Role of a Novel Probiotic in Immune Homeostasis, Microbiome and MicroRNAs' Modulation at the Gut and Brain levels

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Abstract

Numerous studies have focused on identifying novel probiotic-based treatment options for immune homeostasis maintenance and favorable modulation of the gut microbiota which acts as a key regulator of the gut-brain axis. Recently, probiotics interventions are gaining interest as effective approaches to treat neuropsychiatric disorders through the gut-brain axis. However, there is limiting knowledge about probiotics' effects during puberty on the developing brain and immune responses. Probiotic intake could offer a strategy to counteract the immune, microbial and behavioral disturbances induced by inflammatory LPS. Thus, we hypothesized that the intake of a novel probiotic bacterium *Rouxiella badensis* subsp. *acadiensis* would modulate the immune response and that pubertal administration could mitigate LPS- induced inflammation and prevent enduring behavioral changes later in life.

We investigated the interaction of the probiotic with the intestinal mucosa and its ability of modulating the gut mucosal immunity (Article 1). Next, we examined the ability of pubertal treatment with *R. badensis* subsp. *acadiensis* to alleviate LPS-induced anxiety-like and depression-like behaviors in adult male and female mice and to affect the expression of 5HT1A receptors in specific brain areas of adult mice (Article 2). We finally studied the ability of *R. badensis* subsp. *acadiensis* treatment during puberty to mitigate the effects of LPS on the immune system and on the gut microbiome composition (Article 3). These studies have demonstrated the ability of *R. badensis* subsp. *acadiensis* to survive the gastrointestinal conditions, interact with the gut epithelium and modulate the intestinal homeostasis. Pubertal use of the bacterium was associated with sex-specific effects on the acute immune response, microbiome structure, enduring neurobehavioral outcomes and the expression of 5HT1A receptors in specific brain areas, later in life. This dissertation emphasizes on the importance of

puberty as a window of opportunities during which probiotic use can alleviate the long-term neural, behavioral, immunological and microbiome alterations induced by stress.

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Chapter 1: General Introduction

The Gut-Brain Axis

The gut-brain axis consists of bidirectional communication between the enteric and central nervous systems (Carabotti et al. 2015; Bercik, Collins and Verdu 2012). In the last decades, research advances have revealed the importance of the gut microbiota as a critical regulator of the gut-brain axis (Grenham et al. 2011). The interaction between the microbiota and the gut-brain axis involves the immune system, the vagus nerve and the enteric nervous system (Clarke et al. 2013; Grenham et al. 2011). Although, different factors modulate the microbiota composition including diet, host genetic, mode of birth, age, infection, environmental stressors and antibiotics' and probiotics' use (Shreiner, Kao and Young 2016; Rinninella et al. 2019), probiotics-based interventions are gaining interest as an effective strategy to treat neuropsychiatric disorders (Foster 2018; Sherwin et al. 2016; Kelly et al. 2015).

Probiotics

The concept of probiotics was based on Metchnikoff's observations who won Nobel Prize in 1907 and suggested that health could be enhanced, and senility can be slowed down by manipulating the intestinal microbiome with host-friendly bacteria found in yogurt (Mackowiak 2013).

In 2001, the FAO/WHO proposed the first definition to probiotics as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Then, this definition has become the most used and accepted version worldwide. In 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) re-examined the definition of the FAO/WHO and adopted it with slight grammatical modifications and probiotics were re-defined

as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Martín and Langella 2019; Hill et al. 2014).

Probiotics represent the major and continuously growing segment of the functional global food market (ca. 65%) (Holzapfel and Schillinger 2002). Nowadays, the effects of probiotics are investigated either within food matrices or as a mixed or single culture preparations. The most commonly used probiotic micro-organisms belong to the group of lactic acid bacteria (*Lactobacillus*, *Enterococcus*) and to the *Bifidobacterium* genus. However, some Gram-negative bacteria such as *Escherichia coli Nissle 1917* (EcN) and yeast such as *Saccharomyces boulardii* are also used as probiotics (Möllenbrink and Bruckschen 1994; Nissle 1959; Schutz 1989; Saad et al. 2013). Probiotics are regulated as a dietary supplements and food (N. Williams 2010).While the beneficial effect was attributed to improvements in the intestinal microbiota and inducing a balanced and healthy flora, substantial evidence supports the ability of probiotics to modulate different immune functions(Borchers et al. 2009). Claims associated with probiotics include protection against infections and stimulation of the immune system (Holzapfel and Schillinger 2002). Different probiotic strains exert different probiotic potentials and health benefits; even within the same species, each strain can have its specific area of adherence, its specific immunological effects and its unique microbiome modulation abilities (Kekkonen et al. 2008).

Probiotics and the Intestinal Barrier

Many probiotics exert their effects after colonizing the intestinal tract. Some, like *Bifidobacterium longum,* become part of the human intestinal microflora, while others like *Lactobacillus casei* and *B. animalis* do not (Pochart et al. 1992; Sonnenburg, Chen and Gordon

2006); they indirectly exercise their effects either in a transitory way while passing through the intestine or more likely, by influencing the gut microbiota (Sonnenburg, Chen, and Gordon 2006; Preidis and Versalovic 2009). The colon, coecum and distal small bowel colon present high bacterial colonization when compared to more proximal regions (Stephen et al. 1980). The intestinal epithelium forms a barrier that limits direct contact with bacteria, prevent bacterial dissemination into underlying tissue and protects from uncontrolled inflammatory responses. Different mechanisms are involved in the intestinal barrier defenses such as stimulating mucus secretions, antimicrobial peptides production, increasing mucosal secretory IgA response, and improving the epithelial junction adhesion complex (Mcguckin et al. 2009).

Probiotics and the Mucous Layer

The intestinal epithelium is covered by a hydrated gel layer formed by mucins called the mucous layer. The mucous layer allows protection by shielding the epithelium from potentially pathogenic bacteria and damaging antigens and molecules, while ensuring the intestinal lubrication and improving its motility (Ohland and MacNaughton 2010). Mucins are secreted by goblet cells which are specialized epithelial cells, found along the entire length of the intestinal tract. This layer prevents large particles and most bacteria from directly contacting the epithelial cell (Johansson et al. 2008). Goblet cells produced rod-shaped mucins are glycosylated up to 80% wt/wt and are either secreted into the lumen or found located to the cell membrane (Dorothy et al. 1994; Robbe-masselot and Herrmann 2008). MUC2 is the mostly expressed mucin in humans small and large intestines mucous layer, its NH2- and COOH- glycosylated termini provide proteolytic resistance and hydrophilicity while the disulfide bonds between the cysteine residues form the backbone of the mucous layer (Caballero-Franco et al. 2006; Kelsall 2008;

Lievin-Le Moal and Servin 2006). In mice, the inner layer of mucin is densely packed and strongly attached to the epithelium and is bacteria free (Swidsinski et al. 2007). To the opposite, the outer layer is loosely adherent (Phillipson et al. 2008). Thus, the mucus is the first barrier that encounters intestinal bacteria and during infection, pathogens should breach this barrier to reach the epithelial cells. Pathogens that invade epithelial cells can degrade the mucus given their protease activity, ability to reduce mucin disulfide bonds, and glycosidase activity (Aristoteli and Willcox 2003; Bradshaw et al. 1995; Macfarlane, Woodmansey, and Macfarlane 2005). Besides, reduced mucus thickness is observed in areas of inflammation which increases bacterial infiltration (Swidsinski et al. 2007).

Probiotics can regulate mucus secretion and improve the ability of the mucosal layer to exclude pathogens. In vitro studies have shown the ability of certain probiotics like *Lactobacillae* species to enhance mucin expression in human intestinal cell lines; they increase MUC2 expression in Caco-2 cells and MUC2 and 3 in HT29 which blocks the adherence and invasion of *E. coli* invasion (Mack et al. 2009; Mack et al. 2003; Mattar et al. 2002). Although this effect was related to the attachment ability of some probiotics to the cell monolayers, other species like *L. acidophilus A4* cell extract was able to induce the expression of MUC2 in HT29 cells independent to their adhesion ability (Kim et al. 2008).Moreover, VSL#3 which contains a mixture of *Lactobacillae* species enhanced the expression of MUC2, 3, and 5AC in HT29 cells (Otte and Podolsky 2004). In vivo studies revealed the ability of 14 days treatment with probiotic supplement containing the viable microorganisms *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Enterococcus faecium* to increase mucin secretion and goblet cells density in chicks (Smirnov et al. 2005). Besides, 7 days treatment with VSL#3 increased MUC2 expression by 60-fold in rats (Caballero-Franco et al. 2006). Therefore,

Probiotics may significantly contribute to the maintenance of the mucus layer in the intestinal epithelial barrier.

Probiotics and Epithelial Adherence

Probiotic bacteria use multiple pathways to inhibit pathogen adherence to the epithelial barrier which improve the intestinal barrier function against pathogens invasion (Ohland and Macnaughton 2010). They can compete in a strain-specific manner for binding sites to epithelial cells. Components in cell walls can bind to intestinal epithelial cells and initiate anti-inflammatory signaling pathways. (Johnson-Henry et al. 2008). *In vivo,* they can counteract commensal bacterial adherence and translocations(Yuhan et al. 1997) and may prevent pathogens adherence to intestinal epithelium by inhibiting the secretion of virulence factors responsible in adherence (Kim et al. 2001; Altenhoefer et al. 2004). Thus, reducing pathogens adherence to the intestinal epithelium is a mechanism used by for probiotics in preventing intestinal infections.

Probiotics and Secretory IgA

IgA is the most abundantly produced immunoglobulin in mammals and is secreted across mucous membranes of the intestinal, respiratory, biliary and genital tracts(Macpherson et al. 2001). In the intestine, it is mainly produced by IgA-B cells found mainly in Peyer'patches (Ohland and MacNaughton 2010). IL-6, IL-10 and TGFβ boost class switching of IgM B cells to IgA production and terminal differentiation into IgA plasma cells. IgA dimers secreted into the intestinal lumen are called secretory IgA (sIgA) (Macpherson et al. 2001; Macpherson and Uhr 2004; Macpherson et al. 2008). Mucosal IgA plays a key role in protecting the intestinal

epithelium against colonization and invasion by pathogens or commensals. It results in immune exclusion of microorganisms by forming a hydrophilic shell surrounding the bacteria that is repelled by the epithelial glycocalyx (Macpherson et al. 2008; Negrão et al. 2008). Immune exclusion is critical to maintain gut homeostasis as it results in inhibiting the overgrowth of the enteric microflora (Tsuji et al. 2008; Suzuki et al. 2007). Also, sIgA can neutralize intracellular viruses and bacteria by forming an immune complex that is discharged apically (Fernandez et al. 2003). Moreover, the antigen-complexed IgA can bind the receptor FcαRI (CD89) expressed on some immune cells and activate antimicrobial activity, anti-inflammatory or pro-inflammatory signaling and phagocytosis depending on the type of IgA ligand and the immune cells (Monteiro and Winkel 2003; Rodriguez et al. 2000; Cerutti and Rescigno 2008). Titers of rotavirus- and EHEC- specific IgA were increased after pretreatment of mice with *Bifidobacterium bifidum*, *Bifibacterium infantis* and *Bifidobacterium lactis* (Qiao et al. 2002; Shu and Gill 2001). Also, total titers of sIgA in mice increase after probiotic treatment with *B. animalis*(Arantes, Teixeira, and Robert 2009). Nevertheless, probiotics may affect sIgA production differentially. For example, increase in sIgA levels was not seen after administration of a mixture of *L. rhamnosus* and *B. lactis* (Roller, Rechkemmer, and Watzl 2004).

Probiotics and Intestinal Barrier Function

Host cell antimicrobial peptides and probiotics antimicrobial peptides

Probiotics may increase the intestinal production of anti-microbial peptides such as cathelicidins and defensins implicated in host defense against pathogens (Kelsall 2008; Schauber et al. 2021). Butyrate, a short chain fatty acid, produced by some probiotics can stimulate cathelicidin expression that showed a role in treating certain infections by *Shigella* (Raqib et al. 2006). *E. coli*

Nissle, Lactobacilli and VSL#3 (Mondel et al. 2009; Schlee, Harder, and Köten 2008; Wehkamp et al. 2004) were found able to stimulate the expression of hBD-2 (a β-defensin) in Caco-2 cells; In the same line of investigation, *E. coli* strain DSM 17252 S2 G2 was found to increase hBD-2 in humans (Mondel et al. 2009; Schlee, Harder and Köten 2008; Wehkamp et al. 2004). Antimicrobial peptides, α-defensins and β-defensins are expressed by Paneth cells in the intestinal epithelium. These defensins have a wide antimicrobial activity against fungi, viruses and bacteria (Wehkamp et al. 2004; Kelsall 2008; Menendez and Finlay 2007).

Epithelial cell-cell Junctions

Many studies have demonstrated that pretreatment with probiotics is able to reduce tight junctions (TJ) alterations induced by infections, stress and proinflammatory cytokines(Czerucka et al. 2000; Dahan et al. 2003; Liévin et al. 2000; Madsen et al. 2001; Mumy et al. 2021). Probiotics proteins such as with *L. acidophilus* and ECN can change the structure of TJs by modulating the expression of its structural proteins including occludins and claudins family (Hartsock and Nelson 2008; Resta-Lenert and Barret 2003; Ukena et al. 2007).

Probiotics and Immunomodulation

Multiple probiotic strains exert different immunomodulatory activity in different pathological conditions like allergic disease, colitis, rheumatoid arthritis, colorectal cancer, depression, and anxiety (Ganesh and Versalovic 2015; Kang and Im 2015). Growing findings propose that probiotics can contribute to immune homeostasis (Perdigón et al. 2002; Galdeano and Perdigón 2006; G. Vinderola, Matar and Perdigon 2005; Rumbo et al. 2004; Perdigón et al. 2002; Perdigon, Fuller and Raya 2001). At the first line of immune signaling, probiotic bacteria interact with the epithelial and immune cells associated with the gut in order to initiate network

of immune response. Increase in the number of IgA-producing cells is one of the many modulatory activities of probiotics (Vitiñi et al. 2000; Perdigon, Fuller and Raya 2001) IgA plays important physiological roles in the mucosal surface (Macpherson and Uhr 2004). Cytokines like transforming growth factor β (TGF-β), interleukin-4 (IL-4) (Kunimoto, Nordan and Strober 2018; Coffman, Lebman and Shrader 1989) and IL-2, IL-6, and IL-10 were found to promote this switch from IgM to IgA expression (Galdeano et al. 2007). Cytokines such as tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and the regulatory cytokine IL-10, are modulated by probiotic oral administration (Iwasaki and Medzhitov 2004) . Probiotics can promote the production of IL-12, thus polarizing Th1 response and alleviating Th2-related diseases (Atarashi et al. 2013). On the other hand, probiotics can modulate T cells response by their metabolites like Short-chain fatty acids (SCFAs) mainly: acetate, propionate, butyrate and isobutyrate known to contribute to immune homeostasis and up-regulation of T reg cells (Arpaia et al. 2013; Furusawa et al. 2013). In fact, the short fatty acid butyrate stimulates extrathymic generation of Treg cells while de novo Treg-cell generation is potentiated by propionate (Furusawa et al. 2013). Butyrate induces Foxp3+ cells even under Th1- and Th17-polarizing circumstances (Owaga et al. 2015). In addition, probiotics influence Th17 inflammatory cells, by suppressing their signature cytokines IL-17. *B. breve*, *B. longum*, *L. acidophilus*, and *L. gasseri* A5 are known to reduce IL-17 (Owaga et al. 2015).In parallel, IL-23 responsible of the expansion, stabilization and conditioning of Th17 is reduced as per *B. breve* and *L. rhamnosus* GG (Bercik et al. 2011).

Probiotics and Neuroinflammation

Alterations in microglia phenotype results in neuroinflammation which is implicated in a number of psychiatric disorders, including anxiety and depression (Kreisel et al. 2014). Microglia activation and consequent release of inflammatory cytokines plays a causal role in the manifestation of behavioral phenotypes in a variety of rodent stress models (Chunchai et al. 2018). Certain studies found a direct role of probiotic in attenuating microglial activation (e.g *Lactobacillus paracasei* HII01) (Huda et al. 2011). Lactic acid bacteria of fermented milk show a neuroprotective effect; they attenuate the lipopolysaccharide (LPS) induced neuroinflammation via anti-inflammation properties and reduction of pro-inflammatory cytokines (D'Mello et al. 2015). A study reveals the ability of a probiotic mixture to improve abnormal behaviors (like social withdrawal and immobility) that are commonly associated with inflammation (Mello et al. 2015). Probiotics have beneficial effect on brain function and behavior by modulating immune system signals the brain. A meta-analysis reported the association between depression with C-reactive protein, IL-1, and IL-6 (Vojdani, Lambert and Kellermann 2011). Of particular importance is the role of Th17 in neuroinflammation and neuroimmune disorders. IL-17 secreted by Th17 cells, contributes to blood-brain barrier breakdown and the subsequent attraction of macrophages and monocytes into the nervous system (Owaga et al. 2015). The entry of cells along with the local production of inflammatory cytokines contributes to neuroinflammation. Thus, probiotics can indirectly contribute to reduce neuroinflammation at the CNS by reducing Th17, as previously reported (Frank and Pace 2008).

Probiotics and the Gut Ecosystem

During the last two decades, efforts for improving the human health status have concentrated on modulating the indigenous intestinal flora by using live microbial adjuncts or probiotics. The gastrointestinal tract is a complex ecosystem. The mucosal surface allows a huge area that is needed for microbial colonisation and adhesion to the mucosal wall as well as interactions during the digestive process (Holzapfel et al. 1998). The intestine's normal microbiota consists of bacteria, archaea, viruses and eukaryotic microbes. The vast majority of enteric bacteria cannot be cultured *ex vivo* by standard techniques; thus, sequencing of the 16S rRNA-encoding gene and the metagenomic analysis by sequencing the whole microbial DNA in a complex community have helped in studying the genetic potential of the microbial population. Also, studying the microbial transcriptome, proteome, and metabolome have offered supplementary information about the implication of the gut-microbiome in the host physiology (Lozupone et al. 2013; Shreiner, Kao and Young 2016).

The major concentration of microbes and metabolic activity is encountered in the large intestine. The upper bowel is sparsely populated and bacterial concentrations surge from the ileum on, reaching a concentration as high as 10^{11}-10^{12} colony-forming units (CFU)/g in the colon (Isolauri, Salimen and Ouwehand 2004). With an estimated 100 trillion bacteria that constitute the human gut microbiota consisting of up to 40,000 species of bacteria(Frank and Pace 2008) with Bacteriodetes and Firmicutes being the dominant phyla (Lozupone et al. 2013). Given the number of genes which is around 100 times the number of genes in the human genome (Kurokawa et al. 2007) and given the scale of the metabolic and genetic coding capacity of gut microbiome'', it is not surprising that the gut microbiota affects different features of host physiology such as nutrient improved bioavailability, metabolic functions, homeostasis of the

intestinal barrier, and immune system stimulation (O'Hara and Shanahan 2007; Sudo et al. 2004). Interactions between the microbiota and the host immune system are complex, and bidirectional. the gut microbiota is fundamental for mucosal immune stimulation and amplification of immunocompetent cells (Shanahan 1997). Healthy individuals have a stable barrier provided by the gut-associated lymphoid tissues (GALT) that allows protection against infective agents (Shanahan 1997; Holzapfel et al. 1998; Holzapfel and Schillinger 2002). Many environmental factors can lead to alteration in the microbiota composition or dysbiosis such as diet, antibiotics, stress, infections and certain disorders like irritable bowel syndrome. Moreover, dysbiosis has been associated with multiple diseases at the gut level as well as at organs distant from the gut, including but not limited to rheumatoid arthritis, colorectal cancer, obesity, multiple sclerosis, and diabetes (Cryan and Dinan 2012; Holzapfel et al. 1998; Grenham et al. 2011; Frank et al. 2011).

In human gut, the composition of the microbiota changes over the life span (Forde and O'Toole 2013). It has been suggested that the microbiota is relatively stable during adulthood while changes occur mainly during childhood and puberty. Inadequate age-related modifications in the microbiota structure can result in an alteration of microbial diversity that can lead to inflammation and disease development (Claesson et al. 2011; Kumar et al. 2016).

Probiotics, Microbiota and miRNAs

MicroRNAs (small, non-coding RNAs that regulate gene expression post-transcriptionally) are emerging as a key mode for communication between the gut microbiome and host (Zhou, Zhou, and Chen 2017). miRNAs are often differentially expressed in the presence of bacteria and can even be released and taken up by bacteria(Zhou, Zhou, and Chen 2017). Host microRNAs

regulation influences various biological pathways (e.g., innate immune response) via the regulation of host gene expression (Bartel, Lee, and Feinbaum 2004). Interactions between the host and gut microbiome involving microRNAs include mainly three pathways: 1- microRNAs regulate host gene expression, 2- gut microbiota influences host microRNA expression 3-the host influences the gut microbiota through the release of microRNAs (M. R. Williams et al. 2017). In fact, microRNA expression profiles are very different when comparing gut samples collected from colonized mice with germ free mice (Dalmasso et al. 2011; Singh et al. 2011; Xue et al. 2011). Microbiota can influence microRNA expression by toll-like receptors pathways (Xue et al. 2011; Larsson et al. 2012). Probiotics also were found to modulate miRNA for example heat-inactivated *Lactobacillus rhamnosus* GG and *Lactobacillus delbrueckii* subsp. *bulgaricus* down-regulate the effect of miR-146a expression, a negative regulator of immune response, and up-regulate the effect on miR-155 in human monocyte-derived dendritic cells (Kalani et al. 2016). miRNAs are differentially distributed through the gut with most of them found in the ileal lumen(Belcheva 2017). An increasing body of evidence demonstrate that miRNAs are at the root of controlling the inflammatory responses. Remarkably, inflammation is associated with alterations in the expression level of miRNAs. The role of the inflammation-associated miRNAs are reviewed by O'Connell et al. (Ryan M O'Connell, Dinesh S Rao 2012). Thus, miRNAs have been shown to regulate various biological processes involved in inflammation, immune homeostasis and intestinal integrity (Nakata et al. 2017). miRNAs control neuroinflammation in numerous pathologies, including multiple sclerosis, ischemic stroke, and Alzheimer's disease; certain miRNAs could initiate a pro-inflammatory response such as (*miR-155, miR-27b, miR-326*) by preferentially inhibiting the translation of many cellular anti-inflammatory proteins, other are anti-inflammatory (*miR-124, miR-146a, miR-21, miR-223*), and some have mixed

immunomodulatory effects (Gaudet et al. 2017). MiRNA-155 deletion reduces anxiety- and depressive-like behaviours in mice by reducing the inflammatory signature (e.g., decreased IL-6, TNF-a) in the hippocampus (Fonken et al. 2016a).

The Gut Microbiota and Vulnerability to Stress

Stress favorizes the colonization of the gut by pathogens like *Citrobacterium rodentium* and modulates cytokines' levels and secretory IgA leading to changes in the microbiota composition (dysbiosis) and the outcome of bacterial infection (Bailey et al. 2011; Lyte, Vulchanova, and Brown 2011; Powell et al. 2013; Campos-Rodríguez et al. 2013) . In fact, stress increases the gut permeability by disrupting the intestinal barrier (Söderholm et al. 2002; Smith et al. 2010). It is associated with alterations of the gut-brain axis; the HPA axis is activated and corticotrophin-releasing hormone, adrenocorticotrophic hormone and subsequent glucocorticoid are produced (Smith and Vale 2006). Thus, the gut and the brain are implicated in stress-induced modifications. Precisely, the activation of the HPA axis increases intestinal motility and corticotrophin releasing factor influences the gut permeability (Overman, Rivier, and Moeser 2012; Rodiño-Janeiro et al. 2015). Furthermore, exposure to stress can increase the secretion of adrenaline and noradrenaline (catecholamines) via bacterial secretion of neuroactive metabolites (García-Ródenas et al. 2006; O'Mahony et al. 2009; Eutamene et al. 2007). It is important to mention that commensal bacteria contribute to the secretion of neuroactive compounds like catecholamines (Lyte, Vulchanova, and Brown 2011). These findings reveal the role of acute or chronic stress in inducing dysbiosis and modulating the brain biochemistry and mental health (Lyte, Vulchanova and Brown 2011; Barrett et al. 2013; Hsiao et al. 2013). Since probiotics can

improve the intestinal barrier, they may be used to modulate stress-induced changes in the HPA by reducing bacterial translocation (Zareie et al. 2006).

The Gut microbiota and Mental Illnesses

The effect of the gut microbiome on behavior and mental illnesses is one important emerging research paradigm in brain health and disease. Recent studies reveal the key role of gut microbiota to the function of the CNS (Cryan and Dinan 2012). Bidirectional communication between the brain and the gut or gut-brain axis has long been known (O'Mahony et al. 2011). Different established pathways ensure this communication including the autonomic nervous system (ANS), the enteric nervous system (ENS), the neuroendocrine system, and the immune system. Recently, a growing body of experimental data from animal studies focused on the impact of microbiome or commensal bacteria on the CNS function (Cryan and Dinan 2012; Gareau et al. 2011).

The overall balance in the microbiota structure, together with the influence of pivotal species can lead to the modulation of brain function and consequently mood and behavior changes (Borre et al. 2014; Jasarevic, Morrison and Bale 2016; Cryan and Dinan 2012). Numerous studies have linked intestinal dysbiosis to behavioral impairment (anxiety, depression) (Cryan and Dinan 2012). The gut microbiota can affect the host neural development and adult function, both in the enteric nervous system and centrally (Borre et al. 2014; Jasarevic, Morrison and Bale 2016). Dysbiosis during critical periods in neurodevelopment is discovered to be related to a variety of neurological disorders. Prenatal infections was found to be associated with certain neurodevelopment disorders such as autism and schizohphrenia (Mittal et al. 2008; Bale et al. 2010). Also, enduring behavioral disorders such as anxiety-like behaviors were found to be

linked to prenatal exposure to certain pathogens (Bilbo and Schwarz 2009; Goehler et al. 2009). Studies relying on germ-free and conventional animals showed that microbiota is key in driving or mitigating inflammation at the level of the gut and the brain (Erny et al. 2015). Germ-free animals maintained in a sterile environment in gnotobiotic units display less anxiety-like behavior after exposure to new and aversive surroundings conditions compared to controls. In addition, germ-free mice have higher numbers of immature microglia than conventionally colonized mice in the gray and white matters of different brain areas such as the cortex, hippocampus and corpus callosum (Erny et al. 2015). These findings highlight the importance of gut microbes in shaping behavioral response (Diamond et al. 2011; Heijtz et al. 2011; Clarke et al. 2013). However, after colonization of germ-free mice, the reduced anxiety is brought back to normal levels dependent on the age of the mice at the time of colonization (Desbonnet et al. 2014; Gareau et al. 2011).

Furthermore, Autism Spectrum Disorder (ASD) characterized by brain development alterations that affect social and communication skills is strongly associated with gut disorders encountered in autistic children such as diarrhea, constipation, bloating and abdominal pain. An increased number of toxin producing bacteria and neurological toxins is encountered in the feces of children with autism (Hatheway 1990; Parracho et al. 2005; Song, Liu and Finegold 2004; Finegold et al. 2010). Targeting Gram-positive anaerobes by specific antibiotics improves symptoms related to autism (Sandler et al. 2000).

Microbiota Maturity with Focus on Puberty/Adolescence

Infants' microbiota is simple and unstable while adults have a very diverse, and fairly stable gut microbiota (Fouhy et al. 2014). *In utero*, the gut is germ free. Infant gut colonization occurs at

birth and post-delivery and is influenced by the delivery mode, the type of milk (such as breastmilk or formula) and any treatment or exposure early in life (Dominguez-Bello et al. 2010; Prince, Antony and Chu 2014; Mueller et al. 2015; Azad et al. 2013). Puberty/adolescence is a critical developmental window during which microbiota undergoes changes in the composition with an increase in the number of anaerobic species and a reduction in aerobes and facultative anaerobes (Hopkins, Sharp, and Macfarlane 2002) While adults' microbiota is very diverse, adolescents have less diverse microbial gut communities (Agans et al. 2011; Guarino et al. 2012; Ringel-Kulka et al. 2013). Adolescents distal gut demonstrate higher richness of genera *Bifidobacterium* and *Clostridium* when compared to adults (Agans et al. 2011). Also, adults have a higher abundance of Bacteroides when compared to pre-adolescents who have more *Bifidobacterium* (Hollister et al. 2015). In fact, adults and old microbiota are associated with inflammation and metabolism while children and adolescents microbiota play a role in growth and development (Hollister et al. 2015; Borre et al. 2014). Puberty is known for sexual changes and gonadal hormones secretions (Juraska, Sisk, and Doncarlos 2013; Romeo 2003; Marceau et al. 2015). In mice, sex-related microbiota disparities are observed (Jasarevic, Morrison and Bale 2016). These sex differences in the microbiota structure disappear after castration of adult mice, showing a role of gonadal steroid hormones in the microbiota structure of males and females (Yurkovetskiy et al. 2013).

During adolescence, the unstable gut microbiota is susceptible to environmental stressors (like infection, inflammation, poor diet…). Therefore, gut dysbiosis during this critical period of development may lead to the development of enduring brain disorders and psychiatric disturbances later in life (Leclercq et al. 2014; Golubeva et al. 2015; Desbonnet et al. 2015).

Subsequently, crucial interventions during adolescence that prevent microbiota dysbiosis may help improve brain development and mental health in adulthood.

