at 600nm and centrifuged for 10 min at 6000 x g at 4°C. The supernatant was then filtered through a .22µm pore size filter to remove any residual bacteria. Supernatant from *Gardnerella vaginalis*, *Lactobacillus iners* and *Lactobacillus crispatus* cultures were also prepared in this manner and provided to us by Dr. Reid and Dr. Kim (University of Western Ontario, London, Ontario). *Gardnerella vaginalis* was cultured in an identical manner except Mac-Conkey Broth (MCB) was used instead of MRS broth. After 72 hours of trophoblast cell culture, cells were washed using DMEM media and serum starved for 12 hours. Cultured cells were then divided into the following groups: 1) No treatment; 2) Treatment with LPS (*Escherichia coli* O55:B5) alone (200 ng/ml for protein or 100 ng/ml for cytokine measurements) after a further 12h; 3) Treatment with *L. rhamnosus* GR-1, *Gardnerella vaginalis*, *Lactobacillus crispatus* and *Lactobacillus iners* supernatants as well as MRS and MCB broths (1:20 dilution) in different wells for 12h; 4) Pretreatment with *L. rhamnosus* GR-1, *Gardnerella vaginalis*, *Lactobacillus crispatus* and *Lactobacillus iners* supernatants as well as MRS and MCB broths (1:20) in separate wells for 12h and subsequent addition of 200 or 100 ng/ml of LPS to all of them. Preliminary studies established optimal dilution and time of treatments. To carry out these studies, *L. rhamnosus* GR-1 (1:1-1:100) and LPS (0.1-1000 ng/ml) were added to the cells, and media and protein were collected at 2, 4, 8, 12 and 24 hour time points. Controls for the study included the culture medium or MRS alone to confirm that the presence or lack of an effect is due to lactobacilli supernatant itself. All of these control treatments had no effect on the expression of the examined cytokines or proteins. Cell viability was assessed by Trypan Blue Exclusion Test, which revealed minimal cell death as a result of the basic treatments. LDH levels were
also measured in the culture medium using commercially available LDH-Cytotoxicity Assay kit according to manufacturer’s instructions (BioVision, CA, USA). A representative example of the lactate dehydrogenase (LDH) Assay is shown in Figure 3.2 (page 64). The experimental design of the study is presented in Figure 3.3 (Page 65).

3.2.4. **Immunocytochemistry**

Immediately after the culture period, the cells were incubated in 1 ml of 4% PFA (Paraformaldehyde) for 10 minutes. They were then washed with PBS two times, 5 minutes each and were incubated with 0.02% Triton X for 5 minutes. After washing with PBS two times, 5 minutes each, 3-4 drops of DAKO Protein Block Serum (DAKO, Denmark) were added to each well and the cells were then put on an orbital shaker for 30 minutes. Subsequently, they were incubated with Vimentin (DAKO; 1:100), Cytokeratin (DAKO; 1:200) and Negative Mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) primary antibodies separately overnight in 4ºC to stain for fibroblasts and cytotrophoblast cells. After washing with PBS two times, 5 minutes each, cells were incubated with horseradish peroxidase-labeled anti-mouse IgG secondary antibody (DAKO; 1:300) for one hour and washed twice with PBS. Seven drops of DAKO LSAB2 Streptavidin-HRP (DAKO) were added to each well for 30 minutes and cells were subsequently washed with PBS twice, each 5 minutes. DAB (3,3’-diaminobenzidine; DAKO; 2 mL) was added to each well for a brief exposure and then replaced with water. Hematoxilin (Sigma; 1 mL) was added and then replaced with water. Images were assessed using a Leica DMRXE microscope (Leica Microsystems). Different fields were observed for each staining, and a representative image was photographed for each with a Sony D XC-970 MD 3CCD color video camera. The percentage of purity of each cell type was quantified based upon counting each cell type and expressing the value as a percentage of the total number of cells in each field of view. The percentage of purity for duplicate wells of same treatment, with 5 sample field views per well, were then averaged and reported.
3.2.5. Protein extraction and Western blot analysis

Eight hours following LPS treatment, media were collected from 24 well plates. Cells from 60 mm dishes were scraped off with cold RIPA lysis buffer containing Mini EDTA-free protease inhibitors (Boehringer Mannheim Biochemicals, Mannheim, Germany). They were then mixed on a vortex shaker three times, 20 minutes apart, and centrifuged for 30 minutes at 4°C, 12,000 rpm. The supernatant was then collected. Protein concentration was measured by Bradford assay using a protein assay kit (Bio-Rad Laboratories Inc., Mississauga, ON) with bovine serum albumin as standard. The samples were then stored at -80ºC until further analysis. Twenty-five micrograms of the protein extracts were mixed with specific volumes of RIPA and 10% Loading buffer was added to bring the total volume to 20 µl. Samples were then incubated at 55°C for 10 minutes and subjected to standard 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories Inc). The blots were blocked overnight at 4°C with 5% nonfat milk (Nestle, Glendale, CA) in PBS with 0.1% Tween-20 (Bio-Rad Laboratories, Inc.). They were then incubated separately with PTGS2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI; 1:1,000), PGDH polyclonal antibody (Dr. Tai, University of Kentucky, USA; 1: 3,500), and TLR-4 antibody (eBioscience, San Diego, CA; 1:2,000) for 1 hour. The blots were washed 5 times, each for 5 minutes, with PBST (Phosphate Buffered Saline Tween) and incubated for 1 hour with a horseradish peroxidase-labeled anti-mouse IgG (Amersham Pharmacia Biotech, Upsala, Sweden; 1:5,000) in the case of PTGS2 and with anti-rabbit IgG coupled to horseradish peroxidase secondary antibody in case of PGDH and TLR-4 (Amersham Pharmacia Biotech; 1:4,000 and 1:5,000 respectively). Blots were then washed with PBST 6 times, each for 5 minutes. Enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) were added to the membranes for 1 minute, and the blots were then exposed to X-OMAT blue film (Kodak, Rochester, NY) for 5 minutes in the case of PGDH and PTGS2 and 20 minutes in the case of TLR-4. The antibodies revealed signals at 29 kDa and 80-90 kDa for PGDH and TLR-4 respectively. PTGS2 antibody revealed a strong signal at 72 kDa. However, there was evidence of a doublet in this band, which could indicate the presence of glycosylation that is known to shift the PTGS2 band by about 10 kDa (Jang et al., 2007). The
lower band did not change across treatments. The whole range was included in the densitometric analysis to account for both forms of PTGS2. The intensities of protein signals were measured by scanning (6200C scanner, Hewlett Packard Co., Mississauga, ON) and analyzed by using Scion Image software (version 4.0.2, Scion Co., Frederick, MD). All densitometric comparisons were made in the linear range of band intensity. To standardize for protein loading, the blots were stripped and re-probed for β-actin housekeeping protein. The primary antibody was a monoclonal mouse anti-human β-actin antibody (Sigma A-5316; 1:7,000) and the secondary antibody was horseradish peroxidase-labeled anti-mouse IgG (Amersham Pharmacia Biotech; 1:7,000). Blots were incubated for 1 hour with each antibody and then exposed to X-OMAT blue film for one minute. Sigma anti-β-Actin antibody revealed a strong 41 kDa band. Optical densities of PTGS2, PGDH and TLR-4 were divided by the optical density values for β-actin to adjust for protein loading and these relative optical densities (ROD) were then used for analysis.

### 3.2.6. Cytokine measurements

Levels of TNF-α, IL-1β, IL-6 and IL-10 in the culture media were measured using commercially available quantitative ELISA (Enzyme-Linked ImmunoSorbent Assay) kits (Cayman Chemical, Ann Arbor, MI for TNF-α, IL-6 and IL-1β and eBioscience, San Diego, CA for IL-10) according to the manufacturer’s instructions. The minimum detectable limits of the assays were 1.5 pg/ml, 1.5 pg/ml, 7.8 pg/ml and 2 pg/ml for TNF-α, IL-1β, IL-6 and IL-10, respectively. The intra-assay coefficients of variation for these cytokines were <5%. All samples from each experiment were run on the same assay plate.

### 3.2.7. Statistical Analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.). Comparisons between the data were performed using Sigma Stat (Jandel Scientific Software, San Rafael, CA). The criteria for statistical significance were set at p < 0.05 after one-way ANOVA, followed by Student-Newman-Keuls test (for comparison across all treatments) or two-tailed student’s t-test (for comparison between the two sexes). Although the numbers of the
placentae from different fetal sex are different, for any measurement, the n value was the same across all treatments.

3.3. Results

3.3.1. Optimizing time and dose of treatments

Preliminary studies were performed on placental trophoblast cells in which the cells were treated with *L. rhamnosus* GR-1 in dilutions of 1:1, 1:10, 1:20, 1:50 and 1:100 and with LPS in doses of 0.1, 1, 10, 100 and 1000 ng/ml for period of 2, 4, 8, 12 and 24 hours. Optimization experiments showed that the LPS effect in increasing TNF-α output was greatest at 100 ng/ml when cells were treated for a period of 8 hours (Figure 3.4A,B, Page 66). Others have previously shown that the effect of LPS in up-regulating PTGS2 expression in chorion and placental trophoblast cells is greatest at the dose of 200 ng/ml when applied for the same period of time (Li et al., 2007). In the case of *L. rhamnosus* GR-1 supernatant, the effect on TNF-α (Figure 3.5A,B, Page 67) output and PTGS2 expression (Figure 3.6A-D, Page 68) was greatest at 1:20 dilution, when applied at an optimal time of 12 hours. Other dilutions of *L. rhamnosus* GR-1 applied at other time points had a similar trend with the exception of 1:1 dilution at which the syncytiotrophoblast was not formed and the cells died. In previous studies, 1:20 dilution was also consistently the optimal dilution in cultures of mouse macrophages (Kim et al., 2006). *Gardnerella vaginalis, Lactobacillus iners* and *Lactobacillus crispatus* supernatants were applied at the same time as *L. rhamnosus* GR-1 supernatant in order to compare the effects. The time point of measurement for all the cytokines and proteins were also kept the same for comparison studies.

3.3.2. Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on pro-inflammatory cytokines TNF-α, IL-1β and IL-6

LPS caused a significant increase in the output of TNF-α in placental trophoblast cells from pregnancies carrying a male fetus (35.3 ± 7.2 pg/ml to 1386.6 ± 346.6 pg/ml; N=9; p<0.001) and pregnancies with a female fetus (33.3 ± 6.2 pg/ml to 529.4 ± 66.5 pg/ml; N=11; p<0.001). All figures are presenting results relative to control values. Stimulation of TNF-α by LPS in the placental trophoblast cells from women carrying a male fetus was significantly
higher than for females (>2.5-fold, p=0.015, Figure 3.7A, Page 69). *L. rhamnosus* GR-1 supernatant alone caused a small increase in output of TNF-α, but markedly inhibited the LPS-stimulated response in the trophoblast cells from both sexes (5.1-fold decrease in males to a concentration of 271.0 ± 76.2 pg/ml and 6.9-fold decrease in females to a concentration of 76.7 ± 14.0 pg/ml; p<0.001, Figure 3.7A, Page 69). No effect was observed with any of the treatments on IL-1β output (p>0.05; N=6 males and N=5 females, Figure 3.7B, Page 69). LPS and *L. rhamnosus* GR-1 as well as the combination of both treatments increased IL-6 output in placental trophoblast cells from placentae of both male (>6.0-fold, >4.3-fold and >4.6-fold for these treatments respectively, p=0.003; N=6, Figure 3.7C, Page 69) and female (>4.1-fold, >2.6-fold and >3.0-fold respectively for the above treatments, p<0.001; N=9, Figure 3.7C, Page 69) fetuses.

### 3.3.3. Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on anti-inflammatory cytokine IL-10

Similar to TNF-α, LPS stimulated IL-10 output by placental trophoblast cells from pregnancies carrying a male or a female fetus by 12.8-fold and 4.7-fold respectively (5.2 ± 0.5 pg/ml to 66.3 ± 13.5 pg/ml in males (N=12) and 5.2 ± 0.4 pg/ml to 24.4 ± 14.1 pg/ml in females (N=10); p<0.001). The response was 2.7-fold greater in placentae of the male compared to female fetuses (p=0.007, Figure 3.8A, Page 70). *L. rhamnosus* GR-1 supernatant alone increased IL-10 output in the placental trophoblast cells of both sexes (26.9 ± 6.9 pg/ml in males and 13.5 ± 5.6 pg/ml in females, p<0.001, Figure 3.8A, Page 70), but did not further alter the IL-10 responses when used in combination with LPS either in males (92.5 ± 26.2 pg/ml, p<0.001, Figure 3.8A, Page 70) or females (37.6 ± 12.0 pg/ml, p<0.001, Figure 3.8A, Page 70). The endogenous IL-10 concentration in the supernatant was less than that of the control media (Figure 3.8B, Page 70). In addition, we observed that the ratio of TNF-α: IL-10 in control cultures of placentae from pregnancies carrying a male or a female fetus were 7:1 and 11:1 respectively. This is compared to the ratio of 36:1 and 41:1 in LPS-stimulated trophoblast cells. Therefore, the extent of LPS stimulation of IL-10 is much lower than that of TNF-α (N=9 males, p<0.001; N=11 females, p=0.016, Figure 3.9, Page 71). Pre-treatment
with *L. rhamnosus* GR-1 supernatant reduced this ratio to 4:1 and 7:1 for males and females respectively, which were close to the ratios in the control cultures.

### 3.3.4. Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on prostaglandin-synthesizing enzyme, PTGS2

LPS stimulated PTGS2 protein levels (72 kDa) in placental trophoblast cells from both sexes (2.5-fold increase in males (N=8), p=0.004, and 1.2-fold increase in females (N=9), p=0.004). The effect was 2.1-fold greater in males than females (p=0.005, Figure 3.10A,B,C, Page 72). There was no significant effect on PTGS2 with *L. rhamnosus* GR-1 supernatant treatment alone, but the LPS-stimulated expression was reduced in trophoblast cells from male pregnancies by 30% when pretreated with *L. rhamnosus* GR-1 supernatant (p=0.08, Figure 3.10C, Page 72). *L. rhamnosus* GR-1 supernatant alone or in combination with LPS did not change PTGS2 expression in trophoblast cells of the placentae from female fetuses.

