Maternal Probiotic Intervention as a Prophylaxis against the Impact of Neonatal Stress: Implications for Irritable Bowel Syndrome

By

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STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Signed……………………… Date ………………..

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B. Peer reviewed conference abstracts


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Thesis Summary

Neonatal stress is a common early life event, reported in some instances to be associated with adverse physiological alterations that persist into adulthood. This concept has been applied to the ontogeny of functional gastrointestinal disorders such as irritable bowel syndrome (IBS). The use of probiotics in IBS patients has emerged as a treatment approach to improve some IBS symptoms. In addition, new research in rodent models indicates that neonatal probiotic intervention may assist in the prevention of brain-gut axis dysfunctions believed to be associated with IBS. The aim of this study was to determine whether perinatal (both pre and post natal) maternal probiotic supplementation could act prophylactically to block endocrine, immune and gut dysfunctions in rats exposed to neonatal stress (maternal separation) either alone or in combination with adult exposure to stress. This model has been proposed to mimic most of the cardinal features of IBS.

The first series of studies (Chapter 3) examined the effect of maternal probiotic intervention on HPA-axis responses and gut-associated neuroendocrine function including analysis of mRNA expression of corticotropin releasing hormone receptors 1 and 2 (CRH-R1 and CRH-R2), and nerve growth factor (NGF). The results of the study revealed that maternal probiotic intervention induced activation of neonatal stress pathways as indicated by greatly enhanced corticosterone levels, which persisted into adulthood, and exacerbated ACTH responses to stress in adulthood. Maternal probiotic intervention affected gut-associated neuroendocrine gene expression profiles depending on age, gender and stress protocol. These effects include synergism, antagonism and normalisation.

The second series of studies (Chapter 4) examined the effect of maternal probiotic intervention on systemic and gut-associated immune functions. In this chapter plasma levels of cytokines IFN-γ, TNF-α and IL-6, plasma Haptoglobin and IgA, and luminal IgA levels were examined. While the stress protocol did not affect levels of the circulating cytokines in the offspring, maternal probiotic intervention down-regulated IFN-γ production (irrespective of stress conditions) and up-regulated IL-6 responses to neonatal or adult stress. Importantly however, maternal probiotic intervention enhanced immune defence capacity as indicated by increased plasma and luminal IgA. Maternal probiotic intervention was also associated with significant reductions in plasma
haptoglobin levels in all stressed and non-stressed animals to well below the baseline levels indicating enhanced loss of hemoglobin.

The third series of studies (Chapter 5) examined whether maternal probiotic intervention protected against gut microbiota and secretory state alterations induced by neonatal and/or adult stress. Neonatal and/or adult stress disrupted the normal balance of gut microbiota. Maternal probiotic intervention caused shifts in neonatal gut microflora as indicated by fostering an overgrowth of potential negative bacteria such as *E. coli*, enterococci and clostridia in stressed and non-stressed pups, resembling that of neonatally stressed pups in the vehicle subset. In adulthood maternal probiotic intervention was associated with a disruption of the normal balance of gut flora when coupled with neonatal stress, but also restoration of some gut bacterial groups to normal in stressed animals. Maternally separated animals displayed greatly decreased ileal mucin gene expression which was further decreased by exposure to adult stress. Maternal probiotic intervention decreased neonatal ileal MUC2 gene expression. In adulthood however, maternal probiotic intervention reversed the decline in mucin gene expression of stressed males.

Collectively the studies presented in the current thesis are the first to demonstrate the influence of maternal probiotic intervention on the neuroendocrine, immune and gut function in a rat model of irritable bowel syndrome. Maternal probiotic intervention exhibited mixed positive and negative effects on brain, immune and gut function, depending on age, gender and stress protocol applied. By modifying the probiotic preparations utilised (e.g., changes in the composition, dose and method of delivery) and optimising time of use, it might be possible to improve this approach to minimise the adverse outcomes. It is clear however, that maternal probiotic intervention may be a viable means to improve brain-gut outcome in ‘at risk’ neonates exposed to stress in early life and at increased risk of IBS in later life.
Thesis Outline

A brief outline of the thesis is provided here to assist the reader. The thesis comprises six separate chapters.

Chapter 1
Chapter 1 provides a comprehensive review of published literature on early life stress, Irritable Bowel Syndrome and probiotics. It highlights areas of research that have not been explored in this field, and presents the research issues to be addressed in the thesis.

Chapter 2
Chapter 2 provides detail of the general and specific methods used in this thesis.

Chapter 3
Chapter 3 characterises the effect of maternal probiotic intervention on stress-induced alterations to HPA-axis activity and gut-associated neuroendocrine gene profiles.

Chapter 4
Chapter 4 characterises the effect of maternal probiotic intervention on stress-induced alterations to the immune system and gut-immune responses.

Chapter 5
Chapter 5 characterises the effect of maternal probiotic intervention on stress-induced alterations to the normal balance of gut microbiota and intestinal mucin gene expression.

Chapter 6
The thesis closes with Chapter 6, which includes an overall summary of the findings of this work, conclusions and recommendations for future research.
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention-Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous Systems</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen-presenting Cells</td>
</tr>
<tr>
<td>AS</td>
<td>Adult Restraint Stress</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosterone-binding Globulin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous Systems</td>
</tr>
<tr>
<td>CRD</td>
<td>Colorectal Distension</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Threshold Cycles</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>EMS</td>
<td>Emotional Motor System</td>
</tr>
<tr>
<td>ENS</td>
<td>Enteric Nervous System</td>
</tr>
<tr>
<td>Fbgn</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>FGIDs</td>
<td>Functional Gastro-intestinal Disorders</td>
</tr>
<tr>
<td>GF</td>
<td>Germ Free</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal Tract</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>GLMM</td>
<td>Generalised Linear Mixed Model</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>Hp</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic–Pituitary–Adrenal</td>
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<td>HSD2</td>
<td>11ß-Hydroxysteroid Dehydrogenase Type 2</td>
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<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Immunoglobulin A</td>
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<tr>
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<td>Interleukin</td>
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<tr>
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<td>Immunoreactivities</td>
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<td>Lamina Propria</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Major Histocompatibility Complex</td>
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<td>Myenteric Neuronal Plexus</td>
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<tr>
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<td>MRD</td>
<td>Maxidam Recovery Diluents</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>MUC</td>
<td>Mucin</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NS</td>
<td>Neonatal Maternal Separation</td>
</tr>
<tr>
<td>NNS</td>
<td>Non-Neonatal Stress</td>
</tr>
<tr>
<td>OF</td>
<td>Open Field</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Neutrophils</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PVN</td>
<td>Para-Ventricular hypothalamic Nucleus</td>
</tr>
<tr>
<td>RCM</td>
<td>Reinforced Clostridial Medium</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Real Time polymerase chain reaction</td>
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<tr>
<td>SHRP</td>
<td>Stress Hyporesponsive Period</td>
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<tr>
<td>sIL-6R</td>
<td>IL-6 soluble receptor</td>
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<tr>
<td>SNP</td>
<td>Submucosal Neuronal Plexus</td>
</tr>
<tr>
<td>TB</td>
<td>Toulidine Blue</td>
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<tr>
<td>TGF-ß2</td>
<td>Transforming Growth Factor ß2</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Peptide</td>
</tr>
<tr>
<td>YEL</td>
<td>Yeast Extract Lactate</td>
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Chapter I

Introduction
1.1 Background and Aim

There is a growing body of evidence to support the concept that adult health status might be determined by early life events. Early environmental factors acting during the critical and plastic period of the foetal and neonatal development may determine alterations in physiological regulation, promoting critical development and having long-lasting effects on health status. Neonatal stress is a common early life event which alters the development of the endocrine and immune systems. Specifically, exposure to neonatal stress results in alterations to the hypothalamic–pituitary–adrenal (HPA) axis; a neuroendocrine system which is the essential key for the normal stress response to challenges in vertebrates. Perturbations, during early life, in the development of the HPA-axis have been reported to result in neonates who hyper-respond to stress in later life (Kalinichev, Easterling, Plotsky, & Holtzman, 2002; O'Malley, Dinan, & Cryan, 2011; Plotsky & Meaney, 1993; Soderholm, et al., 2002). Recently, this concept has been applied to the ontogeny of functional gastrointestinal (GI) disturbances such as irritable bowel syndrome (IBS). IBS is a chronic relapsing disorder characterised by recurrent abdominal pain and discomfort associated with altered bowel habits causing either diarrhea, constipation or both in the absence of any detectable structural/organic abnormalities. IBS is an extremely prevalent disorder in adulthood and affects up to 3-15% of the population in western countries (Andresen & Camilleri, 2006). While there is not a universal effective treatment for IBS, current IBS therapy involves symptom-oriented approaches. Current IBS medications only bring temporary relief from the symptoms of IBS, and because of the recurrent nature of IBS, they need to be used for prolonged periods. Furthermore, they do not address the entire symptom complex in IBS patients and have side effects which restrict their efficacy. The high prevalence of this disorder and the ineffectiveness of current treatments results in high direct and
indirect costs to society along with considerable pain and distress to sufferers (Cash, Sullivan, & Barghout, 2005). Thus, developing preventive modalities in the management of IBS clearly will have advantages over the current approaches to treatment protocols.

Previous research on human and animal models of IBS has shown that probiotic intake could be a safe and effective treatment approach in improving some symptoms and normalising the bowel movement frequency. Presently there are some available commercial probiotic products claiming to improve some symptoms of IBS (Bausserman & Michail, 2005; Kim, et al., 2005; Niedzielin, Kordecki, & Birkenfeld, 2001; Nobaek, Johansson, Molin, Ahrne, & Jeppsson, 2000). Most recently, administration of probiotics to neonates has been used as a prophylactic strategy to revoke the long term unfavourable imprinting induced on the gastrointestinal system by early life stressors in animal models of human IBS (Gareau, Jury, MacQueen, Sherman, & Perdue, 2007). It is not as yet known however, whether maternal supplementary probiotics may also contribute to improved GI integrity and gut-associated immune functioning in stressed neonates, if these possible improvements persist into adulthood, or how this protective effect may be mediated. The current research is an attempt to link this proposed nutritional approach and its possible preventive effects against GI dysfunctions provoked by neonatal stress.

1.2 The Stress Response

1.2.1 Definition

To understand what stress and stress responses are, how stress impacts on the body and how the body responds to stress, it is an important prerequisite to understand the
concept of ‘homeostasis’. In order for the optimal functioning of the body, the internal environment must be maintained at a dynamic steady state in a process of so-called homeostasis. When an internal or external factor (stressor) threatens the homeostasis of the body, bodily systems initiate compensatory physiological responses to minimise the change, thereby restoring homeostasis (Sherwood, 2009). Therefore, stress is defined as “an acute threat to the homeostasis of an organism, real (physical) or perceived (psychological) and posed by events in the outside world or from within, that evokes adaptive responses that serve to defend the stability of the internal environment and to ensure the survival of the organism” (Mayer, Naliboff, Chang, & Coutinho, 2001).

1.2.2 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

In vertebrates, interactions among the hypothalamus, pituitary and adrenal glands (the hypothalamic-pituitary-adrenal [HPA] axis) function to control and regulate the body’s reaction/response to stressful stimuli. HPA-axis functioning co-ordinates responses of neural, endocrine and immune systems to the factors threatening the homeostatic state of the organism.

As shown in Figure 1.1 in response to brief or sustained periods of stress, two main hormones, corticotropin-releasing hormone (CRH) and vasopressin, synthesised in neurons of the paraventricular hypothalamic nucleus (PVN), are released through the medial parvocellular division into the hypophyseal portal circulation of pituitary. CRH stimulates pro-opiomelanocortin synthesis in the anterior pituitary and adrenocorticotrophin (ACTH) secretion from corticotropes of the anterior lobe of the pituitary gland. ACTH in turn initiates the synthesis of the glucocorticoids (e.g., corticosterone in rodents, and cortisol in primates) in the adrenal cortex, which is then
released into circulation. The HPA axis is regulated by the negative feedback action of glucocorticoids on the secretion of corticotropin from the pituitary and CRH from the hypothalamus (Abel & Majzoub, 2005; Fullford & Harbuz, 2005).

The integrity of the HPA-axis is crucial to the survival of vertebrates. The HPA-axis acts to coordinate neuro-endocrine and immune responses to a wide variety of stressful settings that threaten body homeostasis. The end products of HPA-axis activity are the glucocorticoids that widely influence body functions, including metabolism at cellular levels and amongst other things, immune functioning, cardiovascular homeostasis, intermediary metabolism, and bone turnover (Kino & Chrousos, 2005). Inappropriate glucocorticoid secretion is potentially harmful and may predispose individuals to poor health outcomes and disease pathology (Fullford & Harbuz, 2005). Regulation of the HPA-axis is a complicated process involving coordination of various physiological systems, in part mediated by the immune-neural-endocrine interactions. Individual health status and integrity relies significantly on appropriately integrated stress signals, including central and peripheral pro-inflammatory messages. The functional endogenous balance between pro- and anti-inflammatory responses is important for the appropriate regulation of the HPA-axis and protection against its dysfunction. An imbalance of such variables is characteristic of a wide variety of stress-induced disorders including chronic inflammatory disease (Fullford & Harbuz, 2005).
Figure 1. Components of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Yehuda, 2002)
1.2.3 The Purpose of the Stress Response

The fundamental purpose of the stress response is to prepare organisms to respond effectively to the internal and environmental life-threatening challenges/stressors and the disequilibrium they produce, and to direct individual’s metabolic resources to systems that are needed to deal with impending challenges. The biological changes accompanied by the stress response are mainly designed to allow mobilisation of the body’s energy resources and convert them into a suitable form (e.g., glucose) for immediate use and enhance its transport, along with the additional oxygen required to burn it, to the essential organs (i.e., brain and major exercising muscles) which require it. These responses are mediated by the actions of the glucocorticoids which aim to enable organisms to cope with life-threatening challenges aiding in activation of the ‘fight or flight’ response (Thiel & Dretsch, 2011). However, these events also take place at the expense of some other biological systems such as growth and reproduction. As such, acute responses are typically advantageous to the organism in that they enhance survival and the risk to other functions is short lived and contained. The negative aspects of activation of this system occur as a response to chronic stress, when the ongoing suppression of physiological systems has negative consequences (e.g., suppression of the immune system, inhibition of bone growth, inhibition of reproductive function) (Parker, 2012).
1.3 Programming Concept and Plasticity

Human biological systems are highly plastic during gestation and during the early postnatal period. This developmental period is a critical time during which interactions with environmental stimuli may profoundly influence normal developmental processes and have long-lasting effects on health status. This phenomenon is called ‘developmental programming’ (Carey, 2012). Development is a plastic process, wherein different phenotypes can be produced from a given genotype in response to the internal or external environmental conditions. This genotypic variability is termed ‘plasticity’ (Entringer, Buss, & Wadhwa, 2010; Hochberg, et al., 2011), a process which gives the best opportunity for successful survival and reproduction to the organism facing changing environments (Hochberg, et al., 2011).

The developing embryo/foetus is extremely vulnerable to the environmental conditions during crucial periods of cellular proliferation, differentiation and maturation, resulting in morphological and functional alterations in cells, tissues, and organ systems. These alterations may independently or by interacting with subsequent developments and environments, lead to short- and/or long-lasting outcomes for health and disease susceptibility. These concepts have been brought together in a unifying hypothesis known as the ‘Barker Hypothesis’ (Barker, Bull, Osmond, & Simmonds, 1990; Barker & Osmond, 1987; Barker, Osmond, Golding, Kuh, & Wadsworth, 1989) or the ‘developmental origins of health and disease’ hypothesis (Gluckman & Hanson, 2004). Epidemiological and empirical studies have demonstrated that prenatal and early postnatal adverse events appear to profoundly influence the rate of aging and the risk of developing diseases in later life such as type 2 diabetes, cardiovascular diseases, neurodegenerative diseases and cancer (Burton, Barker, Moffett, & Thornburg, 2011; Carey, 2012; Wolfson, Tacutu, Budovsky, Aizenberg, & Fraifeld, 2008).
underlying mechanisms of developmental programming still remain unclear; however there is a growing body of evidence that epigenetic modifications induced by early life environments play a crucial role (Godfrey, Lillycrop, Burdge, Gluckman, & Hanson, 2007). Epigenetic changes are involved in both normal development and human disease. Where there are defective changes in the epigenome, this can lead to disease through alterations in the global or localised density of DNA methylation, inappropriate histone modifications, or changes in distribution or function of chromatin-modifying proteins. These changes in turn, result in aberrant gene expression without altering DNA sequences (Feinberg, 2007). Recent studies have shown that early life internal or external environmental conditions are linked to long-lasting alterations in the human epigenome (Heijmans, et al., 2008; Tobi, et al., 2009). Epigenetic changes induced by the organism environment during development are typically adaptive and are designed to match an organism’s responses to an eventual predicted (mature) environment (Godfrey, et al., 2007). If, however, there is a high degree of mismatch between the anticipated and actual later-life environments, then the risk of metabolic disease is elevated (Gluckman & Hanson, 2006; Gluckman, Hanson, & Beedle, 2007).

Early life stress is one of the most well documented environmental conditions that profoundly impacts on human long-term health and influences the later-life risk of developing disease. In the following section the effect of early life stress on subsequent disease outcome or predisposition is discussed in more detail.
1.3.1 Prenatal Stress

Prenatal stress has been proposed as a key intrauterine environmental condition/challenge that may profoundly influence the anatomical and physiological integrity, and survival of the developing organism, with significant implications for the developmental programming of long-term health outcomes and susceptibility to disease (Wadhwa, 2005).

There is a growing body of evidence that maternal and foetal glucocorticoids and possibly other stress-related hormones reduce birth weight and have implications for the developing foetal HPA-axis and affective behaviour such as anxiety (Khashan, McNamee, et al., 2008). Low birth weight coupled with lower levels of maternal care associate with reduced hippocampal volume in adult women (Buss, et al., 2007). The children born to mothers experiencing high levels of stress during pregnancy may exhibit temperamental and behavioural problems as toddlers (Gutteling, et al., 2005), impaired attention and concentration (Gutteling, et al., 2006), autism, attention deficit, hyperactivity disorder, language problems, depression (O'Donnell, O'Connor, & Glover, 2009) and increased risk of developing schizophrenia in adulthood (Khashan, Abel, et al., 2008), increased risk of metabolic syndrome (cardiovascular disease and diabetes), asthma and autoimmune disorders, high-risk endocrine profile and impaired working memory performance (Entringer, et al., 2008).

While the number of human studies is quite small in this regard, animal studies suggest that maternal stress during gestation can exert long-lasting impacts on offspring’s central and peripheral systems without changing the birth phenotype (Bailey, Lubach, & Coe, 2004; Bowman, et al., 2004; Coe, Lubach, & Karaszewski, 1999; Coe, Lubach, & Shirtcliff, 2007; Coe, Lulbach, & Schneider, 2002). In rodent models prenatal stress and administration of glucocorticoids reduces birth weight, impairs cognition, increases

It has been suggested that stress-induced maternal-placental-foetal endocrine and immune alterations during gestation are potential underlying mechanisms for the adverse effects of prenatal stress. This is because of the endocrine and immune responsiveness to intrauterine perturbations and their influence on a range of foetal programming targets (Wadhwa, 2005). Maternal-placental-foetal hormones and cytokines play an important role in coordinating key factors underlying brain and peripheral cellular growth, proliferation and differentiation (Cole, et al., 1995; Garbrecht, Klein, Schmidt, & Snyder, 2006; Matthews, 2000; Merrill, 1992; Trejo, Cuchillo, Machin, & Rua, 2000; Zhao & Schwartz, 1998). Therefore, disturbances in both the nature and timing of exposure to specific endocrine and immune effectors are likely to induce structural and functional abnormalities.

Pregnancy itself progressively alters the endocrine and immune functions. These alterations have significant implications for the endocrine and immune responses to external and internal perturbations. Pregnancy induces alterations in hormone and cytokine concentrations and a variety of physiological control mechanisms in order to provide a favourable environment for foetal development and to coordinate physiological systems that facilitate movement of the developing foetus from the internal to the extra uterine environment. The placenta is the primary foetal organ that produces and releases a range of biological effectors including hormones, neuropeptides, growth factors and cytokines, and functions in a way similar to that of a compressed hypothalamic–pituitary–target system (Mastorakos & Ilias, 2003). Glucocorticoid physiology has been considered as an important mediator of foetal
programming, with respect to hormone production and also hormone action mediated by tissue expression of glucocorticoid receptor, sensitivity and affinity, and by maternal–foetal transfer mediated by placental enzyme activity (Harris & Seckl, 2011).

Experiments with adrenalectomised dams have shown that prenatal stress has little effect on the offspring. Replacement of glucocorticoid hormones to adrenalectomised dams restores the adverse effects of prenatal stress on offspring physiology and behaviour (Barbazanges, Piazza, Le Moal, & Maccari, 1996; Ordyan & Pivina, 2003; Zagron & Weinstock, 2006). The findings from these animal studies suggest that maternal glucocorticoid levels are the sole mediator of foetal programming, however data from humans implicates a role of foetal glucocorticoid levels in reducing birth weight, since foetuses in intrauterine growth retardation and in pre-eclampsia have high glucocorticoids levels (Goland, et al., 1993; Tropper, et al., 1992). However, this might also be due to transplacental passage of maternal glucocorticoids.

The placenta plays a vital role in moderating foetal exposure to maternal factors. Glucocorticoid are able to pass freely across the placenta, however they are significantly lower in the foetus than in the mother. This is because the placenta expresses high levels of 11ß-hydroxysteroid dehydrogenase type 2 (HSD2), which has been suggested to provide a protective barrier between the foetus and mother (Edwards, Benediktsson, Lindsay, & Seckl, 1993), shielding glucocorticoid sensitive tissues in the foetus from inappropriately high glucocorticoid levels during development (Meaney, Szyf, & Seckl, 2007). However, up to 20% of maternal glucocorticoids reach the foetus intact (Benediktsson, Calder, Edwards, & Seckl, 1997). Since maternal glucocorticoid levels are so much higher than that of the foetus, modest perturbations in placental HSD2 levels/activity can have a profound impact on foetal glucocorticoid exposure. In the mouse, placental HSD2 mRNA expression drops dramatically towards late gestation.
Brown, et al., 1996) with a subsequent loss of activity, perhaps to allow maternal glucocorticoids to stimulate late foetal maturation. In the rat, the fall in placental HSD2 occurs later in gestation with less loss of activity (Waddell, Benediktsson, Brown, & Seckl, 1998), perhaps because the foetal adrenals contribute more to the maturational surge. In the human placenta, HSD2 levels steadily increase throughout gestation (McTernan, et al., 2001). Since GR expression remains constant in the placenta throughout gestation, natural fluctuations in HSD2 levels may control glucocorticoid action in the placenta as well as the foetus during development (altered GR signalling may alter placental function e.g., efficacy of nutrient transfer).

It has been hypothesised that reduced placental HSD2 activity results in high levels of glucocorticoids reaching the foetus, which retard growth and program disease susceptibility (Benediktsson, et al., 1997; Edwards, et al., 1993; Seckl, 1998). Compatible with this hypothesis, low levels of placental HSD2 activity are correlated with low birth weight in humans (McTernan, et al., 2001; Stewart, Rogerson, & Mason, 1995) and rodent models (Brown, et al., 1996).

Studies in which HSD2 activity is inhibited reveal an important role for a feto-placental barrier in mediating physiological and psychological disease. Administration of HSD2 inhibitors reduces birth weight and produces permanent alterations of the HPA axis and increases anxiety-like behaviour in aversive situations in adult rats (Welberg, et al., 2000). Maternal administration of HSD2 inhibitors throughout pregnancy in rats also leads to the development of hypertension and predisposes to hyperglycaemia in later life (R. M. Lindsay, Lindsay, Seckl, & Waddell, 1996; R. S. Lindsay, Lindsay, Edwards, & Seckl, 1996).
Genetically modified mice with reduced or zero levels of placental HSD2 have resulted in reduced birth weight and increase anxiety relative to the controls (M. C. Holmes, et al., 2006). These experiments establish a clear key role of feto-placental HSD2 in prenatal glucocorticoid programming. Indeed, variation in placental HSD2 levels correlate with altered expression levels of various glucose and amino acid transporter molecules and growth factors in the placenta. These alterations ultimately result in reduced birth weight. Research has shown that placental HSD2 levels/activity are affected by stress. Prenatal stress leads to a reduction in placental HSD2 activity in rats, suggesting that the foetus and placenta are exposed to extra excessive amounts of glucocorticoids (Mairesse, et al., 2007). Dietary protein restriction during pregnancy also reduces placental HSD2 activity (Bertram, Trowern, Copin, Jackson, & Whorwood, 2001; Langley-Evans, et al., 1996).

CRH which is synthesised by placenta in primates and released in abundant quantity into both maternal and foetal circulations regulates glucocorticoid release and ultimately cortisol from the foetal adrenal cortex. While cortisol has a negative feedback on hypothalamic CRH production, it enhances placental CRH production. This phenomenon leads to a progressive increase of both hormones over the period of gestation (Lowry, 1993).

Pregnancy related immune function is adjusted to the extent that the foetus is optimally ‘tolerated’ from an immune perspective inside the mother’s body. To facilitate this, the maternal immune response is lowered, not however to the extent that it increases maternal-foetal susceptibility to infection. The reduction in maternal immune responsiveness during pregnancy is mediated by hormonal alterations, i.e., immunomodulatory molecules expressed by trophoblasts, and results in a progressive switch from a T-helper (Th)1/Th2 balance to a mainly Th2-type pattern of cytokines.
Th lymphocytes subclasses Th1 and Th2 are components of adaptive immunity. Th1 cells primarily secrete IFN-γ, IL-2, and TNFα, which promote cellular immunity, whereas Th2 cells secrete a different set of cytokines, primarily IL-4, IL-10, and IL-13, which promote humoral immunity (Fearon & Locksley, 1996; Mosmann & Sad, 1996; Trinchieri, 2003).

While stress exposure in non-pregnant humans and animals activates HPA and promotes inflammatory responses (Chrousos & Gold, 1992; Elenkov & Chrousos, 1999), pregnancy-related alterations in endocrine and immune systems have been designed to attenuate the systems’ responses to stress. Despite the maternal endocrine alterations induced by pregnancy, the system is responsive to psychosocial stresses (Wadhwa, Dunkel-Schetter, Chicz-DeMet, Porto, & Sandman, 1996). Previous research however has shown that maternal stress responses are progressively attenuated with advancing gestation (Entringer, Buss, Shirtcliff, et al., 2010).

It has been also reported that increased psychosocial stress during pregnancy is associated with elevated concentrations of circulating inflammatory indicators such as C-reactive protein (CRP) and the cytokines IL-1β, IL-6 and TNF-α, with decreased levels of IL-10 and ex-vivo lipopolysaccharide (LPS)-stimulated concentrations of IL-1β and IL-6 (Christian, Franco, Glaser, & Iams, 2009; Coussons-Read, Okun, Schmitt, & Giese, 2005).

In conclusion, it appears that maternal–foetal endocrine and immune systems produce physiological responses to a wide variety of intrauterine disturbances including prenatal stress. It has been recently shown by an increase in the prevalence of several childhood chronic illnesses such as obesity, asthma, and ADHD (Van Cleave, Gortmaker, & Perrin, 2010). From this study, it is speculated that there are common early risks
underlying these conditions that are triggering development of aberrant physiologic pathways, and that adverse early life events impacting stress-sensitive endocrine and immune systems may participate in the onset of illness in children and also predispose them to develop diseases in later life (Halfon & Newacheck, 2010).

1.3.2 Postnatal Stress

There is also a growing body of evidence that early postnatal adverse events such as infection, food restriction and stress exposure have long-lasting impacts on the health. Animal models have been developed for postnatal paradigms such as the impact of maternal care (D. Liu, et al., 1997; Plotsky, et al., 2005) or handling and touch stimulation in the early postnatal period (Meaney, et al., 1991), neonatal infections (Bilbo, Barrientos, et al., 2008; Bilbo, Biedenkapp, et al., 2005; Bilbo, Levkoff, et al., 2005; Bilbo, et al., 2007; Bilbo, Yirmiya, et al., 2008; Breivik, et al., 2002b; Sominsky, Walker, & Hodgson, 2011; Sominsky, Walker, Ong, et al., 2011; Sominsky, et al., 2012; Walker, Hawkins, Sominsky, & Hodgson, 2012; Walker, Hiles, Sominsky, McLaughlin, & Hodgson, 2011; Walker, et al., 2009b; Walker, Nakamura, & Hodgson, 2010), neonatal under-nutrition (Engelbregt, Houdijk, Popp-Snijders, & Delemarre-van de Waal, 2000; Genovese, Nunez, Pombo, & Bielli, 2010; Gilbert, MacPhail, Baldwin, Moser, & Chernoff, 2010; Sloboda, Howie, Pleasants, Gluckman, & Vickers, 2009), and neonatal stress (S. Ishikawa, et al., 2012; Plotsky, et al., 2005; Salzberg, et al., 2007). As the focus of the current thesis is using neonatal maternal separation model in rats, it is discussed below in more detail.

Rodents such as rats and mice give birth to neuro-anatomically immature offspring who develop rapidly in the first two weeks of their postnatal life (Kapoor, Petropoulos, &
Matthews, 2008) (See Figure 1.2). The HPA axis is particularly plastic during this period of development and prone to programming stimuli. While research has shown that CRH-containing neurons exist in the foetal rat (Insel, Battaglia, Fairbanks, & De Souza, 1988), during the postnatal period the HPA-axis is relatively hyporesponsive to stressful stimuli such as noxious stimuli (Levine, 1994). This period has been called the stress hyporesponsive period (SHRP). In this period, the concentration of baseline plasma glucocorticoids is lower than normal levels and is only slightly elevated when pups are exposed to a stressor at this time (Levine, 1994). It seems that the SHRP has evolved to protect the rapidly developing brain of rodent pups from the adverse effects of elevated glucocorticoids. The SHRP appears to be primarily maintained by the presence of the dam which attenuates HPA-axis activity. Neonatal maternal separation (NS) (i.e., separation of pups from the dam for a period of 1-2 hrs per day during the neonatal period) in rodents is, as such, a strong inducer of stress responses, even during the SHRP. Previous research has shown that neonatal maternal separation activates the offspring’s HPA-axis, as indicated by elevated plasma ACTH and glucocorticoids levels (Levine & Wiener, 1988). Animals exposed to maternal separation also exhibited reduced CRH binding sites in pituitary (Anisman, Zaharia, Meaney, & Merali, 1998). Furthermore, animals that had experienced low levels of maternal care displayed a reduction in hippocampal GR levels (D. Liu, et al., 1997) and altered dopamine, serotonin and gamma-aminobutyric acid (GABA) neurochemical pathways in the brain (F. A. Champagne, 2009).

In addition to the HPA axis, maternal separation has been shown to impact on other regions of the brain specifically by increasing the density of CRH binding sites (Anisman, et al., 1998). CRH has been associated with stress-related hippocampal branches and spines loss, which may be important cellular features of stress-related
psychiatric disorders (Fenoglio, Brunson, & Baram, 2006). Furthermore, increased CRH levels in the amygdala and hypothalamus are respectively linked to increased anxiety and HPA axis activity (Schulkin, Gold, & McEwen, 1998). As such the increased CRH-binding sites provoked by maternal separation during the neonatal period have adverse effects over time. The long-term effects of neonatal maternal separation however are age and stress duration dependant. Earlier and longer stress sessions during the postnatal period are associated with greater effects (de Kloet & Oitzl, 2003). While the rodent research provides valuable information about the influence of early-life stress, the brain of the newborn rodent is largely undeveloped compared with that of the human brain. Therefore, translation of the findings from rodent studies to humans is challenging. Research on nonhuman primates however, has provided a bridge in the translation of the findings from rodent studies to the human. Research involving monkeys has demonstrated that neonatal maternal separation (Sanchez, et al., 2005), disrupted maternal feedings (Coplan, et al., 1996) or spontaneous abusive behaviour of mums (Sanchez, 2006) elevates CRH levels in the cerebrospinal fluid and induces short and long-term changes in the HPA-axis activity after the adversity. The latter however, seems to reverse over time after cessation of psychosocial stress (Sanchez, 2006). While diurnal alterations have not been found in rodents, the impacts on higher brain regions of non-human primates appear to be similar to that of rodents (Rosenblum, et al., 2002; Sanchez, Ladd, & Plotsky, 2001; Sanchez, et al., 2005; Siegel, et al., 1993).
In humans, the maturation of the neuroendocrine system occurs in utero and the HPA-axis is highly responsive at birth (See Figure 1.2). There is a growing body of evidence that there may be a similar SHRP in humans that appears in infancy and could extend throughout most of childhood (M. R. Gunnar & Cheatham, 2003). This is in contrast to the finding that the HPA-axis is hyper-responsive to stressors in the newborn infant as indicated by significantly elevated cortisol levels. The HPA axis becomes less responsive to stressors over the first year and this hyporesponsivity of the HPA-axis might reflect the fact that the stress system is moderated by strong social regulation or parental buffering (M. R. Gunnar & Cheatham, 2003). Consistent with this proposal, stressors that involve a lack of parental care or social contact induce a potent stress response in children. While the exact period of the human SHRP has not been as yet
determined, research has shown that HPA-axis becomes more responsive to stressors (e.g., psychosocial stress) in adolescents (M. R. Gunnar & Cheatham, 2003), suggesting that the SHRP is likely to extend throughout childhood.

Human studies have shown that children who attend day care centres exhibited increased glucocorticoid levels over the day with more so in toddlers than in older children (Geoffroy, Cote, Parent, & Seguin, 2006; M. R. Gunnar & Donzella, 2002). This increase however is less pronounced than that of separated rodents and monkeys. It has been reported that the quality of care also impacts the glucocorticoid levels (M. R. Gunnar & Donzella, 2002). While the elevated glucocorticoid levels due to being in day care has not been yet reported to affect development, children with prolonged poor care in early development have displayed behaviour problems later in development (NICHD, 2002). There are other important effectors of child’s HPA axis activity including parent–child interactions and the maternal psychological state. Sensitive parenting during the hyporesponsiveness of the HPA-axis during the first year of life is associated with either smaller increases in or less prolonged activations of the HPA axis to everyday perturbations (Albers, Riksen-Walraven, Sweep, & de Weerth, 2008). Maternal depression during the child’s early years has been associated with an increase in the risk of hyperactivity of the HPA axis (Lupien, King, Meaney, & McEwen, 2000).

While low parental care results in elevated glucocorticoid levels, exposure of children to severe deprivation (e.g., in orphanages), neglect or abuse has been associated with lower basal glucocorticoids levels (M. R. Gunnar & Donzella, 2002). The mechanism underlying this reduction in glucocorticoids levels could be the down-regulation of the pituitary response to continual hypothalamic CRH secretion (Fries, Hesse, Hellhammer, & Hellhammer, 2005), or hypersensitivity of target tissue to glucocorticoids (Yehuda, Yang, Buchsbaum, & Golier, 2006). This situation however could be prevented by
sensitive and supportive care (M. R. Gunnar & Quevedo, 2007). Research on the post-mortem brains of suicide victims has reported that early life adverse events are associated with epigenetic GR regulation (McGowan, et al., 2009).

The effect of early life stress (pre- and postnatal stress) appears to be species-dependant, because early life manipulations may influence different development stages of different species. It is concluded that exposure to stress during the critical period of HPA-axis development results in long-term alterations to the stress system response and may predispose individuals to diseases in later life. The critical period of HPA-axis development occurs during late gestation (last trimester) in the human which analogues the early postnatal period (first two weeks) for rodents.