Probiotics, Microbiota Interactions and the CNS

Probiotic micro-organisms might influence the intestinal microbiota via: competition for adhesion to the epithelium and for nutrients (Vandenbergh 1993); antagonism through the production of antimicrobial substances (Brandão et al. 1998); inhibition of bacterial toxin production (Isolauri et al. 2001) and immunomodulation of the host (Douglas-Escobar, Elliott, and Neu 2013; Bruce-Keller et al. 2015; Aziz et al. 2013; Tillisch and Tillisch 2015; Dinan and Quigley 2011). The modulation of the gut microbiome composition using probiotics may ameliorate the gastrointestinal barrier and decrease the risk of inflammation. It can reduce microglial activation and modulate the biomolecules or neuro-metabolites produced by the microbiome or gut epithelial cells. It may also affect the local immune populations trafficking to the CNS, improve the integrity of the blood brain barrier (BBB) and protect against neuroinflammation (Kelly et al. 2015; Westfall et al. 2017). The effect of the microbiome on behavior and mental illnesses is one important emerging research paradigm in brain health and disease. Numerous studies have linked intestinal dysbiosis to behavioral impairment (anxiety, depression) (Cryan and Dinan 2012). Probiotics may produce directly certain neuroactive metabolites into the circulation and can stimulate the vagus nerve which allows an effect on behavior. For example, secreted GABA by *Lactobacillus* and *Bifidobacterium* results in behavioral and motor activity variations (Collins, Surette and Bercik 2012; Samuel et al. 2008; Clemmensen et al. 2017). Probiotics can affect as well the levels of dopamine, noradrenaline and serotonin (Lyte 2014). Ferulic acid (FA) produced by *L. fermentum NCIMB 5221* and *B.*

animalis is well known to be endowed with neuroprotective properties (Tomaro-Duchesneau and Saha 2012; Szwajgier 2010)(Zhang L, Wang H 2015; Srinivasan, Sudheer, and Menon 2007)·(Mancuso C 2014; Trombino et al. 2013). Recent findings from our lab emphasize on the importance of using probiotics during puberty to prevent long-term induced mental illnesses. These two studies showed that colonizing the gut with specific probiotics during puberty reduces LPS-induced peripheral and central inflammation, counteracts LPS-induced dysbiosis and prevents the long-term anxiety-like and depression-like behaviors in adults in a sex-specific manner (Murray et al. 2019).

Puberty/Adolescence as a Critical Period of Development

Puberty is a critical stage of development that is characterized by transition from a non-reproductive juvenile phase into a reproductive adult (Romeo 2003; Cai et al. 2016) while adolescence is the phase between childhood and adulthood that is characterized by social, cognitive, emotional and reproductive maturation (Sisk and Foster 2004). Thus, puberty and adolescence are periods for crucial physiological and neurological development during which modifications in the hormonal levels (i.e. steroid hormones), development of secondary sexual characteristics, brain reorganization in addition to emotional, behavioral and social changes occur (Schulz and Sisk 2006; Cai et al. 2016; Levitt 2003) . During puberty the hypothalamic-pituitary-gonadal (HPG) axis activates the development of the reproductive state. Gonadotropin-releasing-hormone (GnRH) neurons from the hypothalamus secrete GnRH more frequently and in higher amounts (Marceau et al. 2015; Bourke and Neigh 2011). Intermitted pulses of GnRH release leads to the production of follicle-stimulation hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland which results in gonadal production of steroid hormones:

estradiol and progesterone in females and androgens in males (Juraska, Sisk, and Doncarlos 2013; Marceau et al. 2015). The upsurge of gonadal hormones during puberty results in physiological changes such as the development of secondary sexual characteristics, the onset of reproductive competence such as menstrual cycle and menarche in females and menarche and spermarche in males and fast growth (Patton et al. 1996; Dahl and Gunnar 2009). In humans, puberty starts a bit earlier in girls around the age of 10-11 years old, while in boys puberty begins around 11 to 12 years old (Marshall and Tanner 1969). In mice, the timing of puberty depends on mouse strain and housing environment. In female mice, the onset of puberty starts around postnatal days 30-35 and it is marked by vaginal opening. Puberty ends with the onset of the first estrous cycle around postnatal day 60 (Ojeda et al. 1976; Vandenbergh 1967, 1969). In male mice, the onset pf puberty started with preputial separation around postnatal day 35 and ends around postnatal day 50 followed by a significant increase in circulating androgen levels (Holder and Blaustein 2014; Deboer and Li 2011).

Puberty/Adolescence and Neurodevelopment

During puberty/adolescence, the brain undergoes major neuronal reorganization and remodeling implicated in cognitive function, movement, and emotions (Rager 2019; Herrup and Sunter 1987). These changes involve synaptogenesis, neurogenesis and axonal and dendritic growth which result in the appropriate neuronal amount and synaptic density and are influenced by gonadal steroid hormones (Rager 2019; Herrup and Sunter 1987; Levitt 2003; Sisk and Foster 2004). Adolescence structural changes are connected with modifications in social and cognitive abilities, reward sensitivity, and stress responsivity (Burnett et al. 2011; Spear 2011; Spear 2013; Giedd et al. 1996) . Synapse maturation is important during adolescence as adult synapse density

is typically achieved by mid-adolescence (Herschkowitz, Kagan, and Zilles 1997; Huttenlocher 1979; Petanjek et al. 2011; Glantz et al. 2007). During synaptic refinement, new neural circuits are formed by elimination of certain synapses via the complement system and brain residing microglia and maintaining others (Hua and Smith 2004; Schafer et al. 2012; Sekar et al. 2016). In fact, pruning removes infrequently used synapses while commonly used connections are strengthened (Holtmaat and Svoboda 2009). The prefrontal cortex undergoes an increase in the number of synapses during childhood and puberty, followed by post-pubertal elimination and reorganization of synapse connections (Huttenlocher 1979; Bourgeois, Goldman-Rakic, and P. Rakic 1994; Woo et al. 1997).

During adolescence, axons myelination continues in the frontal cortex which improves neural communication in this area and affects executive functions, cognition, and memory experiences (Markham and Greenough 2004; Fair et al. 2008) (Huttenlocher 1979; Huttenlocher et al. 1982; Benes 1989; Tamminga and Buchsbaum 2004; Miller and Cohen 2001). Myelination also increases in the frontal and parietal lobes which causes modification in gray and white matter volume(Paus, Keshavan and Giedd 2008; E.R. Sowell et al. 2003; Blakemore and Choudhury 2006; Tau and Peterson 2010; Huttenlocher and Dabholkar 1997). Similarly, pruning and alterations in synaptic connectivity can lead to changes in gray matter volume (Paus, Keshavan and Giedd 2008; Sowell et al. 2003; Blakemore and Choudhury 2006; Tau and Peterson 2010; Huttenlocher and Dabholkar 1997). Reductions in cortical gray matter density is observed in the frontal and dorsal regions and an increase in white matters is observed in areas associated with attention and language (Paus et al. 2010; Sowell et al. 2001). Sex differences are observed during puberty with adolescent males showing higher grey-matter density than females in certain cortical regions with testosterones concentrations directly associated to the white matter volume

(Paus et al. 2010; Perrin et al. 2008). In addition, the hippocampus and amygdala volume increase during early pubertal stages (Giedd and Blumenthal 1999; Romeo and Sisk 2001). As a consequence of this significant neuronal reorganization and rewiring, puberty is a highly vulnerable to infection, stress, drugs, dietary deficiencies and immune challenges which increases the risks for the onset of different mental disorders (Paus, Keshavan and Giedd 2008) (Figure 1.1). Mood disorders, anxiety, major depression, eating disorders, psychosis and substance abuse, increase considerably during puberty and adolescence in humans (Jay N. Giedd, Matcheri Keshavan 2008; Kessler et al. 2005; Newman et al. 1996; Xiaojia, R, and Elder 2001). Finally, exposure to inflammation and stress during this critical period can result in lasting effects on adult brain and behaviour (Andersen 2003; Holder and Blaustein 2014).

Puberty and the Emergence of Stress- Related Mental Illnesses

Adolescence is well noted as a period of increasing incidence of numerous classes of psychiatric illnesses, including anxiety, mood disorders, psychosis, personality disorders, eating disorders and substance abuse (Paus, Keshavan and Giedd 2008; Kessler et al. 2005). Results of the National Comorbidity Survey Replication study have showed that the peak age of onset for developing any mental health disorder is 14 years (Kessler et al. 2005). Affective disorders, including depression, are known to particularly affect adolescents. Onset during adolescence is associated with more severe and disabling forms of these illnesses (Leussis and Andersen 2008; Andersen 2003; Tomáš Paus, Keshavan and Giedd 2008). Hormonal changes during adolescence play a role in the predisposition risk for mood and anxiety disorders. Depression, anxiety and panic disorders are more prevalent in females than males at a ratio of 2:1 after puberty instead of equal prevalence before puberty (Angold, Costello and Worthman 1998;

Angold and Costello 2006). Steroid hormones affect the remodeling of brain circuits and trigger permanent structural changes in the brain, leading to sexual dimorphism in various brain regions during puberty (Sisk and Zehr 2005). In fact, sexual dimorphism exists in different postpubertal brain volume and neural density are different between males and females: the amygdala volume is greater in males while the hippocampal volume is larger in girls (Giedd et al. 1996; Hayward and Sanborn 2002). In summary, changes in hormones and hormone receptors, progressively predispose to differential emotional responses to social stimuli. Furthermore, alterations in motivation and reward systems can be at origin of the onset of anxiety and depressive disorders during adolescence. Accordingly, exposure to stress and inflammation or infection during this critical window of neurodevelopment alters neurobehavioral processes and lead to long-term changes in adult brain and behaviour (Andersen 2003; Holder and Blaustein 2014).

Puberty and Stress Response

The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system play a key role in the response to stress. The activation of the sympathetic nerves result in the stimulation of the adrenal medulla and release of catecholamines, which are responsible of stress physiological responses such as pupil dilation, increased heart rate and decreased gut motility (Kvetnansky, Sabban and Palkovits 2009). In parallel, stress activates the HPA axis which results in glucocorticoids production, cortisol in humans and corticosterone in rats and mice: activated hypothalamus produces corticotropin releasing factor (CRF) which binds to specific receptors on the anterior pituitary gland and results in adrenocorticotropic hormone (ACTH) production which stimulates glucocorticoid production by the adrenal cortex into the blood. Different structure such as the amygdala and medial prefrontal cortex (mPFC) may affect this response

(Ulrich-lai and Herman 2014). The HPA axis plays essential roles in the regulation of different physiological functions such as the immune system response, cardiovascular system, reproductive functions and the regulation of metabolism (Pariante and Miller 2001; Vitaliano et al. 2002; Straub et al. 2011; Tafet and Bernardini 2003).

Studies have shown age differences in the corticosterone levels production(Rd. Romeo et al. 2004; Vazquez and Akil 1993). In rats, the basal concentrations of stress hormones are constant during puberty/adolescence (Pignatelli D et al. 2006); however, pubertal rats developed a more extensive rise in the corticosterone levels than adult rats after a physical and psychological stressors (Romeo et al. 2004; Vazquez and Akil 1993). While habituated stress responses after chronic stress exposure are observed in adult rats (Girotti, Pace, and Gaylord 2006; Harris et al. 2004; Magarinos and McEwen 1995; Marti and Armafuo 1997), pubertal rats showed increased ACTH and corticosterone levels directly after chronic stress exposure, but they return back to the normal levels faster than adults group (Romeo et al. 2004; Vazquez and Akil 1993). In mice, pubertal males of outbred or inbred strains produce more intense and extended corticosterone responses than their adult counterparts. These differences are not observed in inbred female mice which develop similar corticosterone responses independent of age (Romeo et al. 2013). The pubertal brain may be more sensitive to corticosterone than adult brain. During acute and chronic stress a greater amount of corticotropin-releasing hormone cells are activated in the paraventricular nucleus of pubertal animals. Where corticosterone significantly increased hippocampal N-methyl-D-aspartate receptor at prepuberty. In addition, different brain regions such as the amygdala, hippocampus and prefrontal cortex continue the neurodevelopment during puberty/adolescence and are highly sensitive to corticosterone (Romeo 2003; Eiland and Romeo 2013; Romeo et al. 2006; Romeo and Mcewen 2006; Lee, Brandy and Koenig 2003). Yet, in the

outbred Swiss-Webster strain, adult females developed a stronger corticosterone response than prepubertal females (Romeo et al. 2013). Sex differences are also observed in mice with adult females developing a strong hormonal reactivity to stress than adult males (Harizi et al. 2007; Goel and Bale 2008). There are species-specific differences in the response to acute stress (Romeo et al. 2013). These age and sex disparities observed in stress responses can be caused by gonadal hormones. While estradiol is known to have an inhibitory effect in the HPA stress response in adult females, the testosterone in male rats diminish the peak and recovery time of the hormonal stress response (Mccormick, Smith, and Mathews 2008; Handa et al. 1994; Mccormick et al. 2002) . Although, gonadal hormones effect on the stress responses is limited to pubertal window as in the absence or presence of gonadal hormones no differences are observed between males and females during pre-puberty(Romeo et al. 2004; Romeo, Lee and Mcewen 2005). These age and sex differences during puberty are not limited to HPA axis reactivity, they are also encountered in the immune response to immune challenges.

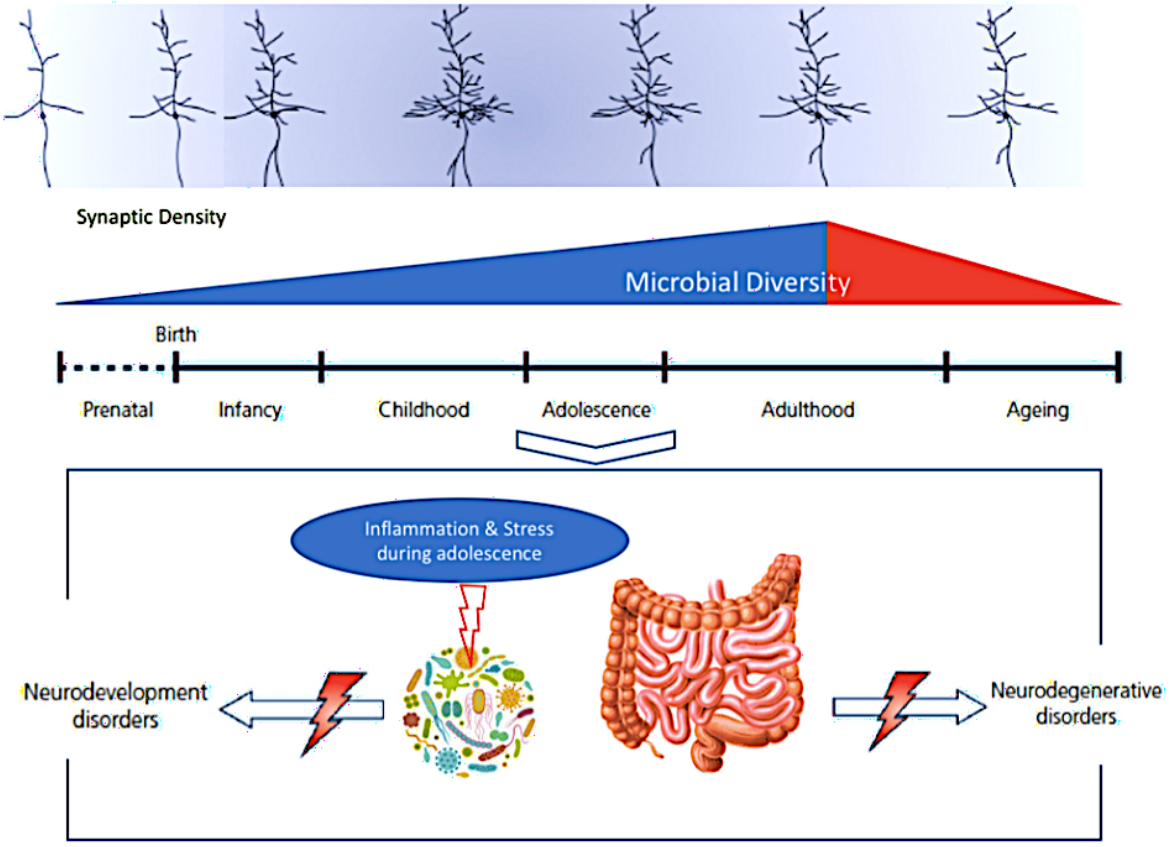

Figure 1.1. Microbiota diversity throughout life span with focus on adolescence

Microbiota diversity and neuronal complexity changes throughout life span. Shaping of the microbiota occurs simultaneously with neurodevelopment and they both have the same critical windows of development. During early adolescence major neuronal rewiring synaptic pruning or elimination of extra synapses occur in parallel with instability and immaturity of gut microbiota leading to a high degree of vulnerability to different pathological stressors (inflammation, stress) (Nour Yahfoufi, Matar, and Ismail 2020)

Puberty and Immune Response to LPS Challenge

Significant sex and age differences are identified in the immune response to challenges between pubertal and adult mice. Previous studies from our laboratory have shown a stronger immune response in term of hypothermia and sickness behaviors in adult males when compared to their pubertal counterparts following LPS treatment. In addition, LPS induced higher levels of pro-inflammatory cytokines at 10 hours post-injections in adult mice of both sexes while pubertal mice produced more anti-inflammatory cytokines (Cai et al. 2016). In fact, pubertal mice showed less body temperature variations, less IL-1β, IFNγ, and TNFα and more IL-10 when compared to adult mice 10 hours post-LPS treatment (Cai et al. 2016). Another study revealed that young-adult mice had less prominent peripheral and central immune responses and a lower expression of TNFα, IL-6, and IL-1β in their microglia and spleen in comparison to middle-aged mice (Nikodemova et al. 2016). Furthermore, LPS raised the corticosterone levels in the serum of all adults and pubertal mice with a greater concentration observed in adult female mice at 2 hours post-injection. LPS exposure increased the expression of c-Fos which is implicated in the suppression of systemic immune-response to endotoxin in a number of forebrain regions of adult mice (male and female) compared to saline-injected controls while no changes in c-Fos were encountered in pubertal mice (Girard-Joyal and Ismail 2017; Ray et al. 2006). Also, puberty is known for an increase in gonadal hormones production which may contribute to sexual dimorphism in immune response as testosterone acts as an immune suppressor while estrogens are immune enhancers and play a role in the maturation of lymphocytes and production of antibodies (Wichmann et al. 1997; Kovats 2016; Lai et al. 2012). In the brain, LPS treatment increased IL-1β, TNFα, and IL-6 mRNA expression in the prefrontal cortex of adults males more than their pubertal counterparts at 8 hours post-LPS treatment while pubertal males had greater

expression of the aforementioned cytokines at 2 hours post-treatment (Sharma et al. 2018). Moreover, peri-pubertal female rats have more activated microglia in the hippocampus, parietal cortex and amygdala than males revealing a higher susceptibility to immune challenges. Also, more activated microglia are encountered in female CD1 mice during puberty than in adulthood (Holder and Blaustein, unpublished observations).

Pubertal Immune Challenge and Mental Illnesses Especially Anxiety and Depression

Pubertal complex stressors such as inflammation, infection, stress or others can alter adult brain and behaviors and contribute to the onset of different mental disorders later in life (Paus, Keshavan and Giedd 2008; Holder and Blaustein 2014). Although microglia act as the first form of active immune defense in the central nervous system (Ginhoux et al. 2010; Bilbo, Smith, and Schwarz 2012; Ling and Wong 1993), continuously activated microglia and astrocytes in the brain can result in neuroinflammation which may cause brain damage (Ginhoux et al. 2010; Bilbo, Smith, and Schwarz 2012; Ling and Wong 1993; Bauer, Kerr and Patterson 2007; Gadient and Otten 1994; Pousset 1994). Neuroinflammation is a common pathway by which adolescent stressors may alter the adult brain and behavior affecting cognition abilities, memory and mental health (Bilbo, Smith and Schwarz 2012; Miller, Maletic, and Raison 2009; Salim, Chugh and Asghar 2012; Cribbs et al. 2012). Recent findings revealed that pubertal mice treated with lipopolysaccharide (LPS) developed a reduced immune response in comparison to adult mice as observed by less hypothermia, symptoms of sickness, and proinflammatory cytokines (Cai et al. 2016; Sharma et al. 2018). In contrast, greater cytokine mRNA expression were induced in different brain regions were observed in pubertal mice when compared to their adults counterparts (Sharma et al. 2018). Microglia are immune cells residing in the brain that produce

cytokines once triggered by stressors and immune insult. The degree of microglial colonization or the number of microglial cells detected is influenced by external stressors (Gomez-Gonzalez and Escobar 2010). Microglia can be activated for long period and have a long life span that allows them to trigger long-term effects after exposure to early stressors (Town, Nikolic and Tan 2005). Furthermore, cytokines are also known to contribute to neurodevelopment, neurogenesis, synapse refinement, synapse formation, neurogenesis and neuronal differentiation (Boulanger 2009; Carpentier and Palmer 2009; Deverman and Patterson 2009; Garay and Mcallister 2010) while microglia are highly involved in the process of synaptic pruning (Schafer et al. 2012; Prinz and Priller 2014). Therefore, neuroinflammation is believed to influence morphological restructuring during critical periods of brain development such as puberty (Aloisi 2001; Kettenmann et al. 2011; Streit 2010) and can be implicated in the development of mental disorder like anxiety, major depression and post-traumatic stress disorder (Capuron and Miller 2011; Miller, Maletic, and Raison 2009; Hou. and Baldwin 2012; Jones and Thomsen 2013). Multiple studies have revealed the long-term behavioral effects induced by immune challenges exposure during adolescence. Pubertal LPS treatment reduces anxiety-like behavior in ovariectomized female C57Bl/6 and CD-1 during puberty later in life (Olesen et al. 2011) as a result of hippocampus injury induced by LPS (Bannerman et al. 2004). In mice, pubertal exposure to LPS at six weeks of age leads to reproductive and non-reproductive long-term behavioral alterations; inbred and outbred strains of mice develop alterations in sexual receptivity (Laroche et al. 2009b, 2009a; Ismail, Garas, and Blaustein 2011) in adulthood. Further alterations in cognitive functioning (Ismail and Blaustein 2013), spatial memory (Kolmogorova et al. 2019) anxiety-like and depression-like behaviors are observed in outbred CD-1 mice later-in life after pubertal LPs exposure (Ismail, Kumlin, and Blaustein 2013; Murray

et al. 2020). These enduring behavioral effects are limited to mice at six weeks of age reflecting mice at puberty period; LPS exposure of younger or older inbred C57B1/6 or outbred CD1 mice did not result in any long-term behavioral alterations in the reproductive and non-reproductive behaviors (Olesen et al. 2011; Ismail and Blaustein 2013; Laroche et al. 2009a).

The immune system is implicated in the pathophysiology of anxiety and depression. Previous findings have demonstrated higher risk for low-grade inflammation and increased concentrations of IL-6, TNFα pro-inflammatory cytokines and c-reactive protein in the blood of depressed and anxious patients (Vogelzangs et al. 2016). Blood levels of pro-inflammatory cytokines are linked to the severity of anxiety symptoms (Vogelzangs et al. 2016). In our laboratory, Murray et al. study have demonstrated that that a single injection of LPS in CD1 mice, at 6 weeks old, a pubertal stress-sensitive period, induces enduring depression-like behaviour in females and anxiety-like behaviour in males (Murray et al. 2019). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and disturbances in the autonomic nervous system are associated with anxiety and depression (Stetler and Miller 2011; Beishuizen and Thijs 2003).

Furthermore, intestinal dysbiosis is encountered in patients with anxiety and depression (Cryan and Dinan 2012). While Bacteroidetes and Firmicutes phyla dominate 9% of the gut microbiota of healthy adults, depressed individuals demonstrated an alteration in the abundance of different genera within the *Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria phyla* (Winter et al. 2018). Recent studies proposed a role of the microbiota in brain development, behavior and mood disorders (Dash et al. 2015).

Probiotics can reduce stress response and may exert anti-depressive and anxiolytic effects by modulating inflammatory responses(Park et al. 2018; Murray et al. 2020; Murray et al. 2019). Recent studies from our laboratory have showed that the consumption of the probiotic

Lactobacillus reuteri during puberty alleviates LPS-induced enduring effects on memory dysfunction, anxiety-like behaviour and stress reactivity in adulthood. Furthermore, probiotics from kefir during property inhibits long-term LPS-induced depression-like behaviour in adult females and anxiety-like behaviors in adult males(Bravo et al. 2011; Murray et al. 2020). However the benefits of probiotics use in mental health and behavioural changes have been investigated, underlying mechanisms and neurochemical alterations related to these effects remain unknown (Bravo et al. 2011; Murray et al. 2020).

Serotonin or 5-hydroxytryptamine (5HT) is one of different monoamine neurotransmitters that play a key role in the pathogenesis of anxiety and depression. The vast majority of the total serotonin is found outside the CNS while less than one in a million CNS neurons produce serotonin (Berger et al. 2018). In the CNS, serotonin is mainly produced in neurons originating in the raphe nuclei located in the midline of the brainstem. However, ascending projections that originate at the brainstem serotonin neurons terminate in in cortical, limbic, midbrain, and hindbrain regions (Berger et al. 2018).

5-HT1A receptors subtype being of major importance as they are targets for many antidepressants and anxiolytic treatments (Cryan and Leonard 2000). These receptors are either presynaptic - autoreceptors that are mainly present within the brainstem or postsynaptic - heteroreceptors that are encountered in different brain regions such as the hippocampus, prefrontal cortex, thalamus, lateral septum, amygdala, and hypothalamic nuclei (Haddjeri, Blier, and Montigny 1998). Depression is associated with an increase in 5HT1A autoreceptors in the dorsal raphe nuclei such as identified in post-mortem depressed patients while a

decrease in 5HT1A heteroreceptors expression is observed in the hippocampus (Boldrini et al. 2008; Moses-kolko et al. 2008; Sargent et al. 2000). Rodent models of depression and anxiety 5HT1A demonstrated an alteration in the expression 5HT1A receptor as a result of chronic stress (Watanabe et al. 1993; Lopez et al. 1998). In fact, greater anxiety-like behaviours are associated with 5HT1A knockout mice while prolonged maternal separation
is related to an increase in depression-like behaviours and a decrease in hippocampal 5HT1A mRNA expression (Maniam and Morris 2010) (Holmes et al. 2003).

Rationale for Study and Objectives

Probiotic administration is considered a key strategy in alleviating immune and neuro disturbances. In our studies, we elect to use a novel probiotic bacterium such as *Rouxiella badensis* subsp. *acadiensis* which was isolated from the microbiota of the wild blueberry fruit (*Vaccinium angustifolium* Aiton) as described in *Luc Martin et al.* publication (Martin and Matar 2005). Several published studies revealed its effective anti-inflammatory properties for neurodegeneration, diabetes, and cancer.(Nachar et al. 2017; Vuong et al. 2016, 2007; Sánchez-villavicencio et al. 2017) and *Rouxiella badensis* subsp. *acadiensis* has been filed in a U.S. Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods" for its potential probiotic effects (Matar et al. 2020). Given the bidirectional communication between the brain and the gut (Bravo et al. 2012); the link between the intestinal microbiota and mental health (Flowers and Ellingrod 2015; Montiel-Castro et al. 2013), probiotics are able to modulate the intestinal homeostasis and immune response (Yahfoufi et al. 2018) and to reduce inflammation at the systemic level as well as at the intestine and brain levels (Isolauri et al. 2001; Dwivedi et al. 2016; Mello et al. 2015). Also, puberty is known as a critical window of neurodevelopment and long-term induced changes in adult brain and behaviors (Kilford, Garrett and Blakemore 2016; Kane and Ismail 2017) which leads to lasting effects of inflammation exposure during adolescence on adult brain and behaviour (Andersen 2003; Holder and Blaustein 2014). Recently, there is increase interest and evidences about probiotics use to manipulate the gut microbiota (O'Toole and Cooney 2008) and to improve mental health (Liu et al. 2016; Murray et al. 2020; Kelly et al. 2015). We hypothesized that *Rouxiella badensis* subsp. *acadiensis* has potential probiotic effect and that LPS during adolescence would cause long-term depression in females and anxiety in male mice while the administration of *Rouxiella badensis*

subsp. *acadiensis (Canan-SV53)* would have an immunoprotective effect in normobiosis conditions or in LPS-induced inflammatory conditions and would prevent enduring behavioral changes later in life.

Thus, we aimed to:

1-investigate the interaction of *Rouxiella badensis* subsp. *acadiensis* with the intestinal mucosa, its localized immune-modulatory characteristics and impact on the morpho-functional aspects at the intestinal level (Article 1).

2- To study age and sex differences related to the mitigating effects of the bacterium adolescent use against long-term LPS-induced behavioral changes in adulthood (Article 2).

3- To examine the effect on the acute immune response and the gut microbiota colonization during adolescence (Article 3).

Chapter 2: Immunomodulation and Intestinal Morpho-Functional Aspects of a Novel Gram-Negative Bacterium *Rouxiella badensis* subsp. *acadiensis*

Preface

The following chapter consists of Article 1 previously published in Frontiers in Microbiology under the title **Immunomodulation and Intestinal Morpho-Functional Aspects of a Novel Gram-Negative Bacterium *Rouxiella badensis* subsp. *acadiensis* by** Nour Yahfoufi[1], Nawal Alsadi[1], Jean Francois Mallet[1], Garima Kulshreshtha[1], Maxwell Hincke[1,2], Nafissa Ismail3 and Chantal Matar[1,4*]

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Frontiers in Microbiology Authors Contribution Statement

NY: experimental design, experiments optimization and running, writing the manuscript, data collection and data analysis. CM: main supervisor, revision of experiments and guidance, data review, and manuscript review. MH and GK: collaboration on antibiotic experiments and correction of the manuscript. NA and JM: helps with samples collection during the Euthanasia day. All authors contributed to the article and approved the submitted version.