### 3.3.5. Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on prostaglandin-metabolizing enzyme, PGDH

LPS did not alter the expression of PGDH protein (29 kDa) in cultured trophoblast cells from pregnancies of either sex. However, *L. rhamnosus* GR-1 supernatant alone, as well as in combination with LPS, increased expression of PGDH (>1.5-fold, and >2.1-fold respectively compared to control non-treated trophoblast cells, p=0.002, N=7), in the placentae of female but not male fetuses (Figure 3.11B,C, Page 73). PGDH expression was significantly higher in placentae of the female fetuses (>1.6-fold for *L. rhamnosus* GR-1 supernatant treatment, p= 0.012, and >2.1-fold for the combination of LPS and supernatant treatments, p=0.006), compared to trophoblast cells from placentae of male fetuses, which did not show a difference in PGDH expression across treatments (Figure 3.11A,C, Page 73; p>0.05, N=9).

### 3.3.6. Effect of fetal sex on TLR-4 expression

Western blotting revealed the presence of a strong 90 kDa band for TLR-4 in placental trophoblast cells (Figure 3.12A, Page 74). Relative optical densities showed a higher
expression of TLR-4 protein in the placental trophoblast cells from pregnancies with a male compared to female fetus (>2.1-fold, p=0.019, N=9 males and N=11 females, Figure 3.12B, Page 74).

3.3.7. Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on cytokines and prostaglandin-regulating enzymes in chorion trophoblast cells

LPS caused an increase in the output of TNF-α in chorion trophoblast cells from pregnancies carrying a male or a female fetus (33.3-fold, N=6; p<0.001 in males and 16.0-fold, N=6; p<0.001 in females). The output of TNF-α from chorion trophoblast cells in control media was 18.93 pg/ml compared to 34.03 pg/ml in placental trophoblast cells. There was no difference in the cytokine responses between the two fetal sexes. *L. rhamnosus* GR-1 supernatant significantly inhibited the LPS-stimulated increase in TNF-α in chorion trophoblast cells from placentae of both male and female fetuses (Figure 3.13A, Page 75).

LPS, *L. rhamnosus* GL-1 supernatant and the combination of both treatments also caused an increase in IL-10 output (2.6-, 1.5-, and 2.5-fold increase respectively in male pregnancies (N=10; p<0.001) and 7.1-, 3.1-, and 4.2-fold increase respectively in female pregnancies (N=7; p<0.001)). The output of IL-10 from chorion trophoblast cells in control media was 8.56 pg/ml compared to 5.00 pg/ml in placental trophoblast cells. Females had a greater response than males as the result of LPS (2.7-fold; p=0.03) and *L. rhamnosus* GR-1 supernatant (2.1-fold; p=0.017) treatments (Figure 3.13B, Page 75).

LPS also caused an increase in the expression of PTGS2 in chorion trophoblast cells in pregnancies carrying either a male or a female fetus (1.7-fold in males, N=7; p<0.001 and 1.5-fold in females, N=8; p=0.002). There was no difference in this response of the chorion trophoblast cells from pregnancies carrying a male or a female fetus and the LPS-stimulated expression of PTGS2 was not down-regulated by pre-treatment with *L. rhamnosus* GR-1 supernatant in placentae from either fetal sex (Figure 3.13C, Page 75).

There was no effect of LPS or *L. rhamnosus* GL-1 supernatant treatment on the expression of PGDH in chorion trophoblast cells in placentae of either fetal sex (Figure 3.13D, Page 75).
3.3.8. Effect of different bacterial strains on cytokines and prostaglandin-regulating enzymes in placental trophoblast cells

The effect of supernatant of different strains of lactobacilli as well as Gardnerella vaginalis was investigated in placental trophoblast cells. *L. rhamnosus* GR-1, *Gardnerella vaginalis*, *Lactobacillus crispatus* and *Lactobacillus iners* were all able to inhibit the LPS-stimulated increase in TNF-α (Figure 3.14A, Page 76, N=6; p<0.001) in placental trophoblast cells. The same bacterial strains with the exception of *Gardnerella vaginalis* increased the output of IL-10 (Figure 3.14B, Page 76, N=5; p=0.01).

Expression of PTGS2 was only partially down-regulated with *L. rhamnosus* GR-1 supernatant treatment while supernatants from other strains of lactobacilli did not have any effect. *Lactobacillus crispatus* caused a slight increase in PTGS2 expression (Figure 3.15A,B, Page 77, N=6; p=0.008). *L. rhamnosus* GR-1 supernatant was also able to increase PGDH expression while other strains’ supernatants did not have any effect on this enzyme (Figure 3.15C,D, Page 77, N=6; p=0.017).

The effect of different broths in which the strains of bacteria were grown in was also investigated in placental trophoblast cells. Neither MRS nor MCB were able to inhibit the LPS-stimulated increase in TNF-α (Figure 3.16A, Page 78, N=4; p<0.001) or PTGS2 expression (Figure 3.16B,C, Page 78, N=6; p<0.001).

3.4. Comment

The present study is the first to our knowledge to identify an effect of fetal sex on the LPS-stimulation of cytokines and prostaglandin-synthesizing enzyme, PTGS2 as well as to investigate the ability of *L. rhamnosus* GR-1 supernatant to interfere with the inflammatory cascade associated with preterm labour.

In this study, we found that LPS markedly stimulated the output of TNF-α, known to play a key role in infection-mediated preterm birth (Challis et al., 2002; Dudley, 1997; Hillier et al., 1993; Romero et al., 2004; Zaga et al., 2004), in both placental and chorion trophoblast cells. This confirms previous findings that in intrauterine tissues, LPS increases the release of pro-
inflammatory cytokines (Zaga et al., 2004). Pro-inflammatory cytokines also increase prostaglandin synthesis and MMP-9 expression, leading to rupture of membranes, uterine contractions and preterm labour (So et al., 1992; Mitchell et al., 1991; Mitchell et al., 1990; Challis et al., 2001a). Down-regulation of TNF-α output will, therefore, significantly interfere with these processes. Here, we have reported sex specific differences in the ability of LPS to increase output of TNF-α, with the male fetuses being more responsive to LPS treatment, promoting an inflammatory state. Potentially, this could account for the higher incidence of neonatal sepsis observed with male fetuses. *L. rhamnosus* GR-1 supernatant markedly inhibited LPS stimulated output of TNF-α in both placental and chorion trophoblast cells of both sexes. Although the same trend exists in the case of IL-6, where LPS increases IL-6 output more in placentae of male pregnancies and *L. rhamnosus* GR-1 supernatant partially down-regulates this stimulation, the effect was not statistically significant nor as potent as with TNF-α. The levels of IL-6 were observed to be much lower than TNF-α in placental trophoblast cells. In contrast to TNF-α, we found minimal output of IL-1β with all of the treatment groups. However, it has previously been reported that there are increased levels of IL-1β in association with chorioamnionitis and preterm delivery (Menon et al., 1995). One possible explanation for our finding could be the tissue-specific expression of this cytokine. It has been shown that IL-1β mRNA is mainly localized in chorionic cells in response to endotoxins such as LPS (Menon et al., 1995). The levels of IL-1β mRNA also decrease markedly during the first 72 hours of primary culture when cytotrophoblast cells differentiate into syncytiotrophoblasts (Stephanou et al., 1995). As our study assessed the output of IL-1β after syncytialization, this may explain the lack of effect of LPS on IL-1β output.

IL-10 is an important immunoregulatory and anti-inflammatory cytokine expressed at the human maternal-fetal interface (Roth et al., 1996). LPS causes an increase in the output of anti-inflammatory cytokines, which counter the local effects of inflammation (Shoji et al., 2007). We have shown in this study that the increase in IL-10 with LPS is more pronounced in trophoblast cells from the male compared to placentae of female fetuses. *L. rhamnosus* GR-1 supernatant alone or in combination with LPS increased IL-10 output. Our findings are consistent with results obtained using mouse macrophages *in vitro* whereby the same *L. rhamnosus* GR-1 supernatant increased IL-10 output (Kim et al., 2006; Pessi et al., 2000).
Although LPS stimulates both TNF-α and IL-10, we found that the ratio of TNF-α: IL-10 after LPS treatment was much greater than in control cultures. This confirms an imbalance of pro- and anti-inflammatory mediators, known to be important in the mechanism whereby exposure to pathogens leads to intrauterine infection (Raghupathy and Kalinka, 2008). L. rhamnosus GR-1 supernatant pre-treatment reduced the ratio of TNF-α to IL-10 to that of control media.

Prostaglandins are important for parturition to occur in an orderly physiological manner. Premature expression of prostaglandins causes weakening of the membranes, softening of the cervix and uterine contraction, all of which can contribute to preterm birth (Challis et al., 2002). Therefore, we examined the role of fetal sex and the effect of L. rhamnosus GR-1 supernatant on the expression of LPS-stimulated PTGS2 as well as PGDH, enzymes responsible for the synthesis and degradation of prostaglandins, respectively. As in the case of TNF-α and IL-10, trophoblast cells from placentae of male fetuses were more responsive to LPS stimulation in expressing PTGS2. These results suggest that there is a greater synthesis of the active prostaglandins in placenta of the male fetuses in a state of inflammation which may explain the higher incidence of preterm birth reported in males. L. rhamnosus GR-1 supernatant attenuated this increase in PTGS2 by 30% in placentae of male fetuses. This suggests a second mechanism, whereby L. rhamnosus GR-1 could interfere with the cascade of prostaglandin output, uterine contractility and preterm birth. L. rhamnosus GR-1 supernatant alone and together with LPS also enhanced the expression of PGDH protein, which was greater in placentae of female fetuses. This protective effect could also contribute to a lower incidence of preterm birth in female fetuses.

The underlying mechanism for the observed sex difference in LPS stimulation of cytokines and prostaglandin-regulating enzymes is not known. Previous studies have suggested a higher expression of TLR-4 in adult male mice (Marriott et al., 2006). TLR-4 is the receptor that primarily mediates the effect of LPS in multiple human models. It signals the translocation of NF-κB to the nucleus to activate transcription of genes encoding cytokines and chemokines (Cavaillon and Adib-Conquy, 2006). We considered that differential expression of this receptor in the placentae of males and females might contribute to our understanding of the sexual dimorphism in LPS stimulation. We found that TLR-4 was
expressed in greater abundance in placental trophoblast cells of male fetuses, which may contribute to the heightened inflammatory response observed in these fetuses. This in turn could also contribute to the increased incidence of preterm birth, sepsis and poorer outcome during fetal and neonatal periods.

The sex specific responses observed in placental trophoblast cells were not evident in chorion trophoblast cells. Chorion is the barrier through which microorganisms ascending through the vaginal tract must pass first in order to access the uterine environment. Even though *L. rhamnosus* GR-1 supernatant inhibited the LPS-stimulated TNF-α and increased IL-10 outputs, it did not play a role in altering the expression of prostaglandin-regulating enzymes as was observed with placental trophoblast cells. Since chorion and placental trophoblast share the same embryological origin, which is the same in a male or a female pregnancy, we propose that the proximity of maternal decidua to chorion and the cross-talk at this interface might play a role in the specificity of the response observed in this tissue. Another plausible explanation could be the interaction of the immune cells in the maternal blood with the placental trophoblast cells. This implies that the response to an infection is different depending on the route through which microorganisms could access the uterine environment. In the case of placenta, this would be by haematogenous means as opposed to an ascending infection, in the case of chorion.

Although *L. rhamnosus* GR-1 is one of the most commonly used probiotic candidates and evidence supports its effectiveness in reversing bacterial vaginosis and initiating an anti-inflammatory cascade (Kim et al., 2006; Reid and Bocking, 2003; Reid et al., 2003a), we investigated whether other strains of lactobacilli, which are found in the normal flora, share the same anti-inflammatory properties. We also compared these effects to the action of *G. vaginalis*, which is considered one of the BV-causing microorganisms. *L. crispatus* and *L. iners* both inhibited the LPS-stimulated TNF-α and increased the IL-10 outputs in placental trophoblast cells. However, unlike *L. rhamnosus* GR-1, they did not down-regulate PTGS2 and up-regulate PGDH expression. This suggests that *L. rhamnosus* GR-1 is possibly the better probiotic candidate for reducing the risk of PTB. *G. vaginalis* was expected to increase the pro-inflammatory cytokine TNF-α and up-regulate PTGS2 expression since it is found in altered vaginal flora associated with bacterial vaginosis. However, this bacterial strain
resulted in minimum TNF-α and IL-10 outputs and cells treated with this supernatant had no detectable expression of PTGS2 and PGDH proteins. LDH analysis further confirmed a high percentage of cytotoxicity in the media of the cells treated with this supernatant, possibly an indication of the harmful nature of this bacterial supernatant for the cells.

Overall, we have reported sex specific differences in the ability of LPS to increase the output of cytokines and the prostaglandin-synthesizing enzyme, PTGS2. Previous studies have shown that placental 11β-HSD2 activity is higher in pregnancies with female fetuses suggesting that placental glucocorticoid metabolism differs according to fetal sex. Since 11β-HSD2 inactivates cortisol to cortisone and cortisol is important in the feed-forward cascade leading to preterm birth (Myatt, 2006), the differential expression of this enzyme in placentae of the two sexes may contribute to the increased incidence of premature birth reported in women carrying male compared to female fetuses. In addition, we have shown that \textit{L. rhamnosus} GR-1 supernatant alone and together with LPS enhanced the expression of PGDH protein in placental trophoblast cells from female fetuses, and up-regulated the anti-inflammatory cytokines in both sexes. Results from this study suggest a mechanism by which lactobacilli might reduce the risk of preterm birth and serve as a potential therapy for prevention of inflammation or infection-mediated preterm birth. As this study only examined trophoblast cells in an \textit{in vitro} setting, further studies are required to determine the interaction between and within different tissues such as amnion, chorion and decidua, all of which may play a role in immune responses. As well, future work is needed to elucidate the specific mechanisms by which \textit{L. rhamnosus} GR-1 exerts its effect in human intrauterine tissues.
Figure 3.1: Characterization of cultured cells.