1.4 Irritable Bowel Syndrome (IBS)

Early life stress alters the development of the endocrine and immune systems. Specifically, exposure to early life stress results in alterations to the hypothalamic–pituitary–adrenal (HPA) axis resulting in offspring who hyper-respond to stress in adulthood. Recently, this concept has been applied to the ontogeny of functional gastrointestinal (GI) disturbances such as irritable bowel syndrome (IBS). This is important given that stress has been reported to be an important causative or precipitative factor in IBS. IBS involves a dysfunctional interaction between the brain and the gut. All essential aspects of the brain–gut axis including the HPA-axis, spinal pathways, the immune system and the balance of enteric microbiota are altered by exposure to early life stress (S. M. O’Mahony, Hyland, Dinan, & Cryan, 2011). IBS is a growing health problem that affects between 3-15% of the population in developed countries (Andresen & Camilleri, 2006). IBS patients display a broad spectrum of severity of symptoms ranging from mild to severe and intractable ones. Principally IBS
is characterised by chronic relapsing or recurrent abdominal pain and discomfort associated with altered bowel habits (diarrhea or constipation) and other gut dysfunction. As a functional gastro-intestinal disorder (FGID), IBS is a disorder for which no detectable structural/metabolic abnormalities can be found to adequately explain its symptoms (Wood, 2012). IBS symptoms may be exacerbated by psychological stress and negative life experiences (Blanchard, et al., 2008; L. Chang, 2011). In the absence of biological markers, symptom-based criteria are applied to define IBS. Rome criteria (Drossman, 1999) are the most extensively used and adopted criteria for IBS. According to predominant bowel habits, IBS is categorised as constipation predominant (IBS-C), diarrhea predominant (IBS-D) and IBS with alternating constipation and diarrhea (IBS-A). The aetiology and physiology of the disorder are not fully understood, but is agreed to be most likely multifactorial. A recent focus of research has been based on the current proposal that IBS involves a dysregulated interaction between the brain and the gut (S. M. O'Mahony, et al., 2011).

The brain–gut axis involves communication between the enteric, autonomic and/or central nervous systems (ENS, ANS and CNS respectively). The brain–gut axis might be impacted by a wide range of events such as genetic and environmental inputs, psychological stress, dysfunctional central processing, autonomic and hormonal events. It seems that peripheral alterations dominate in some IBS patients, while abnormal central processing of signals received from the periphery is seen in others.
1.5 The Brain–Gut Axis

Communication between the central nervous system (CNS) and the gastrointestinal tract (GIT) is a complicated reflex network that is highly relevant to health and disease (Bonaz & Sabate, 2009). The so-called ‘brain–gut axis’ is composed of a variety of specific receptors such as CRH and glucocorticoids receptors, afferent fibres projecting to integrative central areas and efferent fibres projecting to smooth muscle and glands (Gaman & Kuo, 2008). Even though the functioning of the enteric nervous system (ENS) is independent of the CNS (Gershon, Kirchgessner, & Wade, 1994), there is strong bilateral communication between both nervous systems which allows messages from the GIT (e.g., visceral sensation) to the brain to regulate physiological adaptive responses and mood states (Rhee, Pothoulakis, & Mayer, 2009). These inputs in turn impact on GIT functioning, resulting in, for example, alterations in gut motility, secretion states and immune responsiveness (Mayer, Naliboff, & Craig, 2006). As a general consensus, it is known that behavioural and cognitive processes impact brain–gut axis functioning and are implicated in functional gastro-intestinal disorders (FGID) such as IBS (Mayer, et al., 2001). As described in Figure 1.3, brain signals influence GIT function and visceral messages affect brain function. The brain modulates various functions of the gut, as well as the perception of gut stimuli, via a set of parallel outflow systems that are referred to as the emotional motor system (EMS), which include the sympathetic and parasympathetic branches of the autonomic nervous system (ANS), the HPA axis, and endogenous pain-modulation systems (Mayer, 2000). Activation of the EMS can occur via interoceptive and exteroceptive stressors. The enteric microbiota are likely to interact with gut-based effector systems and with visceral afferent pathways, which establish a bidirectional brain–gut–enteric microbiota axis (Rhee, et al., 2009).
Figure 1. 3 Schematic representation of the pattern of bidirectional brain–gut axis (Rhee, et al., 2009). Abbreviations: ANS, autonomic nervous system; CNS, central nervous system; EMS, emotional motor system; GI, gastrointestinal; HPA, hypothalamus–pituitary–adrenal

1.5.1 Neuronal Control of the Brain–Gut Axis

Cortical areas receive afferent GIT messages which are analysed and efferent responses are generated as neural signals being sent through ANS to the GIT (Mulak & Bonaz, 2004; Tougas, 2000). Parasympathetic innervation originates in nuclei in the brainstem and within the spinal cord at the S2-S4 segments. Parasympathetic innervation of the gut is transmitted via the vagus and the pelvic nerves in order to control modulation of gut motility and sensitivity (Drake, et al., 2010), whilst the origin of neurons for afferent pathways responsible for receiving gut information are contained in the nodose ganglion within the brainstem forming a large division of the vagus nerve (Delvaux, 2004; Gaman & Kuo, 2008; Kellow, et al., 2006). Sympathetic efferent fibres which modulate gut function begin in the lateral horn of the thoraco-lumbar spinal cord (T1–L3), while
afferent sympathetic neurons originate in the dorsal root of the ganglia of the thoracic spinal cord (T1–T10 segments). Gut signals are then transferred from the spinal levels by spinothalamic and spinoreticular tracts to sub-cortical structures (Saper, 2002). The spinothalamic tracts and dorsal columns are known as the main pain signalling pathways in the brain–gut axis with descending supraspinal afferents originating from the rostral ventral medulla facilitating the nociceptive signals (Gaman & Kuo, 2008). The limbic system including the hypothalamus, the hippocampus, the amygdala, and several other nearby areas regulates response to emotion impacting the brain–gut axis. The limbic system acts by affecting the endocrine system and the ANS, both intimately involved in bidirectional brain–gut communication (Mulak & Bonaz, 2004; Roze, 1980; Zaiachkivs'ka, Hzhehots'kyi, & Kovalyshyn, 2005).

1.5.2 The Role of HPA Axis in the Brain–Gut axis

The components of the hypothalamic–pituitary adrenal (HPA) axis were previously described (section 1.2.2). Various hormones and neuropeptides are involved in the brain–gut communication (Stanley, Wynne, McGowan, & Bloom, 2005). Among these hormones and neuropeptides, CRH is the stress-related neuropeptide most associated with functioning of the brain–gut axis. Physiological effects of CRH are exerted through activation of the G-protein coupled CRH receptors 1 and 2 (CRH-R1 and CRH-R2) (Chen, Lewis, Perrin, & Vale, 1993; Kostich, Chen, Sperle, & Largent, 1998). CRH receptors have been found within the CNS as well as in the GIT. There is a growing body of evidence suggesting that central CRH as well as CRH released in the GIT plays an important role in the regulation of the brain–gut axis (Tache & Perdue, 2004).

Currently, there are only a limited number of studies examining the involvement of CRH receptors in human intestinal mucosal function. An in vitro study on human
colonic biopsies has shown that CRH regulates macromolecular permeability via CRH-R1 and CRH-R2 of sub-epithelial mast cells (Wallon, et al., 2008). Animal studies have reported the presence of CRH-R1 in the enteric nervous system (ENS) and the colonic mucosa (Chatzaki, et al., 2004; O’Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010; Yuan, Million, Wu, Rivier, & Tache, 2007) where it likely mediates stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O’Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). CRH-R2 has been also found in the colonic mucosa (J. Chang, et al., 2007; Chatzaki, et al., 2004; O’Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010) and in the ENS (Lakshmanan, et al., 2008; Porcher, Juhem, Peinnequin, Sinniger, & Bonaz, 2005). CRH-R2 has been associated with prevention of gastric emptying, suppression of stimulated colonic motor function and protection against the hypersensitivity to colorectal distension (CRD) (Martinez, Wang, Rivier, Vale, & Tache, 2002; Million, et al., 2005; Million, et al., 2006). CRH-R2 has been also suggested to play a role in stress-induced permeability dysfunction and the modulation of colonic mucosal immune and inflammatory responses (Alonso, et al., 2008; Barreau, Ferrier, Fioramonti, & Bueno, 2004; Teitelbaum, Gareau, Jury, Yang, & Perdue, 2008). Its diverse and varied roles confirm that CRH is indeed a key mediator of stress-related changes in GI function.

1.5.3 Immunological Involvement in the Brain–Gut axis

The immune system (systemic and mucosal) participates in the communication between the brain and the gut. It seems that the systemic immune system acts as a communication route between the brain and the gut under conditions such as increased intestinal permeability causing penetration of luminal antigens to GIT mucosa. This even promotes an immune reaction which can influence the brain function through a
humoral pathway (Haulica, Bild, Boisteanu, Ionita, & Mihaila, 2002). The GIT involves the largest component of the body’s immune system. There is a complicated interrelationship between gut-associated immune system, CNS and ENS (Mayer & Collins, 2002). The directly innervated mucosal mast cells have been found to be important regulators of the gut immune function (Farhadi, Fields, & Keshavarzian, 2007). The mast cells mediate an important brain–gut interaction via neuroimmune mechanisms. Immune signalling, for example GIT inflammatory messages, can be transmitted bidirectionally: through a neural (mainly vagal) or humoral route (Haulica, et al., 2002). Psychological stress influences the gut (Blanchard, et al., 2008; Mach, 2004; Park, Jarrett, Cain, & Heitkemper, 2008) probably via the systemic immune system and is able to alter GIT function (Dinan, et al., 2006; S. M. O'Mahony, et al., 2009).

Both the systemic and intestinal mucosal immune systems are involved in brain–gut axis communication and work in a coordinated fashion to maintain gut integrity. Gut permeability has been implicated as a factor in IBS when permeability is enhanced and luminal antigens leak out of the gut (S. M. O'Mahony, et al., 2011). When this occurs, gut permeability initiates an immune reaction, including mucosal (secretory immunoglobulins), cell mediated (macrophages and T cells), and humoral (serum immunoglobulins) immunity (Berg, 1995). Alterations in circulating cytokine levels in IBS patients have been previously reported. Elevated levels of IL-6, IL-8 and IL-6 soluble receptor (sIL-6R) but not TNF-α and IL-10 have been reported in IBS patients compared to controls (Dinan, et al., 2006). In addition to increased plasma levels of IL-6 and IL-8, a recent study by Scully et al. (2010) has demonstrated increased levels of IL-1β and TNF-α in IBS females with extra-intestinal co-morbidities such as
fibromyalgia, premenstrual dysmorphic disorder, and chronic fatigue syndrome. Liebregts et al. (2007) also found significantly higher TNF-α, IL-1β and IL-6 production by peripheral blood mononuclear cells (PBMC) in IBS patients when compared to those of healthy controls. An abnormal IL-10/IL-12 ratio, indicative of a proinflammatory, Th1 state, was also noted in patients with IBS (L. O'Mahony, et al., 2005). At the molecular level, analysis of genotype frequencies of IL-10 using DNA extracted from peripheral blood leucocytes demonstrated significantly lower frequencies of the high producer genotype for IL-10 in IBS patients than controls (Gonsalkorale, Perrey, Pravica, Whorwell, & Hutchinson, 2003). Prevalence of a high producer TNF-α and a low producer IL-10 genotype was also greater in IBS patients relative to controls (van der Veek, van den Berg, de Kroon, Verspaget, & Masclee, 2005).

Apart from alterations in systemic immunity, changes in local immunity particularly in the colon have also been reported in IBS patients. An elevated frequency of peripheral blood CD4+ and CD8+ T cells expressing the gut homing receptor integrin β7 has been reported in IBS patients compared to controls (Ohman, Isaksson, Lundgren, Simren, & Sjovall, 2005). In the same study an increased number of lamina propria CD8+ T cells and a greater expression of mucosal addressing cell adhesion molecule–1+ endothelium, the ligand for integrin β7, were observed in the ascending colon of IBS patients compared with those of control subjects (Ohman, et al., 2005). An increased number of colonic mast cells and proportion of degranulating mast cells, and a higher number of mast cells in close vicinity to nerve endings have been also reported in IBS patients (Barbara, et al., 2004). An increased colonic mucosal myeloperoxidase (MPO) activity, an inflammatory marker, was observed in IBS patients as compared to healthy individuals (Kristjansson, Venge, Wanders, Loof, & Hallgren, 2004).
Studies using animal models of IBS have also shown alterations in circulating cytokine concentrations. A significantly elevated level of TNF-α and IFN-γ was observed in LPS stimulated whole blood samples from rats exposed to neonatal maternal separation (a model of IBS) who were subjected to subsequent acute stress in adulthood compared with the control group (S. M. O'Mahony, et al., 2009). A study by Desbonnet et al. (2010) also demonstrated elevated IL-6 concentrations in the whole blood culture of adult rats exposed to neonatal maternal separation (NS, as a model of IBS, extensively discussed in page 52) following stimulation with concanavalin A but not LPS relative to control animals.

Gut associated immune alterations have been also reported in maternally separated rats. Neonatal maternal separation was associated with significantly elevated activity of colonic MPO (Barreau, Cartier, Ferrier, Fioramonti, & Bueno, 2004; Barreau, Ferrier, et al., 2004). Significant increases in mRNA expression of colonic cytokines IFN-γ, IL-1β, IL-2, IL-4, and IL-10 were also observed in maternally separated adult rats compared with controls (Barreau, Ferrier, et al., 2004).

1.5.4 The Role of Enterochromaffin Cells in the Brain–Gut axis

These cells are important bidirectional communication regulators between the gut and the nervous system (Rhee, et al., 2009). Enterochromaffin cells are the main sensor/effectector cells of the neuroendocrine system diffused in the gut and they regulate a range of important gut functions including secretion, absorption and motility. These cells monitor the gut luminal milieu by microvilli and, once activated, they become involved in the regulation of gut function by serotonin (5-HT) secretion to adjacent mucosa cells and nerve endings in the intestinal submucosa (Crowell, Shetzline, Moses,
Mawe, & Talley, 2004). Afferent innervation of enterochromaffin cells supplied through the vagus provides a direct signalling pathway to neuronal circuits (Lutgendorff, Akkermans, & Soderholm, 2008). This system may play an important role in pain processing (Lutgendorff, et al., 2008; Verdu, et al., 2006). Furthermore, proximity between 5-HT secreting enteroendocrine cells and lymphocytes in the gut mucosa of rhesus monkeys has suggested a role for enterochromaffin cell 5-HT in mucosal immunity (Yang & Lackner, 2004).

1.5.5 Gut Microbiota

There is a growing body of evidence that the gut microbiota plays an important role in the body homeostasis in general and brain–gut axis function. Communication between gut microbiota and the host occurs through multiple routes, including intestinal epithelial cell receptors and, in the case of compromised intestinal permeability, through direct stimulation of host cells in the lamina propria (S. M. Collins & Bercik, 2009; Rhee, et al., 2009; Verdu, et al., 2006). Research on germ-free (GF) mice has provided invaluable information on the effect of gut microbiota on the brain–gut axis. The appropriate mucosal immune system development is largely dependent on a well-balanced neonatal GIT colonisation (Langhendries, 2006). A key study by Sudo et al. (2004) demonstrated the significance of gut microflora in the programming of HPA axis for appropriate responses to stress in adulthood (Sudo, et al., 2004). Exaggerated HPA-axis stress responses to stress were observed in GF mice. Whilst the HPA-axis response to restraint stress was facilitated by intervention with Escherichia coli, this situation was reversed by administration with Bifidobacterium infantis (Sudo, et al., 2004). It has also been shown that bacterial infections can enhance anxiety in mice (Goehler, Lyte, & Gaykema, 2007), while probiotic administration may moderate visceral hypersensitivity.
in mice (Verdu, et al., 2006). These studies and some others indicate the significance of the gut microbiota in health and disease.

There is a growing body of evidence that the intestinal microbiota of IBS patients differs considerably from that of healthy subjects. Analysis of the intestinal microbiota of IBS patients has demonstrated increased relative abundance of lactobacilli, *Bacillus cereus* and *Bacillus clausii*, bifidobacteria, *Clostridium* cluster IX and *Eubacterium rectale*, and decreased abundance of *Bacteroides/Prevotella* group and *Veillonella* genus, and increased pathobionts (gut-resident bacteria which have the potential to cause disease under certain environmental conditions) compared to that of healthy individuals (Maccaferri, et al., 2012). Meta-analysis of gut microbiota in IBS patients has also identified fluctuations in Firmicutes-associated taxa (Salonen, de Vos, & Palva, 2010). Firmicutes along with Bacteroidetes dominate the human gut microbiota. The *Firmicutes* is the largest bacterial phylum containing more than 250 genera. Some of the genera in the *Firmicutes* phyla are *Lactobacillus*, *Mycoplasma*, *Bacillus*, and *Clostridium* (Das, 2011). Other studies have demonstrated alterations in the proportions of Bacteroidetes and Proteobacteria in IBS patients (Kassinen, et al., 2007; Krogius-Kurikka, et al., 2009; Lyra, et al., 2009; Malinen, et al., 2005; Tana, et al., 2010). A recent study has demonstrated that out of 37 IBS patients, 60% of them exhibited an increase of Firmicutes-associated taxa and a depletion of Bacteroidetes-related taxa, while the remaining patients displayed normal-like microbiota composition compared with healthy controls (Jeffery, et al., 2012). A study by Rajilic-Stojanovic et al. (2011) has demonstrated a 2-fold increase in the Firmicutes to Bacteroidetes ratio in IBS patients compared to healthy subjects. This study also showed significantly increased numbers of *Dorea*, *Ruminococcus*, and *Clostridium* spp, but decreased Bacteroidetes, *Bifidobacterium* and *Faecalibacterium* spp in IBS patients (Rajilic-Stojanovic, et al.,
What these studies represent is a disruption to the normal healthy balance of gut bacteria in IBS patients.

1.6 IBS – Treatment versus Prevention

At present, there is no universal satisfactory treatment for IBS. Standard or traditional IBS therapy mainly involves symptom-oriented approaches, for example anti-diarrheal agents for diarrhea, laxatives or soluble fibres for constipation and anti-spasmodic agents or smooth muscle relaxants for pain. Recent and emerging medication strategies have mainly focused on agents which enhance (agonist) or block (antagonist) some specific neuronal receptors. For instance, the 5-hydroxytryptamine3 (5-HT3) receptor antagonists are used for ‘diarrhea predominant IBS’ (IBS-D) and 5-HT4 agonists for ‘constipation predominant IBS’ (IBS-C) (See Table 1.1). There are other speculative novel therapeutic approaches which have been reviewed elsewhere (Andresen & Camilleri, 2006). These include agents acting on the serotonin receptor or serotonin transporter system, novel selective anticholinergics, α-adrenergic agonists, opioid agents, cholecystokinin antagonists, neurokinin antagonists, somatostatin receptor agonists, neurotrophin-3, CRH antagonists, chloride channel activators, guanylate cyclase-c agonists, melatonin and atypical benzodiazepines, probiotics and antibacterials (Andresen & Camilleri, 2006). Current therapies however are ineffective in treating IBS (Andresen & Camilleri, 2006). The high prevalence of this disorder and the ineffectiveness of treatments results in high direct costs in terms of health care utilisation (outpatient medical care, hospitalisation in some cases and medical prescription costs). Moreover, it has high indirect costs or employer costs including missed days from work and loss of productivity while at work (Cash, et al., 2005). As such, this disorder imparts a significant economic, psychological and physiological
burden on the sufferer. Clearly developing preventive modalities and strategies in the management of IBS will have advantages over the current treatment procedures.

**Table 1.1** Drugs in use or in clinical development for IBS

<table>
<thead>
<tr>
<th>Target system</th>
<th>Receptor activity</th>
<th>Compounds</th>
<th>Human physiological effects</th>
<th>Potential or approved indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonergic receptor system</td>
<td>5-HT4-agonists</td>
<td>Prucalopride, AT1-7505 TD-5108</td>
<td>Prokinetic, secretagogue</td>
<td>IBS-C FC</td>
</tr>
<tr>
<td></td>
<td>5-HT3-antagonist</td>
<td>Alosetron, cilansetron</td>
<td>Decrease motility and secretion, increase compliance decrease pain</td>
<td>IBS-D FD</td>
</tr>
<tr>
<td></td>
<td>5-HT3-agonist</td>
<td>DDP-733</td>
<td>Accelerates small bowel transit</td>
<td>IBS-C FC</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>SSRI SNRI</td>
<td>e.g., Venlafaxine</td>
<td>Increase compliance, decrease tone, and sensation</td>
<td>IBS FAP</td>
</tr>
<tr>
<td>Cholinergic system</td>
<td>Selective M3-antagonists</td>
<td>Zamifenacin, darifenacin</td>
<td>Reduce colonic motility</td>
<td>IBS-D FD</td>
</tr>
<tr>
<td>α-Adrenergic system</td>
<td>α 2-agonist</td>
<td>Clonidine</td>
<td>Increases compliance, decreases tone, and sensation</td>
<td>IBS-D FAP</td>
</tr>
<tr>
<td>Opioid system</td>
<td>Peripheral μ-opioid antagonists</td>
<td>Alvimopan, methylaltrexone</td>
<td>Prokinetic Increase laxation</td>
<td>IBS, FAP</td>
</tr>
<tr>
<td></td>
<td>κ-Opioid-agonist</td>
<td>Asimadoline</td>
<td>Decrease sensation</td>
<td>IBS-C FAP, OIC</td>
</tr>
<tr>
<td>Corticoids</td>
<td>CRH antagonist</td>
<td>CRH1 antagonist</td>
<td>Reduce stimulation induced motility and sensitivity</td>
<td>IBS-D FAP</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>2,3-benzodiazepine receptor</td>
<td>Dextofisopam</td>
<td>Reduces stool frequency, Increases stool consistency</td>
<td>IBS-D</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Receptor?</td>
<td>Melatonin</td>
<td>Decreases pain</td>
<td>IBS, FAP</td>
</tr>
<tr>
<td>CCK</td>
<td>CCK antagonists</td>
<td>Loxiglumide, dexloxiglumide</td>
<td>Accelerate gastric emptying, Delay prox. colonic transit</td>
<td>IBS</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Somatostatin-receptor agonist</td>
<td>Octreotide</td>
<td>Slows down transit, decreases secretion</td>
<td>IBS-D FD</td>
</tr>
<tr>
<td>Neurokinin (NK)</td>
<td>NK antagonists 1 and 2</td>
<td>Ezlopitant, nepadutant</td>
<td>Reduce visceral sensation (NK1) and motility (NK2)</td>
<td>IBS FAP</td>
</tr>
<tr>
<td></td>
<td>NK antagonist 3</td>
<td>Talnetant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride channel</td>
<td>Activator</td>
<td>Lubiprostone</td>
<td>Accelerates transit, increases secretion</td>
<td>IBS-C FC</td>
</tr>
<tr>
<td>Guanyltye cyclase-c</td>
<td>Agonist</td>
<td>Linacotide</td>
<td>Decreases stool consistency, increases stool frequency</td>
<td>IBS-C FC</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Neomycin, rifaximin</td>
<td>Overall symptom relief</td>
<td></td>
<td>IBS-C FC</td>
</tr>
<tr>
<td>Probiotics</td>
<td>See 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Camilleri and Andresen (2009)
1.7 Probiotics

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). Potential probiotic strains mainly belong to genera *Lactobacillus* and *Bifidobacterium* (Gibson, Rastall, & Fuller, 2003). However, to a lesser extent strains from genera *Enterococcus*, *Bacillus*, *Streptococcus* and *Propionibacterium* and species *Escherichia coli* have been reported as probiotics. In addition, a strain of yeast *Saccharomyces cerevisiae* marketed invalidly as “*Saccharomyces boulardii*” has been considered as a probiotic (M. E. Sanders, Gibson, Gill, & Guarner, 2007) (Table 1.2).

Many criteria have been considered by several researchers as desirable properties for potential probiotic strains (Salminen, et al., 1998). Probiotics must fulfil a number of safety, functional and technological properties and characteristics to be used in probiotic food products (Table 1.3).
<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactobacillus</strong></td>
<td>acidophilus</td>
</tr>
<tr>
<td></td>
<td>brevis</td>
</tr>
<tr>
<td></td>
<td>delbrueckiiia</td>
</tr>
<tr>
<td></td>
<td>fermentum</td>
</tr>
<tr>
<td></td>
<td>gasseri</td>
</tr>
<tr>
<td></td>
<td>johnsonii</td>
</tr>
<tr>
<td></td>
<td>paracasei</td>
</tr>
<tr>
<td></td>
<td>plantarum</td>
</tr>
<tr>
<td></td>
<td>reuteri</td>
</tr>
<tr>
<td></td>
<td>rhamnosus</td>
</tr>
<tr>
<td></td>
<td>salivarius</td>
</tr>
<tr>
<td><strong>Bifidobacterium</strong></td>
<td>adolescentis</td>
</tr>
<tr>
<td></td>
<td>animalis</td>
</tr>
<tr>
<td></td>
<td>bifidum</td>
</tr>
<tr>
<td></td>
<td>breve</td>
</tr>
<tr>
<td></td>
<td>infantis</td>
</tr>
<tr>
<td></td>
<td>longum</td>
</tr>
<tr>
<td><strong>Propionibacterium</strong></td>
<td>freudenreichii</td>
</tr>
<tr>
<td></td>
<td>acidipropionici</td>
</tr>
<tr>
<td></td>
<td>jensenii</td>
</tr>
<tr>
<td><strong>Streptococcus</strong></td>
<td>thermophilus</td>
</tr>
<tr>
<td></td>
<td>salivarius</td>
</tr>
<tr>
<td><strong>Enterococcus</strong></td>
<td>faecium</td>
</tr>
<tr>
<td><strong>Escherichia</strong></td>
<td>coli</td>
</tr>
<tr>
<td><strong>Bacillus</strong></td>
<td>coagulans</td>
</tr>
<tr>
<td></td>
<td>clausii</td>
</tr>
<tr>
<td><strong>Clostridium</strong></td>
<td>Butyricum</td>
</tr>
<tr>
<td><strong>Saccharomyces</strong></td>
<td>cerevisiae</td>
</tr>
</tbody>
</table>

Table modified from Sanders et al. (2007)

a Probiotic propionibacteria from Cousin et al. (2011)

b *Clostridium butyricum* has been added to the list from Surawicz (2009)
Table 1.3 Selection criteria of probiotic organisms for human use

<table>
<thead>
<tr>
<th>Category</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safety</td>
<td>• Preferably originated from healthy human GI tract</td>
</tr>
<tr>
<td></td>
<td>• Non-pathogenic and not associated with diseases e.g., infective endocarditis or GI disorders</td>
</tr>
<tr>
<td></td>
<td>• Non-inflammatory promoting</td>
</tr>
<tr>
<td></td>
<td>• Not able to deconjugate or dehydroxylate bile salts</td>
</tr>
<tr>
<td></td>
<td>• Not able to carry transmissible antibiotic resistance genes</td>
</tr>
<tr>
<td></td>
<td>• Not having clinical side effects</td>
</tr>
<tr>
<td>Functional</td>
<td>• Resistant to low pH, gastric juice, bile acid and pancreatic juice</td>
</tr>
<tr>
<td></td>
<td>• Adhesion to the intestinal cells and colonisation of the human gut</td>
</tr>
<tr>
<td></td>
<td>• Modulation of immune system</td>
</tr>
<tr>
<td></td>
<td>• Antagonistic against pathogens via competition for adhesion sites and production of antimicrobial metabolites</td>
</tr>
<tr>
<td></td>
<td>• Antimutagenic and antigarcinogenic properties</td>
</tr>
<tr>
<td></td>
<td>• Potential for the delivery of recombinant proteins and peptides to the human GI tract</td>
</tr>
<tr>
<td>Technological</td>
<td>• Reasonable sensory properties</td>
</tr>
<tr>
<td></td>
<td>• Phage resistant</td>
</tr>
<tr>
<td></td>
<td>• Viability during production and storage of the product</td>
</tr>
</tbody>
</table>

1.7.1 Health Benefits of Probiotics

Probiotics have been claimed to be associated with a wide variety of beneficial health effects, however clinical studies have proven to date only a few of these claims. It should be considered that probiotic microorganisms are not alike with regard to their health promoting effects. As a matter of fact, the type and level of health promoting effects of probiotics is strain dependent (M. E. Sanders, 2007). As shown in Table 1.4, the main health benefits of probiotic intake include amelioration of lactose maldigestion, lowering serum cholesterol and triglycerides, improvement of bowel transit, alleviation of IBS symptoms, prevention/treatment of IBD, prevention/treatment of GI and vaginal infections, prevention of systemic infections, modulation of the immune system, prevention of allergic reactions, and protection against colon cancer.
### Table 1.4 Selected recent studies on health benefits of probiotics

<table>
<thead>
<tr>
<th>Effect</th>
<th>Probiotic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amelioration of lactose maldigestion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improvement of symptoms and decrease in hydrogen production intake in lactose-intolerant patients</td>
<td><em>Lactobacillus casei</em> Shirota and <em>Bifidobacterium breve</em> Yakult</td>
<td>(Almeida, Lorena, Pavan, Akasaka, &amp; Mesquita, 2012)</td>
</tr>
<tr>
<td>Reduction in H2 excretion and improvement of the mean clinical Scores in lactose intolerant patients</td>
<td><em>Lb. reuteri</em></td>
<td>(Ojetti, et al., 2010)</td>
</tr>
<tr>
<td>Decrease in symptom scores</td>
<td><em>Bifidobacterium longum or Bif. animalis</em></td>
<td>(He, et al., 2008)</td>
</tr>
<tr>
<td><strong>Lipid modulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease in plasma total cholesterol and LDL-cholesterol concentration</td>
<td><em>Lb. paracasei</em> 37 combined with pentacalcium hydroxytriphosphate</td>
<td>(Trautvetter, Ditscheid, Kiehntopf, &amp; Jahreis, 2012)</td>
</tr>
<tr>
<td>Reduction in LDL-cholesterol, total cholesterol, apolipoprotein B-100 non-HDL-cholesterol in hypercholesterolaeic subjects</td>
<td><em>Lb. reuteri NCIMB 30242</em></td>
<td>(Jones, Martoni, Parent, &amp; Prakash, 2011)</td>
</tr>
<tr>
<td>Decrease in the serum levels of total cholesterol and LDL-cholesterol</td>
<td><em>Lb. paracasei</em> NLB163</td>
<td>(Tanaka-Azuma, et al., 2009)</td>
</tr>
<tr>
<td><strong>Improvement of bowel transit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease in bowel transit in human subjects suffering from longer than desired GI transit time</td>
<td><em>Bif. animalis</em> DN-173010</td>
<td>(Marteau, et al., 2002)</td>
</tr>
<tr>
<td>Improvement of symptoms of constipation in children</td>
<td><em>Lb. rhamnosus</em> Lcr35</td>
<td>(Bu, Chang, Ni, Chen, &amp; Cheng, 2007)</td>
</tr>
<tr>
<td><strong>Alleviation of IBS symptoms (See Table 1.5)</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Prevention/treatment of IBD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease in endoscopic and histological scores in ulcerative colitis (UC) patients</td>
<td>VSL#3</td>
<td>(Miele, et al., 2009)</td>
</tr>
<tr>
<td>Induction of remission in children with mild to moderate acute UC</td>
<td>VSL#3</td>
<td>(Huynh, et al., 2009)</td>
</tr>
<tr>
<td>Improvement in individual scores for systemic and social functions, and decrease in C-reactive protein of outpatients with UC</td>
<td><em>Bif. longum</em> combined with psyllium</td>
<td>(Fujimori, et al., 2009)</td>
</tr>
<tr>
<td><strong>Prevention/treatment of GI infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in the colonisation of <em>Clostridium difficile</em> in critically ill patients treated with antibiotics</td>
<td><em>Lb. plantarum</em> 299v</td>
<td>(Klarin, et al., 2008)</td>
</tr>
<tr>
<td>Decrease in the incidence of antibiotic-associated diarrhea and <em>Cl. difficile</em>-associated diarrhea</td>
<td><em>Lb. casei</em> DN-114 001, <em>Lb. delbrueckii</em> subsp. bulgaricus, and <em>S. thermophilus</em></td>
<td>(Hickson, 2007)</td>
</tr>
<tr>
<td>Suppression of <em>Helicobacter pylori</em> infection in human subjects</td>
<td><em>Lb. reuteri</em> ATCC 55730</td>
<td>(Francavilla, et al., 2008)</td>
</tr>
<tr>
<td>Improvement of the efficacy of triple therapy against <em>H. pylori</em> infection</td>
<td><em>Lb. gasseri</em> OLL2716</td>
<td>(Deguchi, et al., 2011)</td>
</tr>
<tr>
<td><strong>Prevention/treatment of vaginal infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in recurrent urinary tract infection in women</td>
<td><em>Lb. crispatus</em></td>
<td>(Stapleton, et al., 2011)</td>
</tr>
<tr>
<td>Reduction in recurrent bacterial vaginosis and decrease in <em>Gardnerella vaginalis</em> incidence</td>
<td><em>Lb. rhamnosus, Lb. acidophilus, and Streptococcus thermophilus</em></td>
<td>(Ya, Reifer, &amp; Miller, 2010)</td>
</tr>
<tr>
<td>Decrease in the recurrence of bacterial vaginosis</td>
<td><em>Lb. rhamnosus</em></td>
<td>(Marcone, Rocca, Lichtner, &amp; Calzolari, 2010)</td>
</tr>
<tr>
<td>Prevention of Systemic Infections</td>
<td>Bif. breve Yakult and Lb. casei Shirota</td>
<td>(Usami, et al., 2011)</td>
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<tr>
<td>Perioperative probiotic treatment reduced the rate of infectious complications in patients with/without liver cirrhosis who underwent hepatic surgery</td>
<td>Bif. bifidum and Lb. acidophilus</td>
<td>(H. C. Lin, et al., 2008)</td>
</tr>
<tr>
<td>Reduction in the incidence of death or necrotizing enterocolitis (NEC) in very low birth weight preterm infants</td>
<td>Bif. breve and Lb. casei</td>
<td>(Braga, da Silva, de Lira, &amp; de Carvalho Lima, 2011)</td>
</tr>
<tr>
<td>Protection against colon cancer</td>
<td>Lb. casei strain Shirota</td>
<td>(H. Ishikawa, et al., 2005)</td>
</tr>
<tr>
<td>Prevention/moderation of allergic reactions</td>
<td>A combination of Lb. rhamnosus LGG, Lb. rhamnosus LC705, Bif. breve Bb/99 and Propionibacterium freudenreichii ssp. shermanii JS</td>
<td>(Kuitunen, et al., 2009)</td>
</tr>
<tr>
<td>Maternal probiotics intervention prevents IgE-associated allergy in caesarean-delivered children</td>
<td>A combination of Lb. rhamnosus LGG, Lb. rhamnosus LC705, Bif. breve Bb/99 and Propionibacterium freudenreichii ssp. shermanii JS</td>
<td>(Kukkonen, et al., 2007)</td>
</tr>
<tr>
<td>Maternal probiotics intervention prevented eczema and especially atopic eczema in children</td>
<td>Lb. casei strain Shirota</td>
<td>(H. Ishikawa, et al., 2005)</td>
</tr>
</tbody>
</table>
1.7.2 Probiotics and IBS