Abstract

A novel bacterium (*Rouxiella badensis* subsp. *acadiensis*) isolated from the microbiota of wild blueberry fruit was investigated for its immunomodulation capabilities and intestinal morpho-functional aspects. The whole-genome shotgun sequencing of this bacterium led to its new taxonomy and showed absence of pathogenicity genes. Although the bacterium was used for blueberry-fermentation and enhancing its anti-inflammatory effects on neurodegeneration, diabetes, and cancer, no study has assessed the effect of the bacterium on health. In this study, we used several in vitro and in vivo assays to evaluate the interaction of *R. badensis* subsp. *acadiensis* with the intestinal mucosa and its impact on the localized immune response. The strain antibiotic susceptibility has been investigated as well as its tolerance to gastric and intestinal environment and ability to attach to human intestinal epithelial cells (Caco-2 and HT-29). In addition, Balb/c mice were used to explore the immune-modulatory characteristics of the live bacterium at the intestinal level and its impact on the morpho-functional aspects of the intestinal mucosa. *In vitro* assays revealed the ability of *R. badensis* subsp. *acadiensis* to survive the gastric and intestinal simulated conditions and to satisfactorily adhere to the human intestinal epithelial cells. The bacterium was shown to be sensitive to an array of antibiotics. Immuno-modulation studies with mice orally administered with *R. badensis* subsp. *acadiensis* showed a higher number of IgA positive cells in the small intestine, a higher concentration of the anti-inflammatory cytokine IL-10 in the intestinal mucosa, as well as an increase in the number of goblet cells. The anti-inflammatory cytokine miR146a was found to be increased in the ileum and brain. Furthermore, it increases the number of goblet cells which contribute to intestinal barrier integrity.

Taken together, our findings reflect the ability of the tested bacterium to modulates the intestinal homeostasis and immune response. Detailed safety unpublished studies and genome data support our finding. The strain *Rouxiella badensis* subsp. *acadiensis* has been filed in a provisional patent; a U.S. Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods." Future studies are still needed to validate the potential utilization of this strain as functional food and its potential probiotic effect.

Keywords

Probiotic, mucosal immunity, immuno-modulation, micoRNAs (miRNAs), gastro-intestinal tolerance

Introduction

The bacterium *Rouxiella badensis* subsp. *acadiensis* (*R. badensis* subsp. *acadiensis*) explored in this study was previously called *Serratia vaccinii* (Martin and Matar, 2005). The strain was isolated from the surface of lowbush blueberries (Vaccinium angustifolium Aiton) by inoculation of a Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, United States) with whole blueberry fruit as described in Martin and Matar (2005). In this study, inocula were prepared from a Research Cell Bank of the bacterium. *R. badensis* subsp. *acadiensis* is a Gram negative, catalase positive, facultatively anaerobic coccobacillus. It has a fermentative ability of D-glucose, D-fructose, D-mannose, arbutin, esculin, salicin, saccharose, and D-raffinose and under specific conditions and can ferment mannitol, lactose, and trehalose (Martin, 2003; Martin and Matar, 2005). Functionally, the protective properties of a blueberry fermented preparation utilizing this bacterium has shown a wide range of anti-inflammatory effects on neurodegeneration, diabetes, and cancer (Vuong et al., 2016, 2007; Nachar et al., 2017; Sánchez-villavicencio et al., 2017). As per published study the bacterium previously referred to as Serratia vaccinii has been deposited under Accession Number 160 103, at the International Depositary Authority of Canada. They referred to it as Serratia based on its 16S rRNA gene sequence associated with biochemical profile and physical properties (Martin and Matar, 2005). As the 16S rRNA gene sequence analysis was shown to be insufficiently robust to distinguish closely related species and new advanced techniques have emerged, a whole-genome shotgun sequencing and assembly of bacterial strain were performed to determine the phylogenetic position of the isolate. These analyses led to the description of a novel *Rouxiella badensis* subspecies for which we propose the name *R. badensis* subsp. *acadiensis*, following the region

where it was firstly isolated. The type strain is Canan SV-53. The novel bacterium has been deposited at the International Depositary Authority of Canada under Accession Number 160103, (Antioxydant Producing Bacterium and USES Therefore US 8,617,870). A taxonomy description paper of the strain is now under revision. Unpublished data related to genome propose a potential probiotic activity of the bacterium since full genome analysis showed that there are no genes found coding for pathogenicity. A US Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods" has been filed related to the bacterium. Since there are more papers in the process dealing with the bacterium toxicology and safety uses, we have evaluated in this study some in vitro and in vivo characteristics of the Gram-negative *R. badensis* subsp. *acadiensis* and its impact on immunomodulation and intestinal homeostasis and mucosal immunity. Our aim is to study the bacterium ability to interact with the intestinal mucosa and modulate the immune response in order to determine whether future analyses are worth it to investigate potential probiotic activity. In the future, we will be proposing probiotic activity once the safety studies will be published especially that *Rouxiella* belongs to the family of Yerciniacae. While recent findings reveal beneficial immunological effects of certain dead bacteria (Vinderola and Reinheimer, 2003; Mottet and Michetti, 2005), most studies emphasize the importance of colonizing the intestinal epithelium to sustain bacterial beneficial effects (Gopal et al., 2001; Kos et al., 2003; Kumura et al., 2004). Thus, the assessment of bacterial tolerance to human gastric or intestinal juice is of great importance to see if the alive bacterium can reach the intestine (Sanders and Huis In't Veld, 1999; Shah, 2001). Furthermore, beneficial bacteria can adhere to intestinal mucosal surfaces, thus adherence is a key property that shows the ability of bacteria to avoid elimination, to better colonize the intestinal epithelium and to compete with other microorganisms comprising pathogens for nutrients and attachment sites

(Kos et al., 2003). In the host, a complex relationship exists between commensal bacteria, their metabolites, the gut mucosal barrier and the immune and non-immune cells along the intestine (Yahfoufi et al., 2018). The mucosal layer is an important physical barrier; it contains goblet cells which secrete a highly glycosylated mucin and create a net-like layer playing a key role in barrier maintenance. Moreover, they can produce anti-microbial proteins, chemokines, and cytokines with major role in innate immunity. Oral administration of probiotics strengthens the intestinal barrier integrity: they can influence the gut barrier by numerous mechanisms including modulation of mucus production, enhancement of tight junctions, induction of IgA and modulation of the immune response (Hemaiswarya et al., 2013; Scully et al., 2013). Moreover, bacteria can modulate proinflammatory and anti-inflammatory cytokines (Ren et al., 2013; Boonma et al., 2014; Elian et al., 2015). For example, *Lacticaseibacillus rhamnosus, Bifidobacterium longum, Lactobacilus acidophilus, Lactobacillus ruteri, Lacticaseibacillus paracasei*, are able to modulate the T cells response by upregulating T reg cells and IL-10 levels in intestinal contents of mice by indirectly involving short-chain fatty acids (SCFAs) (Atarashi et al., 2013) which are produced by anaerobic bacterial fermentation of prebiotics (Yahfoufi et al., 2018). Secretory IgA is of fundamental importance in host mucosal protection against mucosal pathogens (Perdigon et al., 2001; Perdigón et al., 2002). Numerous studies have reported the role of IgA+ cells in maintaining homeostasis of the intestine (Vinderola G. et al., 2005; Galdeano et al., 2007; Mallet et al., 2014). Hence the importance to investigate the effect of *R. badensis* subsp. *Acadiensis* on the number of IgA positive cells in Peyer's patches and in the lamina propria as well as on the production of secretory IgA. Furthermore, small non-coding RNAs regulate gene expression post-transcriptionally (Zhou et al., 2017). miRNAs have been shown to regulate various biological processes involved in inflammation, immune homeostasis and

intestinal integrity (Gaudet et al., 2017). Certain miRNAs could initiate an anti-inflammatory response such as miR145 and miR146a while other miRNAs play a role in proinflammatory response and some have mixed immunomodulatory effects (Bermudez-Humaran and Langella, 2017). Bacteria like probiotics were found able to modulate miRNAs (O'Connell et al., 2012). Finally, it is important to address concerns that have been raised in the use of some bacterial strains that carry antibiotic resistance genes themselves, as they may pass the antibiotic resistance genes to pathogenic bacteria through horizontal gene transfer. Therefore, antibiotic resistance is often studied in bacterial characterization which can present a challenge to their safe usage. Screening bacterial strains for antibiotic resistance is important to ensure their safety and reduce their potential in contributing to the spread of antibiotic resistance genes (Imperial and Ibana, 2016). Although the most commonly used bacteria for their potential health benefits are Gram-positive (G+) bacteria such as lactic-acid producing bacteria and other that belong to the genus Bifidobacterium, some Gram-negative (G-) bacteria like *Escherichia coli Nissle 1917*(EcN) can exert health efficacy (Saad et al., 2013). When comparing G- and G+ probiotics, G- probiotics show higher activity in reducing the levels of inflammatory mediators during enteric infections (Cross et al., 2004). Referring to existing studies, G- induce higher IL10-responses than G+ probiotics resulting in differential induction of antibody responses. G- EcN has more potent immuno-stimulatory effects than G+ *Lactobacillus rhamnosus GG* in term of inducing mucosal and systemic B cells and results in higher production of IgA at the small intestinal level (Hessle et al., 2000; Kandasamy et al., 2016). The microbe-associated molecular patterns differ between G- and G+ probiotics and can explain the difference in induction of IL-10. In addition, higher antibody response induced by G- when compared to G+ probiotics can be due to different modulation of cytokine milieu (Karlsson et al., 2002; Smits et al., 2004). It is

well known that variations in immunomodulation characteristics are strain-dependent even within G+ probiotics (Christensen et al., 2002). Therefore, we investigated the resistance of this bacterium to commonly used antibiotics, its tolerance to simulated gastric and pancreatic juices, and ability to adhere to intestinal epithelial cells. In vivo, the bacterium was administered to Balb/c mice: IL-6, IL-10, and secretory IgA concentrations in mice intestinal fluid were measured, in addition to the abundance of IL-10 positive cells in the ileum. Moreover, we investigated the morpho-functional changes in the small intestine, and the expression of selected anti-inflammatory miRNAs (miR145 and miR146a).

Materials and Methods

Bacterial Cultures and Media

Stock cultures of the bacterium were maintained at -80ºC in Tryptic Soy Broth (Difco Laboratories, Detroit, MI, United States) supplemented with 30% (v/v) glycerol. The bacterium was grown in Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI, United States) or Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, United States) at 30ºC for 20 h.

Determination of Antibiotic Resistance

Overnight bacterial culture inocula were diluted 1:50 in fresh TSB and grown until the optical density at 600 nm reached 0.2 (~10^8 CFUs/ml). Thereafter, the standardized inoculum (100 ml, OD600 = 0.2–10^8 CFUs/ml) was spread uniformly on TSA. The antibiotic sensi-disks (BD BBL, Sensi-Disc, Becton, Dickinson and Company, United States) used were the following: (ampicillin:10 mg, Tetracyclin: 10 mg, streptomycin: 10 mg, chloramphenicol: 30 mg, erythromycin: 15 mg or neomycin: 5 mg). Blank disks used as negative control were obtained as well from (BD BBL, Sensi-Disc, Becton, Dickinson and Company, United States). While ciprofloxacin 5 mg, trimethoprim-sulfamethoxazole 25 mg were obtained from Oxoid, ON, Canada. Antibiotic disks or blank disks were placed at the center of the TSA plate (one disk per plate). The plates were incubated at 37ºC until even growth of the bacterium was observed. The zones of growth inhibition were measured using a Vernier caliper to categorize the strains as susceptible or resistant to the antibiotic. Experiments were performed three times in duplicates.

Determination of Tolerance to Simulated Gastric Juices and Simulated Pancreatic Juices

Preparation of Simulated Gastric Juice

Simulated gastric juices were prepared by suspending pepsin (Sigma, Saint Louis, MO, United States) to a final concentration of 3 g/l in sterile NaCl (Sigma, Saint Louis, MO, United States) 0.5% weight to volume and adjusting the pH to 2.0, 3.0 or 4.0 with concentrated HCl or sterile 0.1 mol/l NaOH (Huang and Adams, 2004).

Preparation of Simulated Pancreatic Juice

Simulated pancreatic juice was prepared by suspending porcine pancreatin (Sigma, Saint Louis, MO, United States) in sterile saline to a final concentration of 1 g/l, with 0.45% bile salts (Sigma, Saint Louis, MO, United States), and adjusting the pH to 7 or 8 with concentrated HCl or sterile 0.1 mol/l NaOH (Huang and Adams, 2004).

Determination of Tolerance to Simulated Gastric and Pancreatic Juices

A volume of bacterial inoculum corresponding to 10^8 to 10^9 CFU/ml from an overnight culture was subjected to low-speed centrifugation 5000 x *g* for 5 min and the pellet was washed two times in 1X PBS (137 mM NaCl 2.7 mM KCl 10 mM Na2HPO4, 1.8mM mM KH2PO4) pH7. The starting count was determined by standard plate count prior to assay transit tolerance. When screening for gastric or intestinal transit tolerance, 1 ml of simulated gastric juice (pH 2, 3, or 4) or 1 ml of simulated pancreatic juice (pH 7 or 8) and 300 ml of NaCl (0.5% W/V) were added to washed bacterial suspension in 2 ml screw cap sterile microcentrifuge tubes. The preparations were vortexed at low speed for 10 s and incubated at 37ºC. When testing for gastric transit tolerance, aliquots of 100 ml were collected after 60, 90, and 180 min for the determination of total viable counts; while aliquots of 100 ml were collected after 60, 90, 180, and 240 min when

screening for intestinal transit tolerance. To better imitate the passage through the gastro-intestinal tract, bacterial inoculum exposed to simulated gastric juice (pH 2 or 4) for 180 min was centrifuged and re-exposed to simulated pancreatic juice (pH 7 or 8) for 240 min and bacterial count was done after collecting aliquots of 100 ml. Each 100 ml was serially diluted, spread on TSA plates, and incubated at 30ºC for 72 h, followed by determination of CFU count. Results are expressed as Log10 of the CFU/ml.

Adhesion to Epithelial Intestinal Cell Lines

Cell Cultures

Caco-2 cells (ATCC-HTB-37) were grown in minimum essential medium alpha (MEMα; Gibco Canada, Burlington, ON, Canada) containing 10% v/v inactivated fetal bovine serum (FBS; Thermo Fisher Scientific), 1% non-essential amino acids (Gibco) and a mixture of antibiotics at a final concentration of 50 IU/ml penicillin and 50 mg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% v/v CO2. For adherence and invasion assays, confluent Caco-2 cells were harvested by trypsinization in 0.01% EDTA. Caco-2 cells were adjusted to 105 cells/ml by counting in a hemocytometer, seeded in a 24-well tissue culture plate and incubated at 37°C in a 5% CO2 humidified atmosphere until a confluent monolayer formed. Prior to adhesion assay, the cell monolayers were washed twice with 1X PBS (pH 7.4-Sigma, Saint Louis, MO, United States) to remove the antibiotics. HT-29 (ATCC-HTB-38) cell line was maintained in McCoy's medium (Gibco, United States) supplemented with 10% (v/v) heat inactivated bovine fetal serum and a mixture of antibiotics at a final concentration of 50 IU/ml penicillin, 50 mg/ml streptomycin. Cells were grown at 37°C, 5% CO2. Culture media were changed every two days and the cell line was trypsinized with 0.25% trypsin-EDTA solution

(Sigma, Saint Louis, MO, United States). For experiments, 105 cells/ml were seeded in 24-well plates and incubated to confluence. Caco-2 cells were incubated till 15 days old while HT-29 were incubated as per Arboleya et al. to confluence during 13 ±1 day.

Adhesion Assay

Adhesion assay was explored as per Arboleya et al. (2011); bacterial cultures were harvested by centrifugation, washed twice with 1X PBS buffer (Sigma, Saint Louis, MO, United States) and resuspended in MEMα or McCoy's medium supplemented with 10% heat inactivated bovine fetal serum without antibiotics. Prior to adhesion assay, the cell monolayers of Caco-2 or HT-29 were washed three times with 1X PBS (pH 7.4-Sigma, Saint Louis, MO, United States) to remove the antibiotics before adding bacterial suspensions. Bacterial cells were inoculated into wells containing confluent monolayers of Caco-2 or HT-29 cells at a targeted multiplicity of infection (MOI) of 100:1. The actual numbers of bacteria in the inoculum added to the monolayers were confirmed retrospectively by serial dilution and plate counting. Bacteria infected cells were incubated for 4 h at 37°C and 5% CO2 to allow for bacterial adherence. Supernatants were discarded and wells were gently washed three times with 1X PBS buffer to remove the non-attached bacteria. After gentle scraping of cells, the bacterial counts were carried out by plating on TSA to determine the number of adhering bacteria. Serial dilutions were plated and incubated for 48 h at 30°C. The adherence (expressed as a percentage) was calculated by using the ratio of the number of bacterial cells that remained attached to the total number of bacterial cells added initially to each well.

Animals and Bacterial Supplementation

Six to eight-week-old Balb/c female mice weighing 20–25 g were obtained from Charles River (Montreal, Canada). A total of 20 mice were divided into experimental and control groups consisting of 10 mice per group for all experiments except for the qPCR of brain and ileum (21 mice were used further details are provided below). Pairs of mice were housed together in plastic cages kept in a controlled atmosphere (temperature 22 ± 2°C) with a 12 h light/dark cycle. Mice received 10^8 CFU of *R. badensis* subsp. *acadiensis*/mouse by gavage for 7 consecutive days in 1% sucrose, 1X PBS pH 7.4 (sigma). Control mice received the same volume of 1% sucrose in 1X PBS instead. All mice received simultaneously a conventional balanced diet ad libitum and water. Test and control animals were sacrificed after 7 days of probiotic administration. All experimental procedures were approved by the Animal Care Committee of the University of Ottawa (protocol ME-2403-R3) in accordance with guidelines established by the Canadian Council of Animal Care.

Bacterial Translocation Assay

After 7 days, treated and control animals were anesthetized and sacrificed by cervical dislocation; livers were immediately harvested under sterile conditions and homogenized using the Omni TH homogenizer with a 7-mm generator probe (Omni International, Kennesaw, GA, United States) in 5 ml sterile 1X PBS pH 7.4 (Sigma, St. Louis, MO, United States). One milliliter of the liver homogenate was spread onto the surface of MacConkey agar for enterobacteria (Difco Laboratories, Detroit, MI, United States). The plates were then aerobically incubated at 37°C for 48 h. Translocation was considered to have occurred when colonies were observed on the agar plates because the liver is an organ normally devoid of bacteria

(Gregory et al., 1996).

Histological Studies of the Gut

The small intestines of sacrificed mice were removed, washed and the ileum parts were collected and fixed overnight in 4% PFA solution (paraformaldehyde). Afterward, the tissues were dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in paraffin wax using conventional methods (Alturkistani et al., 2016). Then histological slices of 4mm were prepared from paraffin blocks using a rotation microtome (Leica RM2255 Automated Microtome). Processing, paraffin embedding, and histological slices were performed by The University of Ottawa histology core facility.

B Population (IgA+ and IgG+ Cells) Identification by Immunofluorescence

The number of IgA and IgG producing (IgA+ and IgG+) cells was determined on histological slices prepared from the ileum using the direct immunofluorescence method. The immunofluorescence test was performed using (a-chain) antimouse IgA FITC conjugate (Sigma–Aldrich, St. Louis, MO, United States) or (d-chain) anti-mouse IgG FITC conjugate (Sigma–Aldrich, St. Louis, MO, United States). Histological sections were deparaffinized and then rehydrated in a graded ethanol series. Sections were incubated at 37°C in two different solutions of xylene (Sigma–Aldrich, St. Louis, MO, United States) successively for 15 min in each solution followed by incubation in graded ethanol series 95, 70, and 40% for 5 min each at 4°C. Deparaffinized histological samples were incubated with the appropriate antibody dilution (1:100 for IgA or 1:50 for IgG) in 1X PBS solution for 30 min at 37°C in the dark. Samples were then washed two times with PBS solution and examined using a fluorescent light microscope. Dry slides were mounted using Fluoromount (Sigma-Aldrich, St. Louis, MO, United States). The

results were expressed as the number of IgA+ or IgG+ cells (positive = fluorescent cell) per 10-fields (magnification 100X). Data represent the mean of three readings for each animal.

Determination of IL-10+ Cells in Mice Small Intestine

At the end of the 7 days period, the small intestine was removed and processed for histological preparation as described above. The indirect immunofluorescence method was used to label IL-10 positive cells. After deparaffinization of the histological slices, they were rehydrated in a graded series of ethanol -as per described above- and then incubated for 30 min in a 1% blocking solution of bovine serum albumin (Jackson Immuno Research, West Grove, PA, United States) at room temperature. Histological slices mounted on slides were then incubated for 60 min at 37°C with rabbit anti-mouse IL-10 polyclonal antibodies (Peprotech, Inc., Rocky Hill, NJ, United States) at a dilution 1:50. 1X PBS solution was used for two consecutive washes, then sections were treated for 45 min at 37°C with a 1:100 dilution of a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research, West Grove, PA, United States). Finally, sections were washed twice with 1X PBS and examined using a fluorescent light microscope. Dry slides were mounted using Fluoromount (Sigma-Aldrich, St. Louis, MO, United States). The results were expressed as the number of IL10 + cells (positive = fluorescent cell) per 10-fields (magnification 100X).

Determination of Small Intestine Morpho-Functional Changes

Different intestinal segments were collected from treated and control animals. Intestinal segments were fixed in 4% PFA, dehydrated, cleared and embedded in paraffin. Serial sections were cut at 4 mm, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin. For staining neutral glycoconjugates or acid glycoconjugate in the intestinal sections we used periodic acid Schiff (PAS) or Alcian blue (AB), pH 2.5. This staining reveals neutral PAS

reactive and acid AB-reactive glycoconjugates (Smirnov et al., 2005; Martín et al., 2016). All processing, paraffin embedding and histological staining with hematoxylin, eosin, PAS or AB were performed by The University of Ottawa histology core facility. The morphology of different intestinal segments was revealed by staining with hematoxylin and eosin for examination by light microscopy. Positive goblet cells for neutral glycoconjugates or acid glycoconjugates were counted. The results were expressed as the number of goblet cells per 10-fields (magnification 100X). Data represent the mean of three readings for each animal.

Determination of IL-6, IL-10, and Secretory IgA in Mice Intestinal Fluid

The small intestine of treated and control animals was flushed with 5 ml of 1X PBS pH7.4 (Sigma, St. Louis, MO, United States) and this fluid was centrifuged at 10,000 g at 4°C for 10 min to separate particulate material. The supernatant was collected and kept frozen at -80°C until use. IL-6 and IL-10 were determined in supernatant using the corresponding mouse ELISA Set (BD OptEIA, BD Biosciences PharMingen, San Diego, CA, United States) with catalog numbers respectively: 555240 and 555252. For IL-6, capture antibody was diluted at 1:250 in coating buffer (BD OptEIA, BD Biosciences PharMingen, San Diego, CA, United States) while the detection antibody was diluted at 1:259 in assay diluent (BD OptEIA, BD Biosciences PharMingen, San Diego, CA, United States). For Il-10, dilutions were respectively 1:250 dilution in coating buffer for capture antibody while 1:250 dilution in assay diluent for the detection antibody. For secretory IgA, the level of IgA was analyzed by DAS-ELISA using affinity purified goat anti-mouse IgA antibodies (a-chain specific) was added at 1.25 mg/well and horseradish-peroxidase conjugated anti-IgA specific antibodies at 1.25 mg/well (Sigma Chemical

Co., St Louis, MO, United States). Absorbance was read at 450 nm within 30 minutes of stopping reaction with wavelength correction 570 nm.

Real-Time Quantitative Reverse Transcription PCR

Ileum pieces and brains from 11 control: CTR and 10 *R. badensis* subsp. *acadiensis* treated mice: *R. badensis* subsp. *acadiensis* were snap frozen after collection and kept at -80°C until RNA extraction. Samples were extracted using miRNeasy mini kit (Qiagen, Toronto, ON, Canada). Sample purity were verified with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States). Samples underwent a reverse transcription reaction to produce cDNA using individual probes. The cDNA was synthesized by Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen, Canada inc.). The expression of target anti-inflammatory miR-145 and miR-146a was measured by RT-qPCR using Taqman primers for hsamiR- 146a (Thermofisher Taqman miRNA assay ID 000468) and hsa-miR-145 (Thermofisher Taqman assay ID 002278) and a FastStart Taq Polymerase (Roche, Mississauga, ON, Canada) in a CFX96 real-time PCR detection system (Bio-RAD, Laboratories,
Hercules, CA, United States). Gene expression was normalized to gene reference U6 small non-coding RNA (Thermofisher assay ID 001973).

Statistical Analysis

Results are presented as means ±SEM. Results represent three independent experiments. GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States) was employed to carry out statistical analysis. The statistical significance was determined by applying an unpaired two-tailed Student's t-test when comparing two groups, and one-way

analysis of variance (ANOVA) when comparing more than two groups. A difference was considered significant for p-values <0.05.

Results

Antibiotic Susceptibility by Agar Disk Diffusion Method

Rouxiella badensis subsp. *acadiensis* demonstrated susceptibility to different classes of antibiotics like b-lactams, fluoroquinolones, aminoglycosides, chloramphenicols, tetracyclines, and trimethoprim-sulfamethoxazole. *R. badensis* subsp. *acadiensis* is sensitive to ampicillin 10mg (a b-lactam) with a diameter of inhibition zone (di) greater than 17 mm di = 38.67 ± 0.72), the strain is sensitive to ciprofloxacin 5 mg (fluoroquinolones class) with a di = 39.16 ± 1.57 mm > 21 mm. Streptomycin 10 mg (an aminoglycoside) revealed an inhibition zone with a di = 18.08 ± 0.73 > 15 mm reflecting sensitivity of the strain. The bacterium is as well sensitive strain to: chloramphenicols 30 mg with a di = 23.5 ± 1.38 > 15 mm, tetracyclines 10 mg with di = 27.6 ± 1.1 > 15 mm and trimethoprim-sulfamethoxazole 25 mg with a di = 24.5 ± 2.1 > 16 mm while it possessed intermediate sensitivity to erythromycin15 mg (a macrolide) with a di = 20.1 ± 0.6 mm (Table 2.1).

Antibiotic disk	Class of the antibiotic	Diameter (d) of inhibition zone in mm*	Zone diameter (mm) indicating			Conclusion
			S	I	R	
Ampicillin 10μg	β-lactams	38.67 ± 0.72	≥17	14–16	≤13	S
Ciprofloxacin 5 μg	Fluoroquinolones	39.16 ± 1.57	≥21	16–20	≤15	S
Streptomycin 10 μg	Aminoglycosides	18.08 ± 0.73	≥15	12–14	≤11	S
Chloramphenicol 30 μg	Chloramphenicols	23.5 ± 1.38	≥15	13–17	≤12	S
Trimethoprim-sulfamethoxazole 25 μg	Folate pathway antagonists	24.5 ± 2.1	≥16	11–15	≤10	S
Tetracycline 10 μg	Tetracyclines	27.6 ± 1.1	≥15 (30 μg)	12–14	≤11	S
Erythromycin 15 μg	Macrolides	20.1 ± 0.6	≥23	14–22	≤13	I

Comments: S (sensitive), I (intermediate sensitivity), R (resistant).

Table 2.1. Antibiotic susceptibility of *R. badensis* subsp. *acadiensis* to different antibiotics using the agar disk diffusion method

Determination of Tolerance to Simulate Gastric Juices and Simulated Pancreatic Juices

Rouxiella badensis subsp. *acadiensis* showed high stability when incubated in simulated gastric juice. The total duration of incubation tested was 180 min at 37°C which is equivalent to time the bacteria are usually resident at the stomach level. The studies showed that the bacterium displayed high survival and high tolerance to the gastric juice at pH 3 and 4 (Figures 2.1B, C). After incubation for 180 min at pH 2 we observed a significant killing effect $p < 0.05$ (Figure 2.1A). However, the bacterium was still able to survive at high rate. *Rouxiella badensis* subsp. *acadiensis* tolerated the simulated pancreatic juices as well; survival percentages were tested after exposure for 240 min. The bacterium showed highest survival and viability when incubated at pH 7 for 240 min. At pH 8, bacterial growth was observed after 60- and 90- min incubation at 37°C but after 3 and 4 h incubation there were no difference with t = 0 min. The bacterium maintained high survival rate at simulated pancreatic juice (Figures 2.1D, E). After passage of the strain through simulated gastric (pH 2 or 4) for 180 min and subsequent intestinal conditions (pH 7 and 8) for 240 min, we observed higher killing ability of the same tested pHs previously tested (Figure 2.1F).

Adhesion to Human Epithelial Intestinal Cell Lines

Adhesion to cells of the intestinal epithelium is another property that probiotics must possess for successful colonization of the human gastrointestinal tract. The ability of *R. badensis* subsp. *acadiensis* to adhere to intestinal epithelial cells was studied in vitro. Human epithelial colorectal adenocarcinoma Caco-2 and HT-29 cells were used. An adhesion rate of 17.8% ± 2.516 on Caco-2 cells, and 19.92% ± 2.078 on HT29 cells was found (Figure 2.2; Turpin et al., 2012; Argyri et al., 2013).