Fibroblasts and cytotrophoblasts were vimentin (A) and cytokeratin (B) positive respectively. Control is Negative Mouse IgG as shown in column (C). Scale bar 100 mm.
Figure 3.2: Treatment cytotoxicity percentage.

LDH release measured from the media of primary cultures of human placental trophoblast cells treated by *L. rhamnosus* GR-1, LPS and *G. vaginalis* expressed relative to maximum LDH release by Triton X treated cells.
Figure 3.3: Experimental Design

Primary cultures of human placental and chorion trophoblast cells were serum starved for 12 hours after 72 hours of culture. They were pre-treated with *L. rhamnosus* GR-1 supernatant for 12 hours and then treated with LPS. Protein was collected after 8 hours to assess the expression of proteins. Media was collected 8 hours following the LPS addition.
Figure 3.4: Optimizing time and dose of LPS treatment for TNF-α Concentration in placental trophoblast cells.

Histograms showing TNF-α concentration (fold increase relative to control) in media from placental trophoblast cell cultures after treatment with (A) different doses of LPS (0.1, 1, 10, 100 and 1000 ng/ml) for 8 hours and (B) 100 ng/ml of LPS for period of 2, 4, 8, 12 and 24 hours. Results are mean values ± S.E.M. and presented relative to control (N=5). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 3.5: Optimizing time and dose of *L. rhamnosus* GR-1 supernatant treatment for TNF-α concentration in placental trophoblast cells.

Histograms showing TNF-α concentration (fold increase relative to control) in media from placental trophoblast cell cultures after treatment with (A) different dilutions of *L. rhamnosus* GR-1 supernatant (1:10, 1:20, 1:50 and 1:100) for 8 hours and (B) 1:20 dilution of *L. rhamnosus* GR-1 supernatant for period of 2, 4, 8, 12 and 24 hours. Results are mean values ± S.E.M. and presented relative to control (N=5). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
**Figure 3.6**: Optimizing time and dose of *L. rhamnosus* GR-1 supernatant treatment for PTGS2 expression in placental trophoblast cells.

Representative western blots of PTGS2 (72 kDa) and β-actin (41 kDa) and histograms showing RODs of these blots normalized to β-actin expression and expressed relative to control for (A,B) different dilutions of *L. rhamnosus* GR-1 supernatant (1:10, 1:20, 1:50 and 1:100) for 8 hours and (C,D) 1:20 dilution of *L. rhamnosus* GR-1 supernatant for period of 2, 4, 8, 12 and 24 hours. Results are mean values ± S.E.M. (N=4). Statistical significance was present where letters are different (p=0.032) as determined by One-Way-ANOVA.
Figure 3.7: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on output of pro-inflammatory cytokines by LPS-stimulated placental trophoblast cells.

Histograms showing (A) TNF-α (B) IL-1β and (C) IL-6 output (fold increase relative to control) in media (fold increase relative to control) from placental trophoblast cell cultures. Results are mean values ± S.E.M. relative to control (N=9 males, N=11 females for TNF-α, N=6 males, N=5 females for IL-1β and N=6 males, N=9 females for IL-6). Concentration of TNF-α, IL-1β and IL-6 in the media of the control cultures were 34.3, 3 and 20 pg/ml respectively. Statistical significance within gender groups (male: a,b,c and female: a’,b’,c’,d’) was present where letters are different (p<0.001 for all the comparisons except IL-6 output for trophoblast cells from pregnancies carrying male fetuses where p=0.003) by One-Way-ANOVA. Asterisks indicate statistical difference by two-tailed Student t-test *p<0.05.
Figure 3.8: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on IL-10 concentration by LPS-stimulated placental trophoblast cells.

(A) Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=12 males, N=10 females). (B) Histograms showing IL-10 concentration in the *L. rhamnosus* GR-1 supernatant compared to the values in the media from non-treated cells. Concentration of IL-10 in the media of the control cultures was 5.2 pg/ml. Statistical significance within gender groups (male: a,b and female: a’,b’) was present where letters are different (p<0.001) by One-Way-ANOVA. Asterisks indicate statistical difference by two-tailed student t-test **p<0.01.
Figure 3.9: Comparison of TNF-α and IL-10 concentrations in placental trophoblast cells.

Histograms showing the ratio of the concentration of TNF-α to IL-10 in the media of the control, LPS, and combination of LPS and *L. rhamnosus* GR-1 supernatant treated placental trophoblast cells. Results are mean values ± S.E.M. Statistical significance within gender groups (N=9 male: a,b (p<0.001) and N=11 female: a’,b’ (p=0.016)) was present where letters are different by One-Way-ANOVA.
Figure 3.10: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on the expression of prostaglandin-synthesizing enzyme, PTGS2, by LPS-stimulated placental trophoblast cells.

Representative Western blot of PTGS2 (72 kDa) and β-actin (41 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant as well as LPS and *L. rhamnosus* GR-1 supernatant treatments of (A) male and (B) female trophoblast cells. (C) ROD of PTGS2 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=8 males, N=9 females). Statistical significance within gender groups (male: a,b and female: a’,b’) was present where letters are different (p=0.004) by One-Way-ANOVA. Asterisks indicate statistical difference by two-tailed student’s t-test **p=0.005.
Figure 3.11: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on the expression of prostaglandin-metabolizing enzyme, PGDH, by LPS-stimulated placental trophoblast cells.

Representative western blot of PGDH (29 kDa) and β-actin (41 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant as well as LPS and *L. rhamnosus* GR-1 supernatant treatments of (A) male and (B) female trophoblast cells. (C) ROD of PGDH western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=9 males, N=7 females). Statistical significance within gender groups (male: a and female: a’,b’) was present where letters are different (p=0.002) by One-Way-ANOVA. Asterisks indicate statistical difference by two-tailed student’s t-test *p=0.012, ** p=0.006.
Figure 3.12: Effect of fetal sex on TLR-4 expression in cultured placental trophoblast cells.

(A) Representative Western blot of TLR-4 (90 kDa) and β-actin (41 kDa) expression under control conditions. (B) ROD of TLR-4 western blot normalized to β-actin. Results are mean values ± S.E.M. (N=9 males, N=11 females). Asterisk indicates statistical difference by two-tailed student’s t-test *p=0.019.
Figure 3.13: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on cytokines and prostaglandin-regulating enzymes in chorion trophoblast cells.

Histograms showing (A) TNF-α (N=6 males, N=6 females; p<0.001) and (B) IL-10 (N=10 males, N=7 females; p<0.001) concentrations (fold increase relative to control) for different treatment groups in media from chorion trophoblast cell cultures. Histograms showing relative ROD of (C) PTGS2 (72 kDa, N=7 males; p<0.001, N=8 females; p=0.002) and (D) PGDH (N=4 males, N=5 females; p>0.05) normalized to β-actin. Results are mean values ± S.E.M. and represented relative to control. Statistical significance was present where letters are different as determined by One-Way-ANOVA. Asterisk indicates statistical difference by two-tailed student’s t-test *p=0.03 for LPS and *p=0.017 for *L. rhamnosus* GR-1.
Figure 3.14: Effect of different strains on cytokine concentration in placental trophoblast cells.

Histograms showing (A) TNF-α (N=6; p<0.001) and (B) IL-10 (N=5; p=0.01) concentrations (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and represented relative to control. Statistical significance was present where letters are different as determined by One-Way-ANOVA.
Figure 3.15: Effect of different strains on prostaglandin-regulating enzymes in placental trophoblast cells.

(A) Representative western blot of PTGS2 (72 kDa) and β-actin (41 kDa) expression under different treatments. (B) ROD of PTGS2 western blot normalized to β-actin. (C) Representative Western blot of PGDH (29 kDa) and β-actin (41 kDa) expression under different treatments. (D) ROD of PGDH western blot normalized to β-actin. Results are mean values ± S.E.M. (N=6) and represented relative to control. Statistical significance was present where letters are different as determined by One-Way-ANOVA (p=0.008 for PTGS2 and p=0.017 for PGDH).
Figure 3.16: Effect of different bacterial growing broths on TNF-α concentration and PTGS2 expression in placental trophoblast cells.

(A) Histograms showing TNF-α concentration (fold increase relative to control, N=4; p<0.001) for different treatment groups in media from placental trophoblast cell cultures. (B) Representative western blot of PTGS2 (72 kDa) and β-actin (41 kDa) expression under different treatments. (C) ROD of PTGS2 western blot (N=6; p<0.001) normalized to β-actin. Results are mean values ± S.E.M. and represented relative to control. Statistical significance was present where letters are different as determined by One-Way-ANOVA.
Chapter Four

Activation of STAT-3 and p38 are key mechanisms for *Lactobacillus rhamnosus* GR-1-induced IL-10 production in human placental trophoblast cells
Chapter 4

4. Activation of STAT-3 and p38 are key mechanisms for *Lactobacillus rhamnosus* GR-1-induced IL-10 production in human placental trophoblast cells

4.1. Introduction

Bacterial Vaginosis (BV), an alteration in the endogenous vaginal microbiota characterized by decreased levels of certain *Lactobacillus* and elevation of pH, is associated with a 40% increase in risk of preterm birth (McGregor and French, 2000). The events leading to infection/inflammation mediated preterm birth include pathogen invasion of the intrauterine cavity, followed by activation of an inflammatory response that is triggered by endotoxins such as LPS (Goldenberg et al., 2008b; Peltier, 2003). This inflammatory response is accompanied by production of pro- and anti-inflammatory cytokines. Several studies have shown that increased intrauterine cytokine production is associated with both term and preterm labor (Gomez et al., 1997; Dudley, 1997) as cytokines facilitate cervical ripening, and membrane rupture (Challis et al., 2000). The levels of intrauterine cytokines, in particular IL-1β, IL-6, IL-8 and TNF-α are also increased in maternal serum, amniotic and cervico-vaginal fluid of women with threatened preterm labour, particularly those associated with intra-amniotic infection (Hitti et al., 2001; von et al., 2000; El-Bastawissi et al., 2000; Coultrip et al., 1994). We have previously shown that LPS administration mimics a state of infection and increases TNF-α output in human placental trophoblast cells (Yeganegi et al., 2009) (Chapter 3).

Anti-inflammatory cytokines play a key role in pregnancy maintenance. It is known that these cytokines suppress the harmful maternal immune response *in vivo* and protect the allogenic human fetus against maternal immune attack during pregnancy (Bowen et al., 2002; de Moraes-Pinto et al., 1997). It has been also shown that anti-inflammatory cytokines are able to inhibit the production of pro-inflammatory cytokines (Roth et al., 1996). IL-10 was able to inhibit preterm labour and improve fetal outcome by restoring birth weight, increasing liter size and decreasing placental TNF-α when administered into rats, rhesus monkeys and a
number of other models of infection-mediated preterm birth (Peltier, 2003; Rodts-Palenik et al., 2004; Terrone et al., 2001; Rivera et al., 1998).

Due to the ineffectiveness of antibiotics in preventing preterm birth in women with BV, the possibility of using probiotics as an alternative is being considered. Administration of *L. rhamnosus* GR-1 in clinical trials demonstrated significant benefits of *L. rhamnosus* GR-1 on the urogenital health of patients with BV (Falagas et al., 2007; Anukam et al., 2006). In addition, studies suggest that *L. rhamnosus* GR-1 is able to populate the vagina and up-regulate the host’s antimicrobial system after its intravaginal use (Cadieux et al., 2002; Reid et al., 1994b). In both human monocytes and mouse macrophages, *L. rhamnosus* GR-1 supernatant stimulates release of IL-10 which in turn suppresses release of TNF-α (Kim et al., 2006). We have shown recently that *L. rhamnosus* GR-1 supernatant is able to increase IL-10 output in LPS-stimulated human trophoblast cells, providing another line of evidence for its anti-inflammatory properties (Yeganegi et al., 2009) (Chapter 3).

The pathways through which *L. rhamnosus* GR-1 has its anti-inflammatory effects have not been firmly established. However, the literature suggests that two major pathways, the JAK/STAT and RTK-MAPK pathways could play a role in *L. rhamnosus* GR-1 up-regulation of IL-10. STAT-3 signaling is a key mechanism for *L. rhamnosus*-induced suppression of TNF-α production in mouse macrophages (Murray, 2007; Kim et al., 2006). It has also been shown that *L. rhamnosus* GR-1 supernatant up-regulates the phosphorylation of p38. Other signaling pathways are also important in maintenance of pregnancy and hence are altered in infection/inflammation-mediated preterm birth. Several studies have shown that key members of the nuclear factor- NF-κB/Rel family of proteins serve as mediators of cytokine-induced up-regulation of PTGS2 enzyme, leading to an increase in prostaglandin levels (Kniss et al., 2001; Herve et al., 2008). Kim et al also have suggested that the inhibition of JNK, a member of the RTK-MAPK pathway, is a key mechanism by which *L. rhamnosus* GR-1 suppresses TNF-α production in mouse macrophages (Kim et al., 2006).

It has been reported that preterm birth is more frequent in women carrying a male fetus (Ingemarsson, 2003; Zeitlin et al., 2002) and that females are more effective in mounting an immune response and have a better prognosis in septic shock than men in both the neonatal
and adult periods (Schroder et al., 1998; Imahara et al., 2005). In addition, male fetuses are more likely to face intrauterine or neonatal death and other complications (Smith, 2000). We have shown previously that LPS increases TNF-α and IL-10 output in human placental trophoblast cells, an effect that is more prominent in cells derived from placentae of male compared to female fetuses. We propose that the greater response of placentae from male fetuses to LPS is due to the greater expression of its receptor, TLR-4, in these placentae. Our data also suggest that the LPS stimulation of PTGS2 is more pronounced in placentae of the male fetuses whereas the up-regulation of PGDH by lactobacilli is more prominent in placentae of female fetuses (Yeganegi et al., 2009) (chapter 3). Currently, however, the reason behind the sexual dimorphism in lactobacilli action is not understood. Different strains of lactobacilli activate TLRs, which are transmembrane proteins that bind different pathogen-associated molecules. TLR-2 is thought to be the main receptor for peptidoglycan fragments from gram-positive bacteria and its mRNA and protein levels are up-regulated in colonic mucosa after lactobacilli treatment (Voltan et al., 2007).