1.7.2.1 Probiotics for IBS Patients

There are multiple underlying causes of IBS, including the gut resident bacteria (Clavel & Haller, 2007). It is believed that gut bacteria play a role in the pathogenesis of IBS (Othman, Aguero, & Lin, 2008). A consistent finding is that the assemblages of GIT bacteria are disrupted with IBS, and there is evidence that suggests prior enteric infections increase the risk of developing IBS (Cuomo, Savarese, & Gargano, 2007). Fermentations taking place in the colon generate a variable volume of gas. However, some gut bacteria degrade metabolic substrates without producing gas, and even some other species may consume gas, particularly hydrogen. Symptoms of abdominal pain, bloating, and flatulence are commonly seen in patients with IBS. Hypothetically, administration of appropriate bacteria strains in order to restore or improve the GIT bacteria could reduce gas accumulation within the bowel in these patients and induce symptomatic improvement (Guarner, 2009). Table 1.5 shows data studies testing probiotics in patients with IBS. In most studies, both probiotic and placebo treatment decreased the scores of abdominal pain to some extent. This is a common observation in trials with IBS patients, who respond to a placebo at variable rates. However, several studies have shown significant therapeutic gain of probiotics over placebo assessed by increased rate of responders to treatment or increased relief in symptom scores (Guarner, 2009). A consistent finding of the published studies is the reduction of abdominal bloating and flatulence by probiotic treatment (Guarner, 2009). Studies using *Bifidobacterium* strains appear to have a higher rate of therapeutic success in adult IBS patients. Disordered bowel habits (diarrhea, constipation, or alternating habit) are common in subjects with IBS, but efficacy of probiotics for improvement of bowel habit in IBS has not been proven consistently. Probiotics need to be evaluated further,
but they do appear to be useful for the control of the symptoms related with the altered handling or reception of intestinal gas. In order to evaluate the effects of probiotic use in IBS patients, several meta-analysis reviews of the available literature have been conducted. Evaluation of eligible randomised, controlled trials suggests probiotics may be effective for IBS, but additional studies are needed (Ford, Talley, Quigley, & Moayyedi, 2009; McFarland & Dublin, 2008; Nikfar, Rahimi, Rahimi, Derakhshani, & Abdollahi, 2008).
<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Carrier/ Form and Dose (CFU/ml or gr)</th>
<th>Participants</th>
<th>Duration</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb. plantarum MF1298</td>
<td>Capsule containing 10^{10} CFU/capsule</td>
<td>16</td>
<td>3 wk</td>
<td>• Increased weeks with satisfactory relief of symptoms</td>
<td>(Ligaarden, Axelson, Naterstad, Lydersen, &amp; Farup, 2010)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Increased IBS sum score</td>
<td></td>
</tr>
<tr>
<td>A combination of Lb. paracasei ssp. paracasei F19, Lb. acidophilus La5 and Bif. lactis Bb12</td>
<td>Fermented milk-5×10^{7} CFU/mL - 400 ml/day</td>
<td>74</td>
<td>8 wk</td>
<td>• Improved IBS symptom severity only in first 2 weeks of treatment</td>
<td>(Simren, et al., 2009)</td>
</tr>
<tr>
<td>Bif. animalis DN-173 010</td>
<td>Yoghurt-1.25 ×10^{10} CFU/pot</td>
<td>34 female patients with IBS-C</td>
<td>4 wk</td>
<td>• improvements in abdominal girth</td>
<td>(Agrawal, et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• accelerated gastrointestinal transit</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• reduced symptomatology</td>
<td></td>
</tr>
<tr>
<td>Bif. longum LA 101, Lb. acidophilus LA 104, Lb. lactis LA 103 and Strep. thermophilus LA 104</td>
<td>Water-1 × 10^{10} CFU/sachet once daily</td>
<td>100</td>
<td>4 wk</td>
<td>• No difference in relieving symptoms of IBS</td>
<td>(Drouault-Holowacz, et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Decrease in abdominal pain</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>• lower pain score</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>• increase in stool frequency in the constipated sub-group</td>
<td></td>
</tr>
<tr>
<td>A combination of Lb. acidophilus CUL60 and CUL21, Bif. lactis CUL34 and Bif. bifidum CUL20</td>
<td>Capsule- 2.5 × 10^{10} CFU/capsule</td>
<td>52</td>
<td>8 wk</td>
<td>• Improved the symptom severity score of IBS</td>
<td>(E. Williams, et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Improved scores for quality of life, days with pain and satisfaction with bowel habit</td>
<td></td>
</tr>
<tr>
<td>Lb. casei Shirotai (Yakult®)</td>
<td>Fermented milk- 65 mL/day</td>
<td>18</td>
<td>6 wk</td>
<td>• No significant improvement in the symptom score, except for wind</td>
<td>(Barrett, Canale, Gearry, Irving, &amp; Gibson, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Altered fermentation patterns in the small bowel, consistent with reducing small intestinal bacterial overgrowth (SIBO).</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• The loss of early rise in breath hydrogen with lactulose (ERBHAl) was associated with reduced symptoms.</td>
<td></td>
</tr>
<tr>
<td>A combination of Lb. rhamnosus GG, Lb.</td>
<td>Probiotic milk-based drink- 1×10^{7} CFU/</td>
<td>Rome II IBS, 86</td>
<td>5 months</td>
<td>• Decrease in the composite IBS score especially distension</td>
<td>(Kajander, et al., 2008)</td>
</tr>
<tr>
<td>Probiotic</td>
<td>Carrier/ Form and Dose (CFU/ml or gr)</td>
<td>Participants</td>
<td>Duration</td>
<td>Outcomes</td>
<td>Reference</td>
</tr>
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<tr>
<td><em>L. rhamnosus</em> LC705, <em>Propionibacterium freudenreichii</em> ssp. <em>shermanii</em> JS and <em>Bif. animalis</em> ssp. <em>lactis</em> Bb12</td>
<td>mL (each strain)- Once daily 1.2 dL</td>
<td>patients</td>
<td>6 months</td>
<td>Lower incidence of perceived abdominal distention</td>
<td>(Bausserman &amp; Michail, 2005)</td>
</tr>
<tr>
<td><em>Bif. animalis</em> DN-173 010</td>
<td>Yoghurt- 1.25 x10^10 CFU/pot together with yoghurt starters</td>
<td>274 patients</td>
<td>6 wk</td>
<td>Decreased discomfort HRQoL score in C-IBS</td>
<td>(Guyonnet, et al., 2007)</td>
</tr>
<tr>
<td><em>Bif. animalis</em> 35624</td>
<td>Capsule- 1 x 10^8 CFU/cap</td>
<td>362 primary care IBS patients</td>
<td>4 wk</td>
<td>Significant reduction in abdominal pain, the composite score and scores for bloating, bowel dysfunction, incomplete evacuation, straining, and the passage of gas</td>
<td>(Whorwell, et al., 2006)</td>
</tr>
<tr>
<td><em>Lb. reuteri</em> ATCC 55730</td>
<td>Tablet- 1x10^7 CFU/tablet twice a day</td>
<td>54 patients</td>
<td>6 months</td>
<td>No improvement</td>
<td>(Niv, Naftali, Hallak, &amp; Vaisman, 2005)</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> GG</td>
<td>Capsule -10^10 CFU/capsule twice daily</td>
<td>Rome II IBS, 50 children</td>
<td>6 wk</td>
<td>Lower incidence of perceived abdominal distention</td>
<td>(Bausserman &amp; Michail, 2005)</td>
</tr>
<tr>
<td><em>Lb. salivarius</em> UCC4331 or <em>Bif. infantis</em> 35624</td>
<td>Malted milk drink- 1 x 10^10</td>
<td>77 patients</td>
<td>8 wk</td>
<td>Reduction in abdominal pain/discomfort, bloating/distention, and bowel movement difficulty</td>
<td>(L. O'Mahony, et al., 2005)</td>
</tr>
<tr>
<td>A mixture of <em>Lb. rhamnosus</em> GG, <em>Lb. rhamnosus</em> LC705, <em>Bif. breve</em> Bb99 and <em>P. freudenreichii</em> ssp. <em>shermanii</em> JS</td>
<td>Capsule</td>
<td>Rome I or II IBS, 103 patients</td>
<td>6 months</td>
<td>Reduction in the total symptom score (abdominal pain + distension + flatulence + borborygmi)</td>
<td>(Kajander, Hatakka, Poussa, Farkkila, &amp; Korpela, 2005)</td>
</tr>
<tr>
<td>A combination of 29 probiotics and several prebiotics</td>
<td>Capsule- 500mg</td>
<td>Rome II IBS</td>
<td></td>
<td>Significant reductions in subsyndromic factors: general ill feelings-nausea, indigestion/flatulence, and colitis.</td>
<td>(Bittner, Croffut, &amp; Stranahan, 2005)</td>
</tr>
<tr>
<td>VSL#3 *</td>
<td>Yoghurt- 4.5 x 10^11/ sachet twice daily</td>
<td>48 (placebo, n = 24; VSL# 3, n = 24)</td>
<td>4 wk or 8 wk</td>
<td>Significant reduction in flatulence scores and retards colonic transit without altering bowel function in patients</td>
<td>(Kim, et al., 2005)</td>
</tr>
<tr>
<td>Probiotic</td>
<td>Carrier/ Form and Dose (CFU/ml or gr)</td>
<td>Participants</td>
<td>Duration</td>
<td>Outcomes</td>
<td>Reference</td>
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<tr>
<td><em>Lb. plantarum</em> LP0 1 and <em>Bif. breve</em> BR0</td>
<td>Both cultures at a concentration of $5 \times 10^7$ CFU/ml, Rome II IBS, 50 patients (24 males, 26 females), mean age 40 years</td>
<td>4 wk</td>
<td>• Reduction in pain&lt;br&gt;• Decreased the severity score of characteristic IBD symptoms</td>
<td>(Saggioro, 2004)</td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> 299V</td>
<td>fruit drink containing 5% of an oatmeal soup fermented with LP299V- $5 \times 10^7$ CFU/ml, 200 ml twice a day</td>
<td>40</td>
<td>• Resolution of abdominal pain&lt;br&gt;• Normalisation of stools frequency in constipated patients&lt;br&gt;• Improvement in all IBS symptoms</td>
<td>(Niedzielin, et al., 2001)</td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> 299V</td>
<td>lactic acid-fermented oatmeal gruel- $5 \times 10^7$ CFU/ml, 125 ml/day</td>
<td>12</td>
<td>• No alteration in colonic fermentation&lt;br&gt;• No improvement in symptoms</td>
<td>(Sen, et al., 2002)</td>
<td></td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> GG</td>
<td>enterocoated tablet- $10^{10}$ CFU/day</td>
<td>24</td>
<td>• No significant differences in mean symptom scores for pain, urgency or bloating&lt;br&gt;• Reduction in the number of unformed bowel motions for patients with diarrhea.</td>
<td>(M. A. O’Sullivan &amp; O’Morain, 2000)</td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> 299V</td>
<td>rose-hip drink $5 \times 10^7$ cfu/mL, 400 ml/day</td>
<td>60</td>
<td>• Significant reduced flatulence&lt;br&gt;• A better overall GI function at the 12-month follow-up&lt;br&gt;• No change in bloating</td>
<td>(Nobaek, et al., 2000)</td>
<td></td>
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</tbody>
</table>

Yoghurt starter bacteria: *Lb. bulgaricus* and *Strep. thermophilus*

* VSL# 3 is a composite probiotic and each sachet contains 450 billion viable lyophilized bacteria: *Bifidobacterium* (*Bif. longum*, *Bif. infantis* and *Bif. breve*); *Lactobacillus* (*Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii*ssp. *bulgaricus* and *Lb. plantarum*); and *Streptococcus salivarius* ssp. *thermophilus.*
1.7.2.2 Neonatal Probiotic Intervention

A study by Gareau et al. (2007) using a maternally separated rat model of IBS has shown that neonatal probiotic intervention could be a potential prophylaxis against the unfavourable imprinting induced on the brain–gut axis by NS in rats. In this study a probiotic combination containing *Lb. rhamnosus* R0011 (95%) and *Lb. helveticus* R0052 (5%) was administered to Sprague–Dawley rat pups exposed to neonatal maternal separation (3 h/day from PND 4 to 19) during the separation. Animals in the non-stress group received saline. Stressed rats exhibited significantly increased colonic ion transport and macromolecular permeability, increased bacterial adhesion/penetration of total bacteria into the mucosa and reduced *Lactobacillus* species in NS compared with those of non-NS pups. Neonatal probiotic administration was shown to ameliorate the NS-induced gut dysfunctions and bacterial adhesion/penetration one day after cessation of both NS exposure and probiotic administration (PND 20) and in 2 month old rats, and reduced the elevated corticosterone levels at PND 20. The results of this study indicated that neonatal probiotic intervention improved NS-induced leaky gut via normalisation of HPA-axis activity.

1.7.2.3 Maternal Probiotic Intervention

It is not as yet known whether maternal supplementary probiotics may also contribute to prevent or ameliorate IBS related gut-brain axis dysfunction, if possible improvements persist into adulthood, or how this protective effect may be mediated. While no studies have directly investigated such an effect for probiotics, based on indirect evidence we hypothesise that maternal probiotics may prevent or at least attenuate adverse outcomes
of early life stress which may predispose individuals to IBS in later life. The supporting evidence for this hypothesis is as follows:

\[\text{a) Maternal probiotic supplementation impacts positively on gut development and function in the offspring}\]

Findings of most recent animal-model studies have indicated that any manipulation of the maternal gut microbiota has a significant impact on the neonatal GI integrity and functioning. Perinatal maternal exposure to either a combination of the broad-spectrum antibiotics or non-pathogenic \textit{Escherichia coli} was associated with an imbalanced neonatal gut microflora and specific adverse effects on GI growth and functions compared with rat pups from control mothers (Fåk, Ahrné, Molin, Jeppsson, & Weström, 2008). In another study by the same research team a probiotic bacterium, \textit{Lb. plantarum} 299v (Lp299v), was administered through drinking water to pregnant and lactating rat dams until postnatal day 14. The results of this study showed that the probiotic colonised both maternal and neonatal intestines. The weight of the offsprings’ small intestine, liver, spleen, and pancreas was higher in neonatal pups born from probiotic-treated mothers compared to pups from the control group. Maternal probiotic administration also resulted in a significant reduction in intestinal permeability to macromolecules in pups from the maternal probiotic group as compared to the pups from control mothers. Increased intestinal permeability means that the intestinal epithelium is more permeable to pathogens, allergens and other antigens which may induce inflammation and infection. Furthermore, measurement of the plasma level of the acute phase protein haptoglobin, a sensitive indicator of inflammatory responsiveness (Giffen, et al., 2003), indicated that no significant increase in plasma...
Haptoglobin was observed in pups from probiotic treatment mothers compared to the control pups (Fak, Ahrne, Molin, Jeppsson, & Westrom, 2008). Whereas, such an increase has been reported in pups from antibiotic administration or *E. coli* treated mothers (Fåk, et al., 2008). The evidence above clearly indicates that perinatal manipulation of maternal gut microflora impacts on neonatal GI integrity and functioning and that maternal probiotic treatment can have a considerable positive effect in this regard.

**b) Maternal probiotic supplementation affects the development of the HPA-axis stress response through modulation of neonatal gut microbiota**

Numerous studies have demonstrated that early environmental factors acting during developmental plasticity determine alterations in physiological regulation promoting the critical development and survival of the foetus and/or neonate and may predispose individuals to diseases later in life. Previous studies have linked maternal factors such as diet, infection, illness, stress and altered maternal behaviour to altered HPA-axis function. This may be mediated, at least in part, through modulation of neonatal gut microbiota, and, to date, some studies support the role of neonatal microbiota affecting development of the HPA-axis stress response. In line with findings from animal studies, studies in humans have also shown that probiotics administered to pregnant and lactating mothers are able to colonise neonatal gut (Gueimonde, et al., 2006; Schultz, Gottl, Young, Iwen, & Vanderhoof, 2004; Vanderhoof, Iwen, Hinrichs, Bilyeu, & Young, 1999). The foetus are considered sterile and microbial colonisation begins during parturition and rapidly thereafter, maternal microbes and those from the surrounding environment colonise offspring’s GIT. Therefore, maternal consumptions
of probiotics could cause infantile colonisation. A study undertaken by Gueimonde et al. (2006) indicated that maternal probiotic supplementation causes alterations in the offspring’s gut microflora. Maternal probiotic treatment with a probiotic strain *Lactobacillus rhamnosus* GG changed the species-specific percentage and composition patterns of bifidobacterial community in neonatal faeces compared with placebo treatment. Sudo and co-workers (Sudo, 2006; Sudo, et al., 2004) have shown that postnatal microbial colonisation programs the HPA stress response. In this study, exaggerated HPA-axis stress responses were observed in GF mice, while this situation was reversed by a pre-treatment with Bifidobacteria (Sudo, et al., 2004). These works indicate that maternal probiotic supplementation most likely modulates the development of the HPA-axis function through alterations in the microbial environment of the neonatal gut.

c) *Maternal probiotic supplementation affects the development of the neonatal immune response*

Several studies have indicated that prenatal and early postnatal factors that alter neuroendocrine stress responsiveness may also influence some aspects of immune function. Neonatal stress is proposed to alter gut immune function in neonates and in adult animal models of IBS by increasing the number of mast cells and polymorphonuclear neutrophils (PMNs) (Barreau, Cartier, et al., 2004; Barreau, Ferrier, et al., 2004). Furthermore, neonatal stress is associated with raised colonic mRNA expression of several cytokines (e.g., IL-1β, IL-2, IL-4, IL-10, and interferon (IFN)-γ) indicating an increase in both Th-1 and Th-2 cytokines profiles (Barreau, Cartier, et al.,
This may be an indication of systemic inflammation in the gut (Barreau, Ferrier, et al., 2004).

Maternal administration of probiotics has recently emerged as an effective prophylactic approach to the prevention of early immune dysregulation (Blumer, et al., 2007; Rautava, Kalliomaki, & Isolauri, 2002) However, these studies have primarily evaluated the beneficial effect of maternal probiotics on modulation of the immune system aiming to prevent allergic diseases such as eczema and asthma in early life (Blumer, et al., 2007; Rautava, et al., 2002). Some findings in these studies provide peripheral support to our hypothesis.

Blumer et al. (2007) demonstrated that prenatal administration of Lb. rhamnosus GG altered the placental and neonatal cytokine expression patterns in mice derived from perinatally probiotic treated mothers compared with pups from control mothers. Rautava et al. (2002) reported that administration of the same probiotic strain during pregnancy and lactation elevated the level of anti-inflammatory transforming growth factor b2 (TGF-b2) in maternal milk. This factor is a crucial immunoregulatory factor which is involved in promoting secretory immunoglobulin A (sIgA) production in neonatal gut-associated immune system (Ogawa, et al., 2004). Secretory IgA acts to prevent penetration of luminal antigens through the neonatal gut mucosa (Fagarasan & Honjo, 2003).

Therefore, given that maternal probiotic administration produces a positive gastrointestinal immune environment for the offspring, and given that we know that the composition of gut microflora can mediate HPA-axis activity, it could be expected that our proposed hypothesis will elucidate an important, yet currently unknown, link between these two phenomena. That is, maternal probiotic administration is likely to enhance the positive
microflora found in the neonatal gut, which in turn may modulate HPA-axis response to stress. Taking into account these findings, it is concluded that maternal probiotics may protect neonates and adults against intestinal and immune dysfunctions induced by neonatal stress. This means that maternal probiotics may prevent functional GI disorders such as IBS or at least attenuate adverse outcomes of neonatal stress on intestinal barrier function in early and later life. While research has suggested that such a pathway may exist, no studies have directly investigated this relationship. Our hypothesis is an attempt to link this proposed nutritional approach and its possible prophylactic outcomes on GI dysfunctions induced by neonatal stress in early and later life.

1.8 Animal models of IBS

1.8.1 Neonatal Noxious Stimuli Model

The early postnatal period is a time of great plasticity for both somatic and visceral sensory systems. This neuronal plasticity often contributes to adaptive or maladaptive function of the mammalian nervous system, and possibly to the development of chronic pain. The nociceptive neuronal circuits are both formed during embryonic and early postnatal periods when painful stimuli are absent or limited. During this critical period, particularly before the maturation of the descending inhibitory systems (Boucher, Jennings, & Fitzgerald, 1998; Fitzgerald & Koltzenburg, 1986), pain can lead to prolonged structural and functional alterations in nociceptive pathways which can persist into adulthood (Al-Chaer, Kawasaki, & Pasricha, 2000; Fitzgerald & Beggs, 2001; Lidow, Song, & Ren, 2001; Ruda, Ling, Hohmann, Peng, & Tachibana, 2000). The neonatal noxious stimuli model was successfully developed to study the influence of early life noxious stimulation on the gut functioning (Al-Chaer, et al., 2000; C. Lin &
Colonic injury was induced by daily colorectal distension using an angioplasty balloon between PND 8 and 21 in rat pups. Neonatal colorectal distension results in long-term visceral hypersensitivity characterised by allodynia and hyperalgesia in adulthood, along with peripheral and central sensitisation (Al-Chaer, et al., 2000; C. Lin & Al-Chaer, 2003). It has been shown that noxious stimuli such as neonatal ‘gastric suction’ at birth in human newborns could be associated with increased risk of development of functional gastrointestinal disorders (FGID) in adulthood (Anand, Runeson, & Jacobson, 2004). While the neonatal noxious stimuli model provides evidence of the importance of neonatal traumatic experiences, it has been used for investigation of a limited number of cardinal features of IBS including visceral hyperalgesia, somatic pain alterations, depression and/or anxiety and HPA dysfunctions but not alterations to the gut motility, permeability and bacterial translocation (See Table 1.6).

**Table 1.6 Neonatal stress models and irritable bowel syndrome features**

<table>
<thead>
<tr>
<th>Dysfunctions</th>
<th>Neonatal Stress Model</th>
<th>Inflammatory Stimuli</th>
<th>Noxious Stimuli</th>
<th>Neonatal Maternal Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral hyperalgesia</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Motility alterations</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Alterations of gut permeability and bacterial translocation</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Somatic pain alterations</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Depression and/or anxiety</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>HPA dysfunctions</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

Adapted from Barreau et al. (2007)
1.8.2 Neonatal Inflammatory Stimuli

Developmental plasticity in physiological systems is an important mechanism through which organisms can adapt their physiologic responses to better meet environmental demands. Although such alterations may prove some benefit for immediate survival, they may also lead to long-lasting alterations in the physiological responses to environmental challenges, and alter predisposition to pathology later in life (Arborelius, Owens, Plotsky, & Nemeroff, 1999; McEwen & Stellar, 1993; Munck & Naray-Fejes-Toth, 1992). Therefore, researchers have studied the effect of inflammatory stimuli such as exposure to Gram negative bacterial LPS and induction of colonic inflammation during the early postnatal developmental period on adult physiology. Neonatal exposure to LPS derived from Salmonella enteritidis resulted in programming alterations in the development of the HPA-axis and immune system (Shanks, et al., 2000). Neonatal LPS exposure between postnatal days 3 and 5 in rats has been reported to alter the behavioural and clinical course of periodontal disease in adult rats (Breivik, et al., 2002a). Moreover, it has been indicated that single exposure of 14 day old rat pups to LPS resulted in decreased nociceptive thresholds and induced hyper-responsiveness to painful and innocuous stimuli in later life (Boisse, Spencer, Mouihate, Vergnolle, & Pittman, 2005). Neonatal colonic exposure to inflammatory substances such as mustard oil during postnatal weeks two and three resulted in chronic visceral hypersensitivity along with central neural sensitisation in adult rats (Al-Chaer, et al., 2000). Neonatal inflammatory stimuli models highlight the emphasis of the neonatal inflammatory stimuli in the development of some aspects of the human IBS in later life. However, some important IBS features such as alterations to the gut motility, permeability and bacterial translocation have not been yet determined using this model (See Table 1.6).
1.8.3 Neonatal Maternal Separation

There is a growing body of evidence indicating that all aspects of mother–infant interactions play an important role in the development of the newborn. Studies, mainly performed in developing rodents, have demonstrated that the mother behaviour influences various physiologic parameters in the infant as heart rate, sleep/wake cycles, and growth hormone production (Kuhn, Butler, & Schanberg, 1978). Specific physiologic changes in the infant occurring slowly over a relatively protracted period of separation can be tightly linked to specific features of mother infant interaction. Thus, 24 h of NS decreases mean cardiac frequency (Hofer, 1975). This reduction may result from the long duration of mother’s milk deprivation, rather than a lack of other aspects of maternal care such as growth hormone secretion induced by tactile stimulation from the mother.

Previous research has established that early traumatic experience may adversely affect gastrointestinal homeostasis in later life. Early events such as neonatal maternal separation or early weaning have been shown to increase risk of gastric ulcer in adult rats (Ackerman, Hofer, & Weiner, 1978a, 1978b; Glavin & Pare, 1985). NS results in permanent visceromotor and somatic alterations associated with neurochemical changes, altered HPA responsiveness to stressors, and an increased risk of developing depression-like behaviours, thereby mimicking all the main features of IBS in humans (See Table 1.6). Several NS rat models have been developed to study the impacts of early life stress on gut integrity and function in later life (Barreau, et al., 2006; Barreau, Ferrier, et al., 2004; Coutinho, et al., 2002; Gareau, Jury, Yang, MacQueen, & Perdue, 2006; Rosztoczy, et al., 2003; Soderholm, et al., 2002) (See Table 1.7).
Table 1. 7 Long-term consequences of different NMD models on gut functions

<table>
<thead>
<tr>
<th>Rat Stain</th>
<th>Period of separation</th>
<th>Age at experimentation date</th>
<th>GI Disturbance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-Evans</td>
<td>2-14</td>
<td>2 Mo</td>
<td>Visceral hyperalgesia in basal condition and after stress</td>
<td>(Coutinho, et al., 2002; Schwetz, et al., 2005)</td>
</tr>
<tr>
<td>Sparague-Dawley</td>
<td>4-21</td>
<td>2 Mo</td>
<td>Vulnerability of colonic mucosa to acute stress (permeability, ion secretion)</td>
<td>(Soderholm, et al., 2002)</td>
</tr>
<tr>
<td>Sparague-Dawley</td>
<td>4-21</td>
<td>19-30d</td>
<td>Elevated ion secretion</td>
<td>(Gareau, et al., 2006)</td>
</tr>
<tr>
<td>Wistar</td>
<td>1-14</td>
<td>3 Mo</td>
<td>Susceptibility to stress-induced visceral pain</td>
<td>(Rosztoczy, et al., 2003)</td>
</tr>
<tr>
<td>Wistar</td>
<td>2-14</td>
<td>3 Mo</td>
<td>Colonic hyperalgesia</td>
<td>(Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007; Barreau et al., 2006; Barreau, Ferrier, et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Elevated gut and colonic paracellular permeability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bacterial translocation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Susceptibility to TNBS and parasite-induced inflammation</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Barreau et al. (2007)

1.9 Neonatal Maternal Separation model and Gut functions

1.9.1 Gut-Associated Immune Function

Bidirectional communication between the brain and the gut is mediated by neural, immune, and endocrine pathways. Several studies have indicated that early life factors that alter neuroendocrine stress responsiveness may also influence immune function (Barreau, Ferrier, et al., 2004; S. M. O'Mahony, et al., 2009). Both the systemic and intestinal mucosal immune systems are involved in brain–gut axis communication and work in a coordinated fashion to maintain gut integrity. There is a growing body of evidence supporting the existence of a low-grade level of immune activation in individuals suffering from IBS (Clarke, Quigley, Cryan, & Dinan, 2009). Changes in local immunity, particularly in the colon, have also been reported in IBS patients. An elevated frequency of peripheral blood CD4+ and CD8+ T cells expressing the gut homing receptor integrin β7 has been reported in IBS patients compared to controls (Ohman, et al., 2005). In the same study an increased number of lamina propria CD8+ T cells and a greater expression of mucosal addressing cell adhesion molecule–1+
endothelium, the ligand for integrin β7, were observed in the ascending colon of IBS patients compared with those of control subjects (Ohman, et al., 2005). An increased number of colonic mast cells and proportion of degranulating mast cells, and a higher number of mast cells in close vicinity to nerve endings have been also reported in IBS patients (Barbara, et al., 2004). An increased colonic mucosal myeloperoxidase (MPO) activity, an inflammatory marker, was observed in IBS patients as compared to healthy individuals (Kristjansson, et al., 2004). Consistent with this, studies using the maternally separated rat model of IBS have also shown alterations in both the systemic and intestinal mucosal immune systems indicative of inflammation (Barreau, Cartier, et al., 2004; Barreau, Ferrier, et al., 2004). NS was shown to associate with colonic focal hyperemia and mesenteric adherences in adult rats (Barreau, Ferrier, et al., 2004). NS neonates and adults displayed a greater number of colonic mucosal mast cells compared with controls (Barreau, Cartier, et al., 2004). NS adult rats also exhibited an increased number of colonic and jejunal mucosal PMN (Barreau, et al., 2006; Barreau, Ferrier, et al., 2004). Furthermore, neonatal stress was associated with increased colonic mRNA expression of several cytokines including IL-1β, IL-2, IL-4, IL-10, and IFN-γ, indicating an increase in both Th-1 and Th-2 cytokines profiles (Barreau, Ferrier, et al., 2004). This may be an indication of systemic inflammation in the gut. It has been shown that NS compromises immune defence in rats subsequently exposed to the parasite Nippostrongylus brasiliensis. NS rats displayed a higher level of infection and jejunal mucosal MPO activity compared to non-NS animals (Barreau, et al., 2006).

1.9.2 Visceral Sensitivity and Motility Following Neonatal Maternal Separation

Previous research has shown a visceral hyperalgesia and cutaneous hypoalgesia in NS rats under baseline conditions (Coutinho, et al., 2002). NS animals also exhibited a
visceral hyperalgesia and enhanced colonic motility as indicated by increased fecal pellet excretion when exposed to an acute psychological stress in adulthood (Coutinho, et al., 2002; Schwetz, et al., 2005). Neonatally separated adult females displayed greater visceral hypersensitivity when subsequently exposed to rectal distension compared to their male counterparts (Rosztoczy, et al., 2003). Visceral hyperalgesia was also found to be greater in NS animals removed all together from their littermates compared with those removed from half of their littermates, indicating that interaction between dam and pups plays an important role in the long-term effects of NS. This study also showed that only exposure of females to acute restraint stress induces visceral hyperalgesia, and this effect was similar between NS animals from both removal protocols. This study clearly indicated that adverse effects of NS such as long-term visceral hyperalgesia is gender and protocol dependent (Rosztoczy, et al., 2003). Taken together, these studies provide evidence that NS produces long-term gut hypersensitivity both in basal and stressful conditions.

Most experimental studies only use males to remove sexual cycle effect on HPA axis activity. It is known that basal corticosterone levels are significantly higher during proestrus (Mitsushima, Masuda, & Kimura, 2003).

1.9.3 Intestinal Epithelial Permeability

It has been reported that NS animals displayed an immediate temporary increased colonic permeability to macromolecules and increased bacterial adherence and bacterial penetrated into the colonic epithelium (Gareau, et al., 2006). Moreover exposure of NS rats to a mild stress was shown to increase short-circuit current, conductance, and trans-epithelial transport of macromolecules compared to those of control animals (Soderholm, et al., 2002). Increased intestinal permeability means that the intestinal
epithelium is more permeable to pathogens, allergens and other antigens which may induce inflammation and infection. It has also been reported that NS increases long-lasting gut paracellular permeability in rats (Barreau, Cartier, et al., 2004; Barreau, et al., 2006; Barreau, Ferrier, et al., 2004). Adult male Wistar NS pups also exhibited an increase in jejunal and colonic paracellular permeability and increased bacterial translocation from the gut to internal organs (Barreau, Ferrier, et al., 2004). Interestingly, weaning in piglets caused intestinal barrier dysfunction such as reduced trans-epithelial electrical resistance and increased intestinal permeability to mannitol compared with unweaned animals (Moeser, et al., 2007).

1.10 Pathways Involved in the Genesis of Visceral and Permeability Alterations

Previous research has shown that some mediators and cell types contribute to the genesis of NS-induced alterations in visceral sensitivity and gut permeability. Specifically, NGF and CRH (Barreau, Cartier, et al., 2004; Gareau, et al., 2006; Soderholm, et al., 2002), and mast cells (Barreau, Cartier, et al., 2004) have been recognised to play important roles in triggering and maintaining NS-induced gut dysfunction. It has also been demonstrated that polymorphonuclear neutrophils (PMNs), nerve fibres, cytokines (Berin, Yang, Ciok, Waserman, & Perdue, 1999; Ferrier, et al., 2003) and proteases (Coelho, Vergnolle, Guiard, Fioramonti, & Bueno, 2002) may participate in NS-induced gut alterations. PMNs and nerve fibres however, have been reported to be involved in NS-induced visceral hypersensitivity in a close relationship with mast cells (Rice, Farquhar-Smith, & Nagy, 2002; Shu & Mendell, 1999). On the other hand, metabolites secreted from mast cells such as cytokines IFN-γ, TNF-α and
IL-4, and protease II could also enhance visceral pain and elevate intestinal paracellular permeability.

1.10.1 Corticotropin-Releasing Hormone (CRH)
While previous research has shown an important role for CRH in stress-induced adult gut disturbances, recent studies have reported its involvement in NS-induced gut dysfunctions. Exposure of animals to CRH receptor antagonists protects against adverse effects of NS including intestinal permeability, bacterial translocation from gut to internal organs, and visceral hypersensitivity compared to non-treated NS animals (Barreau, Cartier, et al., 2007; Gareau, et al., 2006; Million, et al., 2003; Schwetz, et al., 2005; Soderholm, et al., 2002). These studies confirmed that CRH-R1 mediates NS-induced gut disturbances in rat neonates, a finding previously reported for the crucial role of this receptor in adult stress-induced gut dysfunctions (Greenwood-Van Meerveld, Johnson, Cochrane, Schulkin, & Myers, 2005; Million, et al., 2003; Soderholm & Perdue, 2001). There are controversial data concerning whether CRH antagonists are able to cross the blood-brain barrier. It is also not clear whether central or peripheral mechanisms are involved in CRH mediated NS-induced gut alterations. On the other hand, the origin of CRH is still a matter of debate. While CRH is mainly secreted from CNS, some colonic mucosal cells have been found to be able to release CRH (Cirulli, Micera, Alleva, & Aloe, 1998; Kawahito, et al., 1994). The presence of both CRH precursor and its mature form has been reported in the colonic mucosa. Furthermore, NS animals exhibited an increase in the expression of colonic mucosal CRH precursor peptide (Barreau, Cartier, et al., 2007). Therefore it is more likely that CRH mediates NS-induced gut dysfunctions via peripheral mechanisms.
1.10.2 Nerve Growth Factor (NGF)

Previous research has shown that NGF is able to promote noxious stimuli (Shu & Mendell, 1999) and that NS rats display increased NGF levels in their CNS (Cirulli, et al., 1998). Recent research has shown that NGF plays a role in the genesis of gut dysfunction induced by NS (Barreau, Cartier, et al., 2004). Neutralisation of NGF prior to each NS session suppressed the adverse effects of the stress on visceral responsiveness to rectal distension and gut permeability (Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007). Currently, little is known about underlying central or peripheral mechanisms involved in the effect of NGF on NS-induced gut dysfunction in rats. Nevertheless, as autoimmunisation-induced NGF depletion causes thermal hypoalgesia, along with increased anti-NGF IgG, and absence of the antibody in the cerebrospinal fluid (Chudler, Anderson, & Byers, 1997). Thus, it could be hypothesised that firstly, antibodies against NGF could not cross the blood-brain barrier, and secondly hypoalgesia results from peripheral NGF neutralisation. Therefore, NS-induced gut dysfunctions are more likely generated by a peripheral mechanism. Previous research has also shown an increased colonic NGF mRNA and protein expression in NS rats which could be linked to the gut alterations (Barreau, Cartier, et al., 2004).

1.10.3 Mast Cells (MC)

There is a growing body of evidence to support that mast cells contribute to the genesis of NS-induced gut dysfunctions. Previous research has highlighted the important role of mast cells in stress-induced alterations in intestinal functions (Soderholm & Perdue, 2001) including mucin secretion, ion and water secretion, permeability, and visceral sensitivity. Studies on the exposure of adult mast cell deficient (Santos, Yang,
Soderholm, Benjamin, & Perdue, 2001) or mast cells stabilised (Castagliuolo, et al., 1998; Gue, et al., 1997) rats to stress has proven such an important role for mast cells in stress-induced enhanced intestinal permeability and visceral hyperalgesia. Stabilising mast cells in neonatally separated adult rats suppressed visceral hypersensitivity and enhanced intestinal permeability (Barreau, Cartier, et al., 2004).