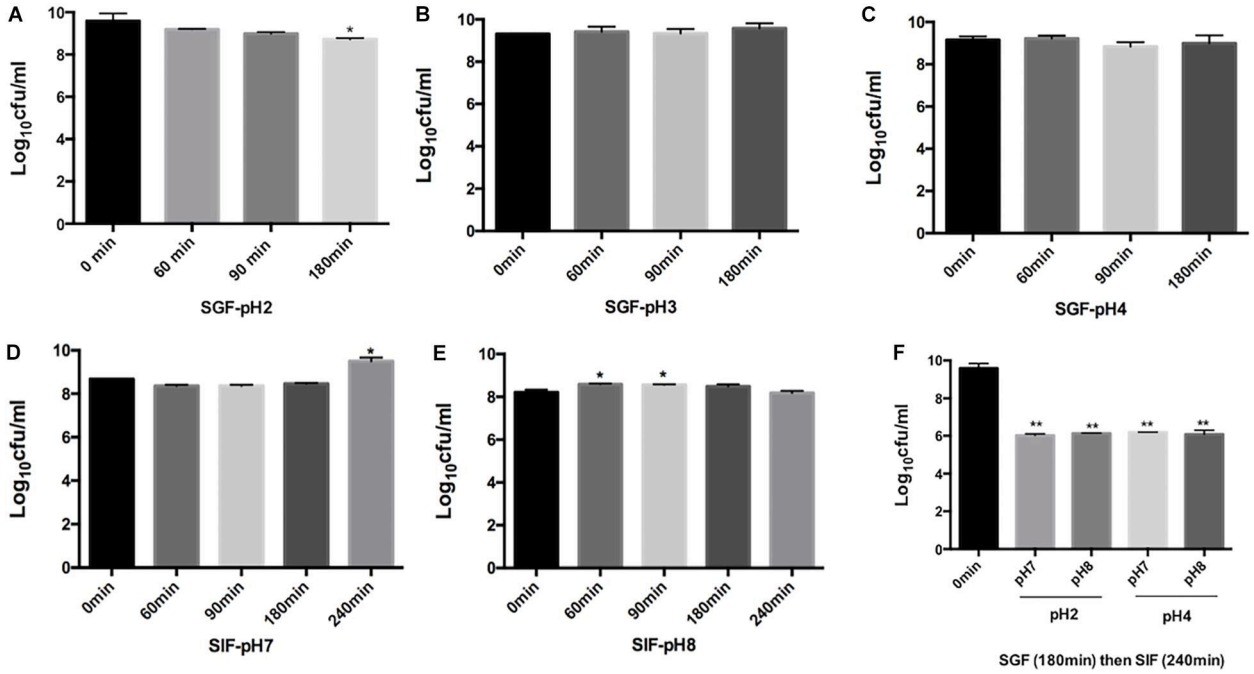

Figure 2.1 Effect of simulated gastric juices SGF [pH 2 (A) pH 3.0 (B), pH 4.0 (C)] and simulated intestinal juice SIF [pH7 (D), pH 8 (E)] on the viability of *R. badensis* subsp. *acadiensis* that was exposed to SGF for 60, 90, and 180 min and to SIF for 60, 90,180, and 240 min. In panel (F) *R. badensis* subsp. *acadiensis* was first exposed to SGF for 180 min then to SIF for 240 min. Data represents mean log10CFU/ml ± SEM (n = 3). Significant difference exists if *$p < 0.05$, **$p < 0.01$.

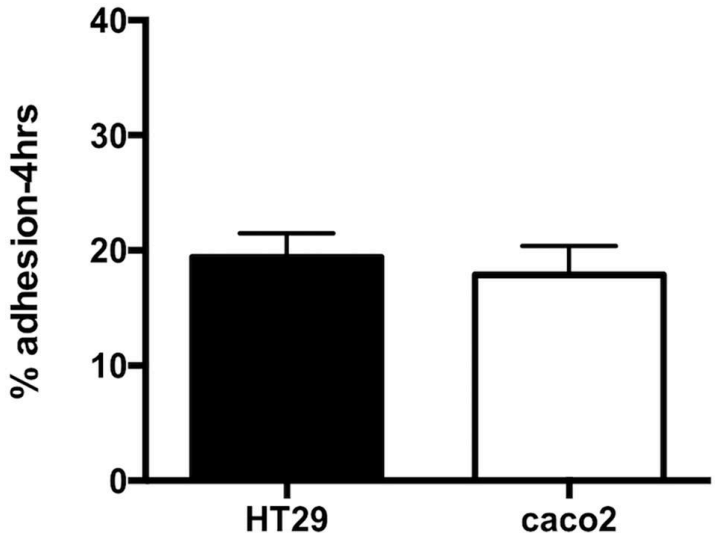

Figure 2.2. Data represents percentage of adhesion of *R. badensis* subsp. *acadiensis* to HT29 and Caco2 cells. Results shown are means of three independent experiments ± SEM.

Bacterial Translocation

Oral administration of *R. badensis* subsp. *acadiensis* did not cause translocation of Enterobacteriacae to the liver in mice treated with 10^8CFU/mouse. No bacterial growth on the MacConckey agar plates was observed for extracts of liver samples collected from Balb/c mice fed the bacterium.

Histological Studies of the Gut
Small Intestine Overall Morphology

The histological study of the small intestine of mice that received *R. badensis* subsp. *acadiensis* for 7 consecutive days showed no edema or mucosal atrophy, compared to control mice. The overall morphology of the small intestine did not show any significant changes when compared to control mice (not shown).

B Cells Population (IgA+ and IgG+ Cells)

The mucosal immunomodulating capacity of *R. badensis* subsp. *acadiensis* was assessed in this study by examining its effects on the IgA+ and IgG+ B cell populations and selected cytokines (pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10) in both the gut mucosa and the intestinal contents. The number of IgA and IgG producing cells (IgA+ and IgG+) in the lamina propria of the small intestine of mice that received *R. badensis* subsp. *acadiensis* showed a significant increase in the number of IgA+ cells in the lamina propria (Figure 2.3) (96.89 ± 1.933) in comparison to the control group (74.23 ± 2.588). The number of IgG+ cells was unchanged (Figure 2.4).

Increase of IL10+ Cells in the Ileum

IL10+ cells were shown to be significantly increased in the intestine of mice receiving the probiotic bacterium (74.83 ± 2.065) versus (49.43 ±1.805) in the control group (Figure 2.5).

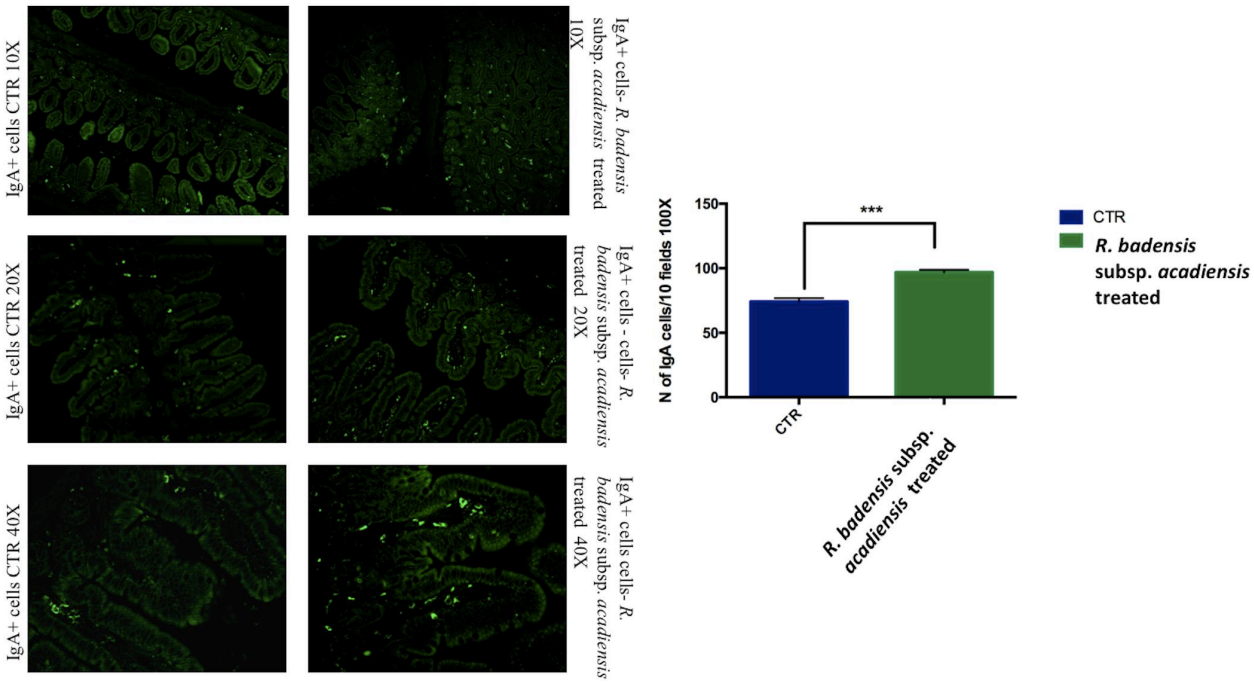

Figure 2.3. Mean ± SEM of the number of IgA positive cells populations in 10 fields of objective 100X in the ileum of mice fed 1% sucrose (CTR) or *R. badensis* subsp. *acadiensis* fed 10^8CFU/mouse/day for 7 days. Significant difference exists if *p < 0.05, representative tissue photos are taken with 10X, 20X, and 40X magnifications.

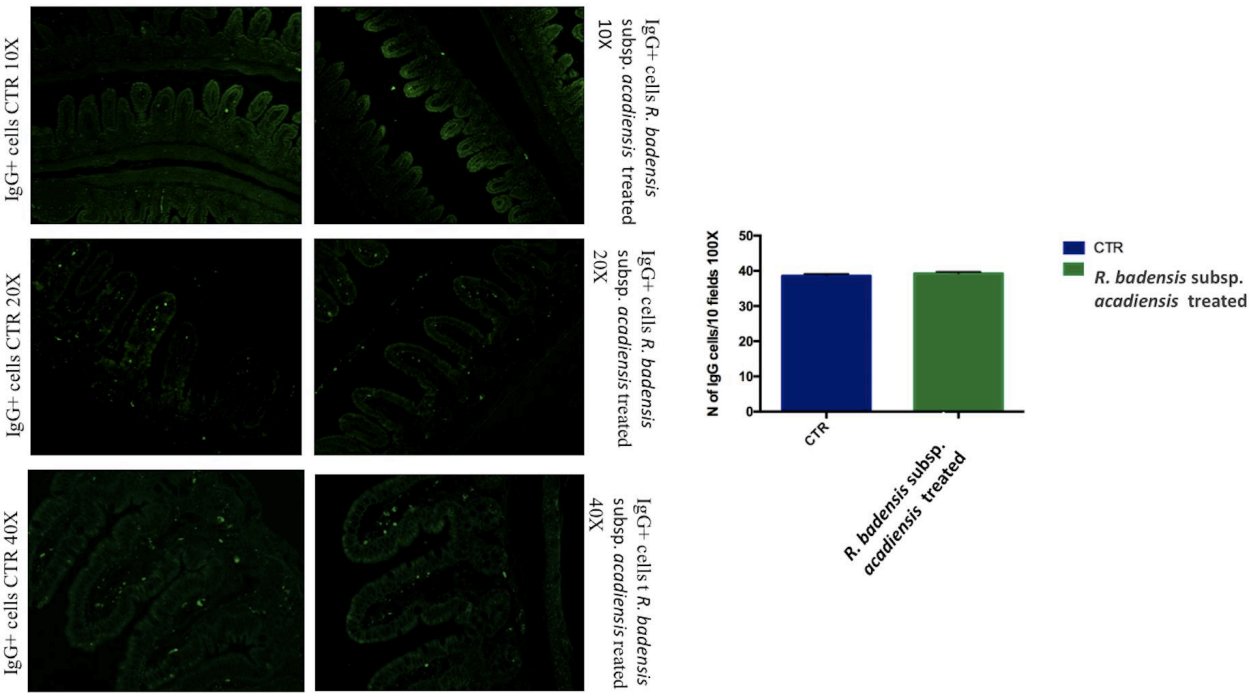

Figure 2.4. Mean ± SEM of the number of IgG positive cells populations in 10 fields of objective 100X in the ileum of mice fed 1% sucrose (CTR) or *R. badensis* subsp. *acadiensis* fed 10^8CFU/mouse/day for 7 days. Significant difference exists if *$p < 0.05$, representative tissue photos are taken with 10X, 20X, and 40X magnifications.

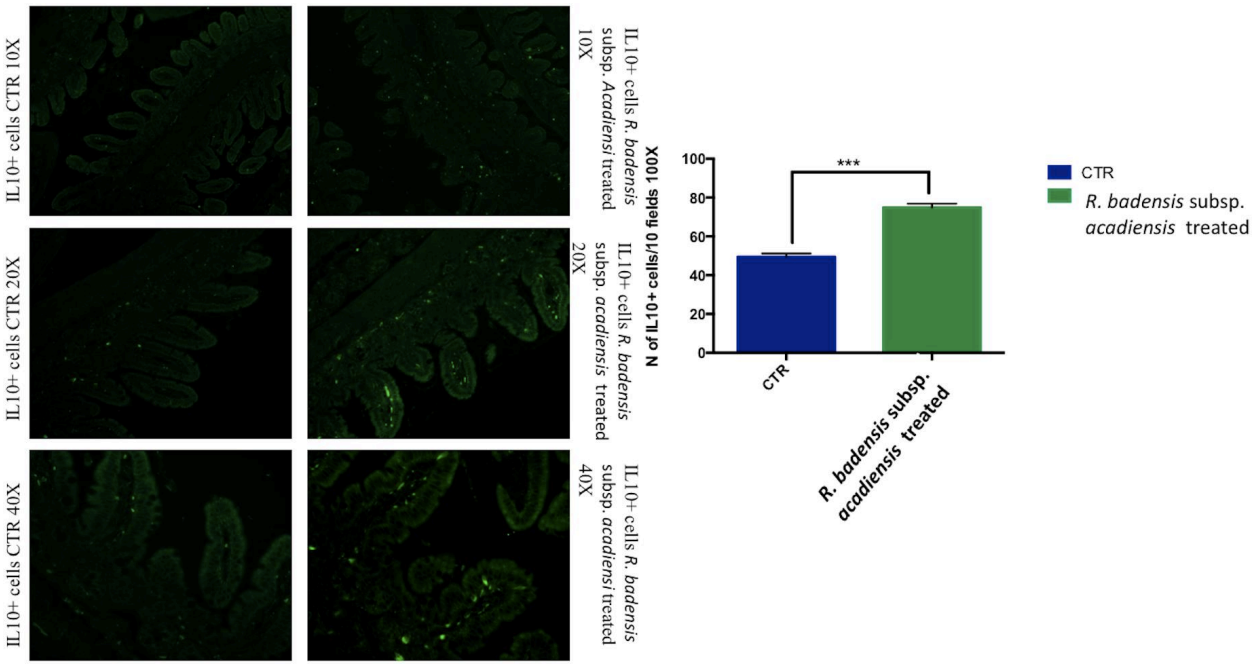

Figure 2.5. Mean ± SEM of the number of IL10 positive cells populations in 10 fields of objective 100X in the ileum of mice fed 1% sucrose (CTR) or *R. badensis* subsp. *acadiensis* fed 108CFU/mouse/day for 7 days. Significant difference exists if *p < 0.05- p is < 0.001 representative tissue photos of IL10 positive cells in the lamina propria are taken with 10X, 20X, and 40X magnifications.

Positive Goblet Cells Determination

Considering the multiple functions of the glycoconjugates that make up intestinal mucins, positive goblet cells for neutral glycoconjugates or acid glycoconjugate were counted after staining intestinal sections with periodic acid Schiff (PAS) and AB (Smirnov et al., 2005; Martín et al., 2016). Tissue histology for small intestine of *R. badensis* subsp. *acadiensis* fed groups displayed a significant increase in the number of PAS stained goblet cells (149.1 ± 3.167) in comparison to the control group (137.9 ± 3.811) with p = 0.0355(Figure 2.6). However, AB staining at pH2.5 did not show any increase in the number of goblet cells positive for acid glycoconjugates (Figure 2.7).

IL-10, IL-6, and Secretory IgA Determination

A significant increase in IL-10 was observed in the intestinal fluid of animals that received *R. badensis* subsp. *acadiensis* for 7-days when compared with control mice (p = 0.042) (Figure 2.8A). There was no change in the levels of IL-6 (Figure 2.8B). Secretory IgA were shown to be increased significantly in the intestinal fluid of mice fed the bacterium (19,232 ng/ml ± 2,431) compared to the control group (12,368 ± 1,429) with p = 0.0255 (Figure 2.8C).

miR145 and miR146a Expression at the Intestinal and Brain Levels

Although no statistically significant changes were observed in the expression of miR145 in ileum or brain, we did observe a trend toward increased expression in the brain. However, a significant increase in miR146a expression was observed in ileum and brain revealing the anti-inflammatory potential of *R. badensis* subsp. *acadiensis* (Figure 2.9).

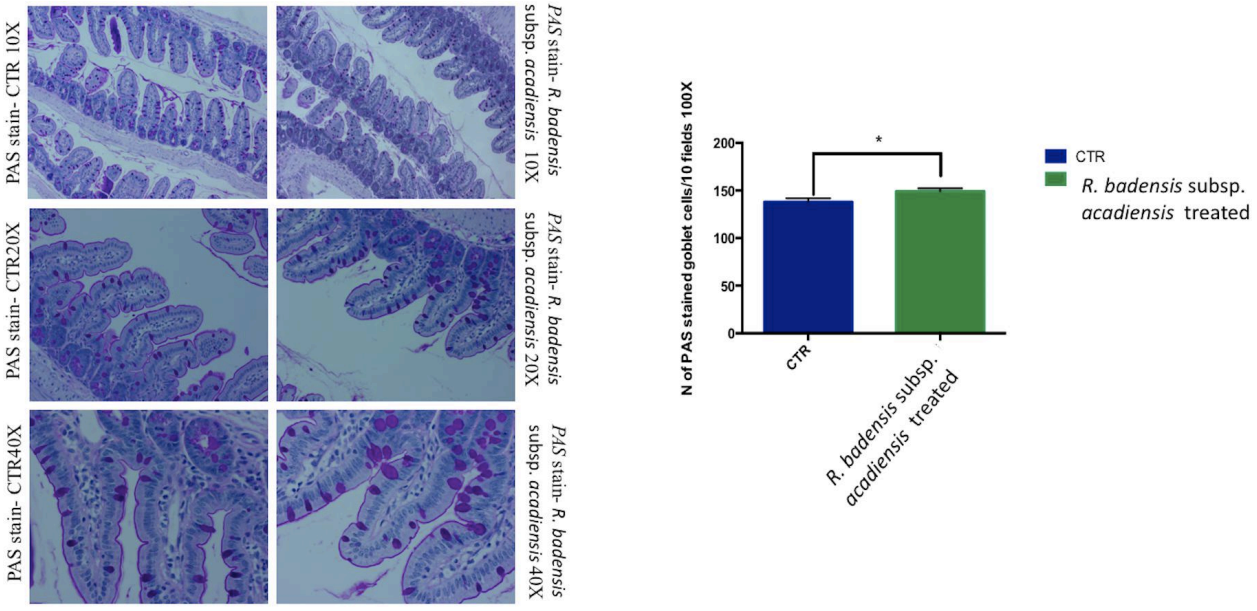

Figure 2.6. Mean ± SEM of the number of goblet cells stained with PAS (Periodic Acid Schiff) in 10 fields of objective 100X in the small intestine of mice fed 1% sucrose (CTR) or *R. badensis* subsp. *acadiensis* fed 108CFU/mouse/day for 7 days. Significant difference exists if *p < 0.05- p = 0.035. Representative tissue photos are taken with objective 10X, 20X, and 40X magnifications of the PAS-stained tissue.

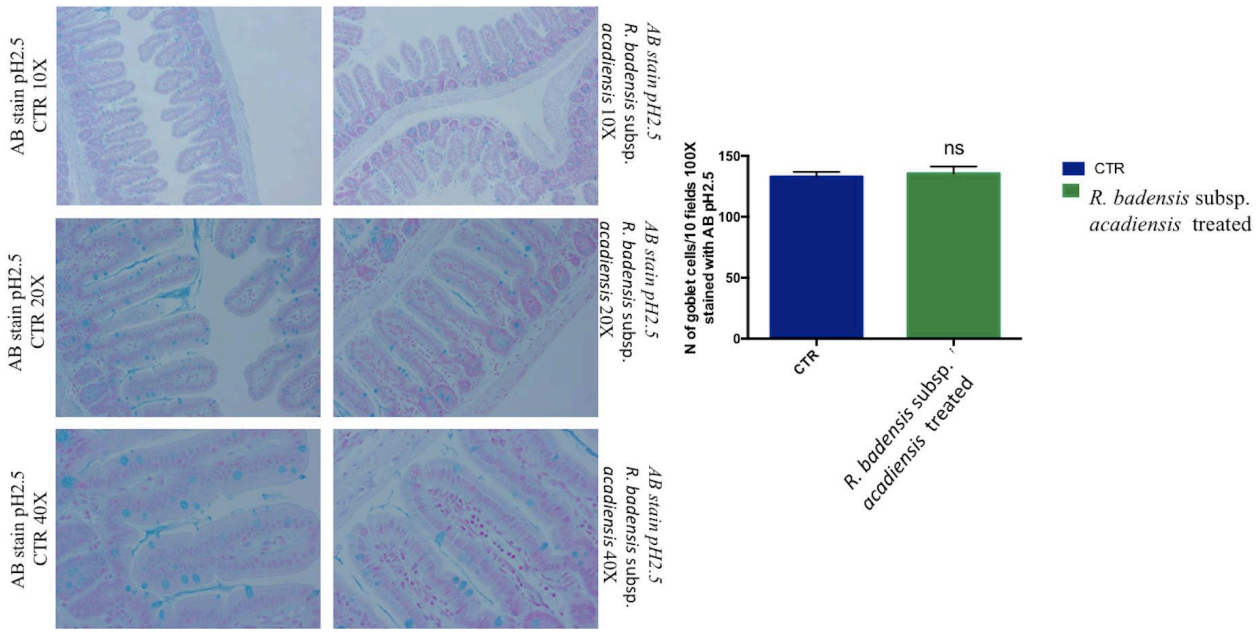

Figure 2.7. Mean ± SEM of the number of goblet cells stained with AB (Alcian Blue) at pH 2.5 in 10 fields of objective 100X in the small intestine of mice fed 1% sucrose (CTR) or *R. badensis* subsp. *acadiensis* fed 10^8CFU/mouse/day for 7 days. Significant difference exists if *p < 0.05- Representative tissue photos are taken with objective 10X, 20X, and 40X magnifications of the PAS-stained tissue. AB stains in blue acid glycoconjugates.

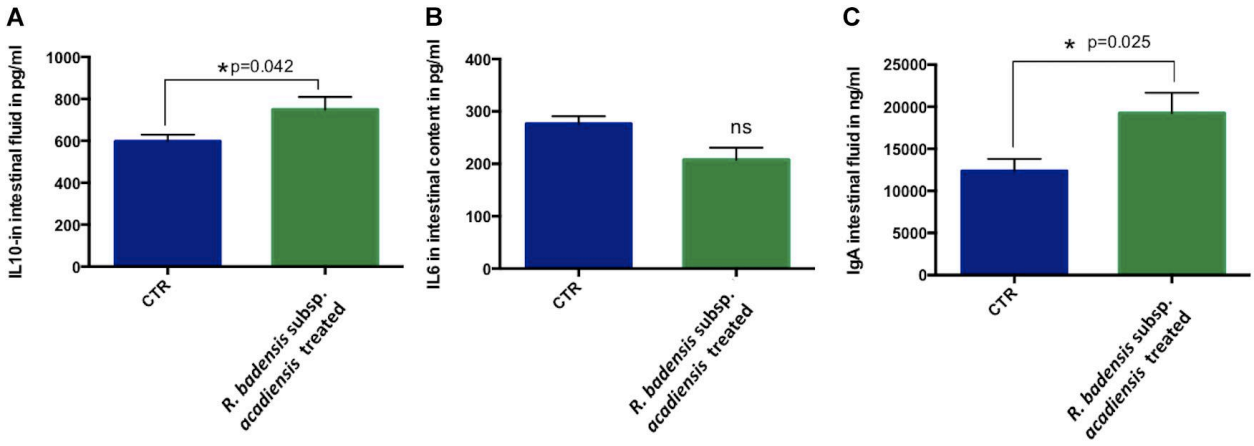

Figure 2.8. Data represents mean ± SEM of concentration of different (A) anti-inflammatory cytokines IL-10, (B) pro-inflammatory cytokine-IL-6 as well a (C) IgA in the intestinal fluid of (CTR) control mice fed 1% sucrose or *R. badensis* subsp. *acadiensis* treated group 10^8CFU/mouse/day of *R. badensis* subsp. *acadiensis* for 7 days. Number of animals per group is n = 10. Difference is considered significant between groups if *$p < 0.05$ ns = non-significant difference when $p > 0.05$.

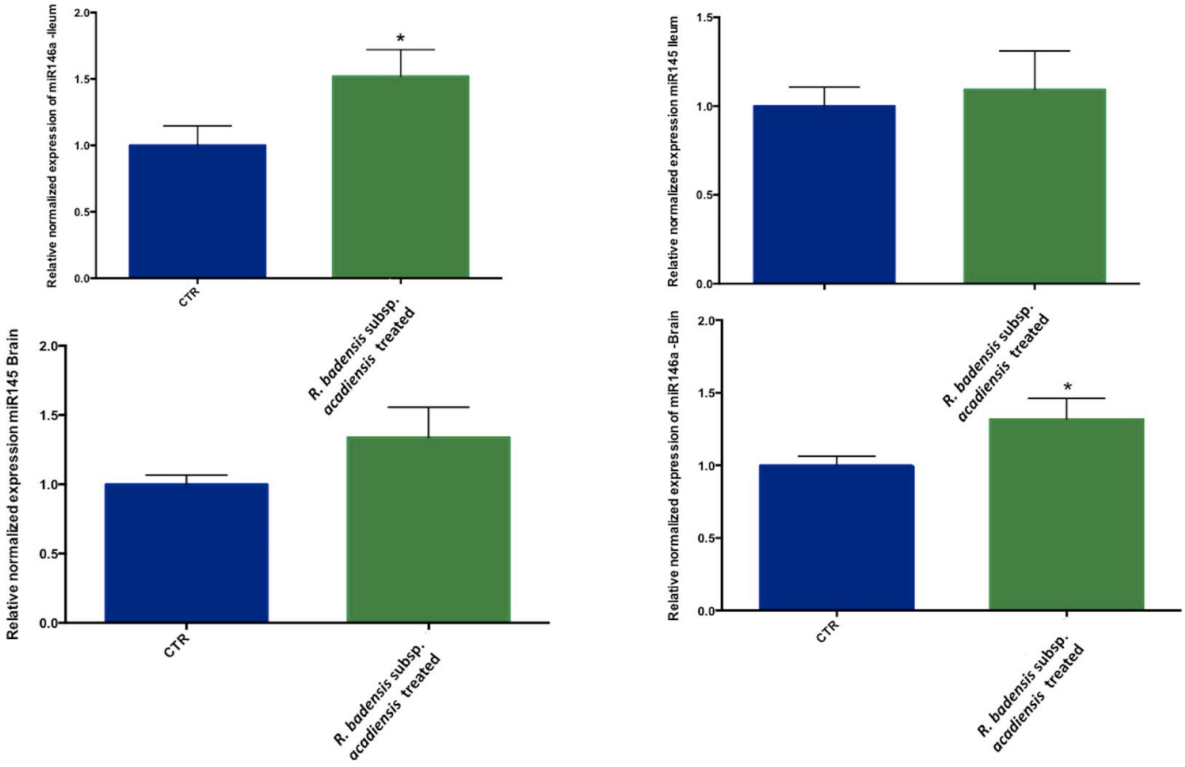

Figure 2.9. Mean ± SEM of relative expression of miR145 and miR146a in the brain and ileum of (CTR) control mice fed 1% sucrose or *R. badensis* subsp. *acadiensis* treated group 10^8CFU/mouse/day of Serratia vaccinia for 7 days. Significant difference between mice exists if *$p < 0.05$.

Discussion

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). They are identified mainly from fermented foods. Although fermented dairy products remain a major source of probiotic bacteria such as lactic acid bacteria and bifidobacteria; some can also be found in fermented vegetable and fruits products (Battcock et al. 1998; Schrezenmeir and De Vrese, 2001). For example, the native microbiota of vegetables involves a wide variety of lactic acid bacteria species implicated in fermentation (Swain et al., 2014). In this study, we characterize the *in vitro* and *in vivo* ability of *R. badensis* subsp. *acadiensis* isolated from the surface of lowbush blueberries (Vaccinium angustifolium Aiton) and used in blueberry-fermented preparation (Antioxydant Producing Bacterium and USES Therefore US 8,617,870) to survive the gastro-intestinal conditions, interact with the lamina propria, modulate the intestinal barrier integrity, affect mucus production and modify the immune response. Since bacteria can act as potential reservoirs for antimicrobial resistance genes, potential bacterial candidates for human and animal feeds should be screened for antimicrobial resistance. The resistant micro-organisms present in food products may result in infections that are difficult to treat. The disk diffusion method showed that *R. badensis* subsp. *acadiensis* does not carry antimicrobial resistance to the various examined antibiotics. Therefore, the bacterium can be considered as safe concerning resistance to antibiotics.