At present, the underlying mechanisms involved in mediating the L. rhamnosus GR-1 up-regulation of IL-10 in human placental trophoblast cells is not known. Therefore, in this study, we sought to determine possible signaling pathways through which L. rhamnosus GR-1 supernatant has its anti-inflammatory effect. We also examined possible sexual dimorphisms in the expression of TLR-2 receptor in order to explain the differential response observed between the placentae from pregnancies carrying a male or a female fetus as a result of L. rhamnosus GR-1 treatment.

4.2. Material and Methods

4.2.1. Samples

Placental tissues were collected as described in 3.2.1.

4.2.2. Placental trophoblast cell culture

Placental trophoblast cells were isolated using established primary culture protocols as outlined in 3.2.2.
4.2.3. Treatment of placental trophoblast cells

Supernatant from *L. rhamnosus* GR-1 cultures was prepared as described previously in 3.2.3. Placental cultured cells were maintained, treated and tested for viability as explained in 3.2.3. These cells were divided into the following groups: 1) No treatment; 2) Treatment with LPS alone (200 ng/ml for protein or 100 ng/ml for cytokine measurements) after a further 12h; 3) Treatment with *L. rhamnosus* GR-1 supernatant alone (1:20 dilution) for 12h; 4) Pretreatment with *L. rhamnosus* GR-1 supernatant (1:20) for 12h and subsequent treatment with 200 or 100 ng/ml of LPS. Preliminary studies established dilution and time of treatments as shown in chapter 3. These four treatments are referred to in this study as the basic treatments because they are the control groups used to compare with the inhibitor treatments, which were the four treatments with addition of either a chemical inhibitor or a neutralizing antibody. The inhibitor treatments were as follow: 1) JAK inhibitor-10^{-5} M (CalBiochem, NJ, U.S.A.) added 9 hours after cell starvation to each of the four basic treatments; 2) p38 inhibitor (SB203580)-10^{-5} M (CalBiochem) added 21 hours after the start of cell starvation to the four basic treatments; 3) ERK Activation Inhibitor-10^{-5} M (Santa Cruz Biotechnology, Inc., CA, U.S.A.) added 21 hours after the start of cell starvation to the four basic treatments; 4) NF-κB inhibitor-10^{-5} M (CalBiochem) added 21 hours after the start of cell starvation to the four basic treatments; 5) JNK inhibitor-10^{-5} M (CalBiochem) added 21 hours after the start of cell starvation to the four basic treatments; 6) IL-10 neutralizing antibody (JES3-9D7)-100µg/ml (eBioscience, San Diego, U.S.A.) added at the start of cell starvation to the four basic treatments; 7) TNF-α neutralizing antibody- 4µg/ml (R&D Systems, Minneapolis, U.S.A.) added at the start of cell starvation to the four basic treatments. All the above inhibitors performed with high specificity at the concentration used with regards to inhibiting their corresponding molecules or kinases. The concentrations used were based on manufacturer’s recommendations and previous studies in our laboratory. Controls for each experiment included culture medium or the inhibitors alone as well the mouse and rat IgG for TNF-α and IL-10 neutralizing antibodies respectively. There was no change in the expression of the examined cytokines or proteins in any of the control studies. Viability of cells treated with these inhibitors and the inhibitor activity were confirmed. The experimental design of the study is demonstrated in Figure 4.1 (Page 90).
4.2.4. Protein extraction and Western blot analysis

Western blot was performed using the extracted protein collected from primary cultures as described in 3.2.5. Membranes were incubated for 1 hour separately with monoclonal rabbit anti-human phospho-STAT3 (Tyr705) antibody (Cell Signalling Technology, MA, U.S.A.; 1:1000) to detect STAT3 phosphorylation, TLR-2 antibody (eBioscience, San Diego, CA; 1:2000) to detect TLR-2 receptor expression, monoclonal mouse anti-human phospho-p38 (Thr180/Tyr182) antibody (Cell Signalling Technology; 1:2000) to detect p38 phosphorylation and PTGS2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI; 1:1000) to detect PTGS2 expression. The blots were washed and incubated for 1 hour with anti-rabbit IgG coupled to horseradish peroxidase secondary antibody in case of phospho-STAT3 (Tyr705) and TLR-2 (Amersham Pharmacia Biotech, Upsala, Sweden; 1:5000) and a horseradish peroxidase-labeled anti-mouse IgG (Amersham Pharmacia Biotech; 1:5000) in the case of phospho-p38 (Thr180/Tyr182) and PTGS2. The antibodies revealed signals at 86 kDa, 43 kDa, 90 kDa and 72 kDa for phospho-STAT3, phospho-p38, TLR-2 and PTGS2 respectively.

4.2.5. Cytokine measurements

Culture media was collected 8 hours after addition of LPS. Levels of TNF-α, and IL-10 in this culture media were measured using commercially available quantitative ELISA kits (Cayman Chemical, Ann Arbor, MI for TNF-α and eBioscience, San Diego, CA for IL-10) according to the manufacturer’s instructions. The minimum detectable limits of the assays were 1.5 pg/ml, and 2 pg/ml for TNF-α and IL-10, respectively. The intra-assay coefficients of variation for these cytokines were <5%. All samples from each experiment were run on the same assay plate.

4.2.6. Statistical Analysis

Statistical analysis was performed as described in 3.2.7.
4.3. Results

4.3.1. *L. rhamnosus* GR-1 stimulated STAT3-Tyr705 and p38 phosphorylation in human trophoblast cells

Levels of STAT3 Tyrosine-705 (STAT3-Tyr705) and p38 phosphorylation were detected in human placental trophoblast cell cultures treated with the four basic treatments: control, LPS alone, *L. rhamnosus* GR-1 supernatant alone, and *L. rhamnosus* GR-1 pre-treatment followed by addition of LPS. STAT3-Tyr705 (86 kDa) (Figure 4.2, Page 91) and p38 (43 kDa) (Figure 4.3, Page 92) phosphorylation were significantly increased (p=0.018; N=8 for STAT3-Tyr705 phosphorylation and p=0.005; N=4 for p38 phosphorylation) in cells treated with *L. rhamnosus* GR-1 in the presence (1.8-fold for STAT-3-Tyr705 and 2.1-fold for p38) or absence (1.7-fold for STAT-3-Tyr705 and 2.1-fold for p38) of LPS. LPS treatment alone also increased p38 phosphorylation by 1.3-fold (p=0.005; N=4), which was significantly less than phosphorylation by *L. rhamnosus* GR-1 alone or with LPS.

4.3.2. *L. rhamnosus* GR-1-induced IL-10 requires activation of the JAK/STAT and p38 pathways

STAT3-Tyr705 and p38 phosphorylation in the four basic treatments were compared with the respective treatments containing the IL-10 neutralizing antibody, JES3-9D7, using western blot analysis. Comparisons were also made for IL-10 concentrations using ELISA between the four basic treatments and respective treatments with addition of either JAK inhibitor or p38 inhibitor (SB203580). There were no significant differences in level of STAT3-Tyr705 and p38 phosphorylation between cultures treated with the four basic treatments (control, LPS alone, *L. rhamnosus* GR-1 alone, *L. rhamnosus* GR-1/LPS) as compared to the respective treatments containing JES3-9D7 (p>0.05, N=8 for STAT3-Tyr705 phosphorylation and N=4 for p38 phosphorylation, Figure 4.4 (Page 93) and Figure 4.5 (Page 94)). A significant decrease in IL-10 output was observed in cultures containing either JAK inhibitor (p=0.028; N=9, Figure 4.6, Page 95) or p38 inhibitor, SB203580 (p=0.02; N=9, Figure 4.7, Page 96), indicating that *L. rhamnosus* GR-1 induction of IL-10 release in human placental trophoblast cells requires an intact JAK/STAT and p38 pathway. The IL-10...
neutralizing antibody completely blocked the IL-10 output in placental trophoblast cells (p<0.001; N=11, Figure 4.8, Page 97). To investigate the role of other members of the RTK-MAPK as well as the NF-κB pathways on the *L. rhamnosus* GR-1 induced up-regulation of IL-10, ERK Activation Inhibitor, JNK inhibitor and NF-κB inhibitor were added to the basic four treatments. No change was observed in the LPS or *L. rhamnosus* GR-1 induced increase in IL-10 as a result of addition of these inhibitors (p>0.05, N=9, Figure 4.9, Page 98).

### 4.3.3. *L. rhamnosus* GR-1-induced down-regulation of TNF-α is independent of JAK/STAT, RTK-MAPK and NF-κB/Rel pathways

There were no significant differences in the level of TNF-α between cultures treated with the four basic treatments (control, LPS alone, *L. rhamnosus* GR-1 alone, *L. rhamnosus* GR-1/LPS), and the respective treatments containing the inhibitors (IL-10 neutralizing antibody, JAK inhibitor, p38 inhibitor, ERK Activation Inhibitor, JNK inhibitor and NF-κB inhibitor) (p>0.05, N=9, Figure 4.10, Page 99). However, the LPS-induced increase in TNF-α was less when inhibitors of the MAPK pathway (ERK, JNK, p38) were added to placental trophoblast cells (p<0.001, N=9, Figure 4.10, Page 99).

### 4.3.4. *L. rhamnosus* GR-1-induced down-regulation of PTGS2 is independent of TNF-α

Western blot analysis revealed that the administration of TNF-α neutralizing antibody did not block LPS-induced PTGS2 (p>0.05, N=7, Figure 4.11, Page 100), indicating that the down-regulation of PTGS2 expression is not directly linked with the decrease seen in TNF-α output.

### 4.3.5. *L. rhamnosus* GR-1-stimulated up-regulation of the TLR-2 receptor in Human Trophoblast Cells

There was no significant difference in the expression of endogenous TLR-2 (p>0.05; N=8 Males, N=5 Females, Figure 4.12A, Page 101) between the trophoblast cells from placentae of male and female fetuses. However, addition of *L. rhamnosus* GR-1 increased the
expression of TLR-2 relative to control values in placental cells from males by 1.2-fold (p=0.044; N=8, Figure 4.12B,C, Page 101) and combination of L. rhamnosus GR-1 and LPS up-regulated this expression relative to control values only in female by 2-fold (p=0.001; N=5, Figure 4.12B,C, Page 101). TLR-2 expression was 2-fold greater in placentae from female fetuses compared to male fetuses after treatment with L. rhamnosus GR-1 and LPS (p=0.011; N=8 Males, N=5 Females, Figure 4.12B,C, Page 101). L. rhamnosus GR-1 and the combination of L. rhamnosus GR-1 and LPS up-regulated TLR-2 by 1.2- and 1.4-fold (p<0.001; Figure 4.12D, Page 101) in cells from pregnancies carrying either a male or a females fetus (N=13).

4.4. Comment

In these studies, we found that L. rhamnosus GR-1 induced phosphorylation of STAT3-Tyr705 in primary human placental trophoblast cell cultures. It is known that the JAK/STAT signaling pathway is activated often in response to cytokines and growth factors and JAKs bind to cell surface receptors, such as to cytokine receptors within the JAK/STAT complex (Murray, 2007). It has been shown previously that IL-10, which binds to a Type II IL-10 cytokine receptor, activates JAK1/STAT3 to mediate its anti-inflammatory effects in macrophages (Murray, 2007). In human monocytes, Kim et al concluded that L. rhamnosus GR-1 treatment stimulated release of G-CSF, which activated JAK2/STAT3 when bound to the Type I-like G-CSF cytokine receptor. The binding of the ligand to the receptor triggers phosphorylation of JAKs and STATs are then tyrosine-phosphorylated by JAKs. Activated STATs will ultimately stimulate transcription of the target genes (Marino and Roguin, 2008). In the case of monocytes, the activation of the JAK/STAT pathway led to the inhibition of TNF-α (Kim et al., 2006).

Members of the RTK-MAPK pathway are also important in infection/inflammation-mediated preterm birth (Shoji et al., 2007). We have shown in this study that L. rhamnosus GR-1 up-regulated phosphorylation of p38. This is in agreement with the work of Kim et al, which found that L. rhamnosus GR-1 up-regulated the phosphorylation of p38 in mouse macrophages (Kim et al., 2006). It has also been suggested that IL-10 may suppress TNF-α levels through inhibition of p38 and that targeting the p38 MAPK pathway may prevent PTB (Shoji et al., 2007). In human choriodecidual cells, inhibition of p38 reduces LPS-induced
TNF-α and the ratio of TNF-α to IL-10 (Shoji et al., 2007). In the present study, activation of p38 was found to be necessary to stimulate *L. rhamnosus* GR-1-induced IL-10 release suggesting that inhibition of p38 may decrease levels of anti-inflammatory cytokines. This discrepancy in findings may be related to cell specific differences between human choriodecidual cells and human placental trophoblast cells.

The anti-inflammatory effects of IL-10 have been proposed to be mediated through activation of the JAK/STAT pathway and inhibition of the p38 pathway (Shoji et al., 2007; Riley et al., 1999). Currently, the relationship between IL-10, the p38 and JAK/STAT pathways in human placental trophoblast cells is unknown. In the present study, we have shown that STAT3-Tyr705 and p38 phosphorylation does not decrease when IL-10 is blocked. However, *L. rhamnosus* GR-1-induced IL-10 release was significantly attenuated in the presence of JAK inhibitor and p38 inhibitor (SB203580), demonstrating the requirement of the JAK/STAT and p38 pathways upstream for IL-10 output. Although IL-10 is thought to be one of the activators of the JAK1/STAT3, we propose that the activation of the JAK/STAT and p38 pathways lead to increased production of IL-10 which is then secreted outside of the cell in order to have a positive feedback on the JAK1/STAT3 receptor to enhance its anti-inflammatory function. No change was observed in *L. rhamnosus* GR-1 up-regulation of IL-10 output when other members of the RTK-MAPK as well as the NF-κB pathways were blocked, suggesting that these members may not play a role in the mechanism through which lactobacilli has its anti-inflammatory effect.