1.10.4 CRH, NGF and MCs, All in One Frame

The preceding sections provided evidence that CRH, NGF and mast cells participate in NS-induced gut dysfunction. Previous research has established an interplay between these three components mediating stress-induced gut disturbances. CRH has been shown to act as one of the most potent secretagogues for the mast cells which are able to produce NGF (Leon, et al., 1994). This inter relationship between CRH, MC, and NGF has been recently proven in NS animals (Barreau, Cartier, et al., 2007) as indicated by the role of CRH in promoting NGF secretion from MCs through CRH-R1 receptor, where the process induced an increase in intestinal permeability (Barreau, Cartier, et al., 2007).

1.11 Neonatal Maternal Separation As a Model for IBS

Recent clinical investigations on intestinal biopsies from patients suffering from IBS have advanced the knowledge and understanding of the underlying mechanisms involved in visceral pain, supporting the observations in rat models of IBS (Barbara, et al., 2004; L. H. Wang, Fang, & Pan, 2004). Biopsies from patients suffering from IBS have indicated an increased intestinal mucosal mast cell density and that mast cells are located in close proximity to nerves compared with control subjects (Barbara, et al., 2004; L. H. Wang, et al., 2004). Immunohistochemistry studies on ileal and recto-
sigmoid mucosal biopsies from IBS patients demonstrated an increase in the density of nerve fibres as clusters around increased mast cell numbers (L. H. Wang, et al., 2004). On the other hand, it has been shown that infiltration of colonic mast cells and secretion of some specific substances in proximity to mucosal innervation may participate in abdominal pain perception in IBS patients (Barbara, et al., 2004). This study has linked the severity and frequency of perceived abdominal painful sensations to the activation of mast cells as indicated by their tryptase release in proximity of colonic nerve endings (Barbara, et al., 2004). Furthermore, stabilisation of mast cells by sodium cromoglycate has demonstrated the important role of mast cells in IBS. Previous studies have demonstrated significantly improved intestinal IBS symptoms by treatment with the mast cell stabiliser (Barau & Dupont, 1990; Grazioli, et al., 1993; Stefanini, et al., 1995).

NS rats also exhibited alterations in the phenotype of colonic mast cells and distribution of nerve terminals, as indicated by increased density of nerve endings density in close proximity of colonic mast cells, in addition to an increase in the release of colonic mucosal mediators (RMCP II) of NS rats. Mast cells released substances such as tryptase and cytokines are known to induce physiological alterations in the enteric nervous system and to enhance visceral sensitivity (Chudler, et al., 1997; Cirulli, et al., 1998). The increased secretion of such mediators happened along with a closer proximity of colonic innervations. This may support the involvement of these anatomical modifications in the sensory-motor dysfunction (Coutinho, et al., 2002; Rosztoczy, et al., 2003). Neonatal separation in rodents seems to be the best animal model of IBS as it results in long-lasting visceromotor and somatic alterations along with neurochemical alterations, HPA-axis dysfunction, and increased depression-like behaviours, mimicking all cardinal features of human IBS (See Table 1.8).
Table 1. Symptoms of brain–gut axis dysfunction in IBS patients and the corresponding alterations induced by early life stress in rodents

<table>
<thead>
<tr>
<th>Brain–gut axis alteration</th>
<th>IBS patients</th>
<th>NS-induced manifestations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIT barrier function</td>
<td>↑Permeability in colonic biopsies</td>
<td>↑Gut paracellular permeability</td>
<td>(Barreau, Cartier, et al., 2007; Barreau, Ferrier, et al., 2007; Gareau, Jury, MacQueen, et al., 2007; Gareau, Jury, &amp; Perdue, 2007; Piche, 2009)</td>
</tr>
<tr>
<td>GIT motility</td>
<td>↓↑↕Transit depending on GI region</td>
<td>↑Colonic transit</td>
<td>(A. Holmes, et al., 2005; S. M. O'Mahony, et al., 2009; Sadik, Bjornsson, &amp; Simren, 2010)</td>
</tr>
<tr>
<td>Visceral sensation</td>
<td>↑Reported sensitivity</td>
<td>↑Pain behaviours during CRD</td>
<td>(Chung, Zhang, Li, et al., 2007; Chung, Zhang, Xu, Sung, &amp; Bian, 2007; Coutinho, et al., 2002; Drossman, et al., 2009)</td>
</tr>
<tr>
<td>Mucosal immune</td>
<td>Gender-dependent mucosal infiltration of immunocytes</td>
<td>Susceptibility to inflammation depends on subsequent stress</td>
<td>(Cremon, et al., 2009; Varghese, et al., 2006; Veenema, Reber, Selch, Obermeier, &amp; Neumann, 2008)</td>
</tr>
<tr>
<td>Enteric microbiota</td>
<td>Altered faecal flora depending on subtype</td>
<td>↑Enterococci and Bacteroides depending on time point</td>
<td>(Garcia-Rodenas, et al., 2006; Kassinen, et al., 2007)</td>
</tr>
<tr>
<td>Systemic immune</td>
<td>↑IL-6+ soluble receptor, IL-8 in plasma depending on subtype</td>
<td>↑TNF-α and IFN-γ in stimulated whole blood</td>
<td>(Dinan, et al., 2006; S. M. O'Mahony, et al., 2009)</td>
</tr>
<tr>
<td>HPA axis</td>
<td>↑HPA axis activity but not correlated with symptoms</td>
<td>↑HPA axis activity depending on sex</td>
<td>(Aisa, Tordera, Lasheras, Del Rio, &amp; Ramirez, 2008; Chung, Bian, Xu, &amp; Sung, 2009)</td>
</tr>
<tr>
<td>Pain matrix</td>
<td>Altered central activation of associated areas depending on sex</td>
<td>Altered activation of spinal and supraspinal areas associated</td>
<td>(Mayer, et al., 2006; Zhang, et al., 2009)</td>
</tr>
<tr>
<td>Emotional areas</td>
<td>Differential activation depending on area</td>
<td>Altered central activation</td>
<td>(Gibney, Gosselin, Dinan, &amp; Cryan, 2009; Mayer, et al., 2006)</td>
</tr>
<tr>
<td>Central neurotransmitters</td>
<td>Enhanced monoaminergic response</td>
<td>Altered monoaminergic levels</td>
<td>(S. O'Mahony, et al., 2008)</td>
</tr>
<tr>
<td>GIT neurotransmitters</td>
<td>↑Mucosal 5-HT-containing enterochromaffin cells</td>
<td>↑Monoaminergic levels post-CRD</td>
<td>(Dunlop, et al., 2005; Ren, et al., 2007)</td>
</tr>
</tbody>
</table>

Symptoms seen in patients depend on subtype and gender, whilst alterations in the MS model are influenced by strain, gender, time points and protocol. ↑=increased, ↓=decreased,↕=alternating

Adapted from O’Mahony et al. (2011)
1.12 Rationale, Aims and Hypothesis

Early environmental factors acting during the neonatal period may determine alterations in physiological regulation, promoting critical development and having long lasting effects on health status. This vulnerability is due to the high degree of plasticity which occurs during this critical period. Rodents such as rats and mice give birth to neuro-anatomically immature offspring who develop rapidly in the first two weeks of their postnatal life (Kapoor, et al., 2008). In the rat, early postnatal life is known as the critical period of development for different systems in particular the hypothalamic–pituitary–adrenal (HPA) axis which is one of the essential physiological systems responsible for the coordination of the normal stress response to challenges in vertebrates. The impact of environmental factors during the early postnatal period has recently been shown to have a wide variety of implications for the developmental trajectory of this system, and under stressful conditions may lead to disease (Gluckman, Cutfield, Hofman, & Hanson, 2005; Gluckman & Hanson, 2004, 2006; Gluckman, et al., 2007; Gluckman, Hanson, Cooper, & Thornburg, 2008). Perturbations, during early life, in the development of the HPA-axis have been reported to result in neonates who hyper-respond to stress in later life (Kalinichev, et al., 2002; O'Malley, Dinan, et al., 2011; Plotsky & Meaney, 1993; Soderholm, et al., 2002). This finding has had implications for the ontogeny of a variety of later life disorders, particularly those whose aetiology is predicated on excessive/dysregulated responses to stress, including functional gastrointestinal disturbances such as irritable bowel syndrome (IBS).

The early postnatal period is also a time when the sterile gut is inhabited and colonised gradually by microorganisms that are likely to reside in the gut throughout life (Inoue & Ushida, 2003). It has been reported that normal gut microbiota is essential for brain development (Diaz Heijtz, et al., 2011) and that postnatal microbial colonisation
programs the HPA-axis stress response (Sudo, et al., 2004). On the other hand, gut microbiota has an important role in the postnatal maturation of the gut immune system (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Round & Mazmanian, 2009). Interactions between external and host factors have also been proposed to impact colonisation profiles in the early postnatal period (Kirjavainen & Gibson, 1999). There is a growing body of evidence that the intestinal microbiota of IBS patients differs considerably from that of healthy subjects. It is also suggested that stress exacerbates IBS (Longstreth, 2005) and alters microbiota (Gareau, Silva, & Perdue, 2008; Phillips, 2009). Animal models have suggested that the early life period may be a critical time in which the presidispistion to IBS is established through the programming of the stress axis and the establishment of gut microbiota (Garcia-Rodenas, et al., 2006; S. M. O'Mahony, et al., 2009).

The high prevalence of this disorder and the ineffectiveness of current treatments results in high direct and indirect costs to society along with considerable pain and distress to sufferers (Cash, et al., 2005). The use of probiotics to replenish the gut microbiota disturbed in the IBS patients has emerged as a nutritional approach to improve some symptoms and normalise the bowel movement frequency in IBS patients (Aragon, Graham, Borum, & Doman, 2010). Neonatal probiotic intervention in a rat model of IBS has been reported as a potential prophylaxis against brain–gut axis dysfunctions by normalisation of HPA-axis activity (Gareau, Jury, MacQueen, et al., 2007). We hypothesised that maternal supplementary probiotics may also contribute to improved brain–gut axis integrity and immune system functioning in neonates at high risk of developing IBS in later life, and that the possible improvements persist into adulthood. The rationale for this hypothesis is based on previous studies showing that maternal introduction of probiotics results in colonisation of the neonatal gastrointestinal tract.
(Buddington, Williams, Kostek, Buddington, & Kullen; Gueimonde, et al., 2006; Schultz, et al., 2004; Vanderhoof, et al., 1999), and also causes substantial alterations in the offspring’s gut microflora (Gueimonde, et al., 2006). Previous research has also shown that postnatal microbial colonisation programs the HPA stress response. Exaggerated HPA-axis stress responses to stress were observed in GF mice. Whilst the HPA-axis stress response was facilitated by intervention with Escherichia coli, this situation was reversed by administration with Bifidobacterium infantis (Sudo, et al., 2004). It has also been reported that maternal administration of probiotics could modulate immune system responsivity in a postive manner in offspring (Blumer, et al., 2007; Rautava, et al., 2002). Therefore, given that maternal probiotic administration produces a positive gastro-immune environment for the offspring, and given that we know that the composition of gut microflora can mediate HPA-axis activity, it could be expected that maternal use of probiotics may confer a more immediate protection against IBS.

To test the hypothesis, research chapters which have comprised this thesis have all employed a well-established animal model of IBS i.e., neonatal maternal separation in Wistar rats (Barreau, Ferrier, et al., 2007; S. M. O'Mahony, et al., 2011) whereby offspring were subjected to intermittent maternal deprivation between postnatal days 2 to 14. In adulthood, the offspring were further exposed to an acute stressor (repeated acute restraint stress) to evaluate the neuroendocrine-immune and gut responsiveness to the stress. Exposure of rats to neonatal maternal separation has been recognised to predispose animals to brain–gut axis dysfunction in response to a subsequent adult stress (Coutinho, et al., 2002; S. M. O'Mahony, et al., 2009; Soderholm, et al., 2002; Welting, Van Den Wijngaard, De Jonge, Holman, & Boeckxstaens, 2005). Essentially these stress models mimic to some extent that which is proposed to account for IBS i.e.,
stress in early life combined with stress in later life (Coutinho, et al., 2002). Essentially these stress models mimic to some extent that which is proposed to account for IBS i.e., stress in early life combined with stress in later life. This can be referred to as a ‘Stress Diathesis’ model in which priming by early stress is compounded by stress in later life. In this study acute restraint stress in adulthood was used as the stress in later life due to its adverse effects on brain-gut functioning in rodents (Israeli, et al., 2008; Julio-Pieper, et al., 2012; Santos, et al., 1999; Sudo, et al., 2004).

A combination of *Bifidobacterium animalis* subsp *lactis* BB-12® and *Propionibacterium jensenii* 702 was used in this study. *Bif. animalis* subsp *lactis* BB-12® was selected as it is the most widely recognised and extensively studied probiotic *Bifidobacterium* strain with a wide range of beneficial effects in human and animal models. In particular, this probiotic strain in combination with other probiotics has been reported to improve IBS symptom severity (Simren, et al., 2009), decrease the composite IBS score, especially distension and abdominal pain, and stabilise gut microbiota in IBS patients (Kajander, et al., 2008). *Bif. animalis* subsp *lactis* BB-12® was combined with *P. jensenii* 702 as the latter has been reported to increase populations of endogenous bifidobacteria in human subjects (Kotula, 2008). An in vitro study also demonstrated a mutual synergistic viability promoting effect of *Bif. animalis* subsp *lactis* BB-12® and *P. jensenii* 702 when co-cultivated (Moussavi & Adams, 2010). This may enhance efficacy of the probiotic preparation.

Therefore the aim of the current thesis was to examine the role of the maternal probiotic intake in preventing the adverse early life stress on brain-immune-gut axis in an animal model of IBS. Using the neonatal maternal separation model, we aimed to determine whether maternal probiotic intervention can contribute to improved HPA-axis, systemic and gut-associated immune integrity, and gut functions including gut microbiota and gut
mucin secretion in stressed neonates, if these possible improvements persist into adulthood, and how this protective effect may be mediated.
Chapter II

Methods and Materials
2.1 General Methods and Approach

2.1.1 Animals and Animal Husbandry

Adult male and female Wistar rats were obtained from the University of Newcastle vivariums and used as breeders. Animals were acclimatised to the housing environment and handled by researchers for two weeks, prior to breeding, in the Behavioural Sciences Animal Facility (BSAF), the University of Newcastle, Australia. Animals were housed in polycarbonate-perspex cages (40×25×12 cm), 2-3 animals per cage lined with chip bedding. Husbandry conditions were 12:12 hour light–dark cycle (lights on at 06:00 am) and an adjusted temperature at 23±1 ºC. Animals had free access to standard rat/mouse pellets (Specialty Feeds, Glen Forrest, WA, Australia) and drinking water. During the last week of gestation, dams were single housed in cages lined with shredded paper. The chow diet was supplemented with sunflower seeds until delivery. After delivery, dams and pups were housed together until weaning at postnatal day (PND) 22. Offspring were then housed with same sex animals, 4-5 animals per box.

2.1.2 Animal Ethics

This study was carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes. The protocol was approved by the Animal Care and Ethics Committee of the University of Newcastle (ACEC No.: 1071). All efforts were made to minimise discomfort and suffering according to the Code of Practice (NHMRC, 2004).
2.1.3 Breeding

Each two virgin females (12-20 weeks of age) were mated to a single male for 10 days. During the period of mating, the males had equal access to the same diet and drinking water as the females. Following successful mating, dams were housed in pairs for up to a week. Dams were single housed for the last week of gestation. The day of birth was designated as postnatal day (PND) one.

2.1.4 Probiotic Preparation

Freeze dried *Bifidobacterium animalis* subsp. *lactis* BB-12® was kindly provided by Chr. Hansen Pty. Ltd. Melbourne, Australia. Lyophilised *Propionibacterium jensenii* 702 culture was obtained from the Laboratory of Food Microbiology, School of Environmental and Life Sciences, the University of Newcastle, Australia. *Propionibacterium jensenii* 702 had been isolated from raw bovine milk and freeze dried commercially by Laboratoires Standa, Caen, France. The probiotic strains were recovered by two consecutive sub-cultures in appropriate media prior to use. *Bif. animalis* subsp. *lactis* BB-12 was grown overnight at 37 °C in Reinforced Clostridial Medium (RCM) broth (Oxoid Australia Pty Ltd, Adelaide, Australia) under anaerobic conditions. *P. jensenii* 702 was grown anaerobically in yeast extract lactate (YEL) medium (Malik, Reinbold, & Vedamuthu, 1968) at 30 °C for 48 h. Bacterial cells were then harvested from fresh probiotic cultures in their stationary phases by centrifugation at 3000 rpm for 10 min and washed three times with Dulbecco’s Phosphate-Buffered Saline (PBS, pH 7.0) (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Bacterial pellets were then resuspended in PBS and refrigerated as probiotic stocks. Bacterial counts of culture stocks were determined by plating 100 µl aliquots of decimal dilutions of
cultures on agar plates. PJ was counted on YEL agar (Malik, et al., 1968) after anaerobic incubation of the plates at 30 °C for seven days. Bb was counted on TOS propionate agar (Yakult Pharmaceutical Ind., Co., Ltd, Tokyo, Japan) following anaerobic incubation of the plates at 37 °C for two days. Fresh stock cultures were prepared every week. Pilot studies carried out in this laboratory has shown that both bacteia could survive refrigerated storage for a week without viability loss. The probiotic drinking water was then prepared by the addition of both cultures to the drinking water so that the doses of Bif. animalis subsp. lactis BB-12 and P. jensenii 702 were approximately 3×10⁹ and 8.0×10⁸ CFU/mL respectively. The probiotic-spiked water was sampled at different times to check for viability. The probiotic drinking water was available in 250-ml bottles. The bottles were replaced with clean bottles containing fresh probiotic drinking water every day.

2.1.5 Study Design

A schematic and timeline of the protocols involved in the animal testing is represented in Figure 2.1. Female breeders were randomly allocated to either ‘vehicle’ or ‘probiotic’ treatment groups. Dams in the probiotic treatment group had free access to the drinking water supplemented with probiotics from 10 days before conception until and including PND 22. Control animals had equal access to water without the probiotics added. Parental rats were then mated within two weeks. After birth, pups were subjected to neonatal maternal separation (NS) (details below) from PND 2 to 14 or left undisturbed. A subset of animals were euthanised at PND 24 with an overdose of pentobarbiton sodium (Lethabarb®, Virbac Pty Ltd, Milperra, NSW, Australia) and blood, tissue and faecal samples were collected. The remaining animals were left undisturbed until PND
83 when they underwent three consecutive days of repeated acute restraint stress exposure (details below) followed by a 30 min isolation session at PND 86. Animals in the no stress condition remained undisturbed. All animals were then euthanised at PND 86 and blood, tissue and faecal samples were collected.
**Figure 2.1** A timeline schematic of experimental procedures. Maternal probiotics administered to subsets of animals from 10 days before mating until weaning (PND 22). Subsets of offspring were subjected to neonatal maternal separation (NS) and/or adult stress (AS). At varying time points (PND 24 and 86) subsets of animals were euthanised for endocrine, immune and gut functional analyses.
2.1.6 Neonatal Maternal Separation (NS)

At PND 2, litters were randomly allocated into either ‘neonatal maternal separation’ (NS), or no stress condition. A slightly modified method of Barreau et al. (Barreau, Ferrier, et al., 2004) was used for NS stress. Pups in the NS condition were removed from their litter mates and placed individually in wire-mesh lidded 925 ml plastic containers lined with several layers of tissue paper (0.5 cm thick) and with holes in the sides allowing free air circulation. Containers were then immediately transferred to a separate heated room with an adjusted temperature of 34±1 °C and remained there for three hours (from 9:00 to 12:00 pm) per day from PND 2 to 14. Following the separation, pups were returned to their dams. Animals in the control groups were left undisturbed.

2.1.7 Restraint Stress (AS)

In adulthood, animals neonatally subjected to NS and non-NS were randomly allocated into either a three-day stress, or no stress condition. Animals allocated to the AS condition underwent a stress protocol which involved three consecutive days of restraint stress and one day of isolation. During the period of restraint stress animals were food and water deprived. Animals were removed from their home cages and placed in a soft wire-mesh tube with closed edges using binder clips (Walker, et al., 2009a) for 30 min per day (from 10:00 to 10:30 am) for three consecutive days (PND 83-85) and isolated for 30 minutes on PND 86. Animals’ eyes were protected from light using a towel and they were monitored on a 10 min basis. Rats in the ‘non-adult-stress’ condition were left undisturbed.
2.1.8 Blood Sample Collection

On PND 24 or 86 and prior to euthanisation, blood was collected from the saphenous vein between 9:30 and 10:30 am, into Vacuette® blood collection tubes containing anticoagulant K$_3$EDTA (Greiner Bio-One GmbH, Kremsmünster, Austria). Following euthanisation, heart blood was also collected via cardiac puncture using a five mL syringe. Animals were then perfused transcardially using 0.9% sodium chloride solution (Baxter, Old Toongabbie, Australia). Blood samples were centrifuged at 1000 ×g for 20 min at 4 °C, and supernatant (plasma) was collected and stored at −20 °C until assayed.

2.1.9 Euthanisation

Animals were euthanised on PND 24 or 86 with an overdose of pentobarbiton sodium (Lethabar® Active constituent: 325mg/ml Pentobarbiton sodium) (Virbac Pty Ltd, Milperra, NSW, Australia).
2.2 Methods for Specific Experiments

2.2.1 Corticosterone and ACTH Assays

Plasma from saphenous blood samples was used for HPA-axis activity assessment by analysing circulating corticosterone and Adrenocorticotropic hormone (ACTH) levels respectively using an ImmuChem™ double Antibody Corticosterone $^{125}$I RIA kit (MP Biomedicals, LLC Diagnostics Division, Orangeburg, NY, USA) and Rat ACTH EIA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) according to manufacturer’s instructions. The minimum detectable doses, inter- and intra-assay variability for the corticosterone assay, were 7.7 ng/mL, ≤7.2% and ≤10.3% respectively. The minimum detectable concentrations, inter- and intra-assay variability for ACTH, were 0.08 ng/ml, <15% and <10% respectively.

2.2.2 Immune Measures

2.2.2.1 Plasma Cytokines TNF-α, IFN-γ and IL-6

The blood samples collected via cardiac puncture were used for the cytokine assays using Quantikine® immunoassay kits (R&D Systems Inc., Minneapolis, MN, USA). Clear samples were analysed for concentrations of tumour necrotising factor alpha (TNF-α, minimum detectable dose, ≤5 pg/mL; inter-assay variability, ≤9.7% and intra-assay variability, ≤5.1%), interferon gamma (IFN-γ, minimum detectable dose, ≤10 pg/mL; inter-assay variability, ≤9.7% and intra-assay variability ≤4.0%) and interleukin 6 (IL-6, minimum detectable dose, ≤21 pg/mL; inter-assay variability ≤10% and intra-assay variability≤8.8%). The optical densities were read at 450 nm wavelength using a Multiskan® EX microtiter plate reader (Thermo Electron Corp., Vantaa, Finland).
Calculations were performed using Ascent software version 2.6 (Thermo Electron Corp., Vantaa, Finland).

### 2.2.2.2 Determination of Plasma Haptoglobin

Plasma obtained from cardiac samples was also used for determination of acute phase protein haptoglobin. Prior to assay, plasma samples were diluted at 1:10,000 using a diluents buffer supplied by the kit. The concentration of plasma haptoglobin was then analysed using an available commercial Rat Haptoglobin ELISA kit (GenWay Biotech Inc., San Diego, CA, USA) according to the manufacturer’s instructions.

### 2.2.2.3 Plasma Immunoglobulin A (IgA)

Plasma samples obtained from heart blood were also used for determination of plasma IgA concentrations. Prior to assay, plasma samples were diluted at 1:500-2000 using a diluents buffer supplied by the kit. Total plasma IgA concentration was then determined by a Rat IgA ELISA kit (GenWay Biotech Inc., San Diego, CA, USA) according to the manufacturer’s instructions. The level of IgA was corrected for the volume of the plasma sample (μg/mL).

### 2.2.2.4 Luminal Immunoglobulin A (IgA)

Following euthanasiation, faecal pellets (1-2 pellets from each animal) were collected into sterile 1.8 mL cryogenic vials (NUNC A/S, Roskilde, Denmark), snap frozen in liquid nitrogen and stored at -80 °C. Prior to IgA assay, samples were thawed at room temperature, weighed, and re-suspended in 9 volume (w/v) phosphate-buffered saline
(PBS). The mixture was incubated at ambient temperature (23 °C) for 15 min and vortex mixed. The suspension was then centrifuged at 12,000 ×g for 10 min. Supernatant was then removed and further diluted 50-200 times (final dilution 500-2000) (Inoue & Ushida, 2003). Total faecal IgA concentration was determined by a Rat IgA ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer’s instructions. The level of IgA were corrected for the weight of the faecal sample (µg/gr).

2.2.3 Gut Microbiota

2.2.3.1 Analysis of Faecal Anaerobic and Aerobic Bacteria

Fresh faecal pellets were collected directly from the rectum and distal colon of euthanised rats into 10mL sterile tubes (Sarstedt, Nümbrecht, Germany) containing 9 times (v/w) of an anaerobic dilution buffer (Kataoka, et al., 2007) and homogenised by vortex mixing. Serial decimal dilutions were then prepared using the anaerobic dilution buffer or maxidam recovery diluent (MRD) (Oxoid, Basingstoke, Hampshire, UK) respectively for analysing anaerobes and aerobes. Amounts of 100 µl of diluted samples were plated on specific agar media. Total aerobes were enumerated on Tryptone Soya Agar (Oxoid) after a week of incubation at 37 °C. Total anaerobes were counted on Glucose Blood Liver agar (Atlas, 2004) after 48 h incubation at 37 °C under anaerobic conditions using AnaeroGen W-Zip Compact (Oxoid). Bacterial counts were expressed as log_{10} CFU per gram of faecal samples.
### Table 2.1 Composition of the anaerobic dilution buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Supplier (Cat. No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2HPO4</td>
<td>0.02925% w/v</td>
<td>SIGMA (P8281)</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.017625% w/v</td>
<td>AJAX (392-500g)</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>0.04425% w/v</td>
<td>SIGMA (A5132)</td>
</tr>
<tr>
<td>CaCl2. 2H2O</td>
<td>0.0045% w/v</td>
<td>CHEM SUPPLY -</td>
</tr>
<tr>
<td>MgSO4</td>
<td>0.00825% w/v</td>
<td>SIGMA (M7506)</td>
</tr>
<tr>
<td>Resazurin sodium salt</td>
<td>0.0001% w/v</td>
<td>SIGMA (R7017)</td>
</tr>
<tr>
<td>L-cysteine hydrochloride monohydrate</td>
<td>0.05% w/v</td>
<td>SIGMA (C7880)</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0.05% w/v</td>
<td>SIGMA (A7506)</td>
</tr>
<tr>
<td>Na2CO3</td>
<td>0.4% w/v</td>
<td>FLUKA (71352)</td>
</tr>
<tr>
<td>Agar Bacteriological (Agar No.1)</td>
<td>0.05% w/v</td>
<td>OXOID (LP0011)</td>
</tr>
<tr>
<td>pH</td>
<td>7.4–7.6</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Kataoka et al. (2007)

### 2.2.3.2 DNA Isolation and Purification

Frozen faecal pellets were also used for molecular analysis of faecal bacterial groups. 100-200 mg of frozen samples were weighed, and re-suspended in 1.4 ml buffer ASL (Qiagen, Hilden, Germany) in a microtube. A 5 mm steel bead (Qiagen) and 300 mg acid washed 425-600 μm glass beads (sigma-Aldrich, Saint Louis, MO, USA) were added. Samples were then completely disrupted and homogenised using a TissueLyser LT (Qiagen) for 5 min at 50 Hz. DNA extraction and purification protocols were then followed as instructed in the QIAamp DNA Stool Mini Kit (Qiagen). Briefly, DNA-damaging substances, PCR inhibitors and impurities present in the lysates were absorbed to InhibitEX matrix (provided in a tablet form). The InhibitEX matrix was then separated by centrifugation and the DNA present in the supernatant was purified on
QIAamp Mini spin columns. The resulting DNA eluate was stored at −20 °C until further analysis.

To generate standard curves, DNA was also isolated from a defined number of control bacterial cultures (as seen in Table 2.2) (serial decimal dilutions 10⁰–10⁹ CFU/mL) using the same kit. The concentration of DNA in final DNA eluate was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). The DNA elute was stored at −20 °C until further analysis.

Table 2.2 Bacterial cultures used for generating RT-PCR standard curves

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>ATCC number</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium coccoides</em></td>
<td>29236</td>
<td>Mouse faeces</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp. <em>animalis</em></td>
<td>25527</td>
<td>Rat faeces</td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em></td>
<td>55739</td>
<td>Colon of conventional rat</td>
</tr>
<tr>
<td><em>Enterococcus ratti</em></td>
<td>700914</td>
<td>Neonatal rat with diarrhea</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>21990</td>
<td>Rat kidney</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>25285</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3.3 Real-Time PCR Quantification of Faecal Microflora

Real-time PCR analysis of gut microflora was performed using a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). Previously reported primer sets and probes (Table 2.3) were used in this study for *Bacteroides* (Layton, et al., 2006), Bifidobacteria (Furet, et al., 2009), Lactobacilli (Delroisse, et al., 2008), Enterococci (Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004), *E. coli* (Huijsdens, et al., 2002) and *Clostridium* cluster XIVa (Matsuki, et al., 2002). Primers and probes were manufactured by Applied Biosystems. Quantitative real-time PCR was performed using
TaqMan method for Clostridia, Bacteroides and Bifidobacteria and lactobacilli, while SYBR-Greens method was adapted for quantification of E. coli and Enterococci. Amplifications were carried out in 96-well plates. Each reaction was run in triplicate in a total volume of 25 µl containing 1 × TaqMan Universal PCR Master Mix or 1 × SYBR® Green PCR Master Mix (Applied Biosystems) 200 nM of both primers, 250 nM probe and 10 µL of diluted purified DNA. Bovine serum albumin (New England Biolabs, Ipswich, MA, USA) was also added to PCR mixtures to a final concentration of 0.1 μg/µl. The amplification conditions were 10 min at 95 °C (1 cycle), followed by 30s at 95 °C (40 cycles) and 1 min at 60 °C (1 cycle). A melting step however was added for SYBR-Greens amplifications. The obtained threshold cycles (CT) were then compared with a standard curve constructed for serially diluted genomic DNA of standard bacterial cultures for each bacterial target group.

Table 2. 3 Primers and probes used for detecting bacterial groups in faecal samples

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sets and probes (5’–3’)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Bacteroides    | F: GAG AGG AAG GTC CCC CAC  
R: CGC TAC TTG GCT GGT TCA G  
P: CCA TTAG ACC AAT ATT CCT CAC TGC TGC CT | TaqMan     | (Layton, et al., 2006)           |
| Bifidobacteria | F: CGG GTG AGT AAT GCG TGA CC  
R: TGA TAG GAC GCG ACC CCA  
P: CTC CTG GAA ACG GGT G | TaqMan     | (Furet, et al., 2009)            |
| Lactobacilli   | F: GAG GCA GCA GTA GGG AAT CTT C  
R: GGC CAG TTA CTA CCT CTA TCC TTC TTT  
P: ATG GAG CAA CGA CGC | TaqMan     | (Delroisse, et al., 2008)        |
| Enterococci    | F: CCC TTA TTG TTA GTT GCC ATC ATT  
R: ACT CGT TGT ACT TCC CAT TGT | SYBR-Green | (Rinttila, et al., 2004)         |
| E. coli        | F: CAT GCC GGG TGT ATG AAG AA  
R: CGG GTA ACG TCA ATG AGC AAA | SYBR-Green | (Huijsdens, et al., 2002)        |
| Clostridium    | F: AAA TGA CGG TAC CTG ACT AA  
R: CTT TGA GTT TCA TCC TTG CGA A | TaqMan     | (Matsuki, et al., 2002)          |
| cluster XIVa   |                                                                 |            |                                  |

F: forward; R: reverse; P: probe
Relative Quantification of Gene Expression

Tissue collection

Ileal fresh tissue samples were collected from euthanised animals into sterile 5mL yellow cap containers (Sarstedt, Nümbrecht, Germany) containing 2-mL RNAlater® solution (Ambion, Austin, TX, USA). Samples were completely immersed in RNAlater® solution. Containers were then stored at 4 °C overnight to allow the solution to thoroughly penetrate the tissue and then moved to –20 °C for long-term storage.

Total RNA Isolation

Total RNA from ileal tissue samples was isolated using an RNeasy® Plus Mini kit according to manufacturer’s instructions (Qiagen Inc., Valencia, CA, USA). Briefly RNAlater stabilised ileal tissue was removed from RNAlater solution. An amount of 20 mg of the tissue was weighed and placed into a 1.7 mL micro-tube. Six hundred (600) μl guanidine-isothiocyanate–containing buffer (Buffer RLT Plus) was then added and the sample was disrupted and homogenised in the buffer using a Polytron® PT 2100 rotor/stator homogeniser and a dispensing aggregate model PT-DA05/2EC-D066 (Kinematica AG, Lucerne, Switzerland) at 25000 rpm for 30-40 sec. Alternatively samples were disrupted and homogenised by adding a 5 mm steel bead (Qiagen) into the tube containing tissue sample and Buffer RLT Plus and using a TissueLyser LT (Qiagen) for 5 min at 50 Hz. The lysate was centrifuged (at 8000 ×g for 30 s) and supernatant was passed through a genomic DNA (gDNA) eliminator spin column. One volume (600 μl) of 70% ethanol was added to flow-through and mixed by pipetting. An amount of 700 μl of the sample was transferred to an RNeasy spin column and...
centrifuged at 8000 g for 15 s. Total RNA binds to the membrane of the RNeasy spin column. Contaminants were then washed away using buffers RW1 and RPE and subsequent centrifugation steps. RNA was then eluted in 30 μl of water. In this procedure all RNA molecules ≥200 nucleotides are isolated and mRNA content is enriched.

2.2.4.3 RNA Quantification and Purity

The concentration of RNA in final RNA eluate was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA eluate was stored at −20 °C until further analysis. The ratio of absorbance at 260 and 280 nm (260/280) obtained by NanoDrop was used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as ‘pure’ for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. In this case, RNA was re-isolated from tissue. Furthermore, ratio of absorbance at 260 nm and 230 nm (260/230) was used as a secondary measure of nucleic acid purity. The 260/230 values for a ‘pure’ nucleic acid are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants. Again in this case, RNA was re-isolated from the tissue.
2.2.4.4 Reverse transcription into complementary DNA (cDNA)

First-strand cDNA was generated using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA, USA) according to manufacturer’s instructions. Briefly, 4 μl 5X VILO™ Reaction Mix, 2 μl 10X SuperScript® Enzyme Mix, 2.5 μg RNA and DEPC-treated water (to 20 μl) in a 200 μl tube were combined, gently mixed and incubated at 25 °C for 10 minutes. Tubes were then transferred to MyCycler™ thermal cycler (Bio-Rad, Hercules, CA, USA) and heated at 42 °C for 60 minutes. The reaction was then terminated at 85 °C for 5 minutes. cDNA was then stored at -20 °C until use. The concentration of cDNA should be 125 ng/μl since the amount of RNA template in the 20-μl reaction was 2.5 μg.