Resistance to human gastro-intestinal transit represents an important in vitro selection criterion to determine the ability of the strain to survive in the intestine and interact with the intestinal mucosa (Goldin et al., 1992). The tested strain must survive passage through the upper gastrointestinal (GI) tract and arrive alive at its site of action in order to better function in the gut environment. In our study, the in vitro methodology was used to simulate in vivo gastric and

small intestinal transit in the presence of bile salts. The investigated strain demonstrated high tolerance to both simulated gastric (pH 3 and 4) containing pepsin and pancreatic juices containing pancreatin and bile salts when tested separately. Nevertheless, the bacterium was more sensitive to simulated gastric juice (pH2) and the exposure to gastric conditions and subsequent intestinal fluid makes it a lot more vulnerable with a survival rate after exposure to both conditions more than 50%. These findings revealed the ability of a good portion of the delivered bacterium to survive in the intestine.

Adhesion to the intestinal epithelial cells permits to assess the ability of the investigated strain to colonize the intestinal mucosa (Fuller et al., 1992; Dunne et al., 2001; Teitelbaum and Walker, 2002). The correlation between intestinal mucosal adhesion and transient colonization is clear between *in vitro* and *in vivo* adhesion in experimental animals as found with several strains of microorganisms (Zárate et al., 2002). A good correlation was reported between the *in vitro* adhesion to Caco-2 cells and the *in vivo* persistence of *Lactobacillus* strains and *Bifidobacterium* strains (Crociani et al., 1995; Jacobsen et al., 1999). Although the ability of a microorganism to adhere to the intestinal mucosa may provide a mean by which the probiotic can modulate the immune system (Helgeland and Brandtzaeg, 2000) and are grounds for competitive exclusion between microorganisms for nutrients and binding sites (Alderberth et al., 2000; Fons et al., 2000), it is now accepted that modulation of immune system and antimicrobial effects could occur independently of the adhesion capabilities of the bacterium. Under the conditions used in our experiment, we have provided evidence that *R. badensis* subsp. *acadiensis* is an *in vitro* adherent strain when tested on Caco-2 and HT-29 cells.

Moreover, the introduction of an intestinal immunomodulating bacteria in the diet must be accompanied by the absence of an adverse inflammatory response (Perdigon et al., 2001).

Bacterial translocation or the passage of viable indigenous bacteria from the gut to the mesenteric lymph nodes, liver and other organs can be promoted by disruption of the ecological gastrointestinal equilibrium or disrupted permeability of the intestinal barrier (Berg, 1992; Vinderola C. G. et al., 2005). In the present study, we performed the translocation assay in the liver of mice after the oral administration of *R. badensis* subsp. *acadiensis*; no translocation was observed. Non-pathogenic commensal microorganisms interact closely with the gut epithelial surface and play a key role in the physiological, anatomical and immunological development of the host. Epithelial cells are the first line of defense; they interact simultaneously with bacteria and bacterial products and neighboring immune cells on their basolateral side. They can distinguish between pathogenic and non-pathogenic microorganisms which is an essential characteristic for the tolerance of the normal microbial microbiota (Galdeano et al., 2005; Vinderola C. G. et al., 2005).

Multiple defense mechanisms are implicated in the surveillance of intestinal mucosal homeostasis. Secretory IgA antibodies and mucus secretions are examples of these mechanisms (Fagarasan and Honjo, 2003). In the gut, IgA+ cells and secretory IgA play a key role as the first line of defense in the host. IgA antibodies cooperate with the innate non-specific defense mechanisms, by exerting immune exclusion (Vinderola et al., 2006) and preventing the invasion and colonization of pathogenic microorganisms through competition for receptors and/or metabolic substrates. We observed that the oral administration of *R. badensis* subsp. *acadiensis* increased the number of IgA + cells in the small intestine lamina propria of mice as well as the secretory IgA content in the intestinal lumen. On the other hand, IgG+ cells and the pro-inflammatory cytokine IL-6 did not increase ruling out persistent immunopathology in the intestinal mucosa (Vinderola et al., 2006).

Mucosal barriers established by intestinal epithelial cells maintain gut homeostasis; to better decipher the beneficial effect of *R. badensis* subsp. *acadiensis* strain on mucus producing cells two different specific stains (AB pH2.5 and PAS) were used. PAS staining representative of tissue histology of small intestine of treated and control groups revealed the increase of goblet cell mucus producing cells in mice gavaged with *R. badensis* subsp. *acadiensis*, pointing out a positive effect of the strain on intestinal mucosa. Goblet cells are responsible for the production and preservation of a protective mucus blanket by synthesizing mucins resulting in a dynamic protective barrier. They constitute, along with epithelial cells, macrophages, and dendritic cells, the main cellular components of the innate defense system (Nakata et al., 2017). Mucin glycoproteins synthesized and secreted by goblet cells are major constituents of the mucus layer which functions as a dynamic defensive barrier (Gaskins, 1998; Mack et al., 1999).

Additionally, mucosal immune response homeostasis can be regulated by the immunoregulatory cytokine interleukin 10 (IL-10) that limits mucosal immune responses and minimizes immunopathology (Glocker et al., 2009; Christopher et al., 2013). In fact, mice with a conditional deletion of IL-10 in the CD4+ T cell subset develop spontaneous inflammation of the intestine (Roers et al., 2004; Rubtsov et al., 2008). During acute mucosal infections, the lack of IL-10 can be protective since it is accompanied with an enhanced inflammatory response and an increased production of pro-inflammatory cytokines like IL-12, TNF, and other cytokines (Arai et al., 1995; Gazzinelli et al., 1996; Dai et al., 1997; Ke and Costa, 2006; Dann et al., 2014). However, the absence of IL-10 could result in inflammation (Gazzinelli et al., 1996; Hunter et al., 1997; Reis e Sousa et al., 1999; Suzuki et al., 2000). IL-10 plays a key role in the context of acute bacterial infection of the intestine in preventing excessive inflammation by limiting innate immunity. Similarly, mice deficient for IL-10 or IL-10 receptor developed inflammation of the

large intestine (Kühn et al., 1993; Spencer et al., 1998; Yen et al., 2006). Therefore, IL- 10 is crucial for maintaining a balance in adaptive immune responses in the mucosae (Denning et al., 2007; Murai et al., 2009; Ueda et al., 2010; Medina-Contreras et al., 2011; Rivollier et al., 2012). Recent studies showed that IL-10 signals drive macrophages to express tolerogenic functions that prevent colitis (Shouval et al., 2014; Zigmond et al., 2014). Our data showed that *R. badensis* subsp. *acadiensis* increased the number of IL-10+ cells in the lamina propria. Secreted IL-10 was also increased in the intestinal fluid. These findings suggest that *R. badensis* subsp. *acadiensis* may actively contribute to mucosal tolerance and may have anti-inflammatory properties (Lee and Mazmanian, 2010).

The role of microRNAs in driving or controlling inflammation is now subject to intense research. More specifically, anti-inflammatory miR-146a is a key regulator of the inflammatory processes involved in Tregs and IL-10 control. The expression of anti-inflammatory molecules like IL-10 is promoted by miR146a overexpression leading to a reduction of the inflammation (Dharap et al., 2009; Liu et al., 2016). On the other hand, recent studies found that the loss of miR-145 stimulates proinflammatory signals in the innate immune system (Akao et al., 2006). In the brain, it plays a role in antioxidant defense (Witwer et al., 2010). Our data supports the ability of *R. badensis* subsp. *acadiensis* to increase the levels of miR146a at the intestine and the brain; this finding highlights its anti-inflammatory activity and its link to IL-10 expression in the ileum.

Our findings reflect the ability of the bacterium to interact with the intestinal mucosa and to modulate the immune response and the gut barrier function. Future papers assessing the bacterium safety will be published in order to assess a potential probiotic activity of *R. badensis* subsp. *acadiensis*.

Conclusion

We demonstrated that *R. badensis* subsp. *acadiensis* is a non-invasive bacterium. It can survive gastrointestinal tract conditions and has resistance to bile salts and digestive enzymes (pancreatin and pepsin). Under the conditions tested, the bacterium exhibits adhesion properties to Caco-2 and HT-29. The immunoregulatory effects of the novel strain was shown by an increase in IL-10, IgA and miR-146. We conclude that *R. badensis* subsp. *acadiensis* participates in specific local immune protection and that its oral administration enhances gut-associated non-specific immunity without an inflammatory outcome. This strain is a good candidate for further investigation to elucidate its potential health benefits.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by Animal Care Committee (ACC)- university of Ottawa.

Authors' Contributions

NY: experimental design, experiments optimization and running, writing the manuscript, data collection and data analysis. CM: main supervisor, revision of experiments and guidance, data

review, and manuscript review. MH and GK: collaboration on antibiotic experiments and correction of the manuscript. NA and JM: helps with samples collection during the Euthanasia day. All authors contributed to the article and approved the submitted version.

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Chapter 3: Adolescent use of potential novel probiotic *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) mitigates pubertal LPS-Induced behavioral changes in adulthood in a sex-specific manner by modulating 5HT1A receptors expression in specific brain areas

Preface

The following chapter consists of Article 2 previously published in Comprehensive Psychoneuroendocrinology under the title **Adolescent use of potential novel probiotic *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) mitigates pubertal LPS-Induced behavioral changes in adulthood in a sex-specific manner by modulating 5HT1A receptors expression in specific brain areas** by Nour Yahfoufi [a,b] , Emily G. Ah-Yen b , Rajini Chandrasegaram [c] , Sarah Aly [b] , Michael Murack [b] , Anthony K. Kadamani [b] , Chantal Matar [a,d] , Nafissa Ismail [b,e],*

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Rajini Chandrasegaram: Investigation, Data Curation

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Michael Murack: Training, Investigation

Anthony Kadamani: Investigation, Data Curation

Chantal Matar: Resources, Funding Acquisition, Writing – Review & Editing, Supervision.

Nafissa Ismail: Conceptualization, Methodology, Formal analysis, Resources, Writing – Review & Editing, Supervision, Project administration

Abstract

Adolescence is a critical period of development during which the brain undergoes significant remodeling that impacts behavior later in life. Exposure to stress, and especially immune challenge, during this period triggers changes in brain function resulting in the development of mental disorders in adulthood, such as depression and anxiety. Previous studies from our laboratory have shown that a single exposure to LPS (lipopolysaccharide) during puberty causes enduring depression-like behaviour in females and anxiety-like behaviours in males. However, administration of probiotics during puberty blocked the enduring effects of LPS on depression-like and anxiety-like behaviors in female and male mice, respectively. These results suggest that the gut microbiome is a mediator of the effects of stress on mental health. The objective of the current study is to examine the effectiveness of a novel probiotic *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) in blocking LPS-induced anxiety like and depression-like behaviors in adult male and female mice. Our results showed that *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) blocked LPS-induced depression-like behavior in female mice. We also found that pubertal treatment with *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) mitigated the LPS-induced decrease in 5HT1A expression in CA1 as well as the LPS-induced increase in 5HT1A expression in the raphe nuclei in female mice. Contrary to our predictions, pubertal LPS treatment at 6 weeks of age did not induce enduring anxiety-like behavior in males. There was also no difference in anxiety-like behavior between the LPS sucrose and LPS-probiotic male groups. However, pubertal LPS treatment increased the expression of 5HT1A receptors in the DRN in males, while probiotic exposure mitigated this increase. Our study highlights the consequences of stress exposure (immune challenge) on mental health in adulthood taking into consideration 5HT1A receptors expression at different regions of the brain. It also emphasizes on

the importance of considering adolescence as window of opportunities during which probiotic use can alleviate the long-term neural and behavioral alterations induced by stress.

Keywords

Probiotic, depression, anxiety, 5HT1A, hippocampus, raphé-nuclei.

Introduction

Adolescence is a critical period of development that stretches between childhood and adulthood and it is defined as social, cognitive, emotional and reproductive maturation (Sisk and Foster 2004). This period is marked by brain remodeling and reorganizing which subsequently impact social cognitive behavior (Burnett et al. 2011). A consequence of this major neuronal transformation and structural reshaping of neural circuits is an increased degree of vulnerability to stressors and immune challenges (Yahfoufi, Matar, and Ismail 2020). More specifically, exposure to stress during adolescence results in short-term and long-term changes in brain neurochemistry, physiology and behavior in adulthood (Andersen 2003; Ismail, Kumlin, and Blaustein 2013). Thus, exposure to certain stressors during this critical period of development can increase the likelihood of developing mental illnesses like depression and anxiety later in life.

The immune system is also involved in the pathophysiology of anxiety and depression. In the past decade, numerous studies have shown higher risk for low-grade inflammation and greater concentrations of pro-inflammatory markers like interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and c-reactive protein in the blood of depressed and anxious patients (Vogelzangs et al. 2016). The severity of anxiety symptoms is positively associated with blood concentration of several pro-inflammatory cytokines (Vogelzangs et al. 2016). Moreover, recent research from our laboratory shows that a single injection of lipopolysaccharide (LPS), a gram negative bacterial endotoxin, at 6 weeks old, a pubertal stress-sensitive period, induces enduring depression-like behaviour in female mice and anxiety-like behaviour in male mice (Murray et al. 2019). Anxiety and depression are associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and disturbances in the autonomic nervous system (Stetler and Miller 2011; Beishuizen and

Thijs 2003). The activation of the HPA axis and the release of glucocorticoids play a key role in modulating inflammation and cytokine response. This cross-talk between the nervous, endocrine, and immune systems serves to maintain physiological homeostasis during inflammatory and stress responses that induce systemic cytokine production (Beishuizen and Thijs 2003). Furthermore, several studies have demonstrated the ability of acute lipopolysaccharide LPS injection to stimulate the hypothalamic-pituitary-adrenal HPA axis and to increase corticosterone concentration in the blood (Takemura *et al.*,1997; Girard-Joyal and Ismail 2017; Rivier and Chizzonite 1989).

Serotonin or 5-hydroxytryptamine (5HT) is one of several monoamine neurotransmitters that contributes to the pathogenesis of anxiety and depression with at least 14 serotonin receptor subtypes involved. 5-HT1A receptors are of primary importance as they are targets for a number of antidepressants and anxiolytic treatments (Cryan and Leonard 2000). These receptors exist both at presynaptic (autoreceptors) and postsynaptic sites (heteroreceptors), with 5-HT1A autoreceptors being located deep within the brainstem and 5-HT1A heteroreceptors localized in various brain regions including the hippocampus, prefrontal cortex, thalamus, lateral septum, amygdala, and hypothalamic nuclei (Haddjeri, Blier, and Montigny 1998). Post mortem analyses in depressed patients revealed an increase in 5HT1A autoreceptor expression in the dorsal raphe nuclei (DRN) (Boldrini et al. 2008) and a decrease in 5HT1A heteroreceptor expression in the hippocampus (Moses-kolko et al. 2008; Sargent et al. 2000). Exposure to chronic stress has also been found to alter 5HT1A receptor expression in the hippocampus in rodent models of depression and anxiety (Watanabe et al. 1993; Lopez et al. 1998). For example, exposure to prolonged maternal separation causes a decrease in hippocampal 5HT1A mRNA expression and an increase in depression-like

behavior in rats (Maniam and Morris 2010). Likewise, 5HT1A knockout mice display greater anxiety-like behavior than do wild-type counterparts (Holmes et al. 2003).

Numerous studies have also linked intestinal dysbiosis to anxiety and depression (Cryan and Dinan 2012). There is a bidirectional relationship between the gut and the brain called the gut-brain axis. The gut -brain axis plays a vital role in maintaining homeostasis and involves complex crosstalk between the endocrine, immune and the autonomic nervous systems (Cryan and Dinan 2012). In healthy adults, 90% of the gut microbiota is dominated by the Bacteroidetes and Firmicutes phyla. In contrast, in depressed individuals, there is an alteration in the abundance of different genera within the Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria phyla (Winter et al. 2018). Exposure to certain stressors like maternal separation, crowding, heat and acoustic stress can harmfully affect the composition of the gut microbiota (Zhou, Zhou, and Chen 2017). However, further research is needed to decipher causality and the importance of microbiota in increasing or decreasing inflammation in the gut and in the brain. Nevertheless, recent microbiome research suggests a key role of the gut microbiota in brain development, behaviour and mood (Dash et al. 2015). The gut microbiome is also important for mental health and for shaping behavioral responses (Heijtz et al. 2011).

Probiotics are live microorganisms that confer a health benefit on the host, when administered in adequate amounts (Hill et al. 2014). They can mitigate the stress response and have anti-depressive and anxiolytic effects by modulating inflammatory responses (Murray et al. 2019; Murray et al., 2020; Park et al. 2018). The interaction of the microbiota with other environmental risk factors, such as stress and diet, opens new horizons in the development of interventions (like probiotics use) targeting the gut microbiota for the prevention and treatment of mental health disorders (Dash et al. 2015). Recent findings from our laboratory demonstrate that

consumption of the probiotic *Lactobacillus reuteri* throughout pubertal development mitigates LPS-induced enduring effects on memory dysfunction, anxiety-like behaviour and stress reactivity in adulthood. Moreover, pubertal exposure to probiotics from kefir blocks LPS-induced depression-like behaviour in females and anxiety-like behaviors in males. Although several studies support the benefit of probiotic interventions in mental health and behavioural modifications, the neurochemical changes or the mechanisms underlying these effects remain unclear. It is also unknown whether our previous findings would extend to other probiotics (Murray et al. 2020; Bravo et al. 2011). LPS is a pyrogenic compound present in the outer membrane of gram-negative bacteria. It activates the innate immune system by binding to toll-like receptors (TLRs) located on innate immune cells (e.g. macrophages, monocytes) (Galic, Riazi, and Pittman 2013). In the brain, these receptors are present on meninges endothelial cells and microglia (Galic, Riazi, and Pittman 2013). TLR-4 is the main receptor involved in the activation of a series of downstream pathways and transcription factors (such as NF-κB) that induce the production of proinflammatory (e.g. interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α) or anti- inflammatory (e.g. IL-10) immunomodulating cytokines (Ribeiro et al. 2019; Andreakos et al. 2004). Central or peripheral administration of LPS can induce neuroinflammation (Layé et al. 1995). Peripheral administration of LPS can cause an inflammatory response in the CNS, either by stimulating *de novo* synthesis of cytokines within the brain (Pyter et al. 2009; Qin et al. 2010; Kempuraj et al. 2017) or by increasing the permeability of the blood-brain barrier (BBB) and permitting infiltration of inflammatory cells such as mast cells, cytokines and chemokines into the brain (Kempuraj et al. 2017; Vitkovic et al. 2000) which contribute to the development of sickness symptoms and behavioral changes (Kolmogorova, Murray and Ismail 2017; Cai et al. 2016).

Therefore, the current study investigated whether the use of a novel probiotic bacterium "*Rouxiella badensis* subsp. *acadiensis"* during puberty can block LPS-induced depression-like and anxiety-like behaviors in adult female and male mice. *Rouxiella badensis* subsp. *acadiensis* has been filed in a U.S. Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods" for its potential probiotic effects (Matar et al. 2020). *Rouxiella badensis* subsp. *acadiensis* is a bacterium isolated from the microbiota of the wild blueberry fruit. Based on its whole-genome shotgun sequencing, there are no pathogenicity genes associated to it (Matar et al. 2020). The bacterium has been used in multiple studies and it was found to be an effective anti-inflammatory agent for neurodegeneration, diabetes, and cancer (Nachar et al. 2017; Vuong et al. 2007, 2016; Martin and Matar 2005). Moreover, *Rouxiella badensis* subsp. *acadiensis* can modulate gut-associated non-specific immunity without an inflammatory outcome (Yahfoufi et al. 2021).

Our study assesses the long-term behavioral impacts of the selected bacterium as a probiotic and its relationship with the expression of 5HT1A receptor in a sex-specific manner. We hypothesize that consumption of *Rouxiella badensis* subsp. *acadiensis.* during pubertal development can block LPS-induced enduring depression-like behavior by modifying the expression of 5HT1A receptors in the raphe nuclei and in the hippocampus in adulthood.

Methods

Animal housing

Ninety-six CD-1 mice (48 male and 48 female) were shipped from Charles River Laboratories (St-Constant, Québec) at 3 weeks of age. Mice were housed in pairs in Polycarbonate cages either in an all-male or an all-female colony room with *ad libitum* access to food (ENGIVO—Teklad Global 18% rodent) and water. Housing rooms were kept at constant temperature (24 ± 2 °C) with a reversed light: dark cycle (14:10; lights off at 10:00am) and 40% (±5%) humidity. Dusk and dawn were gradually induced over a period of 1 h. Cages were bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, US, ¼ inches in diameter). Enrichment in the cages consisted of one square piece of Nestlet (Ancare Corp., Bellmore, NY, US) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, CA). All procedures were approved by the Animal Care Committee of the University of Ottawa.

Probiotics

Stock cultures of the *Rouxiella badensis* subsp. *acadiensis* were maintained at -80^0C in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) supplemented with 30% (v/v) glycerol. The bacterium was grown in TSB at 30 °C for 20h. From 5 to 7 weeks of age, mice in the experimental group received 10^9 CFU of the probiotic in 1% sucrose dissolved in 1X PBS pH7.4 through oral gavage. Control mice received the same volume of 1% sucrose in 1X PBS by oral gavage. Fresh probiotic and 1% sucrose solutions were prepared daily.

Lipopolysaccharide (LPS)

Lyophilized LPS (obtained from Escherichia coli serotype O26:B6; #L3755; Sigma Aldrich) was dissolved into 0.9% (w/v) sterile saline (Ricca Chemical Company) at a concentration of 0.2 mg/ml. At 6 weeks of age, all mice were injected intraperitoneally at the end of the light phase with either 0.9% sterile saline or LPS (1.5 mg/kg). This dose of LPS has previously been shown to cause mild sickness in mice for up to 48 h and to produce enduring impairments in reproductive and non-reproductive behaviors, when administered at 6 weeks of age, during the stress-sensitive pubertal period (N Ismail, Kumlin, and Blaustein 2013; Ismail, Garas, and Blaustein 2011; Laroche et al. 2009a).

Sickness behaviors

Sickness behaviors were monitored at 30 min, 2, 4, 8, 12, 24 and 48 hours following injection by two blinded raters. The assessment was carried out using the protocol described in Kolmogorova et al. (2017) (Kolmogorova et al. 2017); a four-point scale was used to assess mice for the presence of four sickness behaviors (lethargy, huddling, ptosis, and piloerection) at specific time points following exposure to LPS. Scores ranging from 0 to 4 were assigned based on the number of symptoms displayed at each time point. Scores were averaged between the two raters.

Body Weight

Mice were weighed at 5 weeks of age (before the start of the probiotic or control treatment), at 6 weeks of age (before the saline or LPS injection), at 24h and 48h after saline or LPS injection, at 7 weeks of age (upon termination of the probiotic or control treatment), and at 10 weeks of age (in adulthood).

Behavioural testing

At 10 weeks of age, mice were exposed to a battery of behaviour tests to examine anxiety-like and depression-like behaviors. Each test ran over two consecutive days and all mice were exposed to the behavior tests in the same order. Testing began 2h after the start of the dark phase and was conducted under dim red light. All testing was completed within 3.5h each day. To evaluate anxiety-like behavior, the Elevated Plus Maze (EPM) and Open Field Test (OFT) were used, whereas depression-like behavior was assessed using the Forced Swim Test (FST) and the Tail Suspension Test (TST). All mice were given a 48-hour rest period between the behavior tests to avoid any carry-over effects. Panasonic WV-CP284 camera mounted on an Optex T165 tripod and an Ethovision Tracking software were used for tracking and recording.

Anxiety-like behaviour testing

Open Field Test (OFT)

The open field consisted of a square-shaped wall-enclosed area. The open field (100 cm × 100 cm) was divided into a central/inner zone (30 cm × 30 cm) and a peripheral/outer zone (100cm-30cm). During the dark phase of their light cycle, mice were placed in the open field for 5 min. The total distance travelled, time spent in inner zone, latency to first enter inner zone and time spent in outer zone were measured using the Ethovision Tracking software. Less time spent in the inner zone of the maze is associated with greater anxiety (Prut and Belzung 2003).

Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) consisted of four branching arms: two open arms and two closed arms. Each arm is 10 cm × 50 cm. During the test, mice were placed in the center facing one of the open arms for 5 min to explore the maze. The Ethovision Tracking software was used to measure the amount of time spent in each portion of the maze throughout the 5-minute trial. An increased amount of time spent in the closed arms of the maze is linked to greater anxiety (Wall and Messier 2001).

Depression-like behavior testing

Forced swim test (FST)

Mice were placed in a 4L glass cylinder (15cm (diameter) x 30cm (height)) filled with 3L of water at a temperature of 24 °C ± 2 °C and allowed to swim for 5 min. The total time spent climbing (vertical movements along the wall of the cylinder), swimming (active circular swimming) and immobile (absence of movement or floating) was measured by two raters blinded to treatment conditions. Inter-rater reliability factor was within 25%. The results were calculated as the average of the two raters' scores. At the end of the test, mice were removed from the glass cylinder and placed in a recovery cage on a heating pad until they were dry, and then they were returned to their home cages. Increased duration of immobility on the FST is an indication of increased behavioural despair and depression (Porsolt 1979; Castagné et al. 2011).

Tail suspension test (TST)

Mice were suspended 60 cm above the ground within 1cm from the tip of the tail for 5 min. The duration of immobility (absence of any body movement: the mice hung passively and motionless for at least 2s) was measured by two raters blinded to treatment conditions. Inter-rater reliability factor was within 25%. The results were calculated as the average of the two raters' scores. Mice were returned to their home cage upon termination of the testing. Increased duration of immobility on the TST is an indication of increased behavioural despair and depression (Steru et al. 1985).

Tissue collection and immunohistochemistry (IHC)

Mice were intracardially perfused with 20 ml of 0.9% saline followed by 20 ml of 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 4% PFA for 2h then placed in fresh 30% sucrose solution 2 and 24 h after post-fixing and stored at 4 °C until slicing. Brains were sliced at a thickness of 40 µm into 4 series using an automated Leica VT1200 vibratome. Sections were kept in cryoprotectant at -20°C. Free-floating brain sections from one of the series from each mouse were rinsed in 1X TBS, incubated in antigen retrieval solution (0.05M sodium citrate in 1X TBS) for 30 min, rinsed again with TBS and incubated in 0.1M Glycine/1X TBS for 30 min. Following additional TBS rinses, sections were transferred for 30 min in a concentrated blocking solution containing TBS, 20% normal goat serum, 0.3% Triton-X, and 1% H2O2 then incubated for 20h at 4^0C into a solution containing a primary rabbit antibody specific to 5HT1A (ab227165 Abcam; 1:300, Cambridge, MA). Sections were then incubated for 60 min at room temperature in a secondary biotinylated goat anti-rabbit antibody (Vector, BA-1000, IgG; PC38 Millipore 1:500), rinsed for 30 min with 0.2% Trion-X in TBS followed by 60 min incubation in the ABC detection system (Vector, Vectastain ABC kit Elite Pk-6100 standard; Vector Laboratories,

Burlingame, CA, USA). Finally, freshly prepared diaminobenzidine (DAB) solution (DAB Substrate kit, SK-4100 Vector Laboratories) was used to stain targeted 5-HT1A receptors.

Image analysis and cell quantification

The Mouse Brain Atlas was used to locate the various brain regions (Paxinos 2013). The cornu-ammonis (CA) CA1 and CA3 of the hippocampus (i.e., bregma: -2.18 mm) and DRN (i.e., bregma -4.48 mm) were examined for 5HT1A receptor (5HT1A Rc) expression based on one representative section for each mouse. Images were captured at 10X using an Olympus BX51 light microscope connected to a Jenoptik ProRes MF scan camera. Kolor Autopano was used for stitching. Two raters blinded to treatment conditions quantified the number of cells positive for receptors in specific brain regions using unbiased counting on the ImageJ software (version 1.48) with a coefficient of variation $\leq$ 15%.

Procedures

Ninety-six mice arrived at 3 weeks of age. At 5 weeks of age, mice were either exposed to sucrose vehicle (1% sucrose by gavage) or to *Rouxiella badensis* subsp. *acadiensis.* (in 1% sucrose by gavage) for two weeks. At 6 weeks of age, mice were injected with either saline or LPS. Mice were then monitored for sickness behaviour over the next 48h. At 10 weeks of age, mice began behavior testing with EPM, OFT, FST and TST. A 48-hour rest period separated each behavior test to reduce carry-over effects. Mice were euthanized following the completion of all behavior tests.

Statistical Analyses

IBM SPSS V26 software was used to perform statistical analyses. All data were initially screened for outliers using boxplots generated by SPSS. In order to reduce the effects of extreme outliers, cases that fell outside the 1.5 interquartile range were adjusted using winsorization (Pollet and Meij 2017). A four-way mixed ANOVA was used to examine the within-subject effect of time (2, 4, 8, 12, 24 and 48 h), and the between-subject effects of sex, probiotic treatment (control or probiotic *Rouxiella badensis* subsp. *acadiensis*) and immune challenge (saline or LPS) on sickness responses [29,46,54]. Three-way ANOVAs were used to examine the effects of sex, immune challenge and probiotic treatment on the duration of immobility, swimming and climbing in the FST, the duration of immobility in TST, time spent in the inner zone, latency to first enter inner zone, velocity and total distance travelled in the OFT, time spent in the open arms in the EPM, and 5HT1A Rc (s) expression in the CA1 and CA3 regions of the hippocampus and in the DRN. Significant interactions were followed by pairwise comparisons and a Bonferroni correction factor was applied to control for multiple comparisons. The criterion for statistical significance was set at $p < 0.05$.