We also found that TNF-α concentration did not change when inhibitors of several signaling pathways were added to the basic four treatments of the human placental trophoblast cells. However, the levels of LPS-stimulated TNF-α were decreased significantly when inhibitors of the MAPK pathway (JNK, ERK, p38) were added. This is in agreement with other studies that have shown that Mitogen-activated protein kinase proteins regulate LPS-stimulated release of pro-inflammatory cytokines from human gestational tissues (Lappas et al., 2007). Since none of the pathways investigated were linked to the *L. rhamnosus* GR-1 down-regulation of the LPS induced TNF-α and all these pathways function by transcribing the target genes, we propose that the effect of *L. rhamnosus* GR-1 could be post-transcriptional or translational. *L. rhamnosus* GR-1 could also play a role in methylation of the coding
sequence in order to silence the TNF-α gene post-transcriptionally or may interfere with its release from the cells.

Placentae used in this study were collected by elective caesarean sections in order to minimize the confounding effects of clinical infection and/or labour induction. The treatment of placental cells was chosen to mimic a normal uterine environment responding to pathogenic invasion in conditions such as bacterial vaginosis (in LPS treated cultures), and the response of cells to prophylactic probiotic therapy (in *L. rhamnosus* GR-1/LPS treated cells). A limitation in using primary cultures as an experimental model is the chance of having cell contamination within the cultures as well as decreasing cell viability.

The present study is the first to investigate the mechanisms of cytokine regulation in human placenta by *L. rhamnosus* GR-1. We have shown that *L. rhamnosus* GR-1 supernatant acts through the JAK/STAT and MAPK pathways to increase the anti-inflammatory IL-10. Blocking these two pathways did not, however, reverse the down-regulation of TNF-α by *L. rhamnosus* GR-1 supernatant, indicating that there is another pathway through which TNF-α is down-regulated. Up-regulation of the anti-inflammatory cytokine IL-10 could serve as a protective mechanism used by gestational tissues in a healthy urogenital environment to counter the effects of infection. Lactobacilli may, therefore, be a potential candidate in treating BV and potentially preventing the infection/inflammation-mediated preterm birth.
Primary cultures of human placental trophoblast cells were serum starved for 12 hours after 72 hours of culture. They were pre-treated with *L. rhamnosus* GR-1 supernatant for 12 hours and then treated with LPS. Protein was collected after twenty minutes for the measurement of the phosphorylated proteins and after eight hours to assess the expression of PTGS2 and TLR-2. Media was collected eight hours following the LPS addition. Different inhibitors were added to investigate the involvement of different signaling pathways.
Figure 4.2: Effect of *L. rhamnosus* GR-1 supernatant on the expression of STAT3-Tyr705 phosphorylation by LPS-stimulated placental trophoblast cells.

(A) Representative western blot of Phospho-STAT3-Tyr705 (86 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant as well as LPS and *L. rhamnosus* GR-1 supernatant treatments of human placental trophoblast cells. (B) ROD of Phospho-STAT3-Tyr705 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=8). Statistical significance was present where letters are different (p=0.018) as determined by One-Way-ANOVA.
Figure 4.3: Effect of *L. rhamnosus* GR-1 supernatant on the expression of p38 phosphorylation by LPS-stimulated placental trophoblast cells.

(A) Representative western blot of Phospho-p38 (43 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant as well as LPS and *L. rhamnosus* GR-1 supernatant treatments of human placental trophoblast cells. (B) ROD of p38 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=4). Statistical significance was present where letters are different (p=0.005) as determined by One-Way-ANOVA.
Figure 4.4: Effect of IL-10 neutralizing antibody, JES3-9D7, on the expression of STAT3-Tyr705 phosphorylation by *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

(A) Representative western blot of Phospho-STAT3-Tyr705 (86 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant and LPS and *L. rhamnosus* GR-1 supernatant as well as addition of an IL-10 neutralizing antibody to these four basic treatments of human placental trophoblast cells. (B) ROD of Phospho-STAT3-Tyr705 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=8). Statistical significance was present where letters are different (p=0.018) as determined by One-Way-ANOVA.
Figure 4.5: Effect of IL-10 neutralizing antibody, JES3-9D7, on the expression of p38 phosphorylation by *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

(A) Representative western blot of Phospho-p38 (43 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant and LPS and *L. rhamnosus* GR-1 supernatant as well as addition of an IL-10 neutralizing antibody to these four basic treatments of human placental trophoblast cells. (B) ROD of p38 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=4). Statistical significance was present where letters are different (p=0.005) as determined by One-Way-ANOVA.
Figure 4.6: Effect of JAK Inhibitor on IL-10 concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=9). Statistical significance was present where letters are different (p=0.028) as determined by One-Way-ANOVA.
Figure 4.7: Effect of p38 Inhibitor on IL-10 concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=9). Statistical significance was present where letters are different (p=0.02) as determined by One-Way-ANOVA.
Figure 4.8: Effect of IL-10 Inhibitor on IL-10 concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=11). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 4.9: Effect of different signaling pathway inhibitors on IL-10 concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=9). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 4.10: Effect of different signaling pathway inhibitors on TNF-α concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells.

Histograms showing TNF-α concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=9). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 4.11: Effect of TNF-α neutralizing antibody on the expression PTGS2 by LPS-stimulated placental trophoblast cells.

(A) Representative western blot of PTGS2 (72 kDa) and β-actin (43 kDa) expression under control, LPS, TNF-α antibody as well as LPS and TNF-α antibody treatments of human placental trophoblast cells. (B) ROD of PTGS2 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=7). Statistical significance was present where letters are different (p<0.05) as determined by One-Way-ANOVA.
Figure 4.12: Effect of fetal sex on TLR-2 expression in placental trophoblast cells.

(A) ROD of TLR-2 expression under control conditions in placenate of male or female fetuses normalized to β-actin (p>0.05; N=8 Males, N=5 Females). (B) Representative western blot of TLR-2 (90 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant as well as LPS and *L. rhamnosus* GR-1 supernatant treatments of placenta of male or female fetuses. (C,D) ROD of TLR-2 western blot normalized to β-actin for (C) pregnancies carrying a male or a female fetus and (D) Combined samples (N=13). Results are mean values ± S.E.M. and presented relative to control. Statistical significance within gender groups (male: a,b and female: a’,b’) was present where letters are different (C) (p=0.044 for Males and p=0.001 for Females) and (D) (p<0.001) by One-Way-ANOVA. (C) Asterisk indicates statistical difference by two-tailed student’s t-test *<0.05 (p=0.011).
Chapter Five

*Lactobacillus rhamnosus* GR-1 stimulates granulocyte-colony stimulating factor (G-CSF) output in placental trophoblast cells in a fetal sex-dependant manner
Chapter 5

5. **Lactobacillus rhamnosus** GR-1 stimulates granulocyte-colony stimulating factor (G-CSF) output in placental trophoblast cells in a fetal sex-dependant manner

5.1. Introduction

Cytokines are important immunoregulatory mediators at the human maternal-fetal interface. An imbalance of the pro- and anti-inflammatory cytokines is known to play a role in the mechanism whereby exposure to pathogens leads to intrauterine infection (McGregor and French, 2000; Raghupathy and Kalinka, 2008). G-CSF is a protective cytokine with anti-inflammatory effects and is considered to be a major regulator of macrophages, essential for the clearance of bacterial pathogens. G-CSF acts through its main receptor, granulocyte-colony stimulating factor receptor (G-CSFR), detected in the placenta, neurons, endothelial cells, and cardiomyocytes (Panopoulos and Watowich, 2008). Inflammatory stimuli such as endotoxins or cytokines synthesized by a group of specialized cells such as macrophages stimulate the transcriptional and post-transcriptional mechanisms by which G-CSF and subsequent neutrophil production is regulated (Panopoulos and Watowich, 2008). Low levels of G-CSF have been associated with increased incidence and severity of infection in preterm infants (Chirico et al., 1997). Studies have also shown that G-CSF is important in promoting the survival of the granulocytic lineage cells and proliferation and migration of the neutrophils (Panopoulos and Watowich, 2008) as well as trophoblast cells (Fitzgerald et al., 2008; Fitzgerald et al., 2005). In addition, G-CSF promotes angiogenesis and reduces ischemic damage (Ripa et al., 2006; Lee et al., 2005). Recombinant G-CSF has also been widely used for the treatment of neutropenia and several infections in diabetic patients (Panopoulos and Watowich, 2008).

Lactobacilli are the dominant urogenital microflora in healthy females and are implicated in bacterial vaginosis, an inflammatory condition characterized by low numbers of lactobacilli and associated with 1.4-fold increase in risk of PTB (Goldenberg et al., 2008b). Currently, antibiotics have been the most common treatment for BV and prevention of infection-mediated preterm birth. However, these treatments are largely unsuccessful (Simcox et al.,
2007) and in some studies, have actually been shown to increase the risk of preterm birth (Shennan et al., 2006). Probiotic lactobacilli may be an attractive treatment alternative (Kim et al., 2006) mainly because of their ability to replenish vaginal lactobacilli and modulate immunity (Reid et al., 2005; Reid and Bocking, 2003; Reid et al., 2003a). In addition, several studies suggest that *L. rhamnosus* GR-1 is able to populate the vagina and up-regulate the host’s antimicrobial system after its intravaginal use (Cadieux et al., 2002; Reid et al., 1994b). We have shown that *L. rhamnosus* GR-1 is able to down-regulate the pro-inflammatory and up-regulate the anti-inflammatory cytokines in human placental trophoblast cells (Yeganegi et al., 2009) (Chapter 3). It has also been suggested that *L. rhamnosus* GR-1 up-regulates G-CSF production in mouse macrophages and it is the activation of the JAK2/STAT3 by G-CSF that mediates the *L. rhamnosus* GR-1-induced TNF-α suppression (Kim et al., 2006). Furthermore, incubation of the JEG-3 human trophoblastic cell line with G-CSF leads to activation of the STAT3, a member of the JAK/STAT pathway and p38, a member of the MAPK pathway (Marino and Roguin, 2008). Yet, the effect of *L. rhamnosus* GR-1 on G-CSF and its mechanism of action in placental trophoblast cells have not been studied.

The response to bacterial pathogens appears to be sex-dependent with females being more effective in mounting an immune response and having a better prognosis in septic shock than males in both the neonatal and adult periods (Schroder et al., 1998; Imahara et al., 2005). Furthermore, it has been reported that preterm birth is also more frequent in women carrying a male fetus (Ingemarsson, 2003; Zeitlin et al., 2002) and male fetuses are more likely to face intrauterine or neonatal death as well as other complications such as long-term neurological and motor impairments (Smith, 2000; Stevenson et al., 2000) compared to females. We have shown previously that LPS increases TNF-α and IL-10 output in human placental trophoblast cells, an effect that is more pronounced in placentae of the male compared to female fetuses. Our data also indicate that the LPS stimulation of PTGS2 is more pronounced in placentae of male fetuses, but that up-regulation of PGDH by lactobacilli is more prominent in placentae of female fetuses (Yeganegi et al., 2009) (Chapter 3).

At the present time, the effect of *L. rhamnosus* GR-1 on G-CSF in human placental trophoblast cells has not been investigated. Therefore, we sought to investigate possible
sexual dimorphisms in the output of G-CSF as a result of bacterial endotoxin as well as *L. rhamnosus* GR-1 treatments and determined whether this might be associated with differences in G-CSFR receptor expression levels. We further investigated whether G-CSF mediates the down-regulation of the pro-inflammatory and up-regulation of other anti-inflammatory cytokines by *L. rhamnosus* GR-1 supernatant. Finally we investigated the source of G-CSF production by examining the differential response of trophoblast and immune cells to bacterial endotoxin as well as *L. rhamnosus* GR-1 treatments.

5.2. Material and Methods

5.2.1. Samples

Placental tissues were collected as described in 3.2.1.

5.2.2. Placental trophoblast cell culture

Placental trophoblast cells were isolated using established primary culture protocols as outlined in 3.2.2.

5.2.3. Magnetic purification of placental trophoblast cells

Placental trophoblast cells were purified using a magnetic purification technique as described previously (Stenqvist et al., 2008). Briefly, following cell culture, the cell suspension was centrifuged at 1500 x g for 5 minutes, resuspended and allowed to sit in 1ml of magnetic cell sorting buffer (MACS: PBS + 0.25% BSA +2mM EDTA), 10% FCS and 10% human serum for 5 minutes. Cells were then centrifuged at 1500 x g for 5 minutes and resuspended in 60µl of MACS buffer for each 10 million cells. Subsequently, cells were incubated with 20 µl of anti-fibroblast microbeads and 20 µl of anti-CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for each 10 million cells for 30 minutes at room temperature. The column was placed on the magnet and was washed with 3ml of MACS buffer. Five milliliters of MACS buffer were added to the tube containing the cells and after centrifugation at 1500 x g for 5 minutes and resuspension in 3ml of MACS buffer, the cell suspension was allowed to run through the column. The fibroblast/CD45 negative fraction containing pure trophoblast
cells was collected and the column was then washed two times with 3ml of MACS buffer to complete collection. The column was then pulled out of the magnet and was washed with 5ml of MACS buffer to collect the fibroblast/CD45 positive cells which included the leukocyte and fibroblast fraction. Each fraction of cells was then counted and equal number of pure trophoblast cells (10^6), CD45 positive cells (10^6) and the mixture of both (10^6) were seeded in separate wells.