2.2.4.5 Selection of Endogenous Controls

Pre-made TaqMan® Gene Expression controls were used for this study using http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html. Thirty one (31) endogenous controls were found for rats. The search was narrowed by selecting best coverage controls (recommended primer/probe sets for standard gene expression experiments) and those with assay ID ended by _m1 assay. By selecting _m1 controls, performing a -RT control is not necessary as it will not amplify background gDNA, just the transcript. With these filters, the number of controls came to 15. To find the most commonly used endogenous controls in the literature, a scholar Google search was performed. The top three (as seen in Table 2.4) were selected and ordered.
### Table 2.4 Endogenous controls

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Context Sequence</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn00560865_m1</td>
<td>B2m</td>
<td>beta-2 microglobulin</td>
<td>GCTTGCCATTCAGA AAACTCCCCAA</td>
<td>58</td>
</tr>
<tr>
<td>Rn00566655_m1</td>
<td>Gusb</td>
<td>glucuronidase, beta</td>
<td>ATATTACTTCAAGA CGCTGATCGCC</td>
<td>63</td>
</tr>
<tr>
<td>Rn01527840_m1</td>
<td>Hprt1</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
<td>TTCAGGGATTGTG TCAATTTGTG</td>
<td>64</td>
</tr>
</tbody>
</table>

#### 2.2.4.6 Validation of the endogenous controls

The endogenous controls (Table 2.4) need to be validated in samples/tissues to see if they are not regulated (no change in gene expression). For the endogenous control validation six representative cDNA samples (from each biological group) were selected. Then equal amounts of selected cDNA samples should be amplified with the candidate endogenous controls to see which control gives the most stable Ct values across all samples. To gain an idea of how much cDNA to include for this work, generation of some standard curves was needed to determine the dynamic range of samples. To verify that representative cDNA template masses yield results within the linear dynamic range of an assay, a relative standard curve was run. To generate standard curves, the endogenous controls were amplified using a cDNA input range of 25 ng to 40 pg in a series of five-fold dilutions (25 ng, 5 ng, 1 ng, 0.2 ng and 0.04 ng) in triplicate. PCR reaction mix consisted of 1.0 µL 20×TaqMan® Gene Expression Assay (Applied Biosystems), 10.0 µL 2×TaqMan® Gene Expression Master Mix (Applied Biosystems), 1.0 µL cDNA template (25 to 0.04 ng) and 8.0 µL RT-PCR water (Applied Biosystems). The reaction components were then mixed by pipetting. 20 µL of PCR reaction mix was transferred into each well of a 96 well reaction plate. The Plate was then sealed, centrifuged briefly and loaded into a 7500 RT-PCR Fast instrument...
(Applied Biosystems). Amplification and detection were performed with the following thermal cycler conditions: 50 °C for 2:00 min (UNG incubation), 95 °C for 10:00 min (polymerase activation), and 40 cycles at 95 °C for 0.15 min and 60 °C for 1:00 min. Each sample was assayed in triplicate.

As standard curves were generated for each of three endogenous control assays to determine their reaction efficiencies, the validation was also performed whilst determining the dynamic range of cDNA at the same time (see Figure 2.2). It appears that none of the endogenous controls are regulated by the treatment samples so theoretically any of the three controls could be used as the normaliser gene.
Figure 2.2 Standard curves for validation of endogenous controls
2.2.4.7 Relative Quantification of Expression of Target Genes

Quantification-Comparative Ct (ΔΔCt) was run to determine the relative quantification of mRNA expression of CRH-receptors 1 and 2, NGF and MUC-2 in ileal tissue samples. Pre-made TaqMan® Gene Expression targets were purchased from Applied Biosystems (see Table 2.5). PCR reaction mix (20 µL) consisted of 1.0 µL 20×TaqMan® Gene Expression Assay (Applied Biosystems), 10 µL 2×TaqMan® Gene Expression Master Mix (Applied BioSystems), 1.0 µL cDNA template (5 ng/mL), and 8.0 µL RT-PCR water (Applied BioSystems). The endogenous control B2m (Table 2.5) was included in the assay. The reaction components were then mixed by pipetting. 20 µL of PCR reaction mix was transferred into each well of a 96 well reaction plate. The Plate was then sealed, centrifuged briefly and loaded into a 7500 RT-PCR Fast instrument (Applied Biosystems). Amplification and detection were performed with the following thermal cycler conditions: 50 °C for 2:00 min (UNG incubation), 95 °C for 10:00 min (polymerase activation), and 40 cycles at 95 °C for 0.15 min and 60 °C for 1:00 min. Each sample was assayed in triplicate.

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Context Sequence</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn00578611_m1</td>
<td>Crhr1</td>
<td>Corticotropin releasing hormone receptor 1</td>
<td>GACAATGAAAAAGT GCTGGTTTGGCA</td>
<td>58</td>
</tr>
<tr>
<td>Rn00575617_m1</td>
<td>Crhr2</td>
<td>Corticotropin releasing hormone receptor 2</td>
<td>GCATGAGGGCAAT GAGGTCTGGTGC</td>
<td>82</td>
</tr>
<tr>
<td>Rn01498195_m1</td>
<td>Muc2</td>
<td>Mucin 2, oligomeric mucus/gel-forming</td>
<td>TTCAGAAAAAGAAGC CAGATCCCGAAA</td>
<td>77</td>
</tr>
<tr>
<td>Rn01533872_m1</td>
<td>NGF</td>
<td>Nerve growth factor (beta polypeptide)</td>
<td>TGGCCACTCTGAG GTGCATAGCGTA</td>
<td>114</td>
</tr>
</tbody>
</table>
The quantity is expressed relative to a calibrator. The calibrator for this study was a cDNA template from ileal tissue of a male pup (PND 24) and untreated (no-stress and no probiotic). The calibrator, then, becomes the 1X sample, and all other quantities are expressed as an n-fold difference relative to the calibrator.

2.2.5 Histochemistry

Full-thickness ileal and colonic segments were fixed in 10% neutral buffered formalin (Fronine, Lomb Scientific, Taren Point, Australia) until further processing. Tissue samples were then embedded in paraffin, sectioned (5 µm) and stained using hematoxylin and eosin (H&E) and alcian blue-Safranin. Mast cell number and structural damages were evaluated using a Motic light microscopy (Motic, Barcelona, Spain) and image analysis software Motic Images Advanced version 3.2.

2.3 Statistical analysis

Data analyses were performed using SAS® 227 9.2 (SAS Institute Inc., Cary, NC, USA). Generalised linear mixed model (GLMM) was used to analyse the design because of the hierarchical design and the mix of random and fixed variables. The design used in this study was a two-level hierarchical (a split plot design with pups nested within dams) randomised (imperfectly) design, unbalanced, and with incomplete blocks. Dam is a blocking variable which was treated as a random effect variable in the analysis model.

Data sets were natural log (Ln) transformed when data distributions were not normal, so that the data would be approximately normally distributed. In transformed data any
possible observation with standardised residual greater than 2.5 in absolute value was removed as an outlier and the model was refitted.

Planned comparisons of significant interactions were performed using simple effects and post-hoc analyses, when appropriate, were performed with LSD or Tukey’s HSD. All data are presented as means+SE. For all comparisons, a p-value \leq 0.05 was considered significant.
Chapter III

Effect of Maternal Probiotic Intervention on HPA-Axis Activity and Intestinal Gene Expression of Corticotropin Releasing Hormone Receptors 1 and 2, and Nerve Growth Factor in the Maternally Separated Rat Model of Irritable Bowel Syndrome
3.1 Abstract

**Objective:** to examine whether maternal probiotic intervention modulates neonatal maternal separation (NS) and/or adult restraint stress (AS) induced alterations in HPA-axis activity and ileal gene expression of corticotropin releasing hormone receptors 1 and 2, (CRH-R1 and CRH-R2) and nerve growth factor (NGF).

**Design:** Dams had free access to drinking water supplemented with *Bifidobacterium animalis* subsp *lactis* BB-12® (3×10⁹ CFU/mL) and *Propionibacterium jensenii* 702 (8.0×10⁸ CFU/mL) from 10 days before conception until and including weaning day (postnatal day [PND] 22), or control ad lib water. Neonates were subjected to NS from PND 2 to 14 or left undisturbed. From PND 83 to 85, animals underwent 30 min/day AS, or were left undisturbed as controls. On PND 24 and 86, saphenous blood samples were collected for corticosterone and ACTH measurement. Animals were then euthanised and ileal tissue samples were collected to determine mRNA expression of CRH-R1, CRH-R2 and NGF.

**Results:** Neonatal separation significantly increased ACTH levels compared to non-separated animals. AS exposure significantly increased ACTH and corticosterone levels compared to those of non-AS animals. Exposure to combined NS and AS did not appear to affect ACTH and corticosterone levels. Sexually dimorphic effects were observed with adult females exhibiting significantly higher corticosterone levels compared to males (p ≤ 0.05). Maternal probiotic intervention significantly elevated neonatal corticosterone levels which persisted until at least PND 86 in female adults, and also resulted in increased adult ACTH levels.

Ileal CRH-R1 mRNA expression was up-regulated in NS pups and this increase persisted until at least PND 86 in female adults. While none of the stress paradigms (NS
and/or AS) altered CRH-R1 mRNA expression in adult males, significant overexpression of this receptor was observed in both NS and non-NS adult females exposed to AS. Maternal probiotic intervention was associated with increased CRH-R1 gene expression in non-NS pups. NS animals born to probiotic treated dams exhibited a blunted CRH-R1 gene expression response. In adulthood moderate reductions in CRH-R1 gene expression response were observed in females exposed to NS or AS in the probiotic subset. Maternal probiotic intervention however was associated with an underexpression of CRH-R1 gene of females in response to combined NS and AS. An exaggerated ileal CRH-R1 gene expression was observed in males post AS.

NS did not alter ileal CRH-R2 mRNA expression in pups. In adulthood overexpressions were observed in NS rats and all animals exposed to AS. A blunted expression response however was observed in NS animals post AS. Maternal probiotic intervention induced an over- and under-expression in non-NS female pups and NS male pups respectively. In adulthood maternal probiotic intervention was associated with mainly over- and under-expression, with the exception of NS+AS males who displayed a normalised expression.

NS also did not alter ileal NGF mRNA expression in pups. In adulthood overexpressions were observed in females exposed to NS only and all males exposed to AS. Maternal probiotic intervention was associated with an overexpression in both NS and non-NS female pups and an underexpression in NS male pups. In adulthood, maternal probiotic intervention was associated with normalisation of ileal NGF mRNA expression in NS females but an overexpression in NS males.

**Conclusion:** Maternal probiotic intervention induced activation of neonatal stress pathways and exacerbated HPA-axis response to the adult stress. Maternal probiotic
intervention was also associated with a moderation of stress-induced overexpression of ileal CRH-R1 mRNA, while mixed effects including enhancement or suppression of stress-induced expression of CRH-R2 and NGF mRNA depending on sex. These findings suggest that maternal probiotic intervention has a significant impact on the HPA-axis and intestinal endocrine-related gene expression profiles which appears to synergise or antagonise with that of NS or AS.
3.2 Introduction

Early life stress has been reported to be associated with alterations to the development of the hypothalamic–pituitary–adrenal (HPA)-axis; a neuroendocrine system which is involved in the regulation of normal stress responses in vertebrates. Specifically, early postnatal stress provokes alteration to the HPA-axis function characterised by hyper-responsiveness to subsequent stress and long-term hyper-secretion of glucocorticoids (Murgatroyd, et al., 2009). This concept has been applied to the ontogeny of a variety of disorders later in life including functional gastro-intestinal disorders (FGIDs) such as irritable bowel syndrome (IBS) (Gareau, et al., 2008; S. M. O'Mahony, et al., 2011).

Neonatal maternal separation (NS) in rodents is a well-established model of early life stress. NS mimics the cardinal features of human IBS (Barreau, Ferrier, et al., 2007; S. M. O'Mahony, et al., 2011). Rats exposed to NS exhibited significantly elevated corticosterone levels both in basal conditions and in response to a subsequent acute stress compared to the control animals (Barreau, Cartier, et al., 2007; Gareau, Jury, MacQueen, et al., 2007; S. M. O'Mahony, et al., 2009). NS exposure was also associated with gut morphological alterations (O'Malley, Julio-Pieper, Gibney, Dinan, & Cryan, 2010) and dysfunctions (Hyland, et al., 2009; S. M. O'Mahony, et al., 2009) which might be exacerbated by subsequent exposure to an acute stressor (O'Malley, Julio-Pieper, Gibney, Dinan, et al., 2010).

It has been suggested that corticotropin releasing hormone (CRH) which primarily coordinates the body’s overall response to stress (Bale & Vale, 2004; Mayer & Fanselow, 2003), also mediates stress-related abnormalities in gastrointestinal (GI) function (Steckler & Dautzenberg, 2006; C. L. Williams, Peterson, Villar, & Burks, 1987). Physiological effects of CRH are exerted through activation of the G-protein
coupled CRH receptors 1 and 2 (CRH-R1 and CRH-R2) (Chen, et al., 1993; Kostich, et al., 1998).

Currently, there are only a limited number of studies examining the involvement of CRH receptors in human intestinal mucosal function. An in vitro study on human colonic biopsies has shown that CRH regulates macromolecular permeability via CRH-R1 and CRH-R2 of sub-epithelial mast cells (Wallon, et al., 2008). Animal studies have reported the presence of CRH-R1 in the enteric nervous system (ENS) and the colonic mucosa (Chatzaki, et al., 2004; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010; Yuan, et al., 2007) where it likely mediates stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). CRH-R2 has been also found in the colonic mucosa (J. Chang, et al., 2007; Chatzaki, et al., 2004; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010) and in the ENS (Lakshmanan, et al., 2008; Porcher, et al., 2005). CRH-R2 has been associated with prevention of gastric emptying, suppression of stimulated colonic motor function and protection against the hypersensitivity to colorectal distension (CRD) (Martinez, et al., 2002; Million, et al., 2005; Million, et al., 2006). CRH-R2 has been also suggested to play a role in stress-induced permeability dysfunction and the modulation of colonic mucosal immune and inflammatory responses (Alonso, et al., 2008; Barreau, Ferrier, et al., 2004; Teitelbaum, et al., 2008). Neonatal maternal separation has been reported to increase activation of colonic CRH-R1 and CRH-R2 positive cells in rats. Stress-induced alteration in CRH receptor expression was found to be segment dependent. For instance, expression of CRH-R1 and CRH-R2 in the proximal colon was not affected by NS and/or a subsequent exposure to the open field (OF), while distal CRH-R1 and CRH-R2 levels increased in NS rats but significantly reduced after OF exposure. Non-NS rats exposed to CRD
exhibited decreases in the proximal and distal colonic CRH-R1 expression, whereas this was blunted in rats exposed to combined NS and CRD. CRD also increased the functional isoform of CRH-R2 in the distal colon of NS rats (O’Malley, Dinan, & Cryan, 2010).

While most of the research has focussed on CRH/CRH receptors expression and function in the colon, there are only a few reports of such an expression and function occurring in the small intestine. Furthermore there is little or no information regarding effects of stress on CRH receptors gene expression in the small intestine. CRH-R1 and CRH-R2 immunoreactivities (IR) have been detected in ileal myenteric neuronal plexus (MNP) and submucosal neuronal plexus (SNP) of rats (Porcher, et al., 2005) and guinea-pigs (S. Liu, et al., 2006), as well as in nerve fibres of rat ileal muscle layers and mucosal cells (Porcher, et al., 2005). CRH IR was also detected in rat ileal SNP and lamina propria (LP) immune cells and Paneth cells, but not in epithelial cells (la Fleur, Wick, Idumalla, Grady, & Bhargava, 2005). In mice, CRH-R1 mRNA expression has been detected in ileal LP and epithelial cells, while CRH-R2 mRNA expression has only been found in a few cells of LP (Wlk, et al., 2002). An in vitro study has suggested that small intestinal CRH receptors play an important role in the regulation of neural control of small intestinal motility. This study also demonstrated that CRH-like peptides enhance duodenal contractile activity through CRH-R1 receptors while they inhibite ileal phasic contractions through CRH-R2 receptors (Porcher, et al., 2005). Neonatal maternal separation has been reported to induce altered distribution and depletion of duodenal secretory cells including paneth and goblet cells. These alterations were associated with duodenal increased CRH-R2 mRNA but decreased CRH-R1 expression. This study also showed that CRH-R1 and CRH-R2 were involved respectively in the
hyperplasia of duodenal endocrine cells and in the depletion of Paneth cells (Estienne, et al., 2010).

It has been recognised that nerve growth factor (NGF) is another important mediator of stress-induced GI alterations. Previous research has also shown that NS exposure in rats increases NGF levels in CNS (Cirulli, et al., 1998). NGF plays a role in the genesis of NS-induced gut dysfunction (Barreau, Cartier, et al., 2004). Neutralisation of NGF prior to exposure of rat pups to NS suppressed the adverse effects of the stress on visceral responsiveness to rectal distension and gut permeability (Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007). NS rats also exhibited increased colonic NGF mRNA and protein expression (Barreau, Cartier, et al., 2004). It has been reported that CRH enhances NGF secretion from the colonic mast cells of NS adult rats via the CRH-R1 receptor. This was associated with increased gut paracellular permeability (Barreau, Cartier, et al., 2007). Although these studies focused on colonic NGF expression and function, it is possible that this may also be the case for the small intestine.

Previous research on human and animal models of IBS has shown that probiotic intake could be an effective treatment approach in improving some symptoms and normalising the bowel movement frequency. Neonatal probiotic intervention in the maternally separated rat model of IBS has been reported as a potential prophylaxis against the unfavourable imprinting induced on the brain-gut axis by NS in rats. Neonatal probiotic intervention has been shown to normalise NS-induced elevated serum corticosterone levels (Gareau, Jury, MacQueen, et al., 2007). It is not as yet known however, whether maternal supplementary probiotics may also contribute to the impact on HPA-axis activity and mRNA expression of intestinal CRH receptors and NgF expression in stressed neonates, if these possible improvements persist into adulthood, or how this effect may be mediated.
Previous research has shown that maternal introduction of probiotics not only results in colonisation of the neonatal gastrointestinal tract in animal models (Buddington, et al.) and humans (Gueimonde, et al., 2006; Schultz, et al., 2004; Vanderhoof, et al., 1999), but also causes substantial alterations in the offspring’s gut microflora (Gueimonde, et al., 2006). On the other hand, studies by Sudo and co-workers (Sudo, 2006 ; Sudo, et al., 2004) have shown that postnatal microbial colonisation programs the HPA stress response. Therefore, it could be expected that maternal probiotics intake could prevent or at least attenuate the adverse outcomes of neonatal stress on HPA axis activity.

Although the effect of probiotics on gene expression of intestinal CRH receptors and NGF has not been previously reported, a recent study has shown that pre-treatment (oral administration) of female rats with probiotic Lb. farciminis for two weeks protected animals against increases in hypothalamic CRH mRNA expression and CRH positive cells in the PVN induced by partial restraint stress (Ait-Belgnaoui, et al., 2012). It has been reported that commensal bacteria stimulate both CRH protein and CRH mRNA expression in a mouse dendritic cell culture (Hojo, et al., 2011). On the other hand, it has been shown that CRH/CRH receptors gene expression is sensitive to bacterial metabolites such as Clostridium difficile toxin A as indicated by upregulations of ileal mRNA expression of CRH receptors 1 and 2 in mice (Kokkotou, et al., 2006; Wlk, et al., 2002) and CRH mRNA expression in both rats and mice (la Fleur, et al., 2005; Wlk, et al., 2002). Therefore it is possible that probiotics impact ileal mRNA expression of CRH receptors 1 and 2 and NGF.

Based on the available literature, this investigation aimed to address, in particular, the following hypotheses:
1. That HPA-axis activity would be enhanced by exposure of neonates to neonatal maternal separation and this effect would be further exacerbated by exposure to a subsequent acute stressor, restraint stress, in adulthood.

2. That maternal probiotic intervention would protect animals against stress-induced alterations in HPA-axis activity as indicated by attenuated corticosterone and ACTH responses.

3. That ileal mRNA expression of CRH-R1, CRH-R2 and NGF would be increased by exposure of neonates to neonatal maternal separation and a subsequent acute stressor.

4. That maternal probiotic intervention would normalise stress-induced alterations in ileal mRNA expression of CRH-R1, CRH-R2 and NGF.
3.3 Methods and Materials

All general methods are outlined in sections 2.1.1 to 2.1.7 in Chapter II. Methods for specific experiments are also outlined in Chapter II: Corticosterone and ACTH Assays (Section 2.2.1); Ileal Tissue collection (Section 2.2.4.1); RNA Isolation and Reverse Transcription into Complementary DNA (Sections 2.2.4.2 and 2.2.4.4); Real-Time PCR Quantification of Ileal CRH-R1, CRH-R2 and NGF MUC2 mRNA Expression (Section 2.2.4.7). Data were analysed as previously described in section 2.3 in Chapter II.
3.4 Results

3.4.1 ACTH

The model indicated a significant main effect of NS, $F(1, 172) = 5.06, p < 0.026$, and a significant interaction between adult stress (AS) and Probiotic, $F(2, 172) = 5.92, p < 0.003$. ACTH concentrations significantly increased in NS animals compared to non-NS animals ($p \leq 0.05$) (Figure 3.1A). In adulthood (week 12), animals born to both vehicle and probiotic-treated dams exhibited significantly increased plasma ACTH levels when subjected to adult stress compared with non-AS animals ($p \leq 0.05$ in both cases) (Figure 3.1B). Furthermore, maternal probiotic intervention appeared to exacerbate non-AS and AS-induced ACTH response (Figure 3.1B).
**Figure 3.** Effect of maternal probiotic intervention and stress on ACTH levels

A) Effect of neonatal maternal separation (NS) on natural log transformed plasma ACTH concentrations [Ln(ACTH+1), means + SE]. Since some ACTH values were less than 1.0, to avoid dealing with negative numbers, arbitrary constant 1 (one) was added to the entire data set and then Ln transformed. The filled bar represents neonatally separated animals (NS, n = 111) and the hollow bar represents non-separated animals (NNS, n = 105). An asterisk (*) indicates statistical significant difference ($p \leq 0.05$).
B) Effect of adult restraint stress (AS) exposure and maternal probiotic intervention on natural log transformed plasma ACTH concentrations [Ln(ACTH+1), means + SE]. Hollow bars represent animals exposed to no-stress in adulthood (NAS) born to vehicle- (n = 39) or probiotic-treated (n = 35) dams. Filled bars represent AS animals born to vehicle- (n = 40) or probiotic-treated (n = 36) dams. Different symbols indicate statistical significance, $p \leq 0.05$. 
3.4.2 Corticosterone

The model indicated a significant 2-way interaction between AS, maternal probiotics and sex [AS*Probiotic, $F(2, 184) = 20.55, p < 0.001$; AS*Sex, $F(2, 184) = 6.27, p < 0.002$; Probiotic*Sex, $F(1, 184) = 6.71, p < 0.01$]. No significant difference was observed between sexes at PND 24, thus corticosterone concentrations were collapsed across sex. Pups born to probiotic-treated mothers exhibited significantly higher corticosterone levels compared to pups born to vehicle-treated mothers ($p \leq 0.05$) (Figure 3.2). In adulthood, females born to probiotic- and vehicle-treated mothers displayed significantly greater corticosterone levels compared to their male counterparts born to probiotic and non-probiotic treated dams ($p \leq 0.05$ in both cases) (Figure 3.3A). While no difference was observed between males in the vehicle and probiotic subsets, females born to probiotic-treated mothers displayed significantly higher levels of corticosterone compared to females born to vehicle-treated mothers ($p \leq 0.05$). Females exposed to AS or no-AS (NAS) also exhibited significantly higher levels of corticosterone compared to AS and NAS males respectively ($p \leq 0.05$ in both cases) (Figure 3.3C). Moreover, both males and females exposed to AS showed significant greater levels of corticosterone compared to their respective non-AS animals ($p \leq 0.05$ in both cases) (Figure 3.3C). AS exposure of animals born to either vehicle- or probiotic-treated dams significantly increased corticosterone concentrations relative to non-AS animals in vehicle and probiotic subsets ($p \leq 0.05$ in both cases) (Figure 3.3B).
Figure 3.2 Effect of maternal probiotic intervention on neonatal corticosterone levels

Effect of maternal probiotic intervention on natural log (Ln) transformed plasma corticosterone concentrations (LnCort, means + SE) at PND 24. The filled bar represents animals born to probiotic-treated dams (n = 40) and the hollow bar represents animals born to vehicle-treated dams (n = 45). An asterisk (*) indicates statistical significant difference (p ≤ 0.05).
Figure 3. Effect of maternal probiotics, stress and gender on adult corticosterone levels. A) Effect of maternal probiotic intervention and sex on natural log (Ln) transformed plasma corticosterone concentrations (LnCort, means + SE) in adulthood (week 12). Hollow bars represent males: male vehicle (n = 38), male probiotic (n = 33). Filled bars represent females: female vehicle (n = 40), female probiotic (n = 38). B) Effect of maternal probiotic intervention and adult restraint stress on Ln-transformed plasma corticosterone concentrations (LnCort, means + SE) in adulthood (week 12).
Hollow bars represent animals exposed to no-stress in adulthood (NAS): NAS vehicle (n = 39), NAS probiotic (n = 35). Filled bars represent animals exposed to stress in adulthood (AS): AS vehicle (n = 39), AS probiotic (n = 36). C) Effect of adult restraint stress and sex on Ln-transformed plasma corticosterone concentrations (LnCort, means + SE) in adulthood (week 12). Hollow bars represent males: NAS male (n = 35), AS males (n = 36). Filled bars represent females: NAS females (n = 39), AS females (n = 39). An asterisk (*) indicates statistical significant difference (p ≤ 0.05).
3.4.3 CRH-R1 mRNA Expression

A significant 4-way interaction between maternal probiotic intervention, sex, NS and AS was observed for ileal CRH-R1 mRNA expression, $F(2, 44) = 5.05, \ p < 0.0105$.

Planned comparisons revealed both NS-exposed male and female pups (PND 24) born to vehicle-treated dams to exhibit a significantly increased mRNA expression of ileal CRH-R1 compared to their respective non-NS male and female pups born to vehicle treated dams ($p \leq 0.05$) (see Figure 3.4). In addition, sexually dimorphic effects were observed with NS females exhibiting significantly higher CRH-R1 mRNA expression compared to their male counterparts ($p \leq 0.05$). Such increases were also observed in non-NS pups born to probiotic-treated dams compared to non-NS animals in the vehicle subset ($p \leq 0.05$). Interestingly, pups born to probiotic-treated dams and exposed to NS displayed significantly decreased CRH-R1 gene expression compared to both the above animals exhibiting increased CRH-R1 gene expression; however the expression was not restored to the baseline levels in non-NS pups born to vehicle-treated dams.

In adulthood (week 12), in general, the data revealed little if any difference between the results obtained from males exposed to stress paradigms (NS and/or AS) and/or maternally treated with probiotics and that of control males (no-stress animals born to vehicle-treated dams), see Figure 3.5. CRH-R1 gene expression was significantly higher in non-probiotic females exposed to NS or AS relative to non-stress females in the vehicle subset ($p \leq 0.05$). Females exposed to NS combined with AS exhibited significantly less CRH-R1 mRNA expression than females exposed to either NS or AS ($p \leq 0.05$), but still significantly higher than that of non-stress females ($p \leq 0.05$). By comparison, significant reductions in the CRH-R1 mRNA expression were apparent in stressed (NS and/or AS) adult females born to probiotic-treated dams compared with
their respective animals in the vehicle subset ($p \leq 0.05$). In particular, mRNA expression of CRH-R1 in NS+AS females born to probiotic treated dams was found to be significantly less than that of control females (non-stress and non-probiotic) levels ($p \leq 0.05$).
**Figure 3.** Effect of Neonatal maternal separation (NS), sex and maternal probiotic intervention on ileal mRNA expression of CRH-R1 (RQ, means + SE) at PND 24. The filled bar represents female animals (n = 10) and the hollow bar represents male animals (n = 10). An asterisk (*) and number sign (#) respectively indicate statistical significant difference (p ≤ 0.05) relative to male and female animals not exposed to NS and born to vehicle-treated dams. Solid lines joining two bars indicate a significant difference (p ≤ 0.05).
Figure 3. Effect of Neonatal maternal separation (NS), sex, maternal probiotic intervention and adult restraint stress (AS) on ileal mRNA expression of CRH-R1 (RQ, means + SE) at PND 86. The hollow bar represents male animals (M, n = 10), and the filled bar represents female animals (F, n = 10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining males and females in the same treatment group indicate a significant difference ($p \leq 0.05$).
3.4.4 CRH-R2 mRNA Expression

The model indicated a significant 4-way interaction between maternal probiotic intervention, sex, NS and AS, \( F(2, 44) = 421.27, p < 0.0001 \). At PND 24, no significant difference was observed in CRH-R2 mRNA expression between NS and non-NS pups born to vehicle-treated dams. A significant increase was observed in non-NS female pups born to probiotic-treated dams relative to non-NS females in the vehicle subset \( (p \leq 0.05) \). NS males born to probiotic-treated dams displayed dramatically decreased gene expression to well below the control levels \( (p \leq 0.05) \), see Figure 3.6. In adulthood (week 12), CRH-R2 mRNA expression was significantly higher in the maternally vehicle-treated animals exposed to NS and/or AS relative to non-stress animals in the vehicle subset \( (p \leq 0.05) \), see Figure 3.7. The highest increase in CRH-R2 mRNA expression was observed in animals exposed to only NS (~2 and ~3 folds for males and females respectively). Gender-dependent differences were also observed with females displaying significantly higher NS- and lower NS+AS-induced CRH-R2 mRNA expression compared to their male counterparts \( (p \leq 0.05 \) in both cases). In the probiotic subset, CRH-R2 mRNA expression significantly increased in non-stressed, NS and AS males compared with non-stressed males in the vehicle subset. CRH-R2 mRNA expression level was normalised in NS+AS males born to probiotic treated dams. While non-stressed and AS females exhibited significantly higher CRH-R2 mRNA expression, females exposed to NS either alone or in combination with AS displayed significantly lower CRH-R2 mRNA expression levels than that of non-stressed females in the vehicle subset \( (p \leq 0.05 \) in all cases).
**Figure 3.** Effect of Neonatal maternal separation (NS), sex and maternal probiotic intervention on ileal mRNA expression of CRH-R2 (RQ, means + SE) at PND 24. The filled bar represents female animals (n = 10) and the hollow bar represents male animals (n = 10). An asterisk (*) and number sign (#) respectively indicate statistical significant difference (p ≤ 0.05) relative to male and female animals not exposed to NS and born to vehicle-treated dams. Solid lines joining two bars indicate a significant difference (p ≤ 0.05).
Figure 3. Effect of Neonatal maternal separation (NS), sex, maternal probiotic intervention and adult restraint stress (AS) on ileal mRNA expression of CRH-R2 (RQ, means + SE) at PND 86. The hollow bar represents male animals (M, n = 10), and the filled bar represents female animals (F, n = 10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining males and females in the same treatment group indicate a significant difference ($p \leq 0.05$).
3.4.5 NGF mRNA Expression

The model indicated a significant 4-way interaction between maternal probiotic intervention, sex, NS and AS, $F (5, 44) = 3.32, p < 0.0125$. At PND 24, no significant difference was observed in mRNA expression of NGF between NS and non-NS pups born to vehicle-treated dams. In the probiotic subset however, a significant increase was observed in both non-NS and NS females born to probiotic-treated dams relative to non-stressed females in the vehicle subset ($p \leq 0.05$). Moreover, NS males born to probiotic-treated dams exhibited a significant decrease in mRNA expression of NGF compared with the control levels ($p \leq 0.05$), see Figure 3.8. In adulthood (week 12), while exposure of females to AS either alone or in combination with NS did not change NGF mRNA expression in the vehicle subset, females exposed to only NS exhibited a significant increased gene expression compared with non-stressed females born to vehicle-treated dams ($p \leq 0.05$), see Figure 3.9. The increased NGF mRNA expression in NS females was normalised in the probiotic subset. Males exposed to AS either alone or in combination with NS in both vehicle and probiotic subsets displayed comparable significantly increased NGF mRNA expression compared to non-stressed males born to vehicle-treated dams ($p \leq 0.05$). In addition, NS exposure significantly increased NGF mRNA expression in males born to probiotic-treated dams compared to non-stressed males in the vehicle-treated dams ($p \leq 0.05$).
Figure 3. Effect of Neonatal maternal separation (NS), sex and maternal probiotic intervention on ileal mRNA expression of NGF (RQ, means + SE) at PND 24. The filled bar represents female animals (n = 10) and the hollow bar represents male animals (n = 10). An asterisk (*) and number sign (#) respectively indicate statistical significant difference (p ≤ 0.05) relative to male and female animals not exposed to NS and born to vehicle-treated dams. Solid lines joining two bars indicate a significant difference (p ≤ 0.05).
Figure 3.9 Effect of Neonatal maternal separation (NS), sex, maternal probiotic intervention and adult restraint stress (AS) on ileal mRNA expression of NGF (RQ, means + SE) at PND 86. The hollow bar represents male animals (M, n = 10), and the filled bar represents female animals (F, n = 10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining males and females in the same treatment group indicate a significant difference ($p \leq 0.05$).
3.5 Discussion

This study aimed to examine the impact of maternal probiotic intervention on HPA-axis activity, ileal mRNA expression of CRH-R1, CRH-R2 and NGF alterations induced by early life and/or subsequent adult stress in Wistar rats. Previous research has shown that exposure of rats to neonatal maternal separation predisposes adult animals to brain-gut axis dysfunction in response to a subsequent adult stress (Coutinho, et al., 2002; S. M. O'Mahony, et al., 2009; Soderholm, et al., 2002; Welting, et al., 2005). Essentially these stress models mimic to some extent that which is proposed to account for IBS i.e., stress in early life combined with stress in later life. This can be referred to as a ‘Stress Diathesis’ model in which priming by early stress is compounded by stress in later life. In this study acute restraint stress in adulthood was used as the stress in later life due to its adverse effects on brain-gut functioning in rodents (Israeli, et al., 2008; Julio-Pieper, et al., 2012; Santos, et al., 1999; Sudo, et al., 2004).

The data presented here provided evidence of altered HPA-axis activity, and ileal mRNA expression of CRH-R1, CRH-R2 and NGF induced by neonatal maternal separation and/or adult restraint stress. Maternal probiotic intake was associated with activation of neonatal stress pathways which persisted into adulthood, and it appeared to cause alterations in basal and stress-induced ileal CRH-R1, CRH-R2 and NGF gene expression.

3.5.1 HPA axis activity

The current study demonstrates that neonatal maternal separation induced alterations in ACTH but not corticosterone responses. Neonatal maternal separation-induced ACTH elevations have previously been reported in the literature (Liebl, et al., 2009).
Differential ACTH and corticosterone response profiles have also been previously reported. The lack of comparable changes in both corticosterone and ACTH is most likely due to the inability to optimise the timing of blood collection, due to experimental constraints, for both measures (Walker, et al., 2009a). The reason for the lack of neonatal corticosterone response could also be due to the 10-day time lag between cessation of neonatal maternal separation exposure and blood collection. A previous study reporting differences in corticosterone measured circulating corticosterone levels one day after completion of exposure of Sprague-Dawley rats to neonatal maternal separation (Gareau, Jury, MacQueen, et al., 2007). It has been also reported that the corticosterone level is maximal at day 20 (separation between PND 4-19) and the increase persists for 10 days. However corticosterone levels were lower than that of PND 20 (Gareau, et al., 2006). These contrary findings are most likely due to differences in strain and neonatal separation protocols.

In support of previous research, the present study found that exposure of animals to adult stress elevated both ACTH and corticosterone levels (Shanks, Larocque, & Meaney, 1995; Sudo, et al., 2004). Gender-dependent differences were also observed with females displaying significantly higher basal and adult stress corticosterone levels compared to their male counterparts. This is consistent with previous research (Doremus-Fitzwater, Varlinskaya, & Spear, 2009; Galea, et al., 1997; Y. L. Lin, Lin, & Wang, 2012).