Results

Sickness Behaviors and Weight Changes

Pubertal probiotic treatment reduced LPS-induced sickness behaviour in males and females in a time-specific manner

Four-way mixed ANOVA revealed main effects of time ($F_{(3.9, 220.7)} = 117.05$, $p < 0.001$, $\eta_p^2 = 0.68$), LPS treatment ($F_{(1, 56)} = 593.11$ $p < 0.001$, $\eta_p^2 = 0.91$), and probiotic treatment ($F_{(1, 56)} = 23.93$, $p < 0.001$, $\eta_p^2 = 0.30$). There were also significant time x probiotic ($F_{(3.9, 220.7)} = 3.46$, $p < 0.01$, $\eta_p^2 = 0.06$), time x LPS ($F_{(3.9, 220.7)} = 131.88$, $p < 0.001$, $\eta_p^2 = 0.70$), probiotic × LPS ($F_{(1, 56)} = 23.92$, $p < 0.001$, $\eta_p^2 = 0.30$), time × sex × probiotic ($F_{(3.9, 220.7)} = 3.54$, $p < 0.01$, $\eta_p^2 = 0.06$) and time x sex probiotic x LPS ($F_{(3.9, 220.7)} = 2.98$, $p < 0.05$, $\eta_p^2 = 0.06$) interactions (Figure 3.1 (i)).

Pairwise comparison revealed that, as expected, male and female mice treated with LPS displayed more sickness behaviors than saline controls (mean difference (MD) = 1.90, standard error (SE) = 0.11, $p < 0.001$; MD = 1.80, SE = 0.11, $p < 0.001$; respectively). However, pairwise comparisons of sex × probiotic × LPS interaction showed that LPS-treated male and female mice exposed to probiotics displayed less sickness behaviour than LPS-treated mice exposed to sucrose (MD = -0.54, SE = 0.15, $p < 0.05$; MD = -0.95, SE = 0.15, $p < 0.001$; respectively). Moreover, LPS-treated males exposed to probiotic showed more sickness behaviors than LPS-treated female counterparts (MD = 0.33, SE = 0.15, $p < 0.05$,). LPS-treated female mice exposed to sucrose displayed significantly more sickness behavior than LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic at 30min (MD = 0.625, SE = 0,14, $p < 0.001$), 2h (MD = 1.375, SE = 0,34, $p < 0.001$), 4h (MD = 1.44, SE = 0.24, $p < 0.001$), 6h; (MD = 1, SE = 0.25, $p < 0.001$), 8h (MD = 1.125, SE = 0.24, $p < 0.001$), 12h (MD = 0.875, SE = 0.29, $p < 0.001$), and 24h (MD = 1.125, SE = 0.28, $p < 0.001$) following LPS treatment. Similarly, LPS-treated male mice exposed

to sucrose displayed more sickness than LPS-treated males exposed to *Rouxiella badensis* subsp. *acadiensis.* probiotic at 30min (MD=0.875, SE= 0.14, p = 0.001), 6h (MD = 0.875, SE = 0.25, p = 0.001), 8h (MD = 0.81, SE = 0.24, p = 0.001), 12h (MD=1, SE=0.29, p = 0.001) and 24h (MD = 0.94, SE = 0.28, p <0.01) following LPS treatment.

Probiotic Failed to Mitigate LPS-induced Weight Loss

Within subjects tests showed a main effect of time ($F_{(1.5,82.7)}$ = 301.58, p < 0.001, η_p^2 = 0.85) and a time × sex interaction (($F_{(1.5,82.7)}$ = 8.33, p < 0.01, η_p^2 = 0.13). Tests between subjects revealed a main effect of sex ($F_{(1,55)}$ = 6.6, p < 0.05, η_p^2 = 0.1); LPS ($F_{(1,55)}$ = 20.49, p < 0.001, η_p^2 = 0.27) and sex × probiotic interaction ($F_{(1,55)}$ = 4.93, p < 0.05, η_p^2 = 0.08). Pairwise comparison showed that, LPS-treated male mice exposed to sucrose displayed significant weight loss when compared to their saline counterparts at 24h (MD = 7.016, SE = 1.09, p < 0.001), 48h (MD = 7.783, SE = 1.47, p < 0.001), and 1week post-injection (MD = 3.406, SE = 1.36, p < 0.05). Similarly, LPS induced weight loss in females control group at 24h (MD = 7.401, SE = 1.09, p < 0.001), 48h (MD = 7.261, SE = 1.47, p < 0.001), and 1week post-injection (MD = 4.401, SE = 1.36, p < 0.01). Males treated with *Rouxiella badensis* subsp. *acadiensis* and injected with LPS demonstrated more weight loss than saline treated at 24 h (MD = 9.174, SE = 1.09, p < 0.001), 48h (MD = 10.164, SE = 1.47, p < 0.001), and 1week post-injection (MD = 5.018, SE = 1.36, p < 0.05) while females treated with *Rouxiella badensis* subsp. *acadiensis* and injected with LPS demonstrated more weight loss than saline treated at 24 h (MD = 7.124, SE = 1.13, p < 0.001), 48h (MD = 6.086, SE = 1.52, p < 0.001) (Fig.1 (ii)).

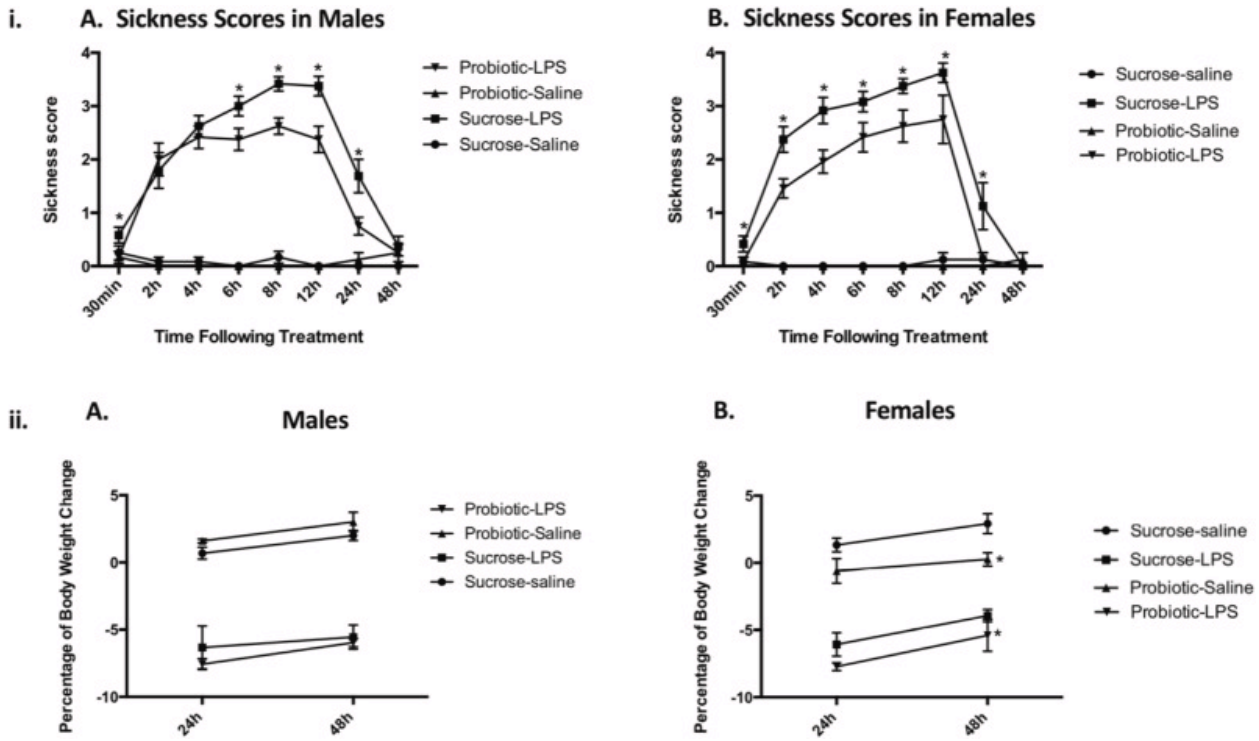

Figure 3.1. (i) Sickness scores of six-week-old (A) male and (B) female mice treated with saline or LPS and exposed to either sucrose or probiotic (*Rouxiella bade*nsis subsp. *acadiensis*). Data represented as mean (±SEM), n = 12/group (30 min – 8 h) and n = 8/group (at 12 h, 24 h and 48 h). The asterisks (*) denote significant difference between LPS treatments (sucrose and probiotic) ($p < 0.05$). (ii) Acute percent body weight change of (A) male and (B) female mice at 24 h and 48 h following saline or LPS treatment and exposed to either sucrose or probiotic treatment. Data represented as mean (±SEM), n = 8/group at 24 h and n = 12/group at 48 h. The asterisks (*) denotes significant difference between treatment groups; *$p < 0.05$.

Probiotic treatment during puberty prevented LPS-induced depression-like behaviour in female mice during adulthood

Forced Swim Test (FST)

Three-way ANOVA revealed a main effect of probiotic treatment on immobility duration ($F_{(1, 94)} = 7.87$, $p < 0.01$, $\eta_p^2 = 0.08$) and a main effect of sex on climbing behavior ($F_{(1,94)} = 11.90$, $p = 0.001$, $\eta_p^2 = 0.12$). The ANOVA also found significant probiotic × LPS treatment interaction on immobility duration ($F_{(1,94)} = 5.88$, $p < 0.05$, $\eta_p^2 = 0.06$) and a significant sex × LPS treatment interaction on swimming duration ($F_{(1,94)} = 4.49$, $p < 0.05$, $\eta_p^2 = 0.05$).

Pairwise comparisons showed that, regardless of sex and LPS-treatment, mice exposed to sucrose control displayed greater duration of immobility than mice exposed to the *Rouxiella badensis* subsp. *acadiensis.* probiotic (MD = 23.12, SE = 8.25, $p < 0.01$). Regardless of probiotic treatment, LPS-treated females displayed longer immobility duration compared to saline-treated females (MD = 23.90, SE = 11.725, $p < 0.05$) (Fig. 2B). No difference was observed in males. Moreover, LPS-treated females exposed to sucrose control displayed longer immobility duration than LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic (MD = 65.37, SE = 16.40, $p < 0.001$). Saline-treated males showed shorter swimming duration than females' counterparts (MD = -37.37, SE = 13.22, $p < 0.01$). Saline-treated females showed longer swimming duration than LPS-treated females (MD = 32.52, SE = 13.22, $p < 0.05$). However, LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis.* probiotic displayed longer swimming duration than females-LPS-treated females exposed to sucrose control (MD = 48.02, SE = 18.50, $p < 0.05$). Lastly, regardless of LPS and probiotic treatments, males displayed longer climbing duration than females (MD = 16,44, SE = 4.76, $p < 0.01$) (Figure 3.2).

Tail Suspension Test (TST)

Three-way ANOVA found mains effect of sex ($F_{(1, 95)} = 4.00$, $p < 0.05$ $\eta_p^2 = 0.04$) and probiotic treatment ($F_{(1, 95)} = 5.73$, $p < 0.05$, $\eta_p^2 = 0.06$) on the duration of immobility in the TST (Figure 3.3).

Pairwise comparisons showed that, regardless of probiotic and LPS treatments, females displayed longer immobility duration than males (MD = 17.11, SE = 8.55, $p < 0.05$). Moreover, regardless of sex and LPS-treatment, mice exposed to *Rouxiella badensis* subsp. *acadiensis* displayed shorter immobility duration than mice exposed to the control (MD = -20.49, SE = 8.55, $p < 0.05$). LPS-treated females exposed to sucrose displayed greater immobility duration than saline-treated counterparts (MD = 37.54, SE = 17.11, $p < 0.05$). However, there was no significant difference in immobility duration between LPS-treated females exposed to the probiotic and saline-treated counterparts. LPS-sucrose mice had more immobility than their LPS-probiotic counterparts (MD = 32.8, SE = 12.1, $p < 0.01$) (Figure 3.3A). Finally, LPS-treated females exposed to probiotic displayed significantly shorter immobility duration than LPS-treated females exposed to sucrose control (MD = -55.02, SE = 17.11, $p < 0.01$) (Figure 3.3A).

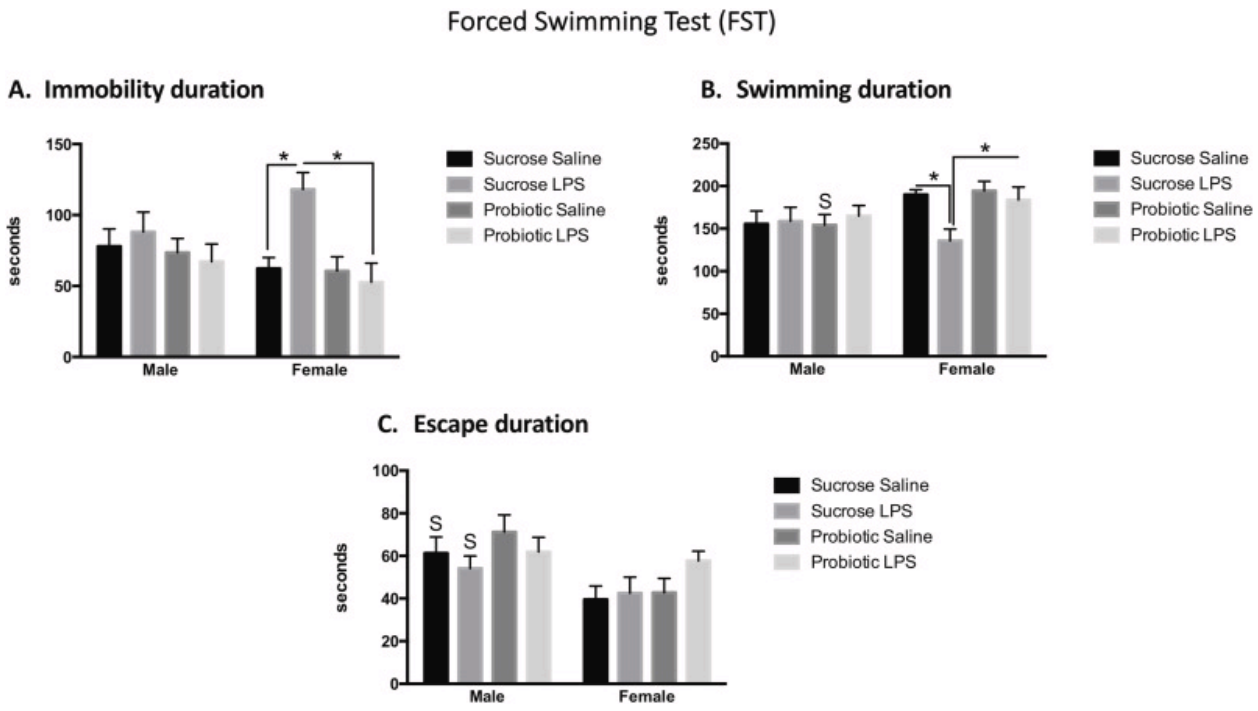

Figure 3.2. Duration of immobility (A), swimming (B) or escape (C) in seconds (sec) during the forced swimming test in adult male and female mice previously treated with saline or LPS at 6 weeks of age and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis (Canan SV-53)* treatment during puberty. Data represented as mean (±SEM), n = 12/group. The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$); S indicates significant difference between males and females ($p < 0.05$).

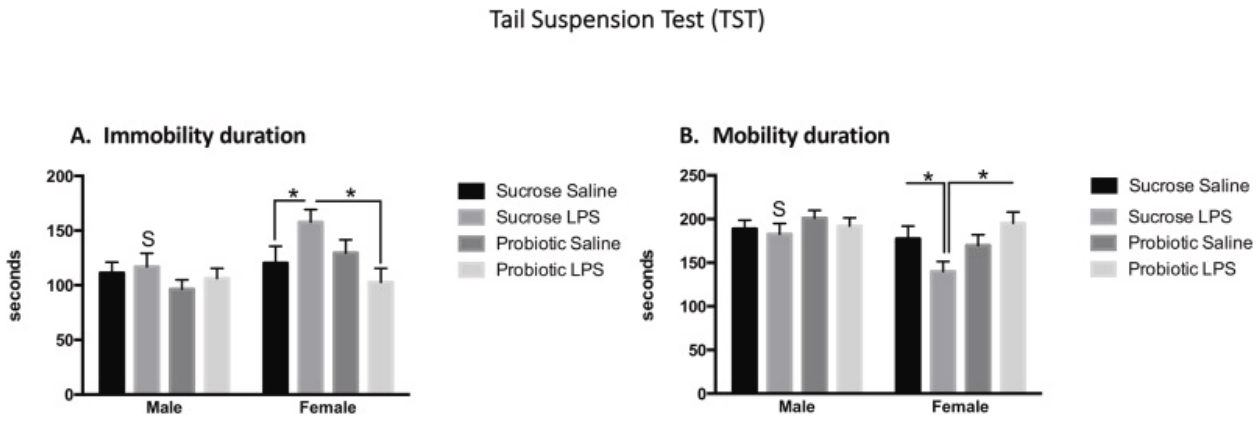

Figure 3.3. Duration of immobility (A) and mobility (B) in seconds (sec) during the tail suspension test in adult male and female mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) treatment during puberty. Data represented as mean (±SEM); The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$); S indicates significant difference ($p < 0.05$) between males and females; n = 12/group.

Pubertal probiotic treatment reduced anxiety-like behaviour

Open Field Test (OFT)

Three-way ANOVA revealed a main effect of probiotic treatment on time spent in the inner zone ($F_{(1, 94)} = 4.87$, $p < 0.05$, $\eta_p^2 = 0.05$). Pairwise comparison showed that regardless of sex and LPS treatment, mice exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic spent more time in the inner zone than mice treated with sucrose control (MD = 1.87, SE = 0.85, $p < 0.05$) (Figure 3.4A). Moreover, LPS-treated females exposed to probiotic spent more time in the inner zone than LPS-treated females exposed to sucrose control (MD = 3.57, SE = 1.69, $p < 0.05$) (Figure 3.4A). Saline-treated males exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic displayed shorter latency to first enter the inner zone than their sucrose counterpart group (MD = -41.0, SE = 20.5, $p < 0.05$) (Figure 3.4B).

Elevated Plus Maze (EPM)

Three-way ANOVA revealed a main effect of sex on time spent in the closed arms (($F_{(1, 94)} = 4.50$, $p < 0.05$, $\eta_p^2 = 0.061$) and a significant sex x LPS treatment interaction on travel velocity ($F_{(1, 94)} = 4.50$, $p < 0.05$, $\eta_p^2 = 0.05$). Pairwise comparisons showed that, regardless of LPS and probiotic treatments, males spent significantly more time in the closed arms than females (MD = 13.94, SE = 5.87, $p < 0.05$). Moreover, LPS-treated female mice displayed reduced travel velocity compared to saline-treated counterparts (MD = 0.715, SE = 0.38, $p < 0.05$) (Figure 3.5C). LPS-treated male mice exposed to the probiotic spent less time in the center zone than their saline-treated counterparts (MD = -25.3, SE = 11.9, $p < 0.05$) (Figure 3.5B).

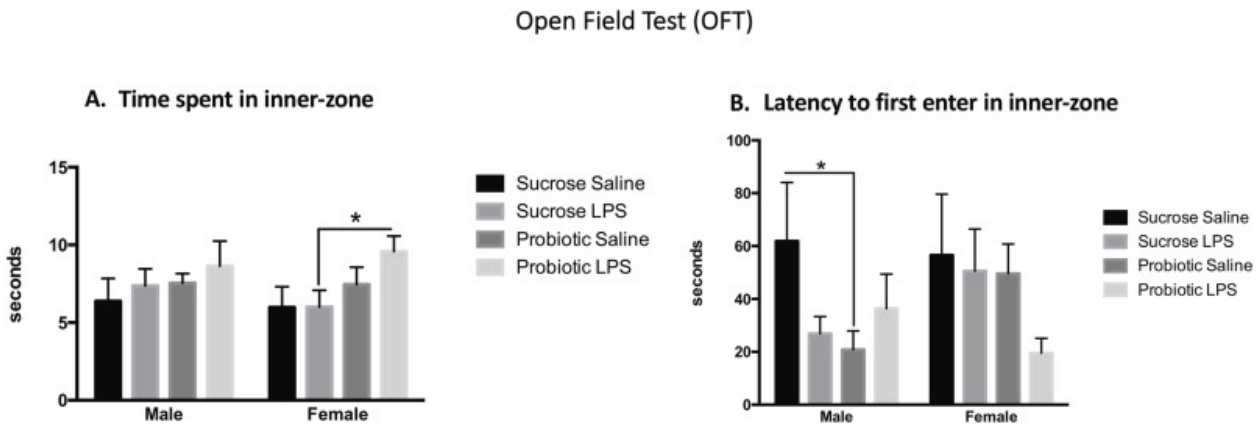

Figure 3.4. Duration of time (sec) spent in inner zone (A) and latency to first enter inner zone (B) during the open field test in adult male and female mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis (Canan SV-53)* treatment during puberty. Data represented as mean (±SEM); The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$); S indicates significant difference ($p < 0.05$) between males and females; n = 12/group.

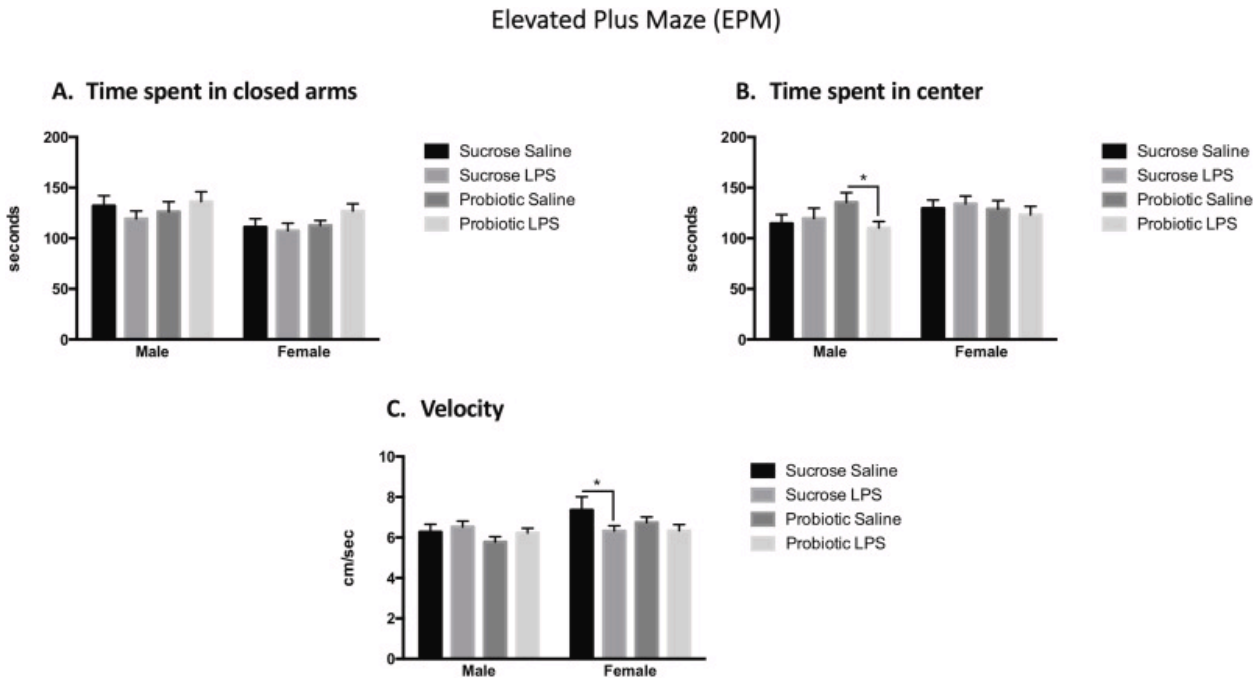

Figure 3.5. Duration of time (sec) spent in closed arms (A) and centre (B), and velocity (cm/sec) (C) during the elevated plus maze test in adult male and female mice previously treated with saline or LPS and exposed to a either sucrose or to *Rouxiella badensis* subsp. *acadiensis (Canan SV-53)* treatment during puberty. Data represented as mean (±SEM), n = 12/group; The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$); S indicates significant difference ($p < 0.05$) between males and females

Pubertal probiotic treatment mitigated LPS-induced changes in 5HT1A receptors expression in specific regions cornu-ammonis (CA1) and (CA3) in the hippocampus and the dorsal raphé nuclei in a sex-specific manner

5HT1A Rc expression in the DRN

Statistical analyses revealed a main effect of probiotic treatment ($F_{(1,77)} = 14.51, p < 0.001$, $\eta_p^2 = 0.17$) and a significant probiotic × LPS interaction ($F_{(1,77)} = 6.15, p < 0.05$ $\eta_p^2 = 0.08$) on 5HT1A Rc expression in the DRN. Pairwise comparisons showed that, regardless of sex and LPS treatment, mice exposed to probiotics has less 5HT1A Rc expression than mice exposed to sucrose control (MD=-11.89, SE = 3.12, $p < 0.05$). Moreover, regardless of sex, LPS treatment increase 5HT1A expression in the DRN in mice exposed to sucrose control (MD=13.23, SE = 4.49, $p < 0.01$). However, LPS treatment failed to alter 5HT1A expression in the DRN in mice exposed to the *Rouxiella badensis* subsp. *acadiensis* probiotic (Figure 3.6).

5HT1A Rc expression in CA1 and CA3 of the hippocampus

Three way ANOVA demonstrated significant probiotic × LPS ($F_{(1,75)} = 5.95, p < 0.05$, $\eta_p^2 = 0.08$) and sex × probiotic × LPS ($F_{(1,75)} = 6.56, p < 0.05$, $\eta_p^2 = 0.09$) interactions on 5HT1A Rc expression in CA1 region, as well as a significant sex x probiotic interaction in the CA3 region ($F_{(1,75)} = 5.43, p < 0.05$, $\eta_p^2 = 0.07$). Pairwise comparison showed that, regardless of sex, LPS treatment decreased 5HT1A Rc expression in the CA1 region of the hippocampus in mice exposed to the sucrose control (MD =16.87, SE = 7.05, $p < 0.05$), but not in mice exposed to the *Rouxiella badensis subsp. acadiensis* probiotic. However, LPS treatment induced a greater decrease in 5HT1A Rc expression in the CA1 region in males exposed to sucrose than in female counterparts (MD = 28.18, SE = 9.78, $p < 0.01$). In addition, regardless of LPS treatment, females exposed to

the probiotic displayed significantly less 5HT1A expression in the CA3 region compared to females exposed to sucrose control (MD = 9.17, SE = 3.94, $p < 0.05$). This difference did not extend to the males (Figure 3.7).

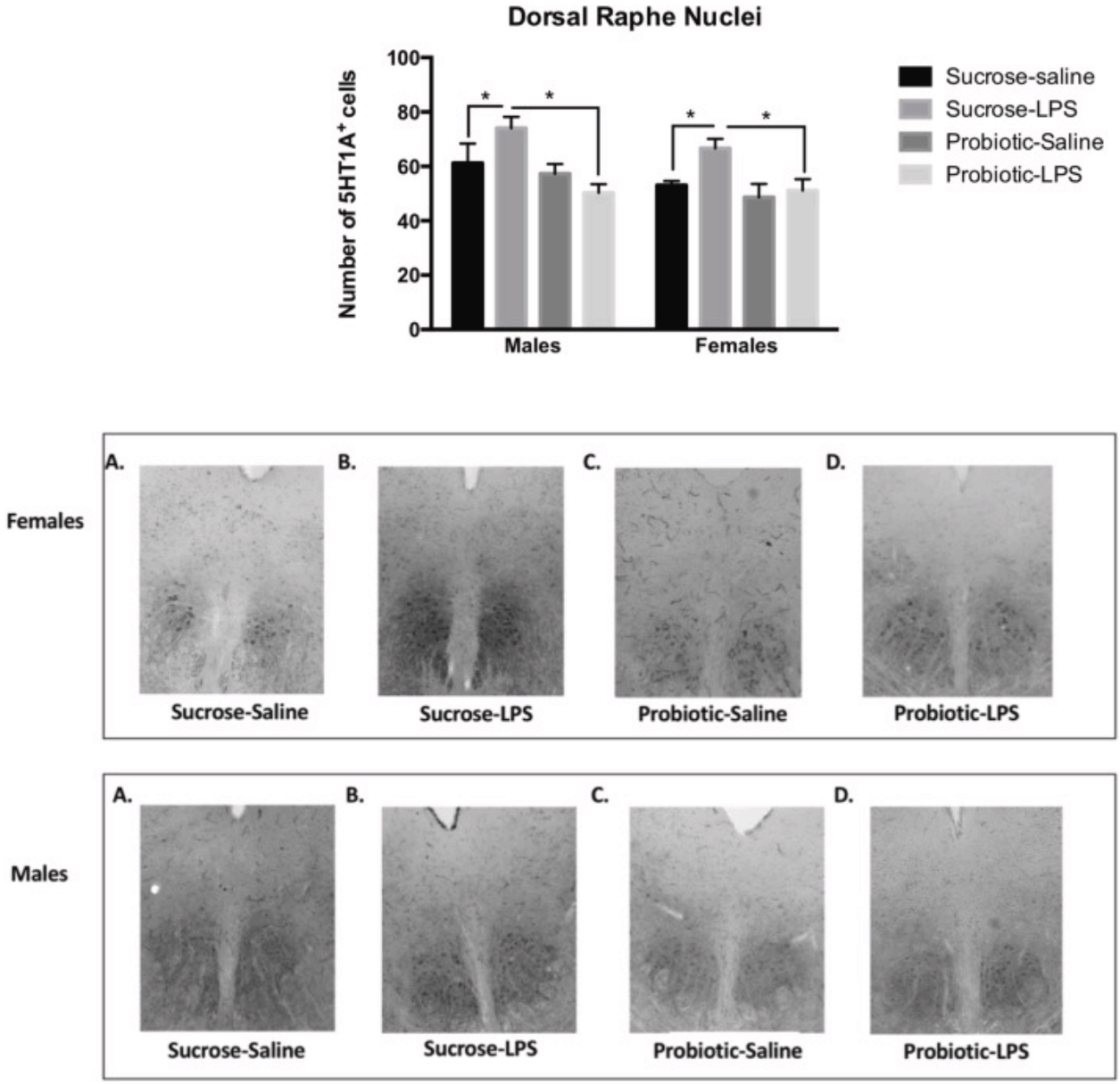

Figure 3.6. Mean (±SEM) expression of 5HT1A in the DRN of mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis (Canan SV-53)* treatment during puberty (A). The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$). Representative photomicrographs of 5HT1A expression in the DRN of adult female and male mice (B): A. Sucrose-Saline, B. Sucrose-LPS, C. Probiotic-Saline, D. Probiotic-LPS.