5.2.4. Treatment of placental and immune cells

Supernatant from *L. rhamnosus* GR-1 cultures was prepared as described previously in 3.2.3. Placental cultures were maintained, treated and tested for viability as described in 3.2.3. These cells were then divided into following groups: 1) No treatment; 2) Treatment with LPS alone (200 ng/ml for protein or 100 ng/ml for cytokine measurements) after a further 12h; 3) Treatment with *L. rhamnosus* GR-1 supernatant alone (1:20 dilution) for 12h; 4) Pretreatment with *L. rhamnosus* GR-1 supernatant (1:20) for 12 h and subsequent treatment with 200 or 100 ng/ml of LPS. These four treatments are referred to in this study as the basic treatments because they are the control groups used to compare with the inhibitor treatments, which were the four treatments with addition of either a chemical inhibitor or a neutralizing antibody. The inhibitor treatments are as follow: 1) JAK inhibitor-10^-5^ M (CalBiochem, NJ, U.S.A.) added 9 hours after cell starvation to the four basic treatments; 2) p38 inhibitor (SB203580)-10^-5^ M (CalBiochem) added 21 hours after the start of cell starvation to the four basic treatments; 3) G-CSF neutralizing antibody-100µg/ml (R&D Systems, Minneapolis, U.S.A.) added at the same time as *L. rhamnosus* GR-1 supernatant. The experimental design of the study is shown in Figure 5.1 (Page 115). G-CSF neutralizing antibody (10µg/mL) neutralizes 50% of the bioactivity of 0.125 ng/ml of recombinant human G-CSF (rhG-CSF). In our cell culture model, 100µg/ml of this antibody was able to completely neutralize the highest concentration of G-CSF (Figure 5.2, Page 116) without any sign of cell death or toxicity. LDH analysis revealed less than 1% cytotoxicity in the culture media of the cells treated with this antibody compared to 100% LDH release by Triton X treated cells. Trypan Blue Exclusion Test also showed no sign of cell lysis after treatment with the antibody. Controls for these experiments included culture medium or the inhibitors alone as well the mouse IgG for G-CSF neutralizing antibody. There was no effect of controls on the
expression of cytokines or proteins. The combination of LPS and G-CSF neutralizing antibody served as a positive control to demonstrate that cells treated with LPS alone or LPS and G-CSF neutralizing antibody were able to increase TNF-α output to the same level, confirming that the antibody does not have any negative effect on the cells (Figure 5.3, Page 117).

5.2.5. Immunocytochemistry

Immunocytochemistry was performed on placenta trophoblast cells as described in 3.2.4. The cells were incubated with CD68 (Novocastra Labs, UK; 1:100), Cytokeratin (DAKO; 1:200) and Negative Mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) primary antibodies separately and with horseradish peroxidase-labeled anti-mouse IgG secondary antibody (DAKO; 1:300) to stain for macrophages and cytotrophoblast cells.

5.2.6. Fluorescent Activated Cell Sorting (FACS) Analysis

FACS analysis was used to identify the presence of any macrophages in the primary cell culture. After the culture, tubes of $10^7$ cells were suspended in 4% PFA and stored in 4°C until analyzed. The cells were centrifuged at 1800 $x$ g for 5 minutes. DAKO Protein Block Serum (DAKO; 1 ml) was added to each tube and cells were all resuspended together. Cells were then divided into 4 eppendorf tubes and centrifuged at 1300 $x$ g for 5 minutes. Perm solution (1:10; 500µl) was added to each eppendorf tube and cells were incubated for 15 minutes. Cells were then centrifuged and resuspended in 200µl of perm solution (1:10) again. The four eppendorf tubes were then incubated with appropriate antibodies as follows: 1) control untreated cells; 2) CD68 antibody (Novocastra Labs; 1:100); 3) yoyo antibody (Invitrogen, CA, U.S.A); 4) combination of yoyo and CD68 antibody. FACS analysis was then performed on the cells using the FACS analysis facility at Mount Sinai Hospital.

5.2.7. Protein extraction and Western blot analysis

Western blot was performed using the extracted protein collected from primary cultures as described in 3.2.5. Membranes were incubated for 1 hour separately with monoclonal rabbit anti-human phospho-STAT3 (Tyr705) antibody (Cell Signalling Technology, MA, U.S.A.;
1:1000) to detect phospho-STAT3-Tyrosine705 (STAT3-Tyr705), G-CSFR antibody (R&D Systems; 1:500) to detect G-CSFR receptor expression and monoclonal mouse anti-human phospho-p38 (Thr180/Tyr182) antibody (Cell Signaling Technology; 1:2000) to detect p38 phosphorylation. The blots were washed and incubated for 1 hour with anti-rabbit IgG coupled to horseradish peroxidase secondary antibody in case of phospho-STAT3 (Tyr705) (Amersham Pharmacia Biotech, Upsala, Sweden; 1:5000) and a horseradish peroxidase-labeled anti-mouse IgG (Amersham Pharmacia Biotech, Upsala, Sweden) in the case of phospho-p38 (Thr180/Tyr182) (1:5000) and G-CSFR (1:3000). The antibodies revealed signals at 86 kDa, 43 kDa, 92 kDa for phospho-STAT3, phospho-p38 and G-CSFR respectively.

5.2.8. Cytokine measurements

Culture media was collected 8 hours after LPS addition. Levels of TNF-α, IL-10 and G-CSF in the culture media were measured using commercially available quantitative ELISA kits (Cayman Chemical, Ann Arbor, MI for TNF-α, eBioscience, San Diego, CA for IL-10 and R&D Systems, Minneapolis, MN for G-CSF) according to the manufacturer’s instructions. The minimum detectable limits of the assays were 1.5 pg/ml, 2 pg/ml and 20 pg/ml for TNF-α, IL-10 and G-CSF, respectively. The intra-assay coefficients of variation for TNF-α and IL-10 were <5% and for G-CSF was <3%. All samples from each experiment were run on the same assay plate.

5.2.9. Statistical Analysis

Statistical analysis was performed as described in 3.2.7. One-way ANOVA, followed by Student-Newman-Keuls test was used for comparison across all treatments and two-tailed student’s t-test was used for comparison between the two sexes or between the responses from different cell types in the magnetic purification experiments.
5.3. Results

5.3.1. *L. rhamnosus* GR-1 stimulated G-CSF output in human placental trophoblast cells of pregnancies with a female fetus

G-CSF concentrations were measured in cultures treated with LPS alone, *L. rhamnosus* GR-1 supernatant alone and the combination of both treatments and then compared to the control in pregnancies carrying a male or a female fetus. Compared to control, cultures treated with LPS, *L. rhamnosus* GR-1 and both treatments increased G-CSF output by 233.1-, 588.7- and 797.1-folds respectively in placentae of female fetuses only (p=0.002; N=6, Figure 5.4, Page 118). The increase in G-CSF output in pregnancies with a female fetus was 47.1-, 65.6-, and 65.7-folds more than those in pregnancies with a male fetus (p=0.004), which did not show any detectable changes across treatments (p>0.05; N=5).

5.3.2. *L. rhamnosus* GR-1-induced up-regulation of G-CSF is dependent on activation of the JAK/STAT and RTK-MAPK pathways in human placental trophoblast cells

G-CSF neutralizing antibody completely absorbed and neutralized the G-CSF secreted by placental trophoblast cells of the female fetuses, which have been shown to have a larger output than males (p<0.001; N=7, Figure 5.2, Page 116). Comparisons were also made for G-CSF concentrations using ELISA between the four basic treatments and respective treatments with addition of either JAK inhibitor or p38 inhibitor (SB203580). There were no significant differences in level of STAT3-Tyr705 and p38 phosphorylation between cultures from placentae of female fetuses treated with the four basic treatments (control, LPS alone, *L. rhamnosus* GR-1 supernatant alone, or both treatments together) and the respective treatments containing G-CSF neutralizing antibody (p>0.05 for STAT3-Tyr705 phosphorylation and N=5 for p38 phosphorylation, Figure 5.5 (Page 119) and Figure 5.6 (Page 120)). A significant decrease in G-CSF output was observed in cultures of placenta from female fetuses containing either JAK inhibitor (p<0.001; N=5, Figure 5.7, Page 121) or p38 inhibitor, SB203580 (p<0.001; N=5, Figure 5.8, Page 122), indicating that *L. rhamnosus*
GR-1 supernatant induces G-CSF release in human placental trophoblast cells through both the JAK/STAT and the p38 pathway.

There were no significant differences in the level of TNF-α (p>0.05, N=4, Figure 5.3, Page 117) or IL-10 (p>0.05, N=4, Figure 5.9, Page 123) between cultures from placentae of the female fetuses treated with the four basic treatments and the respective treatments containing the G-CSF neutralizing antibody.

5.3.3. *L. rhamnosus* GR-1 stimulated up-regulation of the G-CSF receptor in human placental trophoblast cells

Western blot revealed that there is no significant difference in the expression of endogenous G-CSFR (p>0.05; N=8 Males, N=5 Females, Figure 5.10A, Page 124) between the trophoblast cells from male and female fetuses. *L. rhamnosus* GR-1 alone and in combination with LPS, however, increased the expression of G-CSFR in males by 1.8- and 1.8-fold (p=0.015; N=8, Figure 5.10B,C, Page 124) and in female by 1.7- and 2.7-folds (p=0.006; N=5, Figure 5.10B,C, Page 124) respectively. G-CSFR expression was 1.5-fold greater in placentae from male fetuses compared to female fetuses after treatment with LPS (p=0.048; N=8 Males, N=5 Females, Figure 5.10B,C, Page 124). When the results from placental cultures of males and females fetuses were combined (N=13), *L. rhamnosus* GR-1 and the combination of *L. rhamnosus* GR-1 and LPS up-regulated G-CSFR expression by 1.7- and 2.0-fold respectively (p=0.001; Figure 5.10D, Page 124)
5.3.4. The output of cytokines is dependent on both immune and trophoblast cells

In order to investigate the role of immune and trophoblast cells individually on cytokine production, CD45 positive cells were magnetically purified from pure trophoblast cells using the technique described by Stenqvist et al. (Stenqvist et al., 2008). Immunocytochemistry and FACS analysis both showed a 2% macrophage (CD68 positive) contamination in the mixed culture set (pure trophoblast and CD45\textsuperscript{+} cells) and immunocytochemistry confirmed the successful removal of these cells through magnetic purification (Figure 5.11, Page 125). LPS increased the levels of TNF-\(\alpha\) relative to control in mixed, pure trophoblast and CD45\textsuperscript{+} culture sets in both males (\(p=0.029\), \(p=0.023\), \(p=0.015\) respectively; \(N=3\), Figure 5.12B, Page 126), and females (\(p=0.009\), \(p=0.005\), \(p=0.035\) respectively; \(N=4\), Figure 5.12C, Page 126). However, the TNF-\(\alpha\) output detected for the mixed cultures is a combination of those in pure trophoblast and CD45\textsuperscript{+} cells for all treatments, particularly for the LPS (\(p=0.029\)) and combination of \textit{L. rhamnosus} GR-1 and LPS treatments (\(p=0.002\); \(N=7\), Figure 5.12A, Page 126). The same pattern was observed for IL-10 and G-CSF cytokine production with the exception that different treatments did not cause a change in either of these cytokines compared to control levels in pure trophoblast or CD45\textsuperscript{+} culture sets. IL-10 was up-regulated in the mixed culture set of both male (\(p=0.031\); \(N=3\), Figure 5.13B, Page 127) and female (\(p=0.004\); \(N=4\), Figure 5.13C, Page 127) placentae as a result of all treatments. The mixed culture output of IL-10 is a combination of those in pure and CD45\textsuperscript{+} cells (\(p=0.001\); \(N=7\), Figure 5.13A, Page 127) for all treatments. The increase in G-CSF was only observed in the placenta of female fetuses (\(p=0.048\); \(N=4\), Figure 5.14C, Page 128) compared to placenta of male fetuses (\(p=0.033\); \(N=7\), Figure 5.14B, Page 128). Once again, G-CSF output for the mixed culture set was a combination of those in pure trophoblast and CD45\textsuperscript{+} cells with LPS (\(p=0.015\); \(N=7\)), \textit{L. rhamnosus} GR-1 treatment alone (\(p=0.004\); \(N=7\)) and the combination of both treatments (\(p=0.004\); \(N=7\)) (Figure 5.14A, Page 128).

5.4. Comment

In these experiments, we found that \textit{L. rhamnosus} GR-1 caused an up-regulation in the output of the anti-inflammatory cytokine G-CSF in trophoblast cells of placentae carrying a
female fetus. Our findings are consistent with other studies *in vitro* whereby the same *L. rhamnosus* GR-1 supernatant increased G-CSF output in mouse macrophages (Kim et al., 2006) as well as in the JEG-3 human trophoblastic cell line (Marino and Roguin, 2008). Since low levels of G-CSF have been associated with increased incidence and severity of infection in preterm infants (Chirico et al., 1997), our results could serve as another line of evidence for the protective nature of *L. rhamnosus* GR-1 in the intrauterine tissues in preventing preterm birth. LPS also caused an increase in the output of G-CSF as it did for IL-10 (Yeganegi et al., 2009), which we propose may counter the local effects of inflammation. Here, we have reported sex specific differences in the ability of *L. rhamnosus* GR-1 to increase the output of G-CSF, with cells from placentae of female fetuses being strikingly more responsive to LPS, *L. rhamnosus* GR-1 and the combination of treatments. Since preterm birth is less frequent in women carrying a female fetus (Ingemarsson, 2003; Zeitlin et al., 2002) and females are known to be better at mounting an immune response than men (Schroder et al., 1998; Imahara et al., 2005), we propose that our results could account for the lower incidence of preterm birth and neonatal sepsis observed with female fetuses.

Studies suggest that the anti-inflammatory effects of G-CSF are mediated through activation of the JAK/STAT and the MAPK pathways in both mouse macrophages (Kim et al., 2006) as well as in the JEG-3 human trophoblastic cell line (Marino and Roguin, 2008). Currently, the relationship between G-CSF, and the MAPK and JAK/STAT pathways in human placental trophoblast primary cell cultures is unknown. In this study, it was determined that STAT3-Tyr705 and p38 phosphorylation did not decrease when G-CSF was blocked. However, *L. rhamnosus* GR-1-induced-G-CSF output was significantly attenuated in the presence of JAK inhibitor and p38 inhibitor (SB203580), demonstrating the requirement of the JAK/STAT and MAPK pathways upstream for G-CSF output. In human monocytes, Kim et al concluded that *L. rhamnosus* GR-1 treatment stimulated the release of G-CSF, which activated JAK2/STAT3 when bound to the Type I-like G-CSF cytokine receptor to inhibit TNF-α independently of IL-10 (Kim et al., 2006; Panopoulos and Watowich, 2008). In our study, blocking G-CSF with a neutralizing antibody did not reverse the down-regulation of TNF-α or up-regulation of IL-10 by *L. rhamnosus* GR-1, indicating that there must be another pathway through which lactobacilli decreases TNF-α. To determine whether IL-10 and G-CSF mediates *L. rhamnosus* GR-1-induced TNF-α, a knockdown or knockout of the anti-
inflammatory cytokine receptors in human placental trophoblast cells could be performed. Both JAK1, activated by IL-10, and JAK2, activated by G-CSF, pair with STAT3 and phosphorylate STAT3-Tyr705 (Kim et al., 2006; Murray, 2007). However, in human placental trophoblast cells, the opposite effect is seen, where the activation of the JAK/STAT and MAPK pathways may lead to G-CSF production. This may explain why STAT3-Tyr705 and p38 phosphorylation did not change in the current study when G-CSF neutralizing antibody was used to treat human placental trophoblast cells. Our study also shows that adding this neutralizing antibody did not change the L. rhamnosus GR-1 stimulation of IL-10, implying that the increased output of these two anti-inflammatory cytokines by L. rhamnosus GR-1 are independent of each other.