The data therefore provides evidence to support the hypothesis that the HPA-axis activity would be enhanced by exposure of neonates to neonatal maternal separation. However the findings were not supportive of the hypothesis that the effect of neonatal maternal separation would be further exacerbated by exposure to a subsequent acute stressor.
Unexpectedly we observed an increase in corticosterone levels of pups born to probiotic- treated dams which persisted into adulthood, but only for females. Maternal probiotic intervention was also associated with increases in ACTH levels of adult animals. This is an interesting finding, demonstrating maternal probiotic intervention to be capable of inducing long-lasting hyperactivity of the HPA-axis. While research has not yet investigated potential adverse effects of maternal introduction of probiotic bacteria on HPA-axis activity, previous studies have shown such an increase in basal corticosterone levels of adult rats born to dams challenged with Gram negative bacterial LPS (Reul, et al., 1994).

The findings were therefore largely unsupportive of the hypothesis that maternal probiotic intervention would protect animals against stress-induced alterations in HPA-axis activity.

### 3.5.2 Intestinal Gene Expression Profiles

The results suggest, for the first time, that ileal CRH-R1 mRNA expression is up-regulated in neonatally stressed rat pups and this increase persists in female adults. While none of stress paradigms (early and/or later life) altered CRH-R1 mRNA expression in adult males, overexpressions were observed in both neonatally stressed and non-stressed adult females exposed to adult stress.

Pronounced gender-dependent differences of neonatal stress (Barna, et al., 2003; Oreland, et al., 2009; Renard, Rivarola, & Suarez, 2010; Rosztoczy, et al., 2003) or adult stress-induced effects (Doremus-Fitzwater, et al., 2009; Galea, et al., 1997; Y. L. Lin, et al., 2012) have been previously reported. This is of particular relevance to IBS where the incidence is greater for females compared to males (Cain, et al., 2009).
Although there are only a few studies addressing the possible effects of stress on CRH-R1 mRNA expression in the small intestine, most of the research has focussed on the effect of stress on the colon. Previous research has demonstrated overexpression of colonic CRH-R1 mRNA in maternally separated rats (O'Malley, Dinan, et al., 2010; Schwetz, et al., 2005). CRH-R1 mediates stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). CRH-R1 immunoreactivities (IR) have been also detected in the ileum of rats (Porcher, et al., 2005), mice (Wlk, et al., 2002) and guinea-pigs (S. Liu, et al., 2006). It has been shown that small intestinal CRH-R1 plays an important role in the regulation of duodenal contractile activity (Porcher, et al., 2005). Neonatal maternal separation has been associated with altered distribution and depletion of duodenal secretory cells including paneth and goblet cells. These alterations were associated with duodenal decreased CRH-R1 expression. This study also demonstrated that CRH-R1 is involved in the hyperplasia of duodenal endocrine cells (Estienne, et al., 2010). It has been recently reported that CRH receptors are also involved in CRH-mediated intestinal barrier dysfunction such as ileal paracellular permeability in porcine ileum (Overman, Rivier, & Moeser, 2012). Therefore it could be expected that stress-induced ileal alterations in the expression of CRH-R1 expression may be involved in alterations in ileal functions such as motility, permeability and secretory state. However this needs to be further investigated.

The current study also demonstrated that exposure to adult stress induces different effects on CRH-R1 gene expression in non-NS and NS females. Low ileal CRH-R1 gene expression in not neonatally stressed rats was increased by exposure to the later life stressor, whereas higher expression in the neonatally stressed females declined post adult stress. Such divergent effects have been previously reported in colonic CRH-R1
gene expression of non-NS and NS male rats exposed to a later acute stressor, the open field (OF) (O'Malley, Dinan, et al., 2010). Therefore our data suggests that early life stress-induced perturbations may induce dysfunctional CRH-R1 mediated stress responses in the ileum.

Surprisingly, perinatal maternal probiotic intervention was associated with increased CRH-R1 gene expression in non-stressed pups, resembling that of neonatally stressed pups in the vehicle subset. In addition, neonatally stressed animals born to probiotic-treated dams did not reflect their vehicle counterparts and instead exhibited a blunted CRH-R1 gene expression response. Therefore it is suggested that perinatal maternal microbial exposure even with apparently beneficial bacteria may provoke CRH-R1 gene expression mediated stress responses. In adulthood however there was a moderate reduction in CRH-R1 gene expression response of females born to probiotic treated dams to early or later life stresses. While this moderating effect could be considered as a positive effect, maternal probiotic intervention is accompanied by a significantly compromised ileal CRH-R1 gene expression of females in response to combined early and later life stressors. An exaggerated ileal CRH-R1 gene expression was observed in males post adult stress. Overall our findings provide evidence that perinatal maternal probiotic intervention induces overexpression of ileal CRH-R1 mRNA in rat neonates. In addition it was associated with moderation of stress-induced overexpression of CRH-R1 mRNA in adult females. This means that maternal probiotic intervention, to some extent, protects against adverse effects of early or later life stress on ileal CRH-R1 mRNA expression.

Regarding ileal CRH-R2 mRNA expression, maternal probiotic intervention was associated with altered ileal mRNA expression of CRH-R2 including over- and under-expression in non-stressed female pups and neonatally stressed male pups respectively.

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In adulthood a stress-induced overexpression was observed in maternally separated rats, in addition to an overexpression post adult stress. Neonatally stressed animals exposed to adult stress however demonstrated a blunted expression response. Maternal probiotic intervention in adulthood was associated with different effects including mainly over and underexpression, and rarely normalisation of ileal CRH-R2 mRNA expression. Previous research has shown that CRH-R2 is expressed in the rat ileum and that ileal CRH-R2 plays an important role in the control of small intestinal motility as indicated by inhibition of ileal phasic contractions (Porcher, et al., 2005). Therefore alterations in CRH-R2 mRNA expression might disturb small intestinal motility.

Regarding ileal NGF mRNA expression, maternal probiotic intervention was associated with alterations in neonatal NGF mRNA expression including an overexpression in female pups irrespective of neonatal stress and an underexpression in neonatally stressed male pups.

In adulthood (week 12), overexpressions of ileal NGF mRNA were observed in females exposed to only neonatal stress and males exposed to adult stress irrespective of neonatal stress. Maternal probiotic intervention was only associated with normalisation of ileal NGF mRNA expression in neonatally stressed females and no effect in other cases. Maternal probiotic intervention also caused an overexpression in neonatally stressed males.

There is little or no data regarding stress-induced NGF expression and the function in ileum. A study by Belai et al. (1992) has shown that NGF regulates the expression of ileal neuropeptides such as vasoactive intestinal peptide (VIP). VIP is a key neuromediator which is involved in the regulation of mucosal functions as indicated by induction of colonic vasodilatation, modulation of ion secretion and mucin release, and
control of paracellular permeability and intestinal epithelial cell proliferation (Toumi, et al., 2004). It has been reported that psychological stress elevates VIP concentrations in the small intestine of mice (Cao, Wu, Han, & Wang, 2005). Therefore it seems that NGF may be involved in ileal functions through other mediators such as VIP. However this needs to be further investigated. No studies are available regarding ileal NGF mRNA expression. There are few studies focussed on the effects of early life stress on colonic NGF expression and function where neonatal maternal separation was shown to increase colonic NGF mRNA and protein expression in rats (Barreau, Cartier, et al., 2004). NGF has been reported to play a role in stress-induced visceral responsiveness to later acute stressor and gut permeability (Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007). Increased NGF secretion from colonic mast cells of neonatally separated adult rats was shown to be facilitated via CRH/CRH-R1 receptor system. The increase was associated with increased gut paracellular permeability (Barreau, Cartier, et al., 2007). Therefore it could be expected that changes in NGF mRNA expression may be involved in ileal function.

In general, our findings were supportive of the hypothesis that ‘ileal mRNA expression of CRH-R1, CRH-R2 and NGF would be altered by exposure of neonates to neonatal maternal separation and subsequent acute stressor’. However the data clearly did not support the hypothesis that maternal probiotic intervention would protect against stress-induced alterations in ileal mRNA expression of CRH-R1, CRH-R2 and NGF.
3.6 Conclusion

This study does raise concerns about the perinatal use of probiotics given the evidence reported here that they are capable of activating stress pathways and inducing alterations (over- or under-expression) of ileal CRH-R1, CRH-R2 and NGF mRNA. It is possible that probiotics are perceived as an immune challenge inducing disturbances in HPA-axis activity and intestinal expression of neurochemicals and their receptors. Underlying mechanisms of action however need to be further investigated. Moreover, further efforts to examine the effect of maternal probiotic intervention on the role of CRH-R1, CRH-R2 and NGF mRNA expression in ileal functions such as motility, permeability and secretory states under stress conditions are required.
Chapter IV

Effect of Maternal Probiotic Intervention on Systemic and Gut-Associated Immune Responses of Wistar Rats to Neonatal Maternal Separation and Subsequent Adult Stress
4.1 Abstract

Objective: to examine whether maternal probiotic intervention attenuates immune alterations induced by neonatal maternal separation (NS) and/or adult restraint stress (AS).

Design: Dams had free access to drinking water supplemented with *Bifidobacterium animalis* subsp *lactis* BB-12® (3×10⁹ CFU/mL) and *Propionibacterium jensenii* 702 (8.0×10⁸ CFU/mL) from 10 days before conception until weaning day (postnatal day [PND] 22), or control ad lib water. Neonates were subjected to NS from PND 2 to 14 or were left undisturbed as controls. From PND 83 to 85, animals underwent 30 min/day AS, or were set aside as controls. On PND 24 and 86, cardiac blood samples were collected for analysing plasma levels of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-6, Haptoglobin (Hp) and Immunoglobulin A (IgA). Faecal samples were also collected to determine luminal IgA.

Results: Data analysis indicated that neither the maternal separation protocol or the adult stress protocol affected levels of circulating cytokines TNF-α, IFN-γ and IL-6 in adult offspring born to vehicle-treated dams. Maternal probiotic intervention was associated with significantly decreased IFN-γ levels relative to that of non-stress animals in the vehicle subset (*p* ≤ 0.05). Probiotic administration also significantly increased IL-6 levels in neonatally stressed pups and adult animals exposed only to adult stress (*p* ≤ 0.05). Exposure to adult stress either alone or in combination with neonatal stress induced significant increases in haptoglobin levels compared to non-stress animals (*p* ≤ 0.05) in the vehicle subset, whereas administration of probiotics significantly decreased haptoglobin levels in all stressed and non-stressed animals compared with vehicle controls (*p* ≤ 0.05). Neonatal maternal separation was associated
with significant reductions in neonatal plasma circulating IgA levels which persisted into adulthood compared to non-stress animals \((p \leq 0.05)\). Maternal probiotic intervention appeared to restore plasma IgA levels of maternally separated neonates to normal and significantly increase it in maternally separated adults compared to control animals \((p \leq 0.05)\). Maternal probiotic intervention significantly increased luminal IgA levels compared to that of non-probiotic animals.

**Conclusion:** Maternal probiotic intervention induced a down-regulation of plasma IFN-\(\gamma\) and haptoglobin irrespective of stress treatments. It also up-regulated IL-6 responses to neonatal or adult stresses. Importantly however, it improved both plasma and luminal IgA responses to neonatal stress. These findings suggest that maternal probiotics produce mixed immunomodulatory effects including mainly anti-inflammatory effects and enhancement of immune defence capacity in stressed animals.
4.2 Introduction

Early life stress has been associated with alterations to the stress response system and has been suggested as a predisposing factor for the development of several disorders including functional gastrointestinal disorders such as irritable bowel syndrome (IBS) (Gareau, et al., 2008; Soderholm, et al., 2002). The major stress response system that has been extensively investigated in relation to IBS is the hypothalamic–pituitary–adrenal (HPA) axis. Dysregulation of HPA-axis has been reported in IBS patients, with events in early life as an important risk factor. Importantly, early adverse events have been associated with sensitisation of the HPA axis to stress (Murgatroyd, et al., 2009). IBS involves a dysfunctional interaction between the brain and the gut (S. M. O'Mahony, et al., 2011). The bidirectional communication between the brain and the gut is mediated by neural, immune, and endocrine pathways. Several studies have indicated that early life factors that alter neuroendocrine stress responsiveness may also influence immune function (Barreau, Ferrier, et al., 2004; S. M. O'Mahony, et al., 2009). Both the systemic and intestinal mucosal immune systems are involved in brain–gut axis communication and work in a coordinated fashion to maintain gut integrity. Gut permeability has been implicated as a factor in IBS when permeability is enhanced and luminal antigens leak out of the gut (S. M. O'Mahony, et al., 2011). When this occurs, gut permeability initiates an immune reaction, including mucosal (secretory immunoglobulins), cell mediated (macrophages and T cells), and humoral (serum immunoglobulins) immunity (Berg, 1995). Alterations in circulating cytokine levels in IBS patients have been previously reported. Elevated levels of IL-6, IL-8 and IL-6 soluble receptor (sIL-6R) but not TNF-α and IL-10 have been reported in IBS patients compared to controls (Dinan, et al., 2006). In addition to increased plasma levels of IL-6 and IL-8, a recent study by Scully et al. (2010) has demonstrated increased levels of
IL-1β and TNF-α in IBS females with extra-intestinal co-morbidities such as fibromyalgia, premenstrual dysmorphic disorder, and chronic fatigue syndrome. Liebregts et al. (2007) also found significantly higher TNF-α, IL-1β and IL-6 production by peripheral blood mononuclear cells (PBMC) in IBS patients when compared to those of healthy controls. An abnormal IL-10/IL-12 ratio, indicative of a proinflammatory, Th-1 state, was also noted in patients with IBS (L. O’Mahony, et al., 2005). At the molecular level, analysis of genotype frequencies of IL-10 using DNA extracted from peripheral blood leucocytes demonstrated significantly lower frequencies of the high producer genotype for IL-10 in IBS patients than controls (Gonsalkorale, et al., 2003). Prevalence of a high producer TNF-α and a low producer IL-10 genotype was also greater in IBS patients relative to controls (van der Veek, et al., 2005).

Apart from alterations in systemic immunity, changes in local immunity particularly in the colon have also been reported in IBS patients. An elevated frequency of peripheral blood CD4+ and CD8+ T cells expressing the gut homing receptor integrin β7 has been reported in IBS patients compared to controls (Ohman, et al., 2005). In the same study an increased number of lamina propria CD8+ T cells and a greater expression of mucosal addressing cell adhesion molecule–1+ endothelium, the ligand for integrin β7, were observed in the ascending colon of IBS patients compared with those of control subjects (Ohman, et al., 2005). An increased number of colonic mast cells and proportion of degranulating mast cells, and a higher number of mast cells in close vicinity to nerve endings have been also reported in IBS patients (Barbara, et al., 2004). An increased colonic mucosal myeloperoxidase (MPO) activity, an inflammatory marker, was observed in IBS patients as compared to healthy individuals (Kristjansson, et al., 2004).
Studies using the maternally separated (NS) rat model of IBS have also shown alterations in circulating cytokines concentrations. A significantly elevated level of TNF-α and IFN-γ was observed in Lipopolysaccharide (LPS) stimulated whole blood samples from neonatally stressed rats subjected to subsequent open field stress in adulthood compared with the control group (S. M. O'Mahony, et al., 2009). A study by Desbonnet et al. (2010) also demonstrated elevated IL-6 concentrations in the whole blood culture of adult NS rats following stimulation with concanavalin A but not LPS relative to control animals.

Gut associated immune alterations have been also reported in maternally separated rats. Neonatal maternal separation was associated with significantly elevated activity of colonic MPO (Barreau, Cartier, et al., 2004; Barreau, Ferrier, et al., 2004). Significant increases in mRNA expression of colonic cytokines IFN-γ, IL-1β, IL-2, IL-4, and IL-10 were also observed in maternally separated adult rats compared with controls (Barreau, Ferrier, et al., 2004).

Maternal administration of probiotics has recently emerged as an effective prophylactic approach to the prevention of early immune dysregulation (Blumer, et al., 2007; Rautava, et al., 2002). These studies have primarily evaluated the beneficial effects of maternal probiotics on modulation of the immune system aiming to prevent allergic disorders such as eczema and asthma in early life (Blumer, et al., 2007; Rautava, et al., 2002). Blumer et al. (2007) demonstrated that prenatal administration of *Lb. rhamnosus GG* altered the placental and neonatal cytokine expression patterns in mice derived from perinatally probiotic-treated dams compared with pups from control dams. Rautava et al. (2002) reported that administration of the same probiotic strain during pregnancy and lactation elevated the level of anti-inflammatory transforming growth factor b2 (TGF-β2) in maternal milk. This is a crucial immunoregulatory factor which is involved in
promoting secretory immunoglobulin A (sIgA) production in neonatal gut-associated immune system (Ogawa, et al., 2004). Secretory IgA acts to prevent penetration of luminal antigens through the gut mucosa (Fagarasan & Honjo, 2003).

Therefore, given that maternal probiotic administration produces a positive immune environment for the offspring, and given that we know that early life stress alters immune function, it could be expected that maternal probiotic administration may modulate immune system responses to early life stress (neonatal maternal separation) in rats.

Based on the available literature, the current study aimed to address, in particular, the following hypotheses:

1. That systemic and gut-associated immune function will be altered by exposure of neonates to neonatal maternal separation and this effect will persist.

2. That additional stress in adulthood will further exacerbate the effect on systemic and gut-associated immune function.

3. That maternal probiotic intervention will protect animals against stress-induced alterations to systemic and gut-associated immune function as indicated by attenuated immune responses.
4.3 Methods and Materials

All general methods are outlined in sections 2.1.1 to 2.1.7 in Chapter II. Methods for specific experiments are also outlined in Chapter II: Cytokines Assays (Section 2.2.2.1); Haptoglobin Assay (Section 2.2.2.2) and Plasma and Luminal IgA Assay (Section 2.2.2.3 and 2.2.2.4). Data were analysed as previously described in section 2.3 in Chapter II.
4.4 Results

4.4.1 Plasma Cytokines

4.4.1.1 Plasma TNF-α

For all samples, TNF-α values were very low with no significant differences observed between treatment groups. This indicates no notable effect of maternal probiotic intervention, NS, AS and sex on TNF-α response.

4.4.1.2 Plasma IFN-γ

A significant main effect of maternal probiotic intervention was observed for plasma IFN-γ levels, $F(2,35) = 7.57, \ p \leq 0.01$. Planned comparisons revealed animals born to probiotic-treated dams exhibited significantly lower IFN-γ levels compared to animals born to vehicle-treated dams ($p \leq 0.05$) (Figure 4.1).
Figure 4.1 Effect of maternal probiotic intervention on plasma IFN-γ levels (means + SE). The hollow bar represents animals born to vehicle-treated dams (n = 78), and the filled bar represents animals born to probiotic-treated dams (n = 110). An asterisk (*) shows significant difference ($p \leq 0.05$).
4.4.1.3 Plasma IL-6

A significant three-way interaction between maternal probiotic intervention, NS and AS was observed for the plasma IL-6 levels, $F(2,149) = 9.99, \ p < 0.0001$. At PND 24, no difference was observed between NS animals born to vehicle-treated dams or non-NS animals in the probiotic subset and non-NS animals in the vehicle subset. Animals born to probiotic-treated dams and exposed to NS exhibited significantly greater IL-6 concentrations compared to vehicle non-NS animals and all other groups ($p < 0.05$ in all cases) (see Figure 4.2). In adulthood, planned comparisons revealed no differences between treatment groups in the vehicle subset indicating no effect of NS and/or AS on plasma IL-6 levels. In the probiotic subset however, a significant increase was observed in non-NS animals exposed to AS compared with non-NS animals born to vehicle-treated dams ($p < 0.05$). IL-6 levels in this group were also significantly higher than those of animals exposed to NS either alone or in combination with AS in the probiotic subset ($p < 0.05$ in both cases). In addition, in the probiotic subset a significant reduction was observed in animals only exposed to NS compared with non-NS animals exposed or not exposed to AS ($p < 0.05$ in both cases) (see Figure 4.3).
Figure 4. Effect of neonatal maternal separation (NS) and maternal probiotic intervention on plasma IL-6 levels (means + SE) at PND 24. The hollow bar represents non-NS (NNS) animals (n = 9-14), and the filled bar represents NS animals (n = 15-17). An asterisk (*) shows significant difference compared to NNS animals in the vehicle subset (p ≤ 0.05).
Figure 4. 3 Effect of neonatal maternal separation (NS), adult restraint stress (AS) and maternal probiotic intervention on plasma IL-6 levels (means + SE) at PND 86. The hollow bar represents non-NS (NNS) animals (n = 16-17), and the filled bar represents NS animals (n = 16-20). Solid lines joining bars indicate a significant difference (p ≤ 0.05).
4.4.2 Plasma Haptoglobin

Assessment of plasma Haptoglobin revealed a significant interaction between maternal probiotic intervention, sex, NS and AS, $F(6,168) = 2.93$, $p<0.0097)$. At PND 24, Haptoglobin responses did not appear to be affected by probiotic intervention, sex and NS. In adulthood, while previous exposure to NS alone was not associated with any change in Haptoglobin levels, both sexes exposed to AS either alone or in combination with NS exhibited significantly greater Haptoglobin levels compared to their respective vehicle non-stress animals ($p<0.05$ in all cases), see Figure 4.4. A significant decrease however was observed in males exposed to combined NS and AS compared with males exposed to only AS ($p<0.05$ in all cases). Dramatic reductions were evident in Haptoglobin levels of all groups in the probiotic subset compared to their respective animals in the vehicle subset and vehicle non-stress animals ($p<0.05$ in all cases). No significant differences were observed between the groups in the probiotic subset.
Figure 4. Effect of Neonatal maternal separation (NS), sex, maternal probiotic intervention and adult restraint stress (AS) on plasma Haptoglobin levels (means + SE) at PND 86. The hollow bar represents male animals (M, n = 7-10), and the filled bar represents female animals (F, n = 9-10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining males and females in the same treatment group indicate a significant difference ($p \leq 0.05$).
4.4.3 Plasma IgA Response

A significant main effect of sex was observed with males exhibiting significantly greater plasma IgA (PIgA) levels compared to females, $F(1,179) = 7.26$, $p<0.008$ (Figure 4.5A). At PND 24, a significant NS×probiotic interaction was observed, $F(3,179) = 3.6$, $p<0.015$ (Figure 4.5B). NS pups born to vehicle-treated dams and non-NS pups born to probiotic-treated dams displayed significantly lower PIgA compared with non-NS pups born to vehicle-treated mothers ($p \leq 0.05$). There was no significant difference between NS pups in the probiotic subset and non-NS pups born to vehicle-treated mothers. In adulthood, a significant three-way interaction between NS, maternal probiotic intervention and AS was observed, $F(2,179) = 6.75$, $p<0.001$ (Figure 4.5C). Planned comparisons revealed significantly reduced PIgA levels in NS animals either with or without AS exposure in the vehicle subset ($p \leq 0.05$). However, these animals (NS and NS+AS) in the probiotic subset exhibited significant greater PIgA levels compared to control animals ($p \leq 0.05$) (Figure 4.5C).
Figure 4. 5 Effect of maternal probiotic intervention, stress, and gender on plasma IgA levels. A) Effect of sex on Ln-transformed plasma IgA concentrations (LnPIgA, means + SE) in rats. Initial IgA data were expressed as μg/mL plasma. The hollow bar represents males (n = 109) and the filled bar represents females (n = 117). An asterisk (*) indicates statistical significant difference (p ≤ 0.05). B) Effect of maternal probiotic intake and neonatal maternal separation (NS) on Ln transformed plasma IgA concentrations (LnPIgA, means + SE) in rats at PND 24. The hollow bars represent animals exposed to no-NS (NNS): NNS vehicle (n = 15), NNS probiotic (n = 19). The filled bars represent animals exposed to NS: NS vehicle (n = 19), NS probiotic (n = 20). An asterisk (*) indicates statistically significant difference relative to vehicle NNS animals (p ≤ 0.05). C) Effect of maternal probiotic intake, neonatal maternal separation (NS) and adult restraint stress (AS) on Ln-transformed plasma IgA concentrations (LnPIgA, means + SE) in adult rats. Hollow bars represent no-stress control animals or those exposed to adult stress (AS): control vehicle (n = 21), AS vehicle (n = 20), control probiotic (n = 17), AS probiotic (n = 17). Filled bars represent NS or NS+AS animals: NS vehicle (n = 20), NS+AS vehicle (n = 20), NS probiotic (n = 18), NS+AS probiotic (n = 19). An asterisk (*) shows significant difference compared to control animals in the vehicle subset (p ≤ 0.05).
4.4.4 Luminal IgA

Neither neonatal stress, adult stress or a combination of both stresses affected faecal IgA concentrations, whereas a significant main effect of maternal probiotic intervention was observed for faecal IgA concentrations. This was significantly greater for animals born to probiotic-treated dams compared to animals born to vehicle-treated dams, $F(1,38) = 10.56, p<0.002$ (Figure 4.6).

**Figure 4. 6** Effect of maternal probiotic intervention on Ln-transformed faecal IgA concentrations (LnFIgA, means + SE). Initial IgA data were expressed as μg/gr faeces. The filled bar represents animals born to probiotic-treated dams (n = 101) and the hollow bar represents animals born to vehicle-treated dams (n = 113). An asterisk (*) indicates statistical significant difference ($p \leq 0.05$).
4.5 Discussion

There is a growing body of evidence supporting the existence of a low-grade level of immune activation in individuals suffering from IBS (Clarke, et al., 2009). Studies using the maternally separated rat model of IBS have also shown alterations in both the systemic and intestinal mucosal immune systems (Barreau, Cartier, et al., 2004; Barreau, Ferrier, et al., 2004). Thus we analysed whether maternal probiotic intervention could act prophylactically to prevent the alterations to the immune system in rats subjected to maternal separation and subsequent acute stress in adulthood.

We demonstrated that neither maternal separation nor the adult stress protocol affected levels of circulating cytokines TNF-α, IFN-γ and IL-6 in offspring born to vehicle-treated dams. This is consistent with previous reports of no change in circulating TNF-α, IFN-γ and IL-6 levels as measured in the unstimulated whole blood in maternally separated rats (Desbonnet, et al., 2010; S. M. O'Mahony, et al., 2009). The study by O'Mahony, et al. (2009) demonstrated significantly elevated levels of TNF-α and IFN-γ and a trend towards an increase in IL-6 in only LPS stimulated whole blood samples from maternally separated rats with post adult stress. The study by Desbonnet et al. (2010) demonstrated elevated IL-6 concentrations in whole blood culture of adult separated rats following stimulation with concanavalin A but not LPS relative to control animals. This latter study however, failed to find significant differences in IFN-γ or TNF-α levels between neonatally stressed rats following stimulation of whole blood with either concanavalin A or LPS and control animals.

Interestingly, in the current study animals born to probiotic-treated dams displayed decreased pro-inflammatory cytokine IFN-γ levels relative to that of animals in the vehicle subset. IFN-γ has been shown to play important roles in host defence by
exerting pro-inflammatory, immunoregulatory and anti-proliferative activities (Boehm, Klamp, Groot, & Howard, 1997). IFN-γ functions mainly to promote the activity of the components of the cell-mediated immune system. Some of the most important functions of IFN-γ include stimulation of macrophage effector functions, modulation of the expression of class I and class II major histocompatibility complex (MHC) molecules, augmentation of the IL-12-induced Th1 cells differentiation, regulation of Ig class switching, regulation of leukocyte-endothelium interactions (Boehm, et al., 1997; Paludan, 1998), and implication to play a physiologic role in sensory neurons (Neumann, Schmidt, Wilharm, Behrens, & Wekerle, 1997).

Therefore IFN-γ down-regulation induced by maternal probiotic intervention may be of concern for host defence responses against intracellular antigens. In Chapter III, we demonstrated that perinatal use of probiotics is capable of activating stress pathways as indicated by increased corticosterone levels. Previous research has shown that glucocorticoids suppress IFN-γ production in vitro and in vivo in animals and humans (Chrousos, 1995) mainly through the inhibition of IL-12 production by antigen-presenting cells (APCs) and from the loss of IL-12 responsiveness in natural killer (NK) and T helper (Th)1 cells (Elenkov & Chrousos, 1999). Therefore it is possible that maternal probiotic intervention down-regulates IFN-γ through the elevation of corticosterone.

In the current study maternal probiotic intervention was also associated with amplification of circulating IL-6 responses in neonatally stressed pups and adult animals only exposed to adult stress. This demonstrates maternal probiotic intervention to be an immunological challenge capable of sensitising the immune system to neonatal or adult stress. Moreover, we found that maternal probiotic intervention combined with neonatal stress protects against exaggerated IL-6 response to adult stress. IL-6 is a potent
pleiotropic cytokine with a wide variety of biological functions. IL-6 promotes hematopoiesis and regulates the growth and differentiation of various cell types such as hematopoietic stem cells, B- and T-cells. IL-6 also plays an important role in immune responses initiated by infection or injury (Coico & Sunshine, 2009; Elgert, 2009).

As noted previously, maternal probiotic intervention was associated, in this study, with elevated corticosterone levels in early and later life. Previous research has shown that glucocorticoids down-regulate the production of IL-6 by suppressing both the transcription rates of the genes and the stability of their mRNA (Pollack & Kaplowitz, 2006). Therefore up-regulation of both corticosterone and IL-6 is an unexpected finding. An explanation for such discrepancy may lie in the possible cumulative effects of perinatal maternal exposure to probiotic bacteria and early or later life stressors.

The current study also assessed plasma haptoglobin as an inflammation-inducible plasma protein, since it has been reported to be a most sensitive and useful marker of acute inflammation in rats compared with other acute phase reactants including C-reactive protein (CRP) and fibrinogen (Fbgn) (Giffen, et al., 2003). The results indicate that adult stress either alone or in combination with neonatal maternal separation induced increases in haptoglobin levels in animals born to vehicle-treated dams. It seems that neonatal maternal separation did not affect plasma haptoglobin levels in early and later life. This is likely due to measurement of haptoglobin after 10 and 72 days of cessation of neonatal maternal separation. It has been reported that there are increases in haptoglobin during the first 24 hours after neonatal maternal separation in infant primates (Coe, Lubach, & Ershler, 1989).

Surprisingly, haptoglobin levels of all stressed and non-stressed animals born to probiotic-treated dams fell well below the control levels. Decrease in haptoglobin levels
of non-stress offspring born to probiotic-treated dams appears to be inconsistent with
the report of Fak et al. (2008) that maternal consumption of probiotic *Lactobacillus
plantarum* 299v does not change haptoglobin levels in 14 day old Sprague–Dawley rat
pups compared to the control pups from vehicle-treated dams. This contrary finding is
most likely due to differences in rat strain, probiotics or timing of blood collection.

The significant reductions in Haptoglobin levels induced by maternal probiotic
intervention does raise concerns about the perinatal use of probiotics because
haptoglobin plays a crucial role in heme-iron recovery. Haptoglobin has a high affinity
for free hemoglobin, thereby exerts bacteriostatic and antioxidant function. It prevents
loss of hemoglobin through glomerular filtration (Bode, Albrecht, Haussinger, Heinrich,

The change in haptoglobin levels does not appear to be linked to changes in IL-6 which
is known to drive the acute inflammatory response and is a major inducer in the
synthesis of hepatic acute phase proteins in response to inflammation or tissue injury
(Bode, et al., 2012; Coico & Sunshine, 2009; Elgert, 2009). The reason for this lack of
linkage remains unknown.

Finally, the present study importantly found that exposure to neonatal maternal
separation was associated with significant reductions in neonatal plasma circulating IgA
levels which persisted into adulthood. This is supported by previous research that early
life adverse events such as early weaning in piglets resulted in low serum IgA levels
(Levast, et al., 2010). It has been previously demonstrated that neonatal maternal
separation in rats increases bacterial penetration into intestinal mucosa (Barreau,
Cartier, et al., 2007; Gareau, Jury, MacQueen, et al., 2007), which is an initiating event
for bacterial translocation to the bloodstream causing blood and organs infection.
Therefore increased bacterial penetration along with suppressed serum IgA production may potentially lead to an increased risk of infection in neonatally stressed animals. Interestingly the present study demonstrated that maternal probiotic intervention appeared to restore plasma IgA levels to normal in maternally separated neonates and reverse the decline in plasma IgA levels of maternally separated adults to well above control levels. Enhancement of plasma IgA responses to neonatal stress exposure by maternal probiotic intervention has not been previously reported, however previous research has shown that direct ingestion of probiotics significantly increased serum IgA responses to a viral (Tejada-Simon, Lee, Ustunol, & Pestka, 1999) and Gram negative bacterial (Herias, et al., 1999) challenge in rodents. Although the role of plasma IgA is not fully understood (Cunningham-Rundles, 2001), it has been suggested that IgA binds foreign antigens including invasive bacteria into complexes which subsequently are removed by the phagocytic system (Conley & Delacroix, 1987; Morton & Brandtzaeg, 2001; Otten & van Egmond, 2004; van Egmond, et al., 2000). A recent study has shown an increase in bacterial penetration into intestinal mucosa in maternally separated rats (Gareau, Jury, MacQueen, et al., 2007). If this is the case, then increased plasma IgA levels could enhance the elimination of bacteria penetrated into circulation. Therefore, the finding of a contribution of maternal probiotics to increased plasma IgA levels in maternally separated animals is of clinical importance, particularly with respect to improving immune defence capacity against penetrated/translocated bacteria in stressed animals.

The current study also demonstrated that maternal probiotic intervention, independent of stress exposure, induces significant elevations in faecal IgA concentrations. Several studies report that direct administration of probiotics to rodents increase mucosal intestinal IgA concentrations (Ohland & Macnaughton, 2010). On the other hand, a
study by Rautava et al. (2002) has demonstrated that perinatal maternal administration of probiotics during pregnancy and lactation in human subjects elevated the level of anti-inflammatory transforming growth factor β2 (TGF-β2) in maternal milk. This factor is a crucial immunoregulatory factor which is involved in promoting secretory immunoglobulin A (sIgA) production in the neonatal gut-associated immune system (Ogawa, et al., 2004). Intestinal mucosal IgA plays an important role as the first line of defence against antigens at mucosal surface. Mucosal secretory IgA is secreted by IgA-producing plasma cells (differentiated from B cells) which are a main part of adaptive immune cells of the intestinal subepithelium (Cerutti, Chen, & Chorny; Duerkop, Vaishnava, & Hooper, 2009). IgA is transcytosed across the epithelial cells and confines penetration of bacteria across the intestinal epithelial layer (Macpherson, et al., 2000; Macpherson & Uhr, 2004), reduces mucosal-associated bacterial numbers and enhances the homeostasis of intestinal commensal microflora (Suzuki, et al., 2004). Therefore, our finding that maternal probiotics increased luminal IgA levels means that maternal probiotic administration enhances intestinal mucosal immune function in the maternally separated rat model of IBS where there is a high risk of increased intestinal permeability (Barreau, Ferrier, et al., 2004; Gareau, et al., 2006; Soderholm, et al., 2002). Colonic permeability allows the transmigration of luminal antigens including bacteria and toxins across the leaky epithelial barrier and into subepithelial tissues, lamina propria, and circulation.

In conclusion, the current study did not demonstrate that cytokine production was significantly altered by exposure of neonates to neonatal maternal separation. The level of plasma IgA was, however, significantly suppressed by neonatal stress and this did persist into adulthood but was not further affected by exposure to adult stress. The gut-associated immune function also was not affected by any of the stress protocols.
Importantly, the results do support in part the hypothesis that maternal probiotic intervention would protect animals against stress-induced alterations to systemic and gut-associated immune function as indicated by attenuated immune responses. We demonstrated that maternal probiotic use improved plasma IgA responses in early and later life of neonatally stressed rats. Maternal probiotic use was also associated with an improved gut-immune environment as indicated by increased luminal IgA levels.