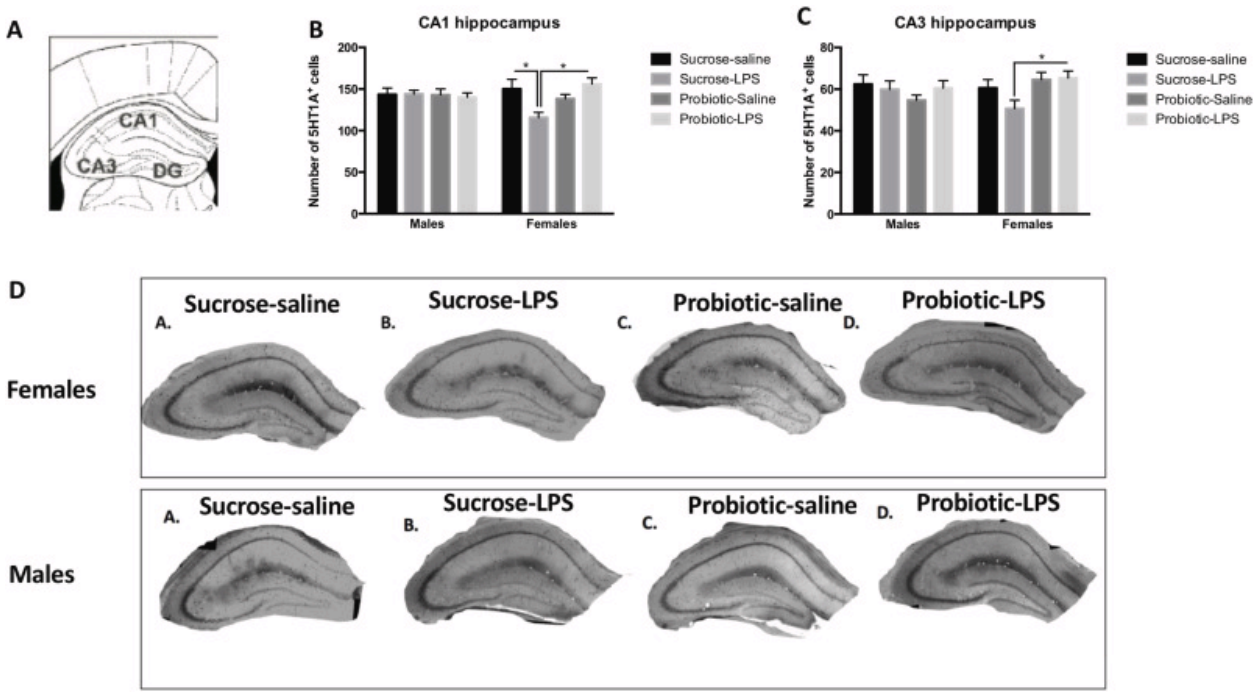

Figure 3.7. A representative schema of the hippocampal areas of interest (A), Mean (±SEM) expression of 5HT1A in the CA1 (B) and CA3 (C) regions of the hippocampus of adult males and females previously treated with saline or LPS at 6 weeks of age and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis (Canan SV-53)* treatment during puberty. The asterisks (*) indicates significant difference between treatment groups ($p < 0.05$). Representative photomicrographs of 5HT1A expression in the hippocampi of adult female and male mice (D): A. Sucrose-Saline, B. Sucrose-LPS, C. Probiotic-Saline, d. Probiotic-LPS.

Discussion

The adolescent brain undergoes significant neuronal reorganization and remodeling of neuronal circuits that are associated with heightened vulnerability to stressors and immune challenges (Burnett et al. 2011; Spear and Ph 2013). Exposure to stress or inflammation during this critical developmental period can affect brain functioning and lead to enduring changes in behaviors and adverse mental health outcomes later in life (Yahfoufi, Matar and Ismail 2020; Cai et al. 2016; Kane and Ismail 2017). Recent findings from our laboratory showed that modulation of the gut microbiota using probiotics during adolescence can mitigate LPS-induced depression-like behaviour in females and anxiety-like behaviours in males (Murray et al. 2019; Park et al. 2018). Although LPS treatment during puberty is known to induce enduring depression-like and anxiety-like behaviors in adulthood, the neural mechanisms underlying these effects remain unclear. There is also limited understanding of the influence of probiotic consumption on the developing pubertal brain. Thus, we investigated the effects of *Rouxiella badensis* subsp. *acadiensis* consumption during adolescence on LPS-induced behavioral changes in pubertal male and female mice. Given the key role of 5HT1A receptors in the pathogenesis of anxiety and depression, we investigated the effect of LPS as well as *Rouxiella badensis* subsp. *acadiensis* on the expression of these receptors in specific brain areas. We found that *Rouxiella badensis* subsp. *acadiensis* reduced LPS-induced sickness behaviours in a time dependent manner and mitigated LPS-induced depression-like behavior in an enduring manner. We also found that it mitigated LPS-induced changes in 5HT1A receptors expression in the hippocampus and in the DRN in a sex-specific manner.

All mice displayed sickness behaviours within 2h following LPS injection with males showing more prolonged sickness behavior compared to female mice, which is consistent with

previous work from our laboratory (Murray et al. 2019; Cai et al. 2016). *Rouxiella badensis* subsp. *acadiensis* also decreased LPS-induced sickness behavior in female mice for up to 24h following exposure to the immune challenge, while in males the improvement started 6h after the injection. These results support our hypotheses and are in line with previous studies showing reduced sickness behavior in mice following probiotic consumption, with the exception of males displaying limited improvement in sickness scores (Murray et al. 2019; Cai et al. 2016). Increased sickness behavior in LPS-treated mice is associated with the rise of pro-inflammatory cytokines production in the blood, including IL-6, TNFα, IL-1β, IFNγ and IL-12 (Murray et al. 2019; Cai et al. 2016). Probiotics consumption has been shown to prevent LPS-induced production of pro-inflammatory cytokines (Murray et al. 2019; Cai et al. 2016) which may explain the reduction in sickness behaviors. The greater sickness behavior observed in probiotic-LPS treated males, compared to female counterparts, at 2h, 4h and 24h post-LPS treatment, suggests a sex-dependent immune response to the bacterium and probably a stronger pro-inflammatory cytokines production in males.

We also found that LPS treatment induced weight loss in males and females at 24h and 48h following LPS treatment and persisted until 1 week following LPS treatment. These results are in line with previous findings (Murray, et al. 2020; Kolmogorova et al. 2017) that showed weight loss at 24h and 48h. Although treatment with *Rouxiella badensis* subsp. *acadiensis* failed to mitigate LPS-induced weight loss at 24h and at 48h, it significantly reduced LPS-induced weight loss 1 week following treatment in both sexes. Moreover, probiotic treatment induced weight loss in saline-treated females at 48h following injection which could be due to changes in gut microbiota diversity and composition affecting metabolism. Future studies should analyze changes in gut microbial composition following probiotic consumption. Nevertheless, *Rouxiella badensis*

subsp. *acadiensis* was effective in reducing LPS-induced weight loss in females at 7 weeks old while no significant change in body weight was observed in male mice at this age.

Furthermore, LPS-injection at 6 weeks of age induced depression-like behaviors in adult female CD1 mice, which replicate our previous findings (Murray, et al. 2020; Murray et al. 2019) and support the fact that pubertal exposure to an immune challenge alters behaviors in a sex-specific manner by affecting distinct pathways. Pubertal treatment with *Rouxiella badensis subsp. acadiensis* mitigated LPS-induced depression-like behaviour in females by eliminating differences in immobility duration (in FST and TST tests) between immune- and non-immune challenged female mice. These results propose that pubertal use of probiotic prevented stress-induced depression-like behaviour in females which is coherent with previous literature related to beneficial effects of probiotic consumption on emotional behaviors such as anxiety- and depression-like behaviours (Murray, et al. 2020; Murray et al. 2019; Bravo et al. 2011). Our findings highlight the protective enduring effects of probiotics use during the pubertal period. Different mechanisms can be at origin of these behavioral changes. For example, a major role of the immune system and neuroinflammation has been associated with the development of major depression and anxiety (Miller, Maletic and Raison 2008; Capuron and Miller 2011). Thus, inflammation induced by pubertal LPS treatment can cause enduring behavioral alterations. Other mechanisms that might be involved in the pathogenesis include alteration of the microbiota composition, transient increase in the blood brain barrier permeability and induction of central inflammation (Murray, et al. 2020; Murray et al. 2019). Protective effect of pubertal probiotic use can involve a reduction in the inflammatory reaction, a modulation of the intestinal microbiota structure, modifications of microbial metabolites levels and improvement of the integrity of the intestinal barrier (Yahfoufi et al. 2018). Previous findings revealed the ability of *Rouxiella*

badensis subsp. acadiensis to modulate the intestinal homeostasis and immune response by increasing the number of IgA positive cells in the small intestine and modulating inflammatory cytokine production. Furthermore, the bacterium has been shown to protect the integrity of the intestinal barrier (Matar et al. 2020).

LPS treatment at 6 weeks of age did not induce anxiety-like behaviors in males. These findings failed to replicate our previous results that LPS treatment induces enduring anxiety-like behaviors in males (Murray, et al. 2020; Murray et al. 2019). However, the absence of anxiety-like behaviors in the current study could be due to chronic stress induced by daily handling and gavage. The discrepancy between our observations and those of Murray et al. (2019) can be further explained by sex-specific effects of chronic adolescent stress on behaviors (Bourke and Neigh 2011). More specifically, chronic social stress (isolation, and change of cage partner) during adolescence was found to increase anxiety-like behaviour in both sexes during adulthood, which can further explain why no difference in anxiety behaviour was observed between LPS- and saline-treated groups (Mccormick, Smith, and Mathews 2008). McCormick and colleagues have found increased anxiety-like behaviour in older males due to a greater increase in corticosterone concentrations in adult males compared to adolescent males after confinement to the open arm of the EPM (Mccormick, Smith, and Mathews 2008). These results could explain the increase of anxiety-like behavior in saline-treated males exposed to chronic stress (gavage) and the absence of a significant difference in anxiety-like behaviors between saline- and LPS-treated males. Additionally, we have observed that saline-treated mice exposed to probiotic showed no change in anxiety-like behaviors which revealed that our probiotic does not have anxiogenic effects. Moreover, the effects of pubertal immune challenge were observed several weeks after LPS exposure, indicating that sex-specific developmental trajectories and vulnerability to mood

disorders may be shaped by immune system activation during critical periods of development, like puberty. Our mouse model and probiotic effects indicate that puberty can be another opportunity to shape brain development either positively or negatively.

Additionally, pubertal LPS treatment increased the expression of 5HT1A receptors in the DRN of both males and females, leading to reduced serotonin release in the projection areas and increased depression-like behaviors. Interestingly, probiotic exposure mitigated the LPS-induced increase in 5HT1A receptor expression in both sexes. Moreover, LPS-treated females exposed to probiotic had lower 5HT1A receptor expression in the DRN compared to LPS-treated females exposed to the control. Thus, *Rouxiella badensis* subsp*. acadiensis* could act as an anti-depressant by decreasing serotonin reuptake in the DRN and increasing serotonin release in projection areas.

LPS treatment also reduced 5HT1A receptor expression in the CA1 region of the hippocampus in females only, highlighting the enduring sex-specific effects of pubertal LPS challenge on 5HT1A receptor expression in this region. *Rouxiella badensis* subsp*. acadiensis* blocked the effects of LPS treatment on 5HT1A expression in the CA1 region and increased 5HT1A receptor expression in the CA1 and CA3 regions in LPS-treated females. These results demonstrate its ability to increase the expression of postsynaptic 5HT1A receptors in specific hippocampal regions. Postsynaptic 5HT1A receptors are found in limbic structures and their activation contribute to a decline in the firing rate of postsynaptic cells, resulting in antidepressant effects (Sprouse JS 1988). A reduction in postsynaptic 5HT1A receptors has been found in the hippocampi of depressed individuals and in animal models of depression (Sargent et al. 2000; Cheetham SC, Crompton MR and Katona CL 1990; Murack et al. 2020). Antidepressant treatment often induce the activation of these receptors (Haddjeri, Blier, and Montigny 1998). These results validate the long-term depressive outcome observed upon pubertal LPS treatment in female mice

and the ability of pubertal probiotic use to alleviate the negative consequences of an immune-challenge, by altering the expression of 5HT1A receptors in the hippocampus in a sex-dependent manner. The absence of depression-like behaviors in males can be due to the fact that LPS treatment did not change the number of 5HT1A postsynaptic receptors in the CA1 and CA3 regions

Conclusion

The current study underscores the key role of puberty as a critical phase during neurodevelopment with heightened level of vulnerability to immune challenges. The results reveal that exposure to an immune challenge during puberty changes 5HT1A receptor expression in different brain regions and causes enduring depression-like behavior in females. Furthermore, our study demonstrates that probiotics like *Rouxiella badensis* subsp. *acadiensis* can mitigate the long-term neural and behavioral alterations induced by pubertal LPS. Further investigations remain necessary to fully elucidate the impact of probiotics during adolescence in the prevention of stress-induced mood disorders.

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Declaration of Interest

We have no conflict to declare

Chapter 4: Pubertal consumption of *R. badensis* subspecies *acadiensis* modulates LPS-induced immune responses and gut microbiome dysbiosis in a sex-specific manner.

Preface

The following chapter consists of Article 3 published in Brain, Behavior and Immunity under the title **Pubertal consumption of *R. badensis* subspecies *acadiensis* modulates LPS-induced immune responses and gut microbiome dysbiosis in a sex-specific manner** by Nour Yahfoufi[1,2], Anthony K. Kadamani[2], Sarah Aly[2], Sara Al Shaarani[2], Jacky Liang[2], James Butcher[3], Alain Stintzi[3], Chantal Matar[1,4] and Nafissa Ismail[2,5]

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Sarah Al Sharani: Investigation, Data Curation
Jacky Liang: Training, Technical support, Data analysis support.
James Butcher: Microbiome analysis, writing- Review & Editing microbiome findings.
Alain Stintzi: writing-Review & Editing
Chantal Matar: Resources, Funding Acquisition, Writing – Review & Editing, Supervision.
Nafissa Ismail: Conceptualization, Methodology, Formal analysis, Resources, Writing – Review & Editing, Supervision, Project administration

Abstract

Puberty is a critical period of development characterized by significant brain remodeling and increased vulnerability to immune challenges. Exposure to an immune challenge such as LPS during puberty can result in inflammation and gut dysbiosis which may lead to altered brain functioning and psychiatric illnesses later in life. However, treatment with probiotics during puberty has been found to mitigate LPS-induced peripheral and central inflammation, prevent LPS-induced changes to the gut microbiota and protect against enduring behavioural disorders in a sex-specific manner. Recent findings from our laboratory revealed that pubertal ***R. badensis*** **subspecies *acadiensis* (***R. badensis* subsp. *acadiensis*) treatment prevents LPS-induced depression-like behavior and alterations in 5HT1A receptor expression in a sex-specific manner. However, the underlying mechanism remains unclear. Thus, the aim of this study was to gain mechanistic insights and to investigate the ability of *R. badensis* subsp. *acadiensis* consumption during puberty to mitigate the effects of LPS treatment on the immune system and the gut microbiome. Our results revealed that pubertal treatment with *R. badensis* subsp. *acadiensis* reduced sickness behaviors in females more than males in a time-specific manner. It also mitigated LPS-induced increases in pro-inflammatory cytokines in the blood and of TNFα mRNA expression in the prefrontal cortex and the hippocampus of female mice. There were sex-dependant differences in microbiome composition that persisted after LPS injection or probiotic consumption. *R. badensis* subsp. *acadiensis* consumption had minimal impacts on the overall microbiota composition and appeared to have a greater impact on male microbiota compositions. In contrast, female microbiotas were more responsive to LPS induced changes. These findings emphasize the sex-specific effects of probiotic use during puberty on the gut microbiome structure and the immune system and highlight the critical role of gut colonization with

probiotics during adolescence on immunomodulation and prevention of the enduring effects of infections.

Keywords: Probiotic, LPS, inflammation, neuroinflammation, cytokines, microbiome, puberty.

Introduction

Puberty is a period of reproductive maturation characterized by changes in gonadal steroid hormones that lay the framework for lifelong physiological differences between both sexes (Wallen and Baum, 2002; Sisk and Foster, 2004). During this period, the brain undergoes extensive remodeling and reorganization which result in increased sensitivity of the central nervous system (CNS) to stressors and immune challenge, like lipopolysaccharide (LPS) (Grant *et al.*, 2003; Levitt, 2003; Sato *et al.*, 2008; Olesen *et al.*, 2011; Ismail and Blaustein, 2013; Holder and Blaustein, 2014). In mice, pubertal exposure to LPS at six weeks of age causes enduring impairments in reproductive and non-reproductive behaviors such as sexual receptivity (Laroche *et al.*, 2009b, 2009a; Ismail, Garas and Blaustein, 2011), cognitive functioning (Ismail and Blaustein, 2013), spatial memory (Kolmogorova, D., Paré, C., Kostuck, S., Hudson, E.C., Lebel, N., Houlding, E. and J.G., Ismail, N., 2019), and anxiety-like and depression-like behaviors in adulthood (Ismail, Kumlin and Blaustein, 2013; Murray, Smith, Stoby, Thomas, Swenson, Arber and Frenette, 2020). These enduring impairments are specific to six weeks of age, as younger or older inbred C57B1/6 or outbred CD1 mice exposed to LPS do not display enduring impairments in reproductive and non-reproductive behaviors (Laroche *et al.*, 2009a; Olesen *et al.*, 2011; Ismail and Blaustein, 2013).

LPS is a pyrogenic compound present in the outer membrane of gram-negative bacteria and is extensively used in research as an immune challenge in a variety of *in vivo* and *in vitro* protocols (Lopes, 2016). LPS activates the innate immune system through toll-like receptors (TLRs) that are encountered on innate immune cells (macrophages, monocytes) (Galic, Riazi and Pittman, 2013). These receptors are also present in the brain and are localized to the

circumventricular organs, meninges endothelial cells and microglia (Galic, Riazi and Pittman, 2013). LPS can induce a pro-inflammatory immune response once detected by the host innate immune system (Lopes, 2016). Peripheral administration of LPS causes inflammation and neuroinflammation (Rivest, 2003) that are dose- and time-dependent (Lopes, 2016). LPS-induced neuroinflammation can be due to *de novo* synthesis of cytokines in the brain by activation of cytokine receptors at the circumventricular organs and the choroid plexus or by the infiltration of peripheral cytokines via increased permeability the blood-brain barrier (Galic, Riazi and Pittman, 2013; Layé *et al.*, 2014).

There are prominent sex differences in the acute immune response to LPS (Cai *et al.*, 2016; Sharma *et al.*, 2018). LPS-treated adult male rats display greater sickness symptoms and prolonged hypothermia compared to LPS-treated adult females that demonstrate a faster recovery from hypothermia and cachexia or body weight loss (Tesfaigzi *et al.*, 2001; Cai *et al.*, 2016). Sex differences in the immune response are also well identified in humans with more robust cellular and humoral immune responses in females who are less affected by certain types of infections, while being more susceptible to autoimmune disorders than males (Bouman, Heineman and Faas, 2005). In rodent *in vivo* models and human cell culture, higher production of TNFα, IL-1β, and IL-6 have been observed in males compared to females following LPS treatment (Moxley *et al.*, 2002; Imahara *et al.*, 2005; Queen *et al.*, 2016). Recent work from our laboratory has shown that adult females display heightened TNFα mRNA expression in the hippocampus at 2h following LPS treatment compared to male counterparts, while adult males display more IL-1β mRNA expression compared to female counterparts in the prefrontal cortex at 8 h following LPS treatment (Sharma *et al.*, 2018). These sex differences are also observed following viral infections. Males display more IL-1β mRNA in the lungs and demonstrate more anorexia and reduced corticosterone

secretion compared to females following influenza infection (Avitsur, Mays and Sheridan, 2011). Gonadal hormones contribute to the sex difference in immune response, since estradiol acts as an immune enhancer and contributes to the maturation of lymphocytes and production of antibodies, while testosterone acts as an immune suppressor (Wichmann *et al.*, 1997; Lai *et al.*, 2012; Kovats, 2016). However, a study from our laboratory revealed that gonadectomy did not eliminate sex differences in LPS-induced immune response (Cai *et al.*, 2016), which suggests that there are additional mechanisms that contribute to these sex differences. Age differences are also observed in the immune response following LPS treatment (Cai *et al.*, 2016; Nikodemova *et al.*, 2016). However, the mechanism underlying these sex and age differences in immune response remains unclear. The gut microbiome may be an important factor contributing to the sex and age differences in immune response. The microbiota and the immune system influence one another to orchestrate host physiology (Bailey and Cryan, 2017). The host immune system identifies microbial presence through a number of microbial receptors called pattern recognition receptors (PRRs) (Grenham *et al.*, 2011; Thaiss *et al.*, 2016). In addition, microbiota-modulated metabolites such as short-chain fatty acids have a major effect on the innate immunity (Thaiss *et al.*, 2016). Thus, the intestinal microbiome acts as a signalling hub that integrates environmental inputs like diet, with genetic, neuroendocrine and immune signals to influence the host's immunity and response to an infection (Thaiss *et al.*, 2016). The gut microbiome undergoes maturation that occurs in parallel with neurodevelopment and has similar critical developmental periods (Yahfoufi, Matar and Ismail, 2020) and its composition is also subject to age and sex differences (Yahfoufi, Matar and Ismail, 2020). Sex differences are induced by gonadal steroid hormones since castration eliminates these sex differences in intestinal microbiota in adult mice (Yurkovetskiy *et al.*, 2013). The gut microbiota changes also throughout life. The gut microbiota is simple and unstable during infancy

and becomes more diverse during adolescence. In adulthood, the microbiota is less diverse but more stable (Agans *et al.*, 2011). In older adults, the reduction in microbial diversity is linked to inflammation (Agans *et al.*, 2011; Claesson *et al.*, 2011; Yahfoufi, Matar and Ismail, 2020). There is also a bi-directional relationship between the gut microbiota and stress response. Several studies have shown that exposure to stressors can induce intestinal dysbiosis which has been linked to behavioral impairment such as anxiety and depression (Zhou, Zhou and Chen, 2017; Cryan and Dinan, 2012).

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.*, 2015). They have been found to improve mental health, cognition, and behavioural outcomes and to also act as immunomodulators (Bercik *et al.*, 2011; Bravo *et al.*, 2011; Messaoudi *et al.*, 2011; Thomas *et al.*, 2012; Yahfoufi *et al.*, 2018). They are able to modulate the gut microbiome composition, secrete neuroactive metabolites into the circulation, regulate local immune populations that cross to the CNS, and stimulate the vagus nerve to impact behavior (Samuel *et al.*, 2008; Collins, Surette and Bercik, 2012; Clemmensen *et al.*, 2017; Azad *et al.*, 2018). In fact, two recent studies from our lab revealed that colonizing the gut with probiotics during puberty mitigates LPS-induced peripheral and central inflammation, prevents LPS-induced changes to the gut microbiota and protects against stress-induced anxiety-like and depression-like behaviors in adulthood in a sex specific manner (Murray, Smith, Stoby, Thomas, Swenson, Arber and Frenette, 2020). However, the mechanisms underlying age and sex differences in the immune response induced by LPS during puberty have not yet been fully elucidated.

Rouxiella badensis subsp. *acadiensis* was first isolated from the microbiota of the wild blueberry fruit and it is involved in its fermentation. It has anti-inflammatory properties and has

been found to be beneficial against neurodegeneration, diabetes, and cancer (Martin and Matar, 2005; Vuong *et al.*, 2007, 2016; Nachar *et al.*, 2017; Sánchez-villavicencio *et al.*, 2017). Given its beneficial properties and that whole-genome shotgun sequencing of the bacterium suggesting the absence of known pathogenic genes (unpublished data by Chantal Matar), it has been filed in a U.S. Provisional Application No. 62/916,921 titled "Probiotics Composition and Methods" for its potential probiotic effects (Matar et al. 2020). Recent findings show that oral administration of this bacterium can interact with the intestinal mucosa and modulate the intestinal immune response and the gut barrier function by increasing the concentrations of IL-10, IgA, miR-146 and the number of goblet cells in the intestine (Yahfoufi N. *et al.*, 2021). Previous studies have shown that *R. badensis* subsp. *acadiensis* is able to maintain the intestinal barrier and to contribute to gut homeostasis and protect against pathogenic bacteria (Matar et al.,2020; Yahfoufi et al., 2021) which could explain its role in mitigating LPS-induced inflammation in female mice. Moreover, exposure to *R. badensis* subsp. *acadiensis* during puberty blocks LPS-induced enduring depression-like behavior and alterations in 5HT1A receptor expression in female mice (Nour Yahfoufi et al. 2021). However, the mechanisms underlying these effects are unknown. One possibility could be that *R. badensis* subsp. *acadiensis* consumption is modifying the effects of LPS treatment on the immune system and/ or the gut microbiome. Thus, the current study was designed to gain mechanistic insight into the sex-dependent effects of *R. badensis* subsp. *acadiensis* consumption during puberty on LPS-induced inflammatory outcomes and changes to the gut microbiome.

In this study, we examined age and sex differences in LPS-induced cytokines in the blood and in the brain and we investigated the mitigating effects of pubertal probiotic treatment on LPS-induced immune response and gut microbiome modulation. We hypothesized that during puberty

sex differences are associated with LPS depletion of specific bacterial genera. We also hypothesized that treatment with *Rouxiella badensis* subspecies *acadensis* starting one week prior to LPS injection would protect against LPS-induced dysbiosis and reduce peripheral and central inflammation by depleting inflammatory cytokines in the plasma and by decreasing inflammatory cytokines mRNA expression in the brain.

Methods

Animal housing

Seventy CD-1 mice (35 male and 35 female) were shipped from Charles River Laboratories (St-Constant, Québec) at 3 weeks of age. Upon arrival, males and females were separated in either an all-male or an all-female colony room. Mice were housed in pairs in Polycarbonate cages except 2 cages which had 3 mice per cage. All mice were given *ad libitum* access to food (ENGIVO—Teklad Global 18% rodent) and water. The rooms were maintained at constant temperature (24 ± 2 °C) with a reversed light: dark cycle (14:10; lights off at 10:00am) and 40% (±5%) humidity. Dusk and dawn were gradually induced over a period of one hour. Cages were bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, US, ¼ inches in diameter). One square piece of Nestlet (Ancare Corp., Bellmore, NY, US) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, CA) were used for enrichment. All procedures were approved by the Animal Care Committee of the University of Ottawa.

Probiotics

Stock cultures of the *Rouxiella badensis* subsp. *acadiensis* were maintained at −80^{0}C in Tryptic Soy Broth (TSB) (Difco Laboratories, Cat. No. 257107.06) supplemented with 30% (v/v) glycerol. *Rouxiella badensis* subsp. *acadiensis* was grown at 30°C in 5 mL of sterile Tryptic Soy Broth (TSB). To prepare bacterial suspensions, the overnight culture was centrifuged at 5000xg for 10 min, washed three times with 1X phosphate-buffered saline solution (1XPBS) and resuspended in 5 mL of sterile 10% (weight to volume) non-fat milk. Bacterial suspensions were diluted 1:30 in

water and provided *ad-libitum* to the experimental group of mice as of 5 weeks of age for 7 days. Control mice received the same preparation without the probiotic. The provided concentration of the bacteria was ≈ 2 ± 1 × 10^9 CFU/mL. Bottles were changed every 24h. We chose a 7-day treatment period since previous work from the NISE Lab has shown that pubertal probiotic (kefir) treatment for 7 days was effective at mitigating LPS-induced immune response at 8h post-LPS injection (Murray *et al.*, 2019).