L. rhamnosus GR-1 and the combination of L. rhamnosus GR-1 and LPS stimulated G-CSFR expression in human placental trophoblast cells. This could indicate that there is an increase binding of G-CSF to its receptor, making it easier for L. rhamnosus GR-1 to activate anti-inflammatory responses to infection as a result of this binding. An alternative explanation for the up-regulation of G-CSFR in placental cell cultures is that L. rhamnosus GR-1-induced-G-CSF may act to increase its own receptor. This effect has been reported in murine bone marrow cells, where treatment of bone marrow cells by G-CSF led to an increase in G-CSFR messenger RNA (Steinman and Tweardy, 1994). It is unknown, however, why placental trophoblast cells from placentae of male fetuses with limited G-CSF output would have an increased G-CSFR expression. We speculated that this may enhance the possibility of binding of other substances to G-CSFR receptor in order to increase a response considering the compromised anti-inflammatory effects observed in these fetuses.

Cytokines are important in homeostasis of the immune system. G-CSF in particular has a direct effect in stimulating the proliferation and survival of granulocytic progenitor cells as well as accelerating the release of neutrophils (Panopoulos and Watowich, 2008). G-CSF is considered to be a major regulator of macrophages and is often studied with regards to its role in immune cell signaling (Kim et al., 2006). Therefore, through magnetic purification of the trophoblast cells, we investigated whether the effect of L. rhamnosus GR-1 supernatant on cytokine production and the subsequent response was from the trophoblast cells, immune cells or the combination of both. Most immune cells that are positive for CD45 are washed
away through our culture process, except for macrophages, which tend to adhere to the bottom of the culture dish. By measuring TNF-α, IL-10 and G-CSF, we observed that the effect of LPS, *L. rhamnosus* GR-1 and the combination of both is an additive effect requiring both pure trophoblast as well as macrophages. Therefore, in an *in vivo* setting, both cell types act together in the maternal-fetal interface in order to respond to bacterial endotoxins or probiotics. In particular, with regards to G-CSF, pure trophoblast cells showed minimum output of this cytokine and macrophages played a more important role in its release. Although primary cultures impose a limitation in terms of possible cell contamination and decreasing cell viability, this model serves as a better representation of the true intrauterine environment than cell lines.

This is the first study to investigate the effect of *L. rhamnosus* GR-1 supernatant on G-CSF output in both placental trophoblast as well as immune cells. From previous (Yeganegi et al., 2009) and current studies, we propose that *L. rhamnosus* GR-1 supernatant acts through the JAK/STAT and MAPK pathways to increase the anti-inflammatory IL-10 and G-CSF. It also independently down-regulates TNF-α. The increase in the production of G-CSF with *L. rhamnosus* GR-1 supernatant is only observed in pregnancies carrying a female fetus and could potentially provide a mechanism for the lower incidence of preterm births in these pregnancies. We also conclude that both trophoblast and immune cells are necessary for the initiation of an anti-inflammatory cascade in the intrauterine environment. Up-regulation of the anti-inflammatory cytokines could be an innate protective mechanism in gestational tissues implemented by the placenta in a healthy urogenital environment to protect against infection. *L. rhamnosus* GR-1 has shown to have beneficial effects for the vaginal microflora (Barrons and Tassone, 2008) and may serve as a suitable alternative to antibiotics in preventing infection/inflammation-mediated preterm birth.
Primary cultures of human placental trophoblast and immune cells were serum starved for 12 hours after 72 hours of culture. They were pre-treated with *L. rhamnosus* GR-1 supernatant for 12 hours and then treated with LPS. Protein was collected after twenty minutes for the measurement of the phosphorylated protein and after eight hours to assess the expression of G-CSFR. Media was collected eight hours following the LPS addition. Different inhibitors were added to investigate the involvement of different signaling pathways.
Figure 5.2: Effect of G-CSF neutralizing antibody on G-CSF concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells of female fetuses.

Histograms showing G-CSF concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=7). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 5.3: Effect of G-CSF neutralizing antibody on TNF-α concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells from placentae of the female fetuses.

Histograms showing TNF-α concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=4). Statistical significance was present where letters are different (p<0.01) as determined by One-Way-ANOVA.
Figure 5.4: Effect of fetal sex and *L. rhamnosus* GR-1 supernatant on concentration of anti-inflammatory G-CSF by LPS and *L. rhamnosus* GR-1-stimulated placental trophoblast cells.

Histograms showing G-CSF concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=5 males, N=6 females). Concentration of G-CSF in the media of the control cultures was 231 pg/ml. Statistical significance within gender groups (male: a and female: a’, b’, c’) was present where letters are different (p=0.002) by One-Way-ANOVA. Asterisks indicate statistical difference by two-tailed student t-test **p=0.004.
Figure 5.5: Effect of G-CSF neutralizing antibody on the expression of STAT3-Tyr705 phosphorylation, by *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells of the female fetuses.

(A) Representative western blot of phospho-STAT3-Tyr705 (86 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant and LPS and *L. rhamnosus* GR-1 supernatant as well as addition of a G-CSF neutralizing antibody to these four basic treatments of human placental trophoblast cells. (B) ROD of phospho-STAT3-Tyr705 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=4). Statistical significance was present where letters are different (p=0.004) as determined by One-Way-ANOVA.
Figure 5.6: Effect of G-CSF neutralizing antibody on the expression of p38 phosphorylation, by *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells of the female fetuses.

(A) Representative western blot of phospho-p38 (43 kDa) and β-actin (43 kDa) expression under control, LPS, *L. rhamnosus* GR-1 supernatant and LPS and *L. rhamnosus* GR-1 supernatant as well as addition of a G-CSF neutralizing antibody to these four basic treatments of human placental trophoblast cells. (B) ROD of p38 western blot normalized to β-actin. Results are mean values ± S.E.M. and presented relative to control (N=5). Statistical significance was present where letters are different (p=0.005) as determined by One-Way-ANOVA.
Figure 5.7: Effect of JAK Inhibitor on G-CSF concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells from placentae of female fetuses.

Histograms showing G-CSF concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=5). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 5.8: Effect of p38 Inhibitor on G-CSF concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells from placentae of female fetuses.

Histograms showing G-CSF concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=5). Statistical significance was present where letters are different (p<0.001) as determined by One-Way-ANOVA.
Figure 5.9: Effect of G-CSF neutralizing antibody on IL-10 concentration from *L. rhamnosus* GR-1 supernatant and LPS-stimulated placental trophoblast cells from placentae of the female fetuses.

Histograms showing IL-10 concentration (fold increase relative to control) for different treatment groups in media from placental trophoblast cell cultures. Results are mean values ± S.E.M. and presented relative to control (N=4). Statistical significance was present where letters are different (p=0.025) as determined by One-Way-ANOVA.
Figure 5.10: Effect of fetal sex on G-CSFR expression in placental trophoblast cells.

(A) ROD of G-CSFR expression under control conditions in placenta of male or female fetuses normalized to β-actin (p>0.05; N=8 Males, N=5 Females). (B) Representative western blot of G-CSFR (92 kDa) and β-actin (43 kDa) expression under LPS, L. rhamnosus GR-1 supernatant as well as LPS and L. rhamnosus GR-1 supernatant treatments of placental trophoblast cells from pregnancies carrying a male or a female fetus. (C,D) ROD of G-CSFR western blot normalized to β-actin for (C) pregnancies carrying a male or female fetus and (D) total samples (N=13). Results are mean values ± S.E.M. and presented relative to control. Statistical significance within gender groups (male: a,b and female: a’,b’) was present where letters are different (C) (p=0.015 for males and p=0.006 for females) and (D) (p=0.001) by One-Way-ANOVA. (C) Asterisk indicates statistical difference by two-tailed student’s t-test *<0.05 (p=0.048).
Figure 5.11: Characterization of cultured cells.

Macrophages and cytrophoblast cells were CD68 and cytokeratin positive respectively. Representative staining for CD68 (A, D, G), cytokeratin (B, E, H) and negative control (C, F, I) in mixed, pure and CD45+ culture sets are shown here (N=4). Scale bar 100 mm. The red arrows indicate macrophages in the mixed and CD45+ culture sets.
Figure 5.12: TNF-α concentration in mixed, pure trophoblast and CD45+ culture sets

TNF-α concentration (fold increase relative to control) for control, LPS, GR-1, and GR-1/LPS treated cells from A) both placentae of male or female fetuses (N=7), B) placenta of male fetuses (N=3) and C) placenta of female fetuses (N=4). Results are mean values ± S.E.M. and presented relative to control. Statistical significance within gender groups (Mixed cells: a,b, Pure trophoblasts: a’, b’,c’ and CD+ cells: a’”, b’”) was present where letters are different by One-Way-ANOVA. For mixed, pure trophoblast and CD45+ culture sets, A) p<0.001, p<0.001, p<0.001; B) p=0.029, p=0.023, p=0.015; and C) p=0.009, p=0.005, p=0.035 respectively. Asterisk indicates statistical difference by two-tailed student’s t-test * = 0.029, ** = 0.002.
Figure 5.13: IL-10 concentration in mixed, pure trophoblast and CD45⁺ culture sets.

IL-10 concentration (fold increase relative to control) for control, LPS, GR-1, and GR-1/LPS treated cells from A) both placentae of male or female fetuses (N=7), B) placentae of male fetuses (N=3) and C) placentae of female fetuses (N=4). Results are mean values ± S.E.M. and presented relative to control. Statistical significance within gender groups (Mixed cells: a,b,c, Pure trophoblasts: a’ and CD+ cells: a’’,b’’) was present where letters are different by One-Way-ANOVA. For mixed culture set, A) p<0.001; B) p=0.031; and C) p=0.004. Asterisk indicates statistical difference by two-tailed student’s t-test *=0.029, **=0.001.
Figure 5.14: G-CSF concentration in mixed, pure trophoblast and CD45\(^+\) culture sets.

G-CSF concentration (fold increase relative to control) for control, LPS, GR-1, and GR-1/LPS treated cells from A) both placentae of male or female fetuses (N=7), B) placentae of male fetuses (N=3) and C) placentae of female fetuses (N=4). Results are mean values ± S.E.M. and presented relative to control. Statistical significance within gender groups (Mixed cells: a,b, Pure trophoblasts: a’ and CD+ cells: a”') was present where letters are different by One-Way-ANOVA. For mixed culture set, A) p=0.033; B) p>0.05; and C) p=0.048. Asterisk indicates statistical difference by two-tailed student’s t-test *=0.015, **=0.004.
Chapter Six

General Discussion
Chapter 6

6. General Discussion

Prior to this thesis research, little was known regarding the effect of probiotics on the mechanisms of preterm birth associated with infection/inflammation. The overall objective of this thesis was to assess the effect of lactobacilli on intrauterine tissues and to determine the signaling mechanism(s) by which it has its effects. We specifically studied 1) the effect of *L. rhamnosus* GR-1 supernatant on cytokine profile and PG-regulating enzyme expression in LPS-stimulated human chorion and placental trophoblast cells; 2) potential signaling pathways in intrauterine tissues through which lactobacilli acts in order to interfere with the cascade leading to preterm birth and 3) the potential role of immune and placental trophoblast cells in initiating an immune response. In addition, we also compared all of these effects in pregnancies with male and female fetuses to determine if there are sexual dimorphisms in the responses.

We have found quite profound sex specific differences in the ability of LPS to increase output of pro- and anti-inflammatory cytokines as well as the prostaglandin-synthesizing enzyme, PTGS2. A previous study showed that placental 11β-HSD2 activity is higher in female fetuses suggesting that placental glucocorticoid metabolism differs according to fetal sex (Clifton, 2005). We found that TLR-4 is expressed in a greater abundance in placental trophoblast cells of male fetuses, which may contribute to the heightened inflammatory response observed in these fetuses. This is in line with the findings of other studies, which show that there is a higher expression of TLR-4 in adult male mice (Marriott et al., 2006). In addition, we found that *L. rhamnosus* GR-1 supernatant alone and together with LPS enhanced the expression of PGDH protein in placental trophoblast cells from female fetuses, and up-regulated the anti-inflammatory cytokine IL-10 in both sexes as well as partially down-regulated the expression of PTGS2 in males. Moreover, we observed that *L. rhamnosus* GR-1 caused an up-regulation in the output of the anti-inflammatory cytokine, G-CSF, but only in the placentae of the female fetuses. Our findings are consistent with other studies *in vitro* whereby the same *L. rhamnosus* GR-1 supernatant increased G-CSF and IL-10 and decreased TNF-α outputs in mouse macrophages (Kim et al., 2006). These results
suggest a mechanism by which lactobacilli might interfere with the inflammatory cascade that is responsible for inflammation/infection-mediated preterm birth. Our findings may also account for the lower incidence of preterm birth observed with female fetuses and explain the observation that females are better at mounting an immune response than males (Ingemarsson, 2003; Zeitlin et al., 2002; Schroder et al., 1998; Imahara et al., 2005).