4.6 Conclusion

Marked differences in humoral (serum IgA) but not systemic immune functions were observed between neonatally stressed animals and control animals. Perinatal maternal exposure to probiotics was sufficient to produce improved immune responses on some (IgA) but not all immune measures. Maternal probiotics use was also associated with down-regulation of plasma IFN-γ and haptoglobin irrespective of stress treatments. It also enhanced IL-6 responses to neonatal or adult stresses. Importantly however, maternal probiotics use improved gut-associated immune function, as indicated by increased luminal IgA levels in the probiotic subset animals. These findings suggest that maternal probiotics are likely perceived as immune challenges inducing significant impacts on the systemic and gut-associated immune functions. These mixed results however, raise concerns about the perinatal use of probiotics.
Chapter V

Effect of Maternal Probiotic Intervention on the Integrity of Gut Microbiota and Intestinal Mucin Gene Expression in the Maternally Separated Rat Model of Irritable Bowel Syndrome
5.1 Abstract

Objective: to examine whether maternal probiotic intervention influences the alterations in gut microbiota and ileal mucin (MUC)-2 mRNA expression induced by neonatal maternal separation in the rodent.

Design: Dams had free access to drinking water supplemented with *Bifidobacterium animalis* subsp *lactis* BB-12® (3×10⁹ CFU/mL) and *Propionibacterium jensenii* 702 (8.0×10⁸ CFU/mL) from 10 days before conception until weaning day (postnatal day [PND] 22), or control ad lib water. Neonates were subjected to neonatal maternal separation (NS) from PND 2 to 14 or were left undisturbed as controls. From PND 83 to 85, animals underwent 30 min/day adult restraint stress (AS), or were set aside as controls. On PND 24 and 86, ileal tissue samples were collected for determining MUC-2 mRNA expression. Ileal and colonic tissue samples were also collected for histological analysis. Faecal samples were collected to analyse the composition of gut microflora.

Results: NS was associated with significantly increased neonatal counts of aerobes, anaerobes, *E. coli*, enterococci and clostridia compared to non-NS animals (*p*≤ 0.05). Exposure to AS significantly decreased aerobes and bifidobacteria, and increased *Bacteroides* and *E. coli* counts compared to non-AS animals (*p*≤ 0.05). Exposure of NS animals to AS significantly decreased anaerobes and clostridia counts compared to the non-stress adult controls (*p*≤ 0.05).

Both control and NS animals born to probiotic-treated dams exhibited neonatal microflora profiles similar to that of animals subjected to NS in the vehicle subset. Maternal probiotic intervention was also associated with significantly increased
enterococci and clostridia in NS adults ($p \leq 0.05$), but it also restored anaerobes, bifidobacteria and *E. coli* to normal in adults.

Maternally separated pups displayed significantly decreased ileal mRNA expression of MUC-2 which persisted into adulthood in males ($p \leq 0.05$). Exposure of maternally separated rats to adult stress significantly decreased MUC-2 gene expression in both sexes compared to the controls ($p \leq 0.05$). Maternal probiotic intervention decreased neonatal ileal MUC2 gene expression compared to the controls ($p \leq 0.05$). In adulthood however, maternal probiotic intervention was associated with significantly increased MUC-2 mRNA expression in stressed males.

**Conclusion:** Maternal probiotic intervention disrupted the normal balance of gut microbiota and decreased ileal MUC2 gene expression of MUC-2 in neonates. Importantly however, it protected adult animals, in part, against stress-induced disturbances in the gut microflora. It also improved intestinal mucin gene expression in stressed adult male rats as indicated by increased MUC2 gene expression. MUC2 mucin gene product is a secreted mucin that has the ability to protect intestinal mucosa against shear stresses resulting from the passage of faecal pellets and also inhibits enteric pathogen epithelial cell adherence.
5.2 Introduction

Irritable bowel syndrome (IBS) involves a dysregulation of the brain–gut axis (S. M. O'Mahony, et al., 2011). Gut microbiota, together with spinal pathways, the hypothalamic-pituitary-adrenal (HPA) axis and the immune system, are essential aspects of the brain–gut axis (S. M. O'Mahony, et al., 2011). There is a growing body of evidence that the intestinal microbiota of IBS patients differs considerably from that of healthy subjects. Analysis of the intestinal microbiota of IBS patients has demonstrated increased relative abundance of lactobacilli, *Bacillus cereus* and *Bacillus clausii*, bifidobacteria, *Clostridium* cluster IX and *Eubacterium rectale*, and decreased abundance of *Bacteroides/Prevotella* group and *Veillonella* genus, and increased pathobionts (gut-resident bacteria which have the potential to cause disease under certain environmental conditions) compared to that of healthy individuals (Maccaferri, et al., 2012). Meta-analysis of gut microbiota in IBS patients has also identified fluctuations in Firmicutes-associated taxa (Salonen, et al., 2010). Firmicutes along with Bacteroidetes dominate the human gut microbiota. The *Firmicutes* is the largest bacterial phylum containing more than 250 genera. Some of the genera in the *Firmicutes* phyla are *Lactobacillus*, *Mycoplasma*, *Bacillus*, and *Clostridium* (Das, 2011). Other studies have demonstrated alterations in the proportions of Bacteroidetes and Proteobacteria in IBS patients (Kassinen, et al., 2007; Krogius-Kurikka, et al., 2009; Lyra, et al., 2009; Malinen, et al., 2005; Tana, et al., 2010). A recent study has demonstrated that out of 37 IBS patients, 60% of them exhibited an increase of Firmicutes-associated taxa and a depletion of Bacteroidetes-related taxa, while the remaining patients displayed normal-like microbiota composition compared with healthy controls (Jeffery, et al., 2012). A study by Rajilic-Stojanovic et al. (2011) has demonstrated a 2-fold increase in the Firmicutes to Bacteroidetes ratio in IBS patients.
compared to healthy subjects. This study also showed significantly increased numbers of *Dorea, Ruminococcus*, and *Clostridium* spp, but decreased Bacteroidetes, *Bifidobacterium* and *Faecalibacterium* spp in IBS patients (Rajilic-Stojanovic, et al., 2011). What these studies represent is a disruption to the normal healthy balance of gut bacteria in IBS patients.

Neonatal maternal separation in rats coupled with subsequent exposure to acute psychological stressors in adulthood has been used to develop an animal model for IBS with high face and construct validity (Coutinho, et al., 2002). The neonatally separated rat model is argued to mimic all of the main features of IBS (Barreau, Ferrier, et al., 2007; S. M. O'Mahony, et al., 2011).

Consistent with human IBS studies, animals subjected to neonatal maternal separation exhibit perturbations in the integrity of their gut microbiota. A significant decrease in faecal lactobacilli counts was reported on day 3 post-separation in infant rhesus monkeys exposed to maternal separation (Bailey & Coe, 1999). A study by Garcia-Rodenas et al. (2006) reported increased counts of total aerobic and anaerobic bacteria, enterobacteria and *Bacteroides* in 5 week old male Long-Evans rats exposed to neonatal maternal separation. Molecular-based analysis of gut microbiota in 7-8 weeks old maternally separated adult male rats demonstrated a markedly altered faecal microbiota compared to their non-stress counterparts (S. M. O'Mahony, et al., 2009).

In addition to alterations in the integrity of gut microbiota of IBS patients, increased epithelial secretory status as indicated by the passage of mucus has previously been described in IBS patients (Mayer & Collins, 2002). Mucus is a complex viscoelastic hydrogel composed of a wide variety of chemical and biochemical substances including proteins, carbohydrates, lipids, salts, antibodies, bacteria, and cellular debris (Ensign,
Cone, & Hanes, 2012). The viscoelastic properties of GI mucus are essential for its protection of underlying epithelial cells from shear stresses. Mucus lubricating properties also ease the passage of faecal pellets (Brownlee, Havler, Dettmar, Allen, & Pearson, 2003). GI mucus also facilitates colonisation by commensal bacteria while acting as a barrier to pathogenic bacteria and trapping inflammatory molecules (Ensign, et al., 2012). GI mucus also contains a wide range of secreted antimicrobial molecules such as defensins, lysozymes and immunoglobulins (McGuckin, Linden, Sutton, & Florin, 2011). The main proteinous component of mucus is mucins which can be either secreted or epithelial cell-bound (Ensign, et al., 2012). Mucins are considered primarily responsible for the gel properties of mucus (Macadam, 1993). Maternally separated rats have exhibited significantly increased secretion of mucus (O'Malley, Julio-Pieper, Gibney, Dinan, et al., 2010), and decreased intestinal mucin (Garcia-Rodenas, et al., 2006). At least twenty proteins have been reported to be encoded in the mucin (MUC) gene family (Lai, Wang, & Hanes, 2009), of which gel-forming mucin MUC2 is the major secretory mucin and is synthesised specifically by goblet cells of the small and large intestine (Audie, et al., 1993; S. K. Chang, et al., 1994; Ho, et al., 1993; van Klinken, et al., 1999). The analysis of MUC2 immunohistochemistry staining in duodenal mucosa of rat pups exposed to neonatal maternal separation (3 h/day between postnatal days 5-20) revealed decreases in the goblet cells number as compared with controls from postnatal day 8 to 24 (Estienne, et al., 2010).

IBS patients also exhibit intestinal morphological changes including increased mast cells in the colon (M. O'Sullivan, et al., 2000) and small intestine (Parkes, et al., 2011; S. H. Wang, et al., 2007), and increased cellularity of colonic lamina propria (Piche, et al., 2008). Consistent with these observations, maternally separated rats also exhibit intestinal morphological changes such as increased mucosal mast cells and goblet cells
Previous research on IBS patients and animal models of IBS has shown that probiotic intake could be an effective treatment approach in improving some IBS symptoms. In particular, consumption of probiotics by IBS patients has been reported to restore disturbed bacterial flora to one resembling that of healthy subjects (Kajander, et al., 2008; Lyra, et al., 2010). In animal studies, administration of probiotics to maternally separated rats restored counts of lactobacilli to that of non-stress controls (Gareau, Jury, MacQueen, et al., 2007). It has been also reported that in vitro exposure of intestinal epithelial cells to the probiotics \textit{Lb. plantarum} 299v and \textit{Lb. rhamnosus} GG increase the expression of MUC2 and MUC3, and mucin secretion (Mack, Ahrne, Hyde, Wei, & Hollingsworth, 2003; Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). Caballero-Franco et al. (Caballero-Franco, Keller, De Simone, & Chadee, 2007) reported significantly increased luminal mucin content in Wistar rats fed the probiotic mixture VSL#3. In vitro exposure of rat colonic loops to the probiotic mixture also stimulated colonic MUC secretion and MUC2 gene expression.

Previous research has also shown that maternal introduction of probiotics not only results in colonisation of the neonatal gastrointestinal tract in animal models (Buddington, et al.) and in humans (Gueimonde, et al., 2006; Schultz, et al., 2004; Vanderhoof, et al., 1999), but also causes substantial alterations in the offspring’s gut microflora (Gueimonde, et al., 2006). Interestingly Sudo and co-workers (2004) have also shown that postnatal microbial colonisation programs the HPA stress response. Exaggerated HPA-axis responses to stress were observed in GF mice. Whilst the HPA-axis response to restraint stress was facilitated by intervention with \textit{Escherichia coli}, this situation was reversed by administration with \textit{Bifidobacterium infantis} (Sudo, et al.,
As such, we have proposed that maternal probiotic intake could protect against adverse outcomes of neonatal stress on the gut microbiota and intestinal barrier function in early and later life.

Based on the available literature, the current study aimed to test the following hypotheses:

1. That normal balance of gut microbiota will be negatively impacted by exposure of neonates to neonatal maternal separation.

2. That ileal gene expression of MUC-2 will be decreased by exposure of neonates to neonatal maternal separation.

3. That neonatal maternal separation will sensitise the integrity of gut microbiota and ileal gene expression of MUC-2 in response to additional stress in adulthood.

4. That maternal probiotic intervention will protect animals against stress-induced alterations to gut microbiota as indicated by restored gut microbiota.

5. That maternal probiotic intervention will protect animals against stress-induced alterations to ileal gene expression of MUC-2 as indicated by increased gene expression.
5.3 Methods and Materials

All general methods are outlined in sections 2.1.1 to 2.1.7 in Chapter II. Methods for specific experiments are also outlined in Chapter II: Analysis of Faecal Anaerobic and Aerobic Bacteria (Section 2.2.3.1); DNA Isolation and Purification (Section 2.2.3.2); Real-Time PCR Quantification of Faecal microflora (Section 2.2.3.3); Ileal Tissue collection (Section 2.2.4.1); RNA Isolation and Reverse Transcription into Complementary DNA (Sections 2.2.4.2 and 2.2.4.4); Real-Time PCR Quantification of Ileal MUC2 mRNA Expression (Section 2.2.4.7) and Histochemistry (Section 2.2.5). Data were analysed as previously described in section 2.3 in Chapter II.
5.4 Results

5.4.1 Gut Microflora

The results obtained from assessment of gut flora composition in animals at PND 24 and week 12 are presented in Figures 5.1 to 5.5. In general, the data revealed little if any difference between sexes for all bacterial groups.

5.4.1.1 Faecal Microflora at PND 24

Examination of microflora data obtained at PND 24 reveals that among the eight faecal bacterial groups, different profiles were evident. There was no difference for lactobacilli, bifidobacteria and Bacteroides across each of these flora. Bacterial counts for these genera appeared to remain largely unaffected by NS, maternal probiotic intervention or gender (Data not shown).

In relation to aerobic and anaerobic bacteria, enterococci and clostridia, a significant interaction between neonatal maternal separation and maternal probiotic intake was observed in each instance [aerobic bacteria, $F(3, 179) = 3.45$, $p < 0.018$; anaerobic bacteria, $F(3, 181) = 4.95$, $p < 0.002$; enterococci, $F(3, 181) = 9.95$, $p < 0.001$; clostridia, $F(3, 170) = 12.09$, $p < 0.001$]. For these classes of bacteria, the number of bacteria significantly increased in NS pups born to vehicle-treated mothers and both non-NS and NS pups from probiotic-treated mothers relative to control pups in vehicle subset ($p \leq 0.05$) (Figure 5.1). There was no significant difference between the three treatment groups with increased bacterial counts.

In the case of E. coli a significant interaction between neonatal maternal separation and maternal probiotic intake was observed, $F(3, 162) = 10.17$, $p < 0.001$. NS pups born to
vehicle-treated mothers and both non-NS and NS pups from probiotic-treated mothers exhibited significantly higher faecal E. coli counts relative to control pups in the vehicle subset \( (p \leq 0.05) \). However a significant reduction was observed in bacterial counts of NS pups born to probiotic-treated mothers compared with that of NS pups born to vehicle-treated mothers \( (p \leq 0.05) \) (see Figure 5.1). In this case E. coli counts did not return to control levels.

To summarise, both neonatal maternal separation and maternal probiotic intervention induced almost a comparable disruption to the normal healthy balance of neonatal gut bacteria as indicated by overgrowth of total aerobic and anaerobic bacteria, and particularly potential negative bacteria E. coli, enterococci and clostridia, while beneficial bacteria such as lactobacilli and bifidobacteria remained unchanged.

### 5.4.1.2 Faecal Microflora in Adulthood

Analysis of the composition of faecal flora in adulthood (week 12) (Figures 5.2-5.5) indicates that faecal counts of lactobacilli were not affected by maternal probiotic intervention, neonatal maternal separation, adult restraint stress or sex (Data not shown). Only a significant main effect of adult stress was observed for faecal bacterial counts of aerobic bacteria and Bacteroides. Adult stress exposure resulted in lower counts of aerobic bacteria \( [F (2, 179) = 4.71, p < 0.01] \), but higher counts of Bacteroides \( [F (2, 171) = 55.92, p < 0.001] \) compared with non-AS animals (Figure 5.2).

Significant three-way interactions between maternal probiotic intake, NS and AS were observed for anaerobes, \( F (3,181) = 4.9, p < 0.003 \); enterococci, \( F (7,181) = 4.27, p <0.001 \) and clostridia, \( F (2,170) = 5.22, p <0.001 \) (see Figure 5.3). NS animals exposed to AS displayed significantly lower anaerobes and clostridia counts compared to vehicle.
controls (p ≤ 0.05 in both cases). Maternal probiotic intervention however was associated with restoration of anaerobes in NS+AS animals and significant increases in enterococci and clostridia in NS and NS+AS animals compared with vehicle-control animals (p≤0.05 in all cases) (see Figure 5.3).

In addition, a significant AS×probiotics interaction was observed for bifidobacteria, F (2,173) = 11.16, p<0.001. Bifidobacteria counts significantly declined in AS animals born to vehicle-treated dams compared with non-AS animals (p≤0.05), but were restored in the probiotic subset (see Figure 5.4).

**Escherichia coli (E. coli)**

In regard to E. coli counts, a significant main effect of sex was observed, F (1,174) = 8.86, p <0.003. Males displayed significantly higher counts of E. coli compared with females (p ≤0.05) (Figure 5.5A). Furthermore, a significant NS×probiotics interaction across time was observed, F (1,174) = 21.44, p <0.001. E. coli counts significantly increased in vehicle NS animals compared with that of non-NS animals (p ≤0.05). While bacterial counts returned to normal in the probiotic subset, probiotic non-NS animals exhibited a higher number of E.coli compared with vehicle non-NS animals (p ≤0.05) (Figure 5.5B). In adulthood, significant two-way interactions between AS and NS or maternal probiotic intervention were observed, AS×NS, F (2,174) = 13.02, p<0.001; AS×probiotics, F (2,174) = 8.34, p<0.003. AS exposure significantly increased counts of E. coli compared to controls (p ≤0.05) (Figure 5.5C). Maternal probiotic intervention was shown to return significantly increased E. coli counts in AS animals to that of non-AS animals born to vehicle-treated dams (Figure 5.5D). Maternal probiotic intervention however was associated with significantly increased E. coli counts in non-AS animals relative to vehicle non-AS animals (p≤0.05).
In summary, while neonatal stress alone did not affect the composition of gut microflora in adulthood, exposure to adult stress only decreased potential beneficial bifidobacteria but increased deleterious bacteria such as *Bacteroides* and *E. coli* counts. Exposure to neonatal stress combined with adult stress disrupted the normal gut microflora as indicated by decreased anaerobes and clostridia counts. Maternal probiotic intervention protected adult animals, in part, against stress-induced disturbances in the gut microflora as indicated by restoration of anaerobes, bifidobacteria and *E. coli* to normal in adults.
Figure 5. 1 Effect of neonatal maternal separation (NS) and maternal probiotic intake on composition of gut microflora (Log CFU/gr, means ± SE) in Wistar rats at PND 24. Hollow bars represent animals exposed to no neonatal stress (NNS): NNS vehicle (n = 14-20), NNS probiotic (n = 19-20). Filled bars represent animals exposed to neonatal stress (NS): NS vehicle (n = 19-20), NS probiotic (n = 20). An asterisk (*) shows significant difference compared to NNS animals in the vehicle subset (p ≤ 0.05).
Figure 5.2 Effect of exposure to stress in adulthood (week 12) on faecal counts of aerobic bacteria and Bacteroides (Log CFU/gr, means + SE). The hollow bars represent animals exposed to no adult stress (NAS): Aerobes NAS (n = 72), Bacteroides NAS (n = 67). The filled bars represent animals exposed to adult stress (AS): Aerobes AS (n = 75), Bacteroides AS (n = 75). An asterisk (*) indicates statistical significant difference (p ≤ 0.05).
Figure 5. 3 Effect of maternal probiotic intake, neonatal maternal separation (NS) and adult restraint stress (AS) on faecal counts of anaerobes, enterococci and clostridia (Log CFU/gr, means + SE) in Wistar rats at week 12. Hollow bars represent no-stress control
animals or those exposed to adult stress (AS): control vehicle (n = 11-19), AS vehicle (n = 18-20), control probiotic (n = 17), AS probiotic (n = 17). Filled bars represent NS or NS+AS animals: NS vehicle (n = 20), NS+AS vehicle (n = 20), NS probiotic (n = 18), NS+AS probiotic (n = 19). An asterisk (*) shows significant difference compared to control animals in the vehicle subset ($p \leq 0.05$).
Figure 5. Effect of maternal probiotic intake and AS on faecal counts of bifidobacteria (Log CFU/gr, means ± SE) in Wistar rats at week 12. Hollow bars represent animals exposed to no adult stress (NAS): NAS vehicle (n = 33), NAS probiotic (n = 35). Filled bars represent animals exposed to adult stress (AS): AS vehicle (n = 39), AS probiotic (n = 36). An asterisk (*) shows significant difference compared to control NAS animals in the vehicle subset (p ≤ 0.05).
Figure 5.5 Effect of sex, neonatal maternal separation, maternal probiotic intake and adult stress on faecal counts of *E. coli* (Log CFU/gr, means + SE) in rats. A) Effect of sex on faecal counts of *E. coli* (Log CFU/gr, means + SE) in rats. The hollow bar represents males (n = 109) and the filled bar represents females (n = 112). An asterisk (*) indicates statistical significant difference (p ≤ 0.05). B) Effect of maternal probiotic intake and neonatal maternal separation (NS) on faecal counts of *E. coli* (Log CFU/gr, means + SE). The hollow bars represent animals exposed to no-NS (NNS): NNS vehicle (n = 52), NNS probiotic (n = 51). The filled bars represent animals exposed to NS: NS vehicle (n = 60), NS probiotic (n = 56). An asterisk (*) indicates statistical significant difference relative to vehicle NNS animals (p ≤ 0.05). C) Effect of NS and adult stress (AS) on faecal counts of *E. coli* (Log CFU/gr, means + SE) in adult Wistar rats (week 12). The hollow bars represent no-stress control animals or those exposed to AS: control (n = 33), AS (n = 37). The filled bars represent animals exposed to NS or NS+AS: NS (n = 38), NS+AS (n = 39). An asterisk (*) indicates statistical significant difference compared to the control (p ≤ 0.05). D) Effect of maternal probiotic intake and AS on faecal counts of *E. coli* (Log CFU/gr, means + SE) in adult Wistar rats (week 12). The
hollow bars represent animals exposed to no-AS (NAS): NAS vehicle (n = 38), NAS probiotic (n = 33). The filled bars represent animals exposed to AS: AS vehicle (n = 40), AS probiotic (n = 36). An asterisk (*) indicates statistical significant difference relative to vehicle NAS animals ($p \leq 0.05$).
5.4.2 mRNA Expression of Mucin 2 (MUC2)

At PND 24, a significant three-way interaction between maternal probiotic intervention, neonatal maternal separation and sex was observed for ileal MUC2 mRNA expression, \( F(7, 44) = 241.35, \ p < 0.0001 \). Data analysis indicated that neonatally stressed pups born to vehicle-treated dams exhibited a significantly decreased ileal mRNA expression of MUC2 compared to non-stressed pups in the vehicle subset (\( p \leq 0.05 \)) (see Figure 5.6). Maternal probiotic intervention was also associated with a significant decrease in MUC2 gene expression in both stressed and non-stressed animals relative to the controls (\( p \leq 0.05 \)). The impact of maternal probiotic intervention appeared to synergise with that of neonatal stress for males. Neonatally stressed females however displayed significantly increased MUC2 gene expression compared with stressed females in the vehicle subset (\( p \leq 0.05 \)). Sexually dimorphic effects were observed in all treatment sets with females exhibiting significantly higher MUC2 mRNA expression compared to their male counterparts (\( p \leq 0.05 \)).

In adulthood, a significant four-way interaction between maternal probiotic intervention, sex, NS and AS was observed for ileal MUC2 mRNA expression, \( F(2, 44) = 1114.03, \ p < 0.0001 \). Neonatally stressed males and females born to the vehicle-treated dams respectively exhibited a significant decrease and increase in MUC2 mRNA expression compared to their respective non-stress controls (\( p \leq 0.05 \)). Contrary to this, exposure to adult stress alone was associated with a significant increase and decrease in MUC2 mRNA expression respectively in males and females compared to their respective non-stress controls (\( p \leq 0.05 \)). When neonatal stress was coupled with the acute stress in adulthood, significant decreases in MUC2 mRNA expression were observed in both sexes compared with their respective non-stress controls (\( p \leq 0.05 \)). In this case no differences between sexes were observed. In the probiotic subset, males
exhibited significantly higher MUC2 mRNA expression compared with their female counterparts \( (p \leq 0.05) \). Maternal probiotic intervention was associated with significantly decreased MUC2 mRNA expression in females in all stress conditions compared with non-stress controls as well as their respective stress-treated females in the vehicle subset \( (p \leq 0.05) \). Males exposed to NS and/or AS however displayed significantly increased MUC2 mRNA expression compared with non-stress controls as well as their respective stress-treated males in the vehicle subset \( (p \leq 0.05) \) (See Figure 5.7).

In summary, maternal probiotic intervention was only associated with improved MUC2 mRNA expression in the stressed (NS and/or AS) adult males as indicated with increased MUC2 mRNA expression.
Figure 5.6 Effect of Neonatal maternal separation (NS), sex and maternal probiotic intervention on ileal mRNA expression of MUC-2 (RQ, means + SE) at PND 24. The filled bars represent female animals (n = 10) and the hollow bars represent male animals (n = 10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining two bars indicate a significant difference ($p \leq 0.05$).
**Figure 5.7** Effect of neonatal maternal separation (NS), sex, maternal probiotic intervention and adult restraint stress (AS) on ileal mRNA expression of MUC-2 (RQ, means + SE) at PND 86. The hollow bars represent male animals (M, n = 10), and the filled bars represent female animals (F, n = 10). Bars having a different letter (same colour) indicate significant differences ($p \leq 0.05$). Solid lines joining males and females in the same treatment group indicate a significant difference ($p \leq 0.05$).
5.4.3 Intestinal Mucosal Morphology

Ileal and distal colonic mucosal morphology including villus length, crypt length and number of goblet cells were not altered by NS, sex or maternal probiotic intervention (See Figures 5.8-5.9).
Figure 5.8 Micrographs showing the effect of neonatal maternal separation (NS) and maternal probiotic intervention on the morphology of ileal epithelium. Tissue sections were stained by haematoxylin and eosin (H&E) or toluidine blue (TB). These micrographs are representative of those obtained in five to 10 pups per group.
Figure 5.9 Micrographs showing the effect of neonatal maternal separation (NS) and maternal probiotic intervention on the morphology of distal colonic mucosa. Tissue sections were stained by haematoxylin and eosin (H&E) or toluidine blue (TB). These micrographs are representative of those obtained in five to 10 pups per group.
5.5 Discussion

This study aimed to examine the impact of maternal probiotic intervention on gut microflora changes and ileal mRNA expression of MUC2 alterations induced by early life and/or subsequent adult stress in Wistar rats. Previous research has shown that exposure of rats to neonatal maternal separation predisposes animals to brain-gut axis dysfunction in response to a subsequent adult stress (Coutinho, et al., 2002; S. M. O'Mahony, et al., 2009; Soderholm, et al., 2002; Welting, et al., 2005). Essentially, these stress models mimic to some extent that which is proposed to account for IBS i.e., stress in early life combined with stress in later life (Coutinho, et al., 2002). This can be referred to as a ‘Stress Diathesis’ model in which priming by early stress is compounded by stress in later life. In this study acute restraint stress in adulthood was used as a later-life stress due to its adverse effects on the brain-gut axis function in rodents (Israeli, et al., 2008; Julio-Pieper, et al., 2012; Santos, et al., 1999; Sudo, et al., 2004).

Consistent with previous studies (Bailey & Coe, 1999; Garcia-Rodenas, et al., 2006; S. M. O'Mahony, et al., 2009), the current study demonstrated that the normal balance of gut microflora is disrupted in maternally separated animals. Maternal separation caused shifts in neonatal gut microflora as indicated by fostering an overgrowth of total aerobic and anaerobic bacteria, and particularly potential negative bacteria E. coli, enterococci and clostridia, while beneficial bacteria such as lactobacilli and bifidobacteria remained unchanged. Interestingly, maternal probiotic use induced comparable neonatal microflora changes to those seen in maternally separated pups in the vehicle subset i.e., increased counts of aerobic and anaerobic bacteria, E. coli, enterococci and clostridia. A previous study has demonstrated similar increases in faecal counts of enterococci and
*Bacteroides* between maternally separated rats that consumed a control diet and both separated and non-separated rats administered a diet containing probiotics (García-Rodenas, et al., 2006). Alterations to neonatal gut microflora could be explained by the fact that postnatal developing microbiota is extremely fragile and susceptible to environmental factors (Kirjavainen & Gibson, 1999). In addition, during this time, luminal IgA known to be capable of promoting the homeostasis of intestinal commensal microflora, dynamically interacts with the development of gut microbiota and sometimes decreases to allow bacterial colonisation (Inoue & Ushida, 2003). Therefore events such as neonatal stress and maternal probiotics may easily affect developing normal bacterial colonisation and lead to an imbalanced microflora.

While adult animals exposed to neonatal stress alone exhibited no change in their gut microflora, exposure of these animals to additional stress in adulthood reduced faecal counts of anaerobic bacteria and clostridia. This supports the double-hit hypothesis of psychopathology, demonstrating that early life stress sensitise, at least in part, gut microbiota to later life stress exposure. In the current study, adult stress exposure alone also adversely affected the normal balance of gut microflora as indicated by the decline in counts of aerobic bacteria and beneficial organisms bifidobacteria while the exposure increased counts of potential deleterious bacteria such as *Bacteroides* and *E. coli*. This was different from that of animals exposed to combined neonatal and adult stress. These findings are consistent with previous research that has shown exposure of mice and rats to restraint stress leads to an imbalance in their gut microflora (Bailey, et al., 2010; Eutamene, Lamine, Chabo, Theodorou, & al., 2007).

A key finding in this study was that perinatal maternal administration of probiotics seemed to restore the altered gut anaerobic bacteria, bifidobacteria and *E. coli* in stressed adults to one resembling that of no-stress animals. In Chapter 4, it was
demonstrated that maternal probiotic intervention increases luminal IgA levels, thus it is likely that, in part, normalisation of gut flora is mediated via increased luminal IgA levels in animals born to probiotic-treated mothers.

Interestingly, maternal probiotic intervention itself was also associated with disruption of the normal balance of gut microbiota of neonatally stressed adults as indicated by increased levels of enterococci and clostridia. This means that early life stress coupled with microbial exposure even with beneficial bacteria could induce long-lasting alterations in the gut microbiota. In this case, maternal probiotic intervention seems to cause an adverse effect on the balance of gut microbiota, but this warrants further investigation.

The microbiota data therefore provides evidence to support the hypothesis that the normal balance of gut microbiota will be altered by exposure of neonates to neonatal maternal separation which also acts to sensitise the integrity of gut microbiota in response to additional stress in adulthood. The current findings were in part supportive of the hypothesis that maternal probiotic intervention acts to protect animals against stress-induced alterations to gut microbiota as indicated by the restored gut microbiota.

In the current study, maternally separated pups also displayed significantly decreased ileal mRNA expression of MUC2 compared to the control. This observation is supported by previous research (Garcia-Rodenas, et al., 2006) that demonstrated decreased intestinal mucin protein secretion in maternally separated rats. Therefore it is concluded that neonatal maternal separation down-regulates both mucin protein secretion and its gene expression. Interestingly results from the present study showed that maternal probiotic intervention, irrespective of stress conditions, also decreased neonatal ileal MUC2 gene expression. Our findings confirm the trend indicated in the
findings by Garcia-Rodenas’s study (2006) who demonstrated that neonatal administration of probiotics decreased intestinal mucin secretion in five week old rats. Our study also adds to this finding in that our results were seen at three weeks caused by perinatal maternal administration of probiotics.

Down-regulation of MUC2 mRNA expression induced by neonatal maternal separation persisted into adulthood in males. Adult females exposed to neonatal stress alone exhibited an increase in MUC2 gene expression. Exposure to adult stress alone increased the gene expression in males but decreased in females. Importantly however, exposure of neonatally stressed rats to adult stress decreased MUC2 gene expression in both sexes. In this case a further suppression was evident in females exposed to combined early and later life stresses compared with females subjected to adult stress alone. This finding partly supports the double-hit hypothesis, demonstrating that early life stress produces a further suppression in the MUC2 gene expression following stress in adulthood. Maternal probiotic intervention, irrespective of stress conditions (neonatal and/or adult stress), down-regulated MUC2 mRNA expression in adult females. Contrary to this, adult males exposed to early and/or later life stress displayed up-regulation of MUC2 gene expression. Increased MUC2 gene expression is consistent with previous reports demonstrating that probiotics increase intestinal in vitro expression of MUC2 and mucin secretion (Caballero-Franco, et al., 2007; Mack, et al., 2003; Mack, et al., 1999). Increased luminal mucin content also has been reported in Wistar rats administered probiotics (Caballero-Franco, et al., 2007). The reason for down-regulation of MUC2 gene expression in adult females born to probiotic-treated dams remains unknown and needs to be further investigated.

The product of up-regulated MUC2 gene expression is secreted gel-forming mucin MUC2 mucin which is the major secretory mucin synthesised by goblet cells of the
small and large intestine (Audie, et al., 1993; S. K. Chang, et al., 1994; Ho, et al., 1993; van Klinken, et al., 1999). Mucins are considered primarily responsible for the mucus visco-elastic gel properties (Macadam, 1993) which are essential for the protection of underlying epithelial cells from shear stresses resulting from the passage of faecal pellets (Brownlee, et al., 2003). On the other hand, increased secretion of mucin inhibit enteric pathogen epithelial cell adherence (Ensign, et al., 2012).

These findings therefore provide evidence to support the hypothesis that ileal gene expression of MUC-2 will be decreased by exposure of neonates to neonatal maternal separation and that additional stress in adulthood will further exacerbate this effect. The findings also support, in part, the hypothesis that maternal probiotic intervention will increase ileal gene expression of MUC-2 in that increased MUC2 gene expression was demonstrated in adult stressed males but not females.

The current study did not demonstrate any intestinal or colonic morphological changes induced by neonatal stress or maternal probiotic intervention in the neonates. These data are in contrast to previous reports that have demonstrated that maternally separated rats exhibit intestinal morphological/structural changes such as increased mucosal mast cells and goblet cells (Barreau, Ferrier, et al., 2004; O'Malley, Julio-Pieper, Gibney, Dinan, et al., 2010). Such discrepancy with the current data may be attributable to differences in strain and stress and staining protocols. O’Malley et al. (2010) used Sprague Dawley and Wistar Kyoto rats, whereas the current study employed the Wistar strain. Barreau et al. (Barreau, Ferrier, et al., 2004) used alcian blue-Safranin for staining of tissue samples whereas we used haemotoxylin and eosin (H&E) and toluidine blue.
5.6 Conclusion

Taken together, these data provide evidence for the long-term effects of neonatal maternal separation on the integrity of the gut microbiota and important intestinal functions such as mucin secretion. Furthermore, our findings suggest that an increased stress sensitivity in maternally separated rats most likely predisposes these animals to disruption of gut flora and dysregulation of intestinal mucin secretion in response to acute stressors in adulthood. Maternal probiotics intervention produced alterations to neonatal gut microbiota and decreased mucin gene expression similar to changes induced by neonatal stress. Notably, maternal probiotic intervention was associated with, to some extent, protection of adult rats against abnormalities in the composition of gut microflora provoked by early and/or later life stress. It also increased mucin gene expression in adult stressed male rats which means improved protection of intestinal mucosa against shear stresses induced by the passage of faecal pellets and also inhibition of enteric pathogen adherence to intestinal epithelial cells. Underlying mechanisms of action however, need to be further investigated. Moreover, further efforts to examine the effect of maternal probiotic intervention on other aspects of brain-immune-gut axis integrity and functioning under stress conditions are required.
Chapter VI

General Discussion
6.1 Background

Early environmental factors acting during the neonatal period may determine alterations in physiological regulation, promoting critical development and having long lasting effects on health status. This vulnerability is due to the high degree of plasticity which occurs during this critical period. Rodents such as rats and mice give birth to neuroanatomically immature offspring who develop rapidly in the first two weeks of their postnatal life (Kapoor, et al., 2008). In the rat, early postnatal life is known as the critical period of development for different systems in particular the hypothalamic–pituitary–adrenal (HPA) axis which is one of the essential physiological systems responsible for the coordination of the normal stress response to challenges in vertebrates. The impact of environmental factors during the early postnatal period has recently been shown to have a wide variety of implications for the developmental trajectory of this system, and under stressful conditions may lead to disease (Gluckman, et al., 2005; Gluckman & Hanson, 2004, 2006; Gluckman, et al., 2007; Gluckman, et al., 2008). Perturbations, during early life, in the development of the HPA-axis have been reported to result in neonates who hyper-respond to stress in later life (Kalinichev, et al., 2002; O'Malley, Dinan, et al., 2011; Plotsky & Meaney, 1993; Soderholm, et al., 2002). This finding has had implications for the ontogeny of a variety of later life disorders, particularly those whose aetiology is predicated on excessive/dysregulated responses to stress, including functional gastrointestinal disturbances such as irritable bowel syndrome (IBS).