Lipopolysaccharide (LPS)

At 6 weeks of age, male (n=35) and female (n=35) mice were injected intraperitoneally at the end of the light phase with either 0.9% sterile saline or LPS (1.5 mg/kg). LPS solution was prepared by dissolving lyophilized LPS (obtained from *Escherichia coli* serotype O26:B6; #L3755; Sigma Aldrich) into 0.9% (w/v) sterile saline (Ricca Chemical Company) at a concentration of 0.2 mg/ml. LPS-treated male (n=18) and female (n=18) mice were injected a dose of 1.5mg/kg of LPS while saline-treated males (n=17) and females (n=17) were injected with an equivalent volume of saline. This dose of LPS has previously been shown by our laboratory to induce mild sickness behavior in mice for up to 48 h (Ismail, Garas and Blaustein, 2011) and to produce enduring impairments in reproductive and non-reproductive behaviors, when administered at 6 weeks of age, during the stress-sensitive pubertal period (Laroche *et al.*, 2009a; Ismail, Kumlin and Blaustein, 2013).

Sickness monitoring

Sickness monitoring consisted of assessing mice for the presence of sickness behaviours (huddling, piloerection, ptosis, and lethargy) at 0.5, 2, 4 and 8 h after exposure saline or LPS injections. Two experienced observers blinded to the treatment conditions individually assigned

a sickness score to each mouse at each time point. Scores range between 0 (no symptoms) and 4 (all four sickness behaviours observed). The final sickness score was calculated by averaging the raters' individual scores at each time point (Kolmogorova, D., Murray, E., Ismail, 2017).

Fecal samples collection

Mice were taken out of their cage and transferred to a new clean cage for a short period of time to induce defection. Fecal samples were collected at two time points: at 6 weeks of age after one week of probiotics treatment (the day before LPS treatment) and at 8h following the LPS injection. Samples were immediately frozen using liquid nitrogen, in Eppendorf tubes, placed on dried ice then stored at −80 °C until DNA extraction.

Euthanasia and blood collection

Mice were euthanized with an intraperitoneal injection of Euthanyl (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirkland, Canada) 8 hours following saline or LPS treatment. Following verification of the absence of reflex, trunk blood was placed into a Microvette CB 300 K2E (Sarstedt, Cat. No. 16.444.100) and was centrifuged for 15 minutes at 1100xg at room temperature and the plasma (top layer) was collected and stored at -80°C.

Multiplex Luminex immunoassay

A multiplex Luminex immunoassay with magnetic beads was used to quantify plasma concentrations of the interleukins (IL)-1β, 6, 10, 12(p70), interferon-γ (IFNγ), and tumour necrosis factorα (TNFα) according to the manufacturers' instructions. A milliplex MAP mouse Th17 magnetic bead panel (Millipore, Cat. No. MT17MAG47k-PX25) was also used measure additional

cytokines (IL-2, IL-4, IL-5, IL-13, IL-15, IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28B, IL-31, IL-33, CD40L, GM-CSF, MIP-3a, TNFβ) in LPS-treated mice according to the manufacturers' instructions. Quality controls and standards were added to the appropriate wells according to the manufacturer's protocol while samples were added in triplicates. Then, an antibody-bead solution was added to the plates. The plates were incubated overnight on a plate shaker at 4°C. Two washes were performed, and detection antibodies were added, and the plates were left to incubate on a plate shaker at room temperature for one hour. Streptavidin-phycoerythrin was added to each well and plates were incubated on a plate shaker at room temperature for 30 min. After washing, beads were resuspended in drive fluid. The assay was read using a MAGPIX software. Inter- and intra-assay variability was < 15%.

Real-time quantitative polymerase chain reaction

Brains were extracted, flash frozen using liquid nitrogen, and stored at −80 °C. Frozen brains were sliced into 350μm thick slices on a Leica CM1900 microtome and slices were mounted onto Fisherbrand *Superfrost Plus* microscope slides. The prefrontal cortex (PFC), hippocampus, and hypothalamus were collected by punching the prepared slices; specific brain areas were determined as per guidelines from *The Mouse Brain Atlas in Stereotaxic Coordinates* (Paxinos, 2013). Messenger RNA (mRNA) was extracted from punched tissues using PureLink® RNA Mini Kit, Cat. No. 12183025. Genomic DNA contamination in mRNA samples was eliminated using gDNA wipeout buffer and cDNA synthesis was achieved using the QuantiTect Reverse Transcription kit (Qiagen, Cat. No. 205311). Relative gene expression was measured using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, Cat. No. 172-5271) in 10 μL reactions on a CFX96TOUCH real time PCR machine (Bio-Rad). Primers for β-actin, IL-1 β, IL-6 and TNFα

were ordered through Integrated DNA Technologies. After running standard curves, all primer pairs in this experiment achieved reaction efficiencies between 90% and 110%. Primers were diluted to a final concentration of 0.5 μM for (β-actin, IL-1 β, IL-6) and 0.3 μM for TNFα. RT-qPCR samples were run in triplicates. Bio-Rad CFX Maestro version 3 was used to view RT-qPCR results. The $2^{-\Delta\Delta Ct}$ method was used to calculate fold change in gene expression, using β-actin as the reference/housekeeping gene.

The sequences of the qPCR primers (Table 1) were as follows:

Table 4.1. Summary of qPCR Primer sequences.

TARGET GENE	**FORWARD** 5'-3'	**REVERSE** 5'-3'
B-ACTIN	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAGG
IL-1B	TCTTGGGACTGATGCTGGTG	CAGAATTGCCATTGCACA ACTC
TNFA	GCCTATGTCTCAGCCTCTTCTC	GCCATTTGGGAACTTCTCATCC
IL-6	GCCTTCTTGGGACTGATGCT	GCCATTGCACAACTCTTTTCTC

β-Actin was used as the reference/housekeeping gene for all samples. No significant difference in β-actin mRNA expression was found between experimental groups. For each reaction, the quantitative threshold amplification cycle number (Cq) was determined by taking the average across groups, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression. Geometric mean of the fold change were computed and statistical analyses were performed on the log transformed normalized expression as per Taylor et al. 2018 (Taylor *et al.*, 2018).

Metagenomic DNA extraction

A FastDNA spin kit (MP Biomedicals, Cat. No. 116540600) was used to extract DNA. Fecal samples were homogenized with two mechanical lysis cycles in a cell lysis solution and lysing matrix using a Mixer Mill 400 at 25hz for 40s with 5 min cooling on ice between the two cycles. Negative (no feces) and positive (ZymoBIOMICS Microbial Community Standard (D6300)) controls were included for extraction and subsequent PCR reactions. The DNA was then isolated and purified with the Fast DNA Kit according to manufacturer's recommendations. Qubit fluorometer (Invitrogen, cat No Q33238) and Qubit HS DNA assay kit (Invitrogen, Cat. No. Q33230) were used to quantify the extracted DNA. Aliquots of 5ng/µL were prepared and stored at -80°C until used.

16S rRNA V6 library construction for Ion Torrent sequencing and Analysis

V6 region of 16S rRNA gene library construction and sequencing were performed as previously described (Mottawea *et al.*, 2016). Briefly, the V6 hypervariable region of the extracted metagenomic DNA was amplified in PCR reaction using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Cat. No. F548L) with modified universal 16S V6 primers that included Ion Torrent adaptors and barcode sequence. The reaction was heated to 98°C for 30 s and then subjected to 10 cycles of 98 °C for 5 s, 61 °C for 15 s and 72 °C for 15 s with 1 °C drop each cycle, followed by additional 15 cycles of 98 °C for 5s, 61 °C for 15s and 72 °C for 15s, followed by a final extension step at 72 °C for 5 min. 1.5% agarose gel was used to verify successful amplification with correct amplicon size. Each amplicon was quantified with the Qubit fluorometer

(Invitrogen, Cat. No Q33238) using Qubit BR DNA assay kit (Molecular Probes, Cat. No. Q32850) according to the manufacturer's instructions. Equal masses from all amplicons were pooled together. The amplicon pool was purified using a Purelink PCR purification kit (ThermoFischer Scientific, Cat. No. K310001), following by a size selection using AMPure XP DNA purification beads (Beckman Coulter, Cat. No. A63880) before sequencing on a Proton Ion Torrent sequencer using HiQ reagents according to the manufacturer's instructions.

Raw sequencing reads were demultiplexed using cutadapt (Martin, 2011) discarding reads without both universal primer sequences present, reads with ambiguous bases and reads <100 in length after primer removal. Demultiplexed reads have been deposited to NCBI SRA under project accession PRJNA818974. Amplicon Sequence Variants (ASVs) were identified using the dada2 (Callahan *et al.*, 2016) workflow and subsequent analysis performed using phyloseq(Mcmurdie and Holmes, 2013). Briefly, reads were quality filtered to remove those with expected errors >1 and the error profile determined using the recommended settings for Ion Torrent datasets. Reads were denoised and chimera filtered in dada2 with sample pooling. Taxonomy was also assigned in dada2 using the default parameters against the Silva 132 database. Resulting ASVs were inserted into a properly formatted Silva 132 backbone tree using SEPP with the default settings (Mirarab, Nguyen and Warnow, 2012) to calculate phylogenic distances. The ASVs belonging to the administered probiotic were identified by BLAST ('Coordinators NR. Database resources of the National Center for Biotechnology Information.', 2018) searching the ASVs against the probiotic's published rRNA sequence (Martin and Matar, 2005) and the NCBI reference genome (NZ_CP060592.1). The rRNA genes for this strain are present in 7 copies with two alleles in the V6 hypervariable region at ratio of 6:1 that differ from each other by a single nucleotide

substitution. Two ASVs were identified with 100% matches to the primary (ASV00301) and minor (ASV00801) allele (Supplemental Table S1) and present at a median ratio of 9.43. The taxonomy of these putative probiotic ASVs were originally assigned as an undefined *Hafnia-Obesumbacterium* genera; which closely mimics the original taxonomy assigned to this strain (Martin and Matar, 2005). As the taxonomy of this strain has since been revised (Yahfoufi *et al.*, 2021); we manually updated the taxonomy to match its current designation. ASVs annotated as Cyanobacteria or Mitochondria were removed as off-target amplicons and technical contaminants identified and removed using decontam (Davis *et al.*, 2018). Outlier samples were identified and removed by manual inspection of principal co-ordinate ordinations of the Bray-Curtis dissimilarity, weighted Unifrac distance, and unweighted Unifrac distance. ASVs were subsequently filtered to only keep those with ≥5 counts at least 5% of the final sample dataset, and samples were rarefied to 130,000 reads prior to analysis. Differences in alpha diversities were assessed using the Chao1 index and Shannon diversity using paired Mann Whitney tests. Sample beta diversity clustering was assessed using the adonis function in vegan (Oksanen *et al.*, 2016). For simplicity, only the unweighted Unifrac results are presented herein as this phylogenetically aware metric more suitable for detecting subtle impacts on microbiome composition (Lozupone *et al.*, 2013). Potentially differentially abundant taxa between various metadata were identified using MaAsLin2 (Mallick *et al.*, 2021) using the default parameters and controlling for sex and probiotic administration with caging as a random effect term. Post-injection samples also included injection type as a covariate. Results were considered significant with a p-value <0.05 and, where appropriate, after controlling for multiple hypothesis testing using the Benjamini and Hochberg approach.

Procedures

Mice were received at 3 weeks of age. As of 5 weeks of age, mice were either exposed to the probiotic treatment or to control. At 6 weeks of age, mice were treated with either saline or LPS. Mice were euthanized 8 h following treatment to examine peripheral cytokine concentrations and central cytokine mRNA expression. Fecal samples were collected the day before LPS treatment and at 8h following LPS injection. Sickness scores were assessed at 0.5, 2, 4 and 8 h after exposure saline or LPS treatment (Figure 4.1).

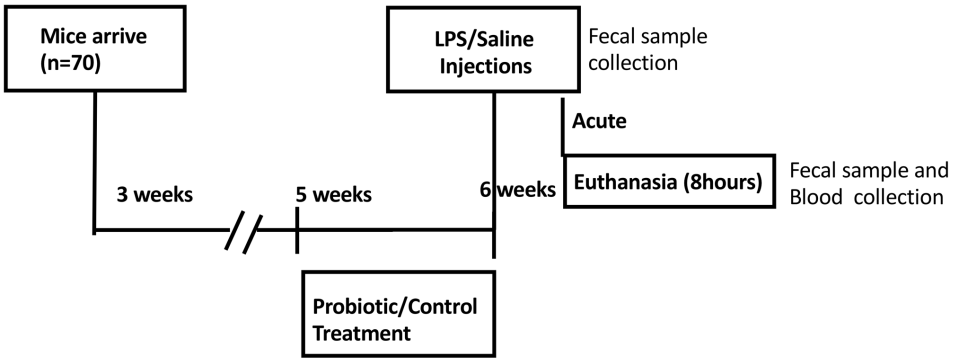

Figure 4.1. Study plan showing experimental timeline, groups and sample collection of mice examined for LPS-induced immune response 8 h post-injection (acute effects).

Results

Sickness Monitoring: Pubertal *Rouxiella badensis* subsp. *acadiensis* treatment reduced LPS-induced sickness behaviour in a sex and time-specific manner.

Our results revealed that pubertal treatment with *R. badensis* subsp. *acadiensis* mitigated LPS-induced sickness behaviors in females more than males in a time-specific manner.

Four-way mixed ANOVA revealed main effects of time ($F_{(2.78, 172.45)} = 429.58$, $p < 0.001$, $\eta_p^2 = 0.87$), LPS ($F_{(1, 62)} = 3656.07$ $p < 0.001$, $\eta_p^2 = 0.98$), *R. badensis* subsp. *acadiensis* ($F_{(1, 62)}) = 7.26$, $p < 0.01$, $\eta_p^2 = 0.10$) and sex ($F_{(1, 62)}) = 12.05$, $p < 0.001$, $\eta_p^2 = 0.16$) on sickness behaviors (Figure 4.2). We also observed significant *R. badensis* subsp. *acadiensis* x LPS ($F_{(1, 62)}) = 8.72$, $p < 0.01$, $\eta_p^2 = 0.12$), sex x LPS ($F_{(1, 62)}) = 4.74$, $p < 0.05$, $\eta_p^2 = 07$), time x LPS ($F_{(2.78, 172.45)} = 422.58$, $p < 0.001$, $\eta_p^2 = 0.87$), time x sex ($F_{(2.78, 172.45)} = 6.45$, $p < 0.001$, $\eta_p^2 = 0.09$) and time x sex x LPS ($F_{(2.78, 172.45)} = 6.97$, $p < 0.001$, $\eta_p^2 = 0.10$) interactions. Pairwise comparisons showed that, regardless of sex, mice exposed to *R. badensis* subsp. *acadiensis* and treated with LPS (probiotic-LPS) displayed less sickness behaviors than mice exposed to placebo and treated with LPS (control-LPS) (MD = -0.23, SE = 0.06, $p < 0.01$). Moreover, *R. badensis* subsp. *acadiensis* -LPS females demonstrated less sickness than control-LPS females (MD = 0.28, SE = 0.08, $p < 0.001$) at 30min (MD = -0.19, SE = 0.07, $p < 0.01$), 6h (MD = -0.32, SE = 0.13, $p < 0.05$) and 8h (MD = -0.17, SE = 0.09, $p < 0.05$) following LPS treatment. In comparison, *R. badensis* subsp. *acadiensis* -LPS males displayed less sickness behavior than control-LPS males at 2h following LPS treatment (MD = 0.38, SE = 0.17, $p < 0.05$). Moreover, control-LPS and *R. badensis* subsp. *acadiensis* -LPS males developed more sickness than their females' counterparts (MD = 0.17, SE = 0.08, $p <0.05$*;* MD = 0.28, SE = 0.07, $p <0.001$, respectively), with *R. badensis* subsp.

acadiensis -LPS males showing significantly more sickness behavior than *R. badensis* subsp. *acadiensis* -LPS females at 6h (MD = 0.55, SE = 0.13, p <0.001) and 8 h (MD = 0.85, SE = 0.07, p <0.001) following LPS treatment.

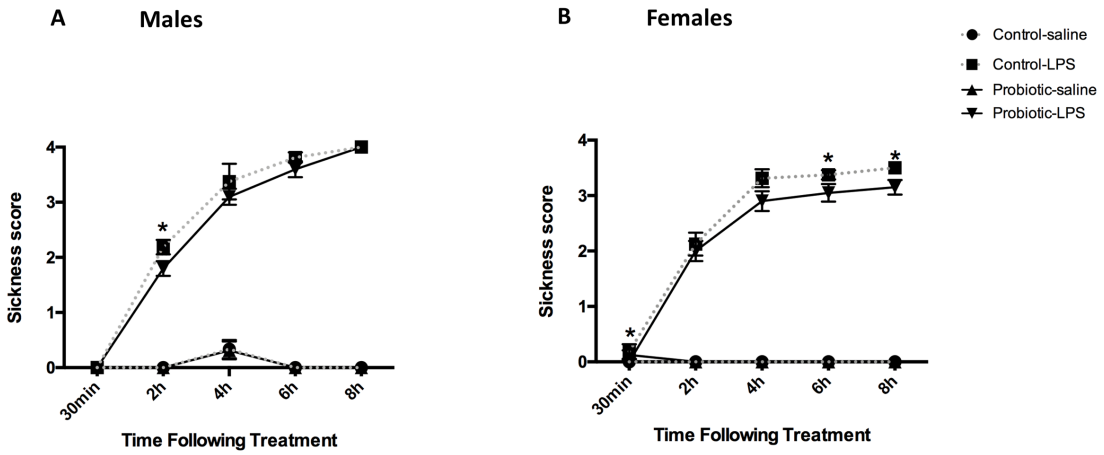

Figure 4.2. Sickness scores of six-week-old (A) male and (B) female mice treated with saline or LPS and exposed to either control or probiotic (*Rouxiella badensis* subsp. *acadiensis*) as a function of time. Data represented as mean (±SEM), of Control-saline (n = 9), Control-LPS (n = 8), Probiotic-saline (n = 8) and Probiotic-LPS (n = 10) (30 min – 8 h). The asterisks (*) denote significant difference between LPS treatments (control and probiotic) ($p < 0.05$).

***Rouxiella badensis* subsp. *acadensis* prevented peripheral LPS-induced increases in pro-inflammatory cytokines in females.**

LPS treatment increased plasma IL-6, TNFα, IL-1β, IFNγ, IL-10, and IL-12 concentrations in males and females. LPS-induced greater concentrations of the anti-inflammatory cytokines IL-10 and IL-4 and of the pro-inflammatory cytokines IFNγ and IL-1β in females than in males. However, *R. badensis* subsp. *acadiensis* mitigated LPS-induced increases in plasma of IL-1β and IL-12 concentrations in female mice. Three-way ANOVA revealed main effects of LPS on interleukin-6 (IL-6) ($F_{(1,65)} = 15.2$, $p < 0.001$, $\eta^2_p = 0.21$), interferon-γ (IFNγ) ($F_{(1,66)} = 39.09$, $p < 0.001$, $\eta^2_p = 0.40$), interleukin-12 (IL-12) ($F_{(1,67)} = 44.47$, $p < 0.001$, $\eta^2_p = 0.43$), interleukin-10 (IL-10) ($F_{(1,65)} = 59.84$, $p < 0.001$, $\eta^2_p = 0.51$), tumour necrosis factor-α (TNFα) ($F_{(1,64)} = 59.20$, $p < 0.001$, $\eta^2_p = 0.51$), interleukin-1β (IL-1β) (($F_{(1,64)} = 27.3$, $p < 0.001$, $\eta^2_p = 0.32$) concentrations (Figure 4.3). A significant sex x *R. badensis* subsp. *acadiensis* interaction was found on IL-12 ($F_{(1,67)} = 5.4$, $p < 0.05$, $\eta^2_p = 0.08$) and IL-1β ($F_{(1,64)} = 4.7$, $p < 0.05$, $\eta^2_p = 0.08$) concentrations. A sex x LPS x *R. badensis* subsp. *acadiensis* interaction was found on IL-12 ($F_{(1,67)} = 4.74$, $p < 0.05$, $\eta^2_p = 0.07$) and IL-1β ($F_{(1,64)} = 4.26$, $p < 0.05$, $\eta^2_p = 0.07$) concentrations. Regardless of sex and probiotic treatment, LPS-treated mice showed higher concentrations of IL-6, IFNγ, IL-12, IL-10, TNFα, IL-1β than their saline-treated counterparts (MD = 12063.93, SE = 3095.25, $p < 0.001$; MD = 10186.13, SE = 1629.19, $p < 0.001$; MD = 68.99, SE = 10.35, $p < 0.001$; MD = 232.12, SE = 30, $p < 0.001$; MD = 82, SE = 10.66, $p < 0.00$; MD = 88.21, SE = 16.89, $p < 0.001$, respectively). *R. badensis* subsp. *acadiensis* -LPS female mice displayed lower concentrations of IL-1β and IL-12 compared to control-LPS females (MD =104.58, SE =33.13, $p < 0.01$; MD =63.27, SE =20.14, $p < 0.01$, respectively).

Pairwise comparisons also showed that *R. badensis* subsp. *acadiensis* -LPS males have greater concentration of IL-12 compared to their female counterparts (MD =76.83, SE =18.99, $p < 0.001$). Control-LPS males displayed lower IL-1β concentrations compared to their female counterparts (MD =78.37, SE =37.40, $p < 0.05$) while *R. badensis* subsp. *acadiensis* -LPS males showed higher IL-1β concentrations compared to their female counterparts (MD =64.5, SE =30.06, $p < 0.05$).

For the milliplex ® MAP mouse Th17 magnetic bead panel, two-way ANOVA revealed a main effect of *R. badensis* subsp. *acadiensis* on IL-4 ($F_{(1,30)} = 4.92$, $p < 0.05$, $\eta^2_p = 0.15$) in LPS-treated mice (Figure 4.4). There was also a sex x *R. badensis* subsp. *acadiensis* interaction on IL-4 concentration ($F_{(1,30)} = 4.55$, $p < 0.05$, $\eta^2_p = 0.14$). Pairwise comparisons showed lower IL-4 concentration in *R. badensis* subsp. *acadiensis* -LPS female mice compared to control-LPS female mice (MD = -0.65, SE = 0.21). Moreover, control-LPS males had lower IL-4 concentration than their female counterparts (MD = 0.51, SE = 0.21, $p < 0.05$).

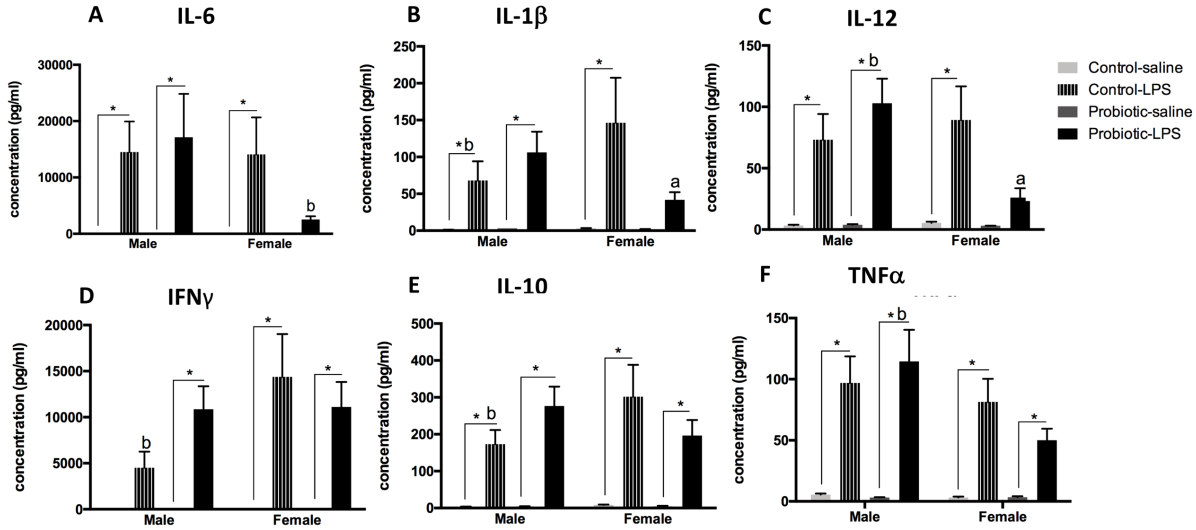

Figure 4.3. Pro-inflammatory and anti-inflammatory cytokine concentrations (pg/mL) from blood in male and female mice treated with saline or LPS and at 8 h post-injection and following 1 week of either control or probiotic (*Rouxiella badensis* subsp. *acadiensis*) treatment. Data represented as mean (±SEM) of Control-saline (n = 8/sex; 8 males, 8 females), Control-LPS (n = 8/sex), Probiotic-saline(n = 8/sex) and Probiotic-LPS (n = 10/sex); The asterisks (*) denotes $p < 0.05$ between saline and LPS treated groups, 'a' denotes a significant difference ($p < 0.05$) between control-LPS and Probiotic-LPS treatment groups, 'b' denotes a significant difference ($p < 0.05$) between males and females of the same treatment group.

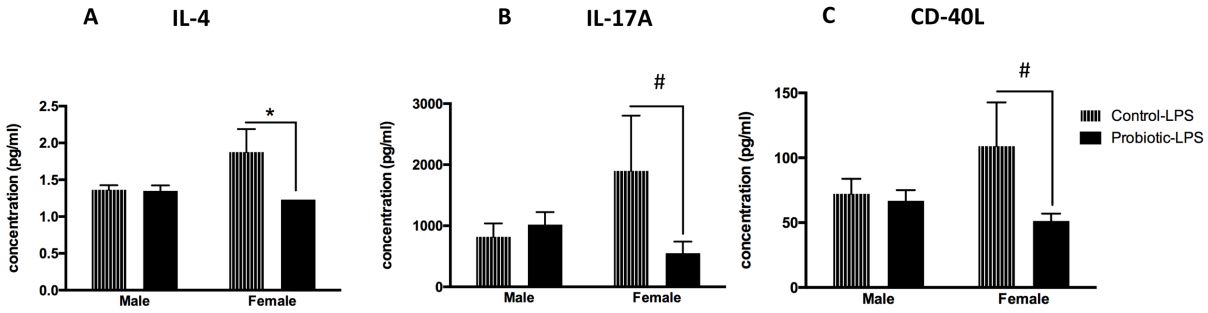

Figure 4.4. Selective cytokine concentrations (pg/mL) from blood using milliplex MAP mouse Th17 magnetic bead panel in male and female mice injected with saline or LPS and at 8 h post-injection and following 1 week of either control or probiotic (*Rouxiella badensis* subsp. *acadiensis*) treatment. Data represented as mean (±SEM) of Male Control-LPS (n = 8), Male Probiotic-LPS (n = 8), Female Control-LPS (n = 7) and Female Probiotic-LPS (n = 9); The asterisks (*) denotes $p < 0.05$, (#) denotes a trend.

***Rouxiella badensis* subsp. *acadensis* did not reduce LPS induced IL-1β mRNA expression in the brain.**

LPS treatment increases IL-1β expressions in the PFC, hypothalamus, and hippocampus in a sex-dependent manner. Three-way ANOVA revealed a main effect of LPS on the hippocampus and hypothalamus ($F_{(1,37)} = 44.27$, $p < 0.001$, $\eta^2_p = 0.6$; $F_{(1,36)} = 24.59$, $p < 0.001$, $\eta^2_p = 0.46$; respectively) and main effects of LPS and sex on IL-1β mRNA expression in the prefrontal cortex ($F_{(1,36)} = 13.92$, $p < 0.01$, $\eta^2_p = 0.33$; $F_{(1,36)} = 8.03$, $p < 0.01$, $\eta^2_p = 0.22$, respectively) (Figure 4.5). Regardless of sex and *R. badensis* subsp. *acadiensis* treatment, LPS-treated mice demonstrated greater IL-1β mRNA expression in the prefrontal cortex, the hippocampus and hypothalamus than saline-treated mice (MD = 1.66, SE = 0.44, $p < 0.001$; MD = 2.53, SE = 0.38, $p < 0.001$; MD = 2.38, SE = 0.48, $p < 0.001$; respectively). Also, males showed greater IL-1β mRNA expression than females in the prefrontal cortex (MD = 1.26, SE = 0.44, $p < 0.01$).

***Rouxiella badensis* subsp. *acadensis* reduced LPS-induced TNFα mRNA expression in specific brain regions in a sex-dependent manner.**

LPS treatment increases TNFα mRNA expressions in the PFC, hypothalamus, and hippocampus in a sex-dependent manner. However, *R. badensis* subsp. *acadiensis* mitigated LPS-induced increase in TNFα mRNA expression in the PFC of female mice. Three-way ANOVA revealed a main effect of LPS in the prefrontal cortex, hippocampus and hypothalamus ($F_{(1,36)} = 6.91$, $p < 0.05$, $\eta^2_p = 0.19$; $F_{(1,37)} = 7.24$, $p < 0.05$, $\eta^2_p = 0.19$; $F_{(1,37)} = 48.29$, $p < 0.001$, $\eta^2_p = 0.62$, respectively) (Figure 4.5). A significant LPS x *R. badensis* subsp. *acadiensis* interaction was found in the prefrontal cortex and in the hippocampus ($F_{(1,36)} = 4.45$, $p < 0.05$, $\eta^2_p = 0.13$; $F_{(1,37)} = 4.6$, $p < 0.05$, $\eta^2_p = 0.13$). There was also a significant sex x *R. badensis* subsp. *acadiensis*

interaction on TNFα mRNA expression in the hypothalamus ($F_{(1, 37)}$) = 4.57, $p < 0.05$, $\eta_p^2 = 0.13$) and a significant sex x LPS x *R. badensis* subsp. *acadiensis* ($F_{(1,37)} = 5.4$, $p < 0.05$, $\eta_p^2 = 0.15$) interaction on TNFα mRNA expression in the hippocampus. LPS-treated mice displayed higher TNFα mRNA