Although, the same pattern of response was observed for the effect of lactobacilli on cytokine output in chorion trophoblast cells as placental trophoblast cells, there was no sex specific difference in the production of cytokines, and lactobacilli had no effect on prostaglandin-regulating enzymes in chorion trophoblast cells. This is of interest since both placental and chorion trophoblast cells share the same embryological origin. The chorion lies in close proximity to the maternal decidua, and the cross-talk at this interface might play a role in the specificity of the responses observed in this tissue. Another plausible explanation could be the interaction of the immune cells in the maternal blood with placental trophoblast cells. The syncytiotrophoblast cells that line the chorionic villi are in contact with the maternal blood and, therefore, contain a significant number of CD45+ cells, which are surface molecules that are on all the members of the leukocyte group (Riley, 2008). In addition, Immunoglobulins that are present in fetal blood and pass to the placenta may play a role in the differential response observed (Blanc, 1979). We have found that in our mixed culture sets, there exists a sexual dimorphism pattern in cytokine output that favors a lower inflammatory response in placentae of female fetuses. However, upon removal of the immune cells which could potentially come from the maternal blood, the sexual dimorphism in cytokine production, particularly G-CSF, which is secreted in significant amounts from macrophages, was abolished. This suggests that the response to infection and inflammation may be different depending on the route through which microorganisms access the uterine environment such as by haematogenous routes as opposed to an ascending infection. In addition, we also observed that in a setting where both pure trophoblast cells and macrophages are present, LPS, \textit{L. rhamnosus} GR-1 and the combination of both treatments lead to a response that is greater than the responses observed from individual cell types. Therefore, in an \textit{in vivo} setting, both cell types are required to act together in the maternal-fetal interface in order to respond effectively to bacterial endotoxins. Another possible explanation for the difference in results between placental and chorion trophoblast cultures is that placental trophoblast cells
differentiate and form a syncytium whereas chorion trophoblast cultures remain as single cells throughout the experiment. hCG is a marker of syncytialization that is present in greater abundance in maternal serum and cord blood of women carrying female fetuses than males (Gol et al., 2004). hCG is known to inhibit LPS-induced septic shock as well as Th1 cytokine levels in mice (Khan et al., 2002). However, it has been shown that the difference in maternal serum and cord blood hCG levels in relation to fetal sex is not associated with single cytotrophoblast cell activity (Gol et al., 2004). In the human, glucocorticoids are another marker of syncytialization and its levels are regulated by the enzyme 11β-HSD2, which is known to have a greater activity in premature female neonates than males (Stark et al., 2009). This provides further evidence for the presence of sexual dimorphism within a syncytium compared to single cell cytotrophoblast cells of chorion. The sex difference in cytokine output pattern observed in our mixed culture set could also be due to the fact that macrophages express receptors for gonadal hormones (androgens and estrogen). Estrogen usually has Th2 cell activity while androgens foster Th1 cell activity (Namazi, 2009). In addition, the enzymes responsible for regulation of Th1 and Th2 cells could themselves be controlled by an X-linked gene, which could explain the sexual dimorphism in cytokine output pattern observed in our studies. Epigenetic modifications in target gene promoters such as increased histone acetylase transferase activity, histone acetylation and DNA demethylation could also lead to receptor regulation (Weaver, 2009), leading to sexual dimorphism observed in cytokine and/or prostaglandin-regulating enzyme expression. Since epigenetic modifications are tissue specific, this could also account for the differential response of placental trophoblast cells and chorion cytotrophoblasts to different treatments. We further propose that the sexual dimorphism observed in our results in placenta trophoblast cells could also be due to epigenetic modifications or differential expression of products of certain genes. It has been suggested that the differential expression of glucose-6-phosphate dehydrogenase (G6PD), an X-linked gene, between male and female mouse embryos causes the females to grow slower under conditions of stress and to have better survival through lowering their metabolic rates (Perez-Crespo et al., 2005). Another study has shown that interferon-tau (IFN-τ), a product of an autosomal gene known to down-regulate PGs and up-regulate G-CSF, is secreted two-fold greater by female bovine blastocysts compared to males (Kimura et al., 2004), providing a possible explanation for the
sexual dimorphism observed in G-CSF output in our experiments. In addition, epigenetic studies in bovine blastocysts have revealed that male embryos have a shorter telomere length and a higher methylation in a sequence close to a variable number of tandem repeats minisatellite region (Bermejo-Alvarez et al., 2008). These epigenetic differences could, therefore, affect development and metabolism of the embryo as early as implantation and could provide another explanation for the sexual dimorphism observed in our studies.

We have proposed a mechanism of action through which lactobacilli could have its anti-inflammatory effects. We found that *L. rhamnosus* GR-1 induced phosphorylation of STAT3-Tyr705 and p38 in primary human placental trophoblast cell cultures. This is in agreement with previous studies, which showed that the *L. rhamnosus* GR-1 supernatant up-regulated the phosphorylation of both STAT3 and p38 in mouse macrophages (Kim et al., 2006). Furthermore, we showed that *L. rhamnosus* GR-1-induced IL-10 and G-CSF release were significantly attenuated in the presence of a JAK inhibitor and SB203580, a p38 inhibitor, demonstrating the role both the JAK/STAT and p38 pathways play upstream of IL-10 and G-CSF production. Blocking different members of these and the NF-κB pathway or neutralization of the anti-inflammatory cytokines, however, had no effect on the down-regulation of TNF-α by lactobacilli, suggesting that there might be another pathway through which lactobacilli down-regulates pro-inflammatory cytokines. In addition, there was no difference in the phosphorylation of STAT3-Tyr705 and p38 in placental cells from the two fetal sexes. Yet, there was a sex specific difference in the output of both IL-10 and G-CSF downstream of these signaling pathways. This could be due to the presence of different phosphorylation sites for STAT3, since our antibody only detected the phosphorylation of the Tyr 705 residue. However, antibodies for other sites of STAT3 such as Ser 727 are also available and could show a sexual dimorphism in their phosphorylation. As well, LPS also induces the secretion of cytokines via post-transcriptional mechanisms (Falkenburg et al., 1991). For instance, G-CSF transcripts contain conserved AU-rich sequences in the 3’ untranslated region, which are thought to be signals for rapid degradation. This is shown from the very short half-life of G-CSF. However, different factors such as IL-17 could alter this half-life and stabilize G-CSF mRNA. Therefore, even though there was no sexual dimorphism in the phosphorylation of the factors that lead to transcription of the cytokines in our studies, there could be other differences between the two fetal sexes in the production of
IL-17 or other factors that help stabilize the mRNA of these cytokines post-transcriptionally and account for the differences we have observed.

Our signaling pathway studies were conducted using inhibitors or neutralizing antibodies. Although, the specificity of the inhibitors and antibodies were examined in our system fully, alternative approaches could be used in order to verify that suspected pathways are involved. Gene knock-downs of the players in the signaling pathways by using siRNA is one strategy to examine if lactobacilli can still exert its effect independently or if it acts through a certain pathway to have its effect. Another strategy would be to use a competing inhibitor, which does not interfere with the phosphorylation of other kinases. For instance, Stattic is a small molecule known to inhibit the dimerization and nuclear translocation of STAT-3 and hence can be used as its competing inhibitor, without interfering with other pathways (Samavati et al., 2009). Finally, in-vitro kinase assays could also be used in order to observe whether a kinase is phosphorylated without inhibiting its phosphorylation.

In contrast to TNF-α, we also found minimal output of IL-1β and IL-6 with all of the treatment groups. Previous studies suggest that the mRNA of some pro-inflammatory cytokines decreases markedly during the first 72 hours of primary culture when cytotrophoblast cells differentiate into syncytiotrophoblasts (Stephanou et al., 1995). As our study assessed the output of IL-1β and IL-6 after syncytialization, this may explain the lack of effect. Moreover, blocking the pro-inflammatory cytokine TNF-α did not decrease the expression of PTGS2, implying that the down-regulation of TNF-α and PTGS2 by lactobacilli or their up-regulation by LPS are independent of each other.

Finally, we have provided evidence, which indicates that other strains of lactobacilli, such as *L. crispatus* and *L. iners* also inhibit LPS-stimulated TNF-α and increase IL-10 output in placental trophoblast cells. However, they do not play any role in modulating the expression of the prostaglandin-regulating enzymes. We also found that addition of *G. vaginalis*, which is considered to be one of the BV-causing microorganisms, resulted in cell death. These findings support the hypothesis that *L. rhamnosus* GR-1 may be one of the most effective strains for altering the course of vaginal and, therefore, intrauterine inflammation or infection in pregnancy as proposed previously (Reid and Bocking, 2003; Reid et al., 2003a).
In primary cell culture experiments, complete purity of the interested cell type is hard to achieve. There is always a contamination of other cells, such as fibroblasts, red blood cells, macrophages, neutrophils and other cell types. Washing of the cells with DMEM in our study, ensured that most of these cells were eliminated. Collection of the right band at the percoll gradient and scraping the tissues (especially decidua from the chorion) also helped to eliminate most of the undesired cell types. Finally histochemical staining was performed to assess the purity of the cells. Primary cultures also have limitations in terms of decreasing cell viability over time, which was assessed by Trypan Blue Exclusion Test. In addition, our data must be interpreted with caution as the cell culture experiments do not consider all cell to cell interactions and other factors that might contribute to a different result in an in vivo environment. However, we have tried to mimic an in vivo setting by considering different cells types and their interaction at the maternal-fetal interface. Our primary culture cell model serves as a good representation of the true intrauterine environment when compared to immortalized cell lines.

Future experiments are required to elucidate the precise mechanisms by which L. rhamnosus GR-1 exerts its effect in human intrauterine tissues. Studies could be performed where separate placental trophoblast cells and immune cells are combined in different proportions and their production of cytokines is compared with the original mixed population. Other experiments could be performed to identify the component of the lactobacilli that is responsible for its anti-inflammatory properties. Fractionation of the supernatant and treatment of cells could identify the portion with anti-inflammatory such as G-CSF producing properties. The fractions with maximum activity could then be subjected to chromatography and analyzed by SDS-PAGE gels. Each band could then be excised and undergo mass spectrometry in order to identify the substance based on the homology against current databases. As well, its effect on cytokines could be studied in a greater detail to investigate whether the changes are due to regulation at a transcriptional, post-transcriptional, translational or secretary level. Also, since G-CSF, IL-10 and TNF-α neutralizing antibodies made complexes with the secreted cytokines to prevent their action, knockout models lacking the gene for these cytokines could better investigate whether they play a role in regulation of prostaglandin-regulating enzymes. To investigate the sexual dimorphism, promoters on the gene for specific cytokines could be looked at for any silencing, which might explain the lack
of any effect in placentae of the male fetuses for G-CSF. In addition, it is known that caspase-12 confers susceptibility to infection and estrogen can alter its expression, since caspase-12 is a transcriptional target of estrogen receptor alpha (Yeretssian et al., 2009). Therefore, its differential expression in placentae of male and female fetuses could account for the sexual dimorphism observed. In order to mimic a better in vivo model, co-culture models could explore the interaction between decidua cells and the chorion trophoblast cells in order to understand the communication at the maternal-fetal interface and its role in regulation of cytokines and prostaglandins. Alternatively, decidual cells could be treated with the conditioned medium from lactobacilli and LPS-treated chorion trophoblast cells and expression of cytokines and PGs could then be measured. Since most spontaneous births that happen in early gestation (20-28 weeks) are associated with histological chorioamnionitis (Gray et al., 1992), it is thought that lactobacilli should be given early in gestation in order to achieve its maximal beneficial effect. Our culture model, however, mimics an infection/inflammation at term. Explant cultures could be performed on early gestation placentae from terminated pregnancies, which would reflect an in vivo setting and consider cell to cell interactions. Our cell culture model also reflects a prophylactic treatment of bacterial infection, whereas in some women, they present with an acute or repeated bacterial infections. Therefore, cells could be treated with lactobacilli before and after repeated doses of LPS and changes in cytokines or prostaglandin-regulating enzymes could be measured. Cultures could also be done on preterm placentae or placentae associated with other pathologies to determine whether lactobacilli have an effect on these tissues. Finally, to take the above findings to a more in vivo setting, lactobacilli could be given to animal models of preterm birth such as mice, rat, rabbit and monkey (Adams Waldorf et al., 2008; Holmgren et al., 2008; Yakuwa et al., 2007; Celik and Ayar, 2002) and the health of the fetuses as well as the presence of cytokines and chemokines in plasma, cervicovaginal fluid, cord blood and amniotic fluid can be investigated.

The studies performed as part of this doctoral thesis are the first to our knowledge to identify an effect of fetal sex on the LPS-stimulation of cytokines and prostaglandin-regulating enzymes as well as to investigate the ability of L. rhamnosus GR-1 and other probiotic strain supernatants to interfere with the inflammatory cascade associated with preterm labour. We have shown (Figure 6.1, Page 138) that L. rhamnosus GR-1 supernatant acts through the
JAK/STAT and MAPK pathways to increase the anti-inflammatory IL-10 and G-CSF. It also independently down-regulates PTGS2 and TNF-α and up-regulates PGDH. The increase in the production of G-CSF and PGDH is only observed in pregnancies carrying a female fetus and could potentially provide a mechanism for the lower incidence of preterm births in these pregnancies. We also conclude that both trophoblast and immune cells are necessary for the initiation of an anti-inflammatory cascade in the human placenta. Up-regulation of the anti-inflammatory cytokines could be an innate protective mechanism in gestational tissues used by the placenta in a healthy urogenital environment to protect against infection. *L. rhamnosus* GR-1 has been shown to have beneficial effects for the vaginal microflora (Anukam et al., 2006; Reid, 2001; Reid et al., 2001) and could be used in combination with antibiotics in preventing infection/inflammation-mediated preterm birth. The evidence for therapeutic benefit of lactobacilli will help to create novel non-invasive strategies with significant potential for developing new therapeutic targets for inflammatory diseases during pregnancy. In addition, there is the potential for improving the diagnosis and management of preterm labour, allowing clinicians to more accurately target prevention and treatment strategies for this important health issue.
Figure 6.1: Potential mechanism of action of *L. rhamnosus* GR-1.

Red lines indicate the inhibitory actions of *L. rhamnosus* GR-1.
7. Reference List


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