The early postnatal period is also a time when the sterile gut is inhabited and colonised gradually by microorganisms that are likely to reside in the gut throughout life (Inoue & Ushida, 2003). It has been reported that normal gut microbiota is essential for brain development (Diaz Heijtz, et al., 2011) and that postnatal microbial colonisation
programs the HPA-axis stress response (Sudo, et al., 2004). On the other hand, gut microbiota has an important role in the postnatal maturation of the gut immune system (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Round & Mazmanian, 2009). Interactions between external and host factors have also been proposed to impact colonisation profiles in the early postnatal period (Kirjavainen & Gibson, 1999). There is a growing body of evidence that the intestinal microbiota of IBS patients differs considerably from that of healthy subjects. It is also suggested that stress exacerbates IBS (Longstreth, 2005) and alters microbiota (Gareau, et al., 2008; Phillips, 2009). Animal models have suggested that the early life period may be a critical time in which the predisposition to IBS is established through the programming of the stress axis and the establishment of gut microbiota (Garcia-Rodenas, et al., 2006; S. M. O’Mahony, et al., 2009).

The high prevalence of this disorder and the ineffectiveness of current treatments results in high direct and indirect costs to society along with considerable pain and distress to sufferers (Cash, et al., 2005). The use of probiotics to replenish the gut microbiota disturbed in the IBS patients has emerged as a nutritional approach to improve some symptoms and normalise the bowel movement frequency in IBS patients (Aragon, et al., 2010). Neonatal probiotic intervention in a rat model of IBS has been reported as a potential prophylaxis against brain-gut axis dysfunctions by normalisation of HPA-axis activity (Gareau, Jury, MacQueen, et al., 2007). We hypothesised that maternal supplementary probiotics may also contribute to improved brain-gut axis integrity and immune system functioning in neonates at high risk of developing IBS in later life, and that the possible improvements persist into adulthood. The rationale for this hypothesis is based on previous studies showing that maternal introduction of probiotics results in colonisation of the neonatal gastrointestinal tract (Buddington, et al.; Gueimonde, et al., 2006; Schultz, et al., 2004; Vanderhoof, et al., 1999), and also causes substantial
alterations in the offspring’s gut microflora (Gueimonde, et al., 2006). Previous research has also shown that postnatal microbial colonisation programs the HPA stress response. Exaggerated HPA-axis stress responses to stress were observed in GF mice. Whilst the HPA-axis stress response was facilitated by intervention with Escherichia coli, this situation was reversed by administration with Bifidobacterium infantis (Sudo, et al., 2004). It has also been reported that maternal administration of probiotics could modulate immune system responsivity in a postive manner in offspring (Blumer, et al., 2007; Rautava, et al., 2002). Therefore, given that maternal probiotic administration produces a positive gastro-immune environment for the offspring, and given that we know that the composition of gut microflora can mediate HPA-axis activity, it could be expected that maternal use of probiotics may confer a more immediate protection against IBS.

To test the hypothesis, research chapters which have comprised this thesis have all employed a well-established animal model of IBS i.e., neonatal maternal separation in Wistar rats (Barreau, Ferrier, et al., 2007; S. M. O'Mahony, et al., 2011) whereby offspring were subjected to the intermittent maternal deprivation between postnatal days 2 to 14. In adulthood, the offspring were further exposed to an acute stressor (repeated acute restraint stress) to evaluate the neuroendocrine-immune and gut responsiveness to the stress. Exposure of rats to neonatal maternal separation has been recognised to predispose animals to brain-gut axis dysfunction in response to a subsequent adult stress (Coutinho, et al., 2002; S. M. O'Mahony, et al., 2009; Soderholm, et al., 2002; Welting, et al., 2005). Essentially these stress models mimic to some extent that which is proposed to account for IBS i.e., stress in early life combined with stress in later life (Coutinho, et al., 2002).
The data presented in this thesis demonstrates that maternal probiotic intervention produces a number of immediate and long-lasting consequences in neonatally stressed offspring pertaining to neuroendocrine, immune system and gut functions. These outcomes are discussed in greater details in the following sections.

### 6.2 Overview of Findings

#### 6.2.1 Impact of Maternal Probiotic Intervention on Endocrine Function in the Neonatally Stressed Rats

**6.2.1.1 Impact of Maternal Probiotic Intervention on HPA-Axis**

The role of HPA-axis in IBS has been well documented with human IBS studies showing perturbations in cortisol release in IBS suffers (L. Chang, et al., 2009; Dinan, et al., 2006). Animal models of IBS have also exhibited significantly elevated corticosterone levels both in basal conditions and in response to a subsequent acute stress compared to control animals (Barreau, Cartier, et al., 2007; Gareau, Jury, MacQueen, et al., 2007; S. M. O'Mahony, et al., 2009). As described in Chapter 5 and our paper (Barouei, Moussavi, & Hodgson, 2012), neonatal maternal separation was shown to significantly increase ACTH levels compared to non-separated animals. No difference was observed in corticosterone levels. Elevated ACTH levels induced by neonatal maternal separation is consistent with the literature (Liebl, et al., 2009). Differential ACTH and corticosterone response profiles have been previously reported and are not unexpected. The different response profiles of corticosterone and ACTH levels are most likely due to the inability to optimise the timing of blood collection, due to experimental constraints, for both measures. Another explanation could be utilisation differences between corticosterone and ACTH as it would be appear that different levels
of ACTH have resulted in equal corticosterone release. Hence it could have been due to different levels of corticosterone-binding globulin (CBG) but this was not assessed in this study. This will be assessed in future studies.

In support of previous research, we also found that exposure of animals to adult stress alone elevated both ACTH and corticosterone levels (Shanks, et al., 1995; Sudo, et al., 2004). These response patterns were not observed in neonatally stressed animals exposed to adult stress who demonstrated a blunted corticosterone and ACTH response. This is also consistent with the literature (Roman, Gustafsson, Berg, & Nylander, 2006).

Unexpectedly we observed that maternal probiotic intervention increased neonatal corticosterone levels which persisted into adulthood, but only for females. Maternal probiotic intervention was also associated with increases in ACTH levels of adult animals. This is an interesting finding, demonstrating maternal probiotic intervention to be capable of inducing long-lasting hyperactivity of the HPA-axis. While research has not yet investigated potential adverse effects of maternal introduction of probiotic bacteria on HPA-axis activity, this finding may raise concerns about the perinatal use of probiotics given the evidence reported here that they are capable of activating stress pathways.

As described in Chapter 5 and our paper (Barouei, et al., 2012), maternal probiotic intervention causes long-lasting shifts in the gut microflora, in particular fostering an overgrowth of potential negative bacteria such as E. coli, enterococci and clostridia in neonates. On the other hand previous research has shown that the composition of gut microflora can mediate HPA-axis activity (Sudo, et al., 2004). Therefore, the microbiota data may provide a potential explanation for the probiotic-induced activation of stress pathways. That is, the mechanism to be responsible is the adverse effects of maternal
probiotic administration on the neonatal gut microbiota which in turn affect the HPA-axis activity.

6.2.1.2 Impact of Maternal Probiotic Intervention on Ileal mRNA Expression of Corticotropin Releasing Hormone (CRH) Receptors 1 and 2 (CRH-R1, CRH-R2) and Nerve Growth Factor (NGF)

In addition to coordinating the body’s overall response to stress (Bale & Vale, 2004; Mayer & Fanselow, 2003) CRH mediates stress-related abnormalities in gastrointestinal function (Steckler & Dautzenberg, 2006; C. L. Williams, et al., 1987). CRH effects are exerted through activation of the G-protein coupled CRH-R1 and CRH-R2 (Chen, et al., 1993; Kostich, et al., 1998). CRH-R1 likely mediates stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). In Chapter 3 we demonstrated that neonatally stressed pups exhibited an over-expression of ileal CRH-R1 gene which persisted until at least 10 weeks after cessation of neonatal maternal separation in females but not in males. An over-expression CRH-R1 gene was also observed only in females exposed to adult stress. Neonatal stress-induced CRH-R1 gene over-expression was antagonised by exposure of females, but not males, to adult stress. However, expression levels did not return to the control levels and remained elevated. CRH-R1 has been reported to mediate stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). Therefore, it could be expected that stress-induced ileal alterations in the expression of CRH-R1 expression may also be involved in alterations in important ileal functions such as motility, permeability and secretory state. Pronounced gender-dependent differences are of particular relevance to IBS where the incidence is greater for females compared to males (Cain, et al., 2009).
Maternal probiotic intervention was also associated with increased CRH-R1 gene expression in non-stressed pups, resembling that of neonatally stressed pups in the vehicle subset. However, neonatal stress-induced over-expression of ileal CRH-R1 gene was antagonised by maternal probiotic intervention in both sexes. Maternal probiotic intervention was also associated with moderate reductions in CRH-R1 gene expression response in stressed females. An exaggerated ileal CRH-R1 gene expression was observed in males exposed to adult stress alone. Overall our findings provide evidence that perinatal maternal probiotic intervention induces neonatal over-expression of ileal CRH-R1 mRNA and has limited suppressive effects on stress-induced over-expression of CRH-R1 mRNA in adult females.

CRH-R2 has been shown to play an important role in the control of small intestinal motility (Porcher, et al., 2005). Our data did not demonstrate any difference in ileal CRH-R2 gene expression between the neonatally stressed and control neonates born to the vehicle-treated dams; whereas maternal probiotic intervention caused changes up to 0.4 fold in the gene expression as indicated by an up-regulation in non-stressed female pups and a down-regulation in neonatally stressed male pups. In adulthood all stressed animals exhibited significant over-expressions of CRH-R2 gene levels with animals exposed to neonatal stress alone appearing maximal at approximately 2-3-folds (for males and females respectively) above the control levels. Neonatal stress coupled with adult stress significantly reduced gene expression but not to the level of the controls. Maternal probiotic intervention induced significant reductions in CRH-R2 gene expression of neonatally stressed adults. In particular, this was associated with normalisation and under-expression of the CRH-R2 gene respectively in males and females exposed to combined neonatal and adult stress. However, it was associated with over-expression in non-stressed adult animals and females exposed to adult stress alone.
These findings indicated that maternal probiotic intervention affects CRH-R2 gene expression in a sex-dependent and stressor-specific fashion. Maternal probiotic intervention was associated with attenuation of neonatal stress-induced over-expression of CRH-R2 gene. Ileal CRH-R2 plays an important role in the control of small intestinal motility as indicated by inhibition of ileal phasic contractions (Porcher, et al., 2005). Therefore, maternal probiotic intervention may protect stressed animals against altered intestinal motility via attenuation of stress-induced over-expression of CRH-R2 gene.

NGF has been reported as an additional potential mediator of stress-induced gut dysfunctions such as visceral responsiveness and gut permeability (Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007). Neonatal maternal separation has been reported to increase colonic NGF mRNA and protein expression in rats (Barreau, Cartier, et al., 2004). The current study did not however demonstrate any difference in ileal NGF mRNA expression between neonatally stressed pups and the controls. Maternal probiotic intervention did however alter NGF gene expression as indicated by less than 10% increases in both stressed and non-stressed females and 45% decrease in the stressed male pups. In adulthood increased expression of ileal NGF gene was observed in females exposed to neonatal stress alone and males exposed to adult stress. Maternal probiotic intervention restored ileal NGF mRNA expression only in neonatally stressed females to normal, but increased the gene expression in their male counterparts. Our data therefore provides evidence of a positive effect of maternal probiotic intervention on neonatal stress-induced ileal NGF mRNA expression in adult females, in that it normalised ileal NGF mRNA expression in neonatally stressed females.
6.2.2 Impact of Maternal Probiotic Intervention on Immune Function in the Neonatally Stressed Rats

Bidirectional communication between the brain and the gut is mediated by neural, immune, and endocrine pathways. Several studies have indicated that early life factors that alter neuroendocrine stress responsiveness may also influence immune function (Barreau, Ferrier, et al., 2004; S. M. O'Mahony, et al., 2009). Both the systemic and intestinal mucosal immune systems are involved in brain–gut axis communication and work in a coordinated fashion to maintain gut integrity. There is a growing body of evidence supporting the existence of a low-grade level of immune activation in individuals suffering from IBS (Clarke, et al., 2009). Consistent with this, studies using the maternally separated rat model of IBS have also shown alterations in both the systemic and intestinal mucosal immune systems indicative of inflammation (Barreau, Cartier, et al., 2004; Barreau, Ferrier, et al., 2004). In Chapter 4, we explored the possibility that neonatal and/or adult stress alters immunity and whether maternal probiotic intervention could block these changes. The following sections will describe the immune data collected.

6.2.2.1 Impact of Maternal Probiotic Intervention on Cytokines

Contrary to our expectations, findings from the current study (Chapter 4) indicated that neither the neonatal maternal separation protocol or the adult stress protocol affected levels of the circulating cytokines TNF-α, IFN-γ and IL-6 in offspring born to vehicle-treated dams. This is consistent with previous reports of no change in circulating TNF-α, IFN-γ and IL-6 levels as measured in the unstimulated whole blood assay in maternally separated rats (Desbonnet, et al., 2010; S. M. O'Mahony, et al., 2009).
Interestingly however, offspring born to the probiotic-treated dams, irrespective of stress conditions, exhibited significantly decreased expression of the pro-inflammatory cytokine IFN-γ below baseline levels. A potential explanation for decreased IFN-γ levels in animals in the probiotic subset is the suppressive effect of elevated corticosterone levels on the production of IFN-γ. In Chapter 3, we demonstrated that perinatal maternal use of probiotics is capable of activating stress pathways as indicated by increased corticosterone levels. Previous research has shown that glucocorticoids suppress IFN-γ production \textit{in vitro} and \textit{in vivo} in animals and humans (Chrousos, 1995) mainly through the inhibition of IL-12 production by antigen-presenting cells (APCs) and from the loss of IL-12 responsiveness in natural killer (NK) and T helper (Th)1 cells (Elenkov & Chrousos, 1999). Therefore it is possible that maternal probiotic intervention down-regulates IFN-γ through the elevation of corticosterone. IFN-γ down-regulation to levels below baseline levels may be of concern for host defence responses against intracellular antigens because this cytokine plays important roles in host defence by exerting pro-inflammatory, immunoregulatory and anti-proliferative activities (Boehm, et al., 1997).

Maternal probiotic intervention was also associated with amplification of circulating IL-6 responses in neonatally stressed pups. This effect however did not persist into adulthood. A significant increase in IL-6 was also observed in adult animals exposed to adult stress alone. Interestingly maternal probiotic intervention was associated with normalisation of IL-6 in neonatally stressed animals exposed to adult stress. Immediate elevation of IL-6 in animals born to probiotic-treated dams following exposure to neonatal or adult stress may demonstrate maternal probiotic intervention to be an immunological challenge capable of sensitising the immune system to stress. In Chapter 3 we showed that maternal probiotic intervention was associated with elevated
corticosterone levels in early and later life. Previous research has shown that glucocorticoids down-regulate the production of IL-6 (Pollack & Kaplowitz, 2006). This up-regulation of both corticosterone and IL-6 is an unexpected finding. An explanation for such discrepancy may lie in the possible cumulative effects of perinatal maternal exposure to probiotic bacteria and early or later life stressors. Probiotics seems to be an immunological challenge which induces increased levels of IL-6 in animals exposed to neonatal or adult stress. Increased circulating IL-6 levels has been previously reported in rats exposed to neonatal maternal separation following an immunological challenge (Desbonnet, et al., 2010). A recent study has shown that increased IL-6 is associated with gastrointestinal dysfunctions such as altered motility and secretory dysfunctions (O'Malley, Liston, Hyland, Dinan, & Cryan, 2011). This would seem to suggest that the probiotics program the immune system (at least IL-6) response, such that it responds more to neonatal or adult stress.

6.2.2.2 Impact of Maternal Probiotic Intervention on haptoglobin

The current study also assessed plasma haptoglobin as an inflammation-inducible plasma protein. Elevated levels of haptoglobin reflect an inflammatory state (Giffen, et al., 2003). Neonatal haptoglobin levels were not affected by probiotic intervention, sex, or neonatal maternal separation. However, in adulthood, exposure to adult stress either alone or in combination with neonatal stress increased haptoglobin levels. Interestingly maternal probiotic intervention significantly suppressed haptoglobin levels in all stressed and non-stressed adult animals compared with vehicle controls. The significant reduction in haptoglobin levels induced by maternal probiotic intervention does raise concerns about the perinatal use of probiotics because haptoglobin plays a crucial role
in the prevention of loss of haemoglobin through glomerular filtration (Bode, et al., 2012). The change in haptoglobin levels does not appear to be linked to changes in IL-6 which is known to drive the acute inflammatory response and is a major inducer in the synthesis of hepatic acute phase proteins in response to inflammation or tissue injury (Bode, et al., 2012; Coico & Sunshine, 2009; Elgert, 2009). This means that maternal probiotic intervention may enhance loss of haemoglobin via suppression of haptoglobin production. Underlying mechanisms however need to be further investigated.

6.2.2.3 Impact of Maternal Probiotic Intervention on plasma IgA levels

Previous research has shown that early life adverse events such as early weaning in piglets results in low serum IgA levels (Levast, et al., 2010). This is an important issue because plasma IgA plays an important role in host defence by binding foreign antigens such as invasive bacteria into complexes which subsequently are removed by the phagocytic system (Conley & Delacroix, 1987; Morton & Brandtzaeg, 2001; Otten & van Egmond, 2004; van Egmond, et al., 2000). On the other hand, previous research has demonstrated that neonatal maternal separation in rats increases bacterial penetration into intestinal mucosa (Barreau, Cartier, et al., 2007; Gareau, Jury, MacQueen, et al., 2007) which is an initiating event for bacterial translocation to the bloodstream causing blood and other organ infection. Therefore, increased bacterial penetration along with suppressed serum IgA production may potentially lead to an increased risk of infection in neonatally stressed animals. In Chapter 4 and published data derived from this chapter (Barouei, et al., 2012) we showed that neonatal maternal separation was associated with significant reductions in neonatal plasma circulating IgA levels which persisted into adulthood. Interestingly the current study demonstrated that maternal probiotic intervention appeared to restore plasma IgA levels to normal in the maternally
separated neonates and reversed the decline in plasma IgA levels of maternally separated adults to well above control levels.

6.2.2.4 Impact of Maternal Probiotic Intervention on luminal IgA levels

Intestinal mucosal IgA also plays an important role as the first line of defence against antigens at mucosal surface. IgA confines penetration of bacteria across the intestinal epithelial layer (Macpherson, et al., 2000; Macpherson & Uhr, 2004), reduces mucosal-associated bacterial numbers and enhances the homeostasis of intestinal commensal microflora (Suzuki, et al., 2004). The data in the current study demonstrated that neonatal and adult stress did not affect luminal IgA levels; however maternal probiotic intervention induced significant elevations in faecal IgA concentrations. Previous research has shown that administration of probiotics to rodents increases mucosal intestinal IgA concentrations (Ohland & Macnaughton, 2010). Chapter 4 and published data derived from this chapter (Barouei, et al., 2012) also reports increased luminal IgA levels in offspring in the case where the probiotics were given to the dams. Therefore, our finding that maternal probiotics increased luminal IgA levels means that maternal probiotic administration enhances intestinal mucosal immune function in the maternally separated rat model of IBS where there is a high risk of increased intestinal permeability (Barreau, Ferrier, et al., 2004; Gareau, et al., 2006; Soderholm, et al., 2002). Increased intestinal permeability allows the transmigration of luminal antigens including bacteria and toxins across the leaky epithelial barrier into sub-epithelial tissues, lamina propria, and circulation. Thus, maternal probiotic intervention may confer a more immediate protection against increased intestinal permeability to antigens (e.g., pathogenic
bacteria, microbial toxins and viruses) in the stressed neonates via enhanced mucosal IgA secretion.

6.2.3 Impact of Maternal Probiotic Intervention on the Gut Microbiota and Intestinal Mucin gene Expression in the Neonatally Stressed Rats

6.2.3.1 Impact of Maternal Probiotic Intervention on the Gut Microbiota

There is a growing body of evidence that the intestinal microbiota of IBS patients differs markedly from that of healthy subjects. The rats exposed to neonatal maternal separation also have exhibited perturbations in the integrity of their gut microbiota (Garcia-Rodenas, et al., 2006; S. M. O'Mahony, et al., 2009). Consistent with these studies, we demonstrated in Chapter 5 that the normal balance of gut microflora is disrupted in the maternally separated animals. In Chapter 5 and published data derived from this chapter (Barouei, et al., 2012) we reported that maternal separation caused shifts in neonatal gut microflora as indicated by fostering an overgrowth of total aerobic and anaerobic bacteria, and particularly potential negative bacteria *E. coli*, enterococci and clostridia. Unexpectedly, maternal probiotic use also induced microflora changes comparable to those seen in the maternally separated pups born to vehicle-treated dams. A previous study has demonstrated that neonatal administration of probiotics induced similar increases in faecal counts of enterococci and *Bacteroides* between maternally separated rats that consumed a control diet and both separated and non-separated rats administered a diet containing probiotics (Garcia-Rodenas, et al., 2006). Alterations to neonatal gut microflora could be explained by the fact that postnatal developing microbiota is extremely fragile and susceptible to environmental factors (Kirjavainen &
Gibson, 1999). Therefore events such as neonatal stress and maternal probiotics could potentially affect normal bacterial colonisation and lead to an imbalance in microflora.

Microbiota alterations in the neonatally stressed animals born to vehicle-treated dams appeared to return to normal levels in adulthood. However, exposure of these animals to an acute stressor in adulthood reduced faecal counts of anaerobic bacteria and clostridia. This means that early life stress sensitises specific gut microbiota to later life stress exposure. Adult stress exposure alone also disrupted the normal balance of gut microflora as indicated by declined counts of aerobic bacteria and beneficial organisms bifidobacteria while there were increased counts of potential deleterious bacteria such as Bacteroides and E. coli. This is consistent with previous research showing that exposure of adult mice and rats to restraint stress leads to an imbalance in their gut microflora (Bailey, et al., 2010; Eutamene, et al., 2007).

Interestingly neonatally stressed animals born to the probiotic treated dams exhibited increased levels of enterococci and clostridia when exposed to stress in adulthood. This would appear to suggest that early life stress coupled with microbial exposure even with beneficial bacteria could induce long-lasting alterations in the gut microbiota. On the other hand, perinatal maternal administration of probiotics appeared to restore the altered gut anaerobic bacteria (animals exposed to neonatal stress combined with adult stress), bifidobacteria (adult stress alone) and E. coli (adult animals exposed to either neonatal or adult stress) to one resembling that of no-stress animals. In Chapter 4, we demonstrated that maternal probiotic intervention increases luminal IgA levels, thus it is likely that normalisation of gut flora is mediated via increased luminal IgA levels in animals born to probiotic-treated mothers. To summarise, maternal probiotic intervention was shown to disrupt the normal balance of gut microbiota in neonates and
in most cases in adulthood. Importantly however, it also normalised some stress-induced disturbances in the gut microflora in adulthood.

6.2.3.2 Impact of Maternal Probiotic Intervention on the Intestinal Mucin Gene Expression of the Neonatally Stressed Rats

An important function of intestinal mucosa is secretion of mucus which has viscoelastic and lubricating properties, essential for the protection of intestinal epithelial cells from shear stresses and the easing of passage of faecal pellets (Brownlee, et al., 2003). Mucus also facilitates colonisation by commensal bacteria while acting as a barrier to pathogenic bacteria and trapping inflammatory molecules (Ensign, et al., 2012). GI mucus also contains a wide range of secreted antimicrobial molecules such as defensins, lysozymes and immunoglobulins (McGuckin, et al., 2011). Mucins, primarily responsible for the gel properties of mucus (Macadam, 1993), are the main proteinous component of mucus (Ensign, et al., 2012). Gel-forming mucin MUC2 is the major secretory mucin and is synthesised specifically by goblet cells of the small and large intestine (Audie, et al., 1993; S. K. Chang, et al., 1994; Ho, et al., 1993; van Klinken, et al., 1999).

In Chapter 5, we demonstrated that maternally separated pups displayed decreased ileal mRNA expression of MUC2 compared to the control. A previous study has indicated a decrease in the intestinal mucin protein secretion in the maternally separated rats (Garcia-Rodenas, et al., 2006). Therefore it is concluded that a decreased level of intestinal mucin content results from down-regulation of mucin gene expression. In addition, we found that that maternal probiotic intervention, irrespective of stress conditions, also decreased neonatal ileal MUC2 gene expression. Our findings confirm
the trend indicated in the findings by Garcia-Rodenas’s study (2006) who demonstrated that neonatal administration of probiotics decreased intestinal mucin secretion.

Down-regulation of MUC2 mRNA expression induced by maternal separation persisted into adulthood in males. Importantly however, exposure of neonatally separated rats to adult stress decreased MUC2 gene expression in both sexes. This finding, in relation to MUC2, supports the double-hit hypothesis, demonstrating that early life stress produces a suppressed MUC2 gene expression following subsequent stress in adulthood. Maternal probiotic intervention, irrespective of stress conditions, down-regulated MUC2 mRNA expression in adult females. Contrary to this, adult stressed males (exposed to neonatal and/or adult stress) exhibited up-regulation of MUC2 gene expression. Increased MUC2 gene expression could be supported by previous research which demonstrated that probiotics increase intestinal in vitro expression of MUC2 and mucin secretion (Caballero-Franco, et al., 2007; Mack, et al., 2003; Mack, et al., 1999). Increased luminal mucin content also has been reported in Wistar rats administered probiotics (Caballero-Franco, et al., 2007). The reason for these sex-dependent effects of maternal probiotics on MUC2 gene expression in adult animals remains unknown and needs to be further investigated.

6.3 Summary of Key Findings and Significance

To our knowledge, the research detailed in this thesis was the first study to investigate the influence of maternal probiotic intervention on the neuroendocrine, immune and gut function in a rat model of irritable bowel syndrome. There have been several studies that have focussed on the use of probiotics in the maternally separated rat model of IBS but the intervention has only been during the neonatal period (Gareau, Jury, MacQueen,
et al., 2007) or in adulthood (Desbonnet, et al., 2010; Eutamene, et al., 2007). The current studies have demonstrated that maternal probiotic intervention induces activation of neonatal stress pathways, which persist into adulthood, and exacerbates HPA-axis responses in these same animals when exposed to stress in adulthood. Maternal probiotic intervention was also associated with suppression of neonatal stress-induced over-expression of ileal CRH-R1 gene but an up-regulation in non-stressed neonates. It was also associated with a sex-dependant moderation in stress-induced over-expression of CRH-R1 mRNA in adulthood. Increased CRH-R1 expression has been linked to stress-induced enhanced colonic motility, permeability and visceral pain sensitivity (Larauche, et al., 2009; O'Malley, Julio-Pieper, Gibney, Gosselin, et al., 2010). Maternal probiotic intervention also reversed neonatal stress-induced over-expression of CRH-R2 gene in adult rats. CRH-R2 plays an important role in the control of small intestinal motility (Porcher, et al., 2005). Sex-dependent effects of maternal probiotic intervention were also observed with expression of ileal nerve growth factor (NGF) gene as indicated by normalisation of the gene expression in neonatally stressed females, but an increase in expression in the male counterparts. NGF is a mediator of stress-induced gut dysfunctions such as visceral responsiveness and gut permeability (Barreau, Cartier, et al., 2004; Barreau, Cartier, et al., 2007).

In regard to immune functions, while neither the neonatal or the adult stress protocols affected levels of circulating cytokines TNF-α, IFN-γ and IL-6 in adult offspring born to vehicle-treated dams, maternal probiotic intervention induced a down-regulated IFN-γ production (irrespective of stress conditions) and up-regulated IL-6 responses to neonatal or adult stresses. Down-regulation of IFN-γ and up-regulation of IL-6 induced by maternal probiotic intervention are of concern and could be indications of compromised host defence and inflammation respectively. Importantly however,
maternal probiotic intervention increased plasma IgA levels in neonatally stressed animals where there is an increased risk of bacterial translocation from the gut to the bloodstream and causing blood and organs infection. Maternal probiotic intervention, irrespective of stress conditions, increased luminal IgA concentrations known to confine penetration of bacteria across the intestinal epithelial layer. Maternal probiotic intervention was also associated with significant reductions in plasma haptoglobin levels, an inflammation-inducible plasma protein, in all stressed and non-stressed animals to well below the control levels. These marked reductions raise concerns because haptoglobin plays a crucial role in the prevention of loss of haemoglobin through glomerular filtration (Bode, et al., 2012).

The current study also reports changes in gut microflora. Neonatal stress was associated with an imbalance in gut microflora as indicated by overgrowth of total aerobic and anaerobic bacteria, E. coli, enterococci and clostridia. Unexpectedly both control and stressed pups born to the probiotic-treated dams exhibited altered microflora profiles similar to that of neonatally stressed pups in the vehicle subset. Maternal probiotic intervention was also associated with significantly increased potential negative bacteria such as enterococci and clostridia in animals exposed to neonatal stress either alone or in combination with stress in adulthood. Exposure of neonatally stressed animals to adult stress decreased anaerobic bacteria and clostridia counts. Maternal probiotic intervention restored only anaerobic bacteria to normal in this case. Maternal probiotics were also able to restore altered bifidobacteria and E. coli in adults exposed to adult stress only. Probiotic-induced alterations in the gut microbiota could be, at least in part, explained by increases in luminal IgA levels known to regulate the composition and homeostasis of intestinal commensal microflora (Suzuki, et al., 2004). Growth
promoting or inhibitory metabolites secreted by probiotic bacteria have also the potential to influence the composition of the gut microbiota.

Finally, neonatal stress was associated with down-regulation of neonatal ileal mucin 2 gene expressions which persisted into adulthood in males and was further declined in both sexes after exposure to adult stress. Maternal probiotic intervention, irrespective of stress conditions, further decreased neonatal ileal mucin 2 gene expression. Sex-dependent effects of maternal probiotic intervention were observed in adult animals as indicated by decreased gene expression in females but significantly increased mucin gene expression in stressed adult males. Increased mucin gene expression means improved protection of intestinal mucosa against shear stresses induced by the passage of faecal pellets and also inhibition of enteric pathogen adherence to intestinal epithelial cells.

Collectively the findings of the current thesis suggest that maternal probiotic intervention induces profound long-lasting effects on neuroendocrine, immune and gut functions in the maternally separated rat model of IBS. These data raise some concerns about the perinatal maternal use of probiotics (at least the probiotic preparation used in this research) given the evidence that they are capable of activating stress pathways. On a positive note however maternal probiotics use was associated with enhancement of immune defence capacity, anti-inflammatory effects, and moderation of stress-induced altered intestinal gene expression profiles which are involved in intestinal functions such as visceral pain sensitivity, permeability, motility and mucus secretion, and to some extent protection against abnormalities in the composition of gut microflora. Underlying mechanisms of these impacts in the offspring, whether they are caused directly by neonatal altered gut microbiota and/or indirectly by mediators produced in the dams and transferred to the offspring, are the subject of ongoing investigations.


6.4 Conclusion

Probiotics are currently used by some IBS patients as a nutritional approach to improve some symptoms and normalise the bowel movement frequency in IBS patients (Aragon, et al., 2010). The use of probiotics in neonates is a common intervention aimed to improve health and gut function. Neonatal probiotic intervention has also been proposed as a potential prophylaxis against brain-gut axis dysfunctions (Gareau, Jury, MacQueen, et al., 2007). The studies detailed in this thesis are the first to demonstrate the influence of maternal probiotic intervention on the neuroendocrine, immune and gut function in a rat model of irritable bowel syndrome. Using a probiotic combination administered perinatally to pregnant and lactating dams we showed that maternal probiotics may be useful in preventing some adverse outcomes of neonatal stress on brain-immune-gut function in early and later life. Improvements were shown in regard to enhancement of immune defence capacity as indicated by increased plasma IgA levels in stressed animals and increased luminal IgA contents, moderation of stress-induced over-expression of ileal CRH-R1 gene in adult females, reversal of neonatal stress-induced over-expression of CRH-R2 gene in adult rats, normalisation of NGF gene expression in neonatally stressed females, restoration of altered bifidobacteria and E. coli in stressed adults and increased mucin gene expression in stressed adult males. However our findings also indicated some adverse effects of maternal probiotic intervention. Adverse effects include long-lasting activation of stress pathways, alterations in neonatal CRH-R1, CRH-R2 and NGF gene expression, and under-expression of CRH-R1 gene of neonatally stressed adult females, exaggeration of ileal CRH-R1 gene expression in stressed adult males, alterations in CRH-R2 gene expression in stressed adults, over-expression NGF gene in neonatally stressed adult males, down-regulation of IFN-γ and haptoglobin and up-regulation of IL-6 compared to baseline levels, alterations in
neonatal gut microbiota, increased negative bacteria such as enterococci, clostridia and *E. coli* in adults, and decreased mucin 2 gene expression in adult females. By modifying probiotic preparation (e.g., changes in the composition, dose and method of delivery) and also optimising time of use, it might be possible to improve this approach to minimise the adverse outcomes. It is clear however, that maternal probiotic intervention may be a viable means to improve brain-gut outcome in ‘at risk’ neonates exposed to stress in early life and at increased risk of IBS in later life. Maternal probiotic treatment potentially could improve brain-gut integrity and functioning in the neonate and ultimately it would appear that improvements may persist into adulthood. Thus, this nutritional strategy may confer a more immediate protection against IBS. The use of maternal probiotic treatment can give the offspring a healthy start to life by improving GI integrity and functioning both in the short and long term and may offer protection from some chronic gastrointestinal conditions such as IBS.
6.5 Future direction

- Further studies are clearly required to elucidate the underlying mechanisms of action of maternal probiotic intervention, and examine how maternal probiotics affect neuroendocrine, immune and gut functions in the offspring. Of particular interest is whether or not maternal probiotics transfer to and colonise neonatal gut and how long they reside there and how probiotics interact with the gut microbiota.

- In order to more clearly ascertain the effect of maternal probiotic intervention it will be neccessary to continue to explore the outcomes of maternal probiotic administration on stress-induced specific protein and related gene expression profiles in the brain, immune system and gut. Of particular interest is assessment of intestinal cytokine mRNA expression since previous research has shown that neonatal stress induces alterations in the colonic cytokine mRNA expression profiles (Barreau, Ferrier, et al., 2004) and that some cytokines such as IL-6 are involved in gastrointestinal dysfunction induced by neonatal stress (O'Malley, Liston, et al., 2011). Moreover examination of central protein and gene expression of CRH and IL-1β and also CRH receptors known to be responsible for regulating the neuroendocrine system will provide insight into central mediation of HPA axis functioning in stressed animals who have received probiotics.

- A worthwhile future research direction is investigation of the effect of different probiotic preparations, doses and timing of probiotic use (e.g., pregnancy, lactation and neonatal period and adulthood) on the neuroendocrine, immune and gut functions of the rat model of IBS.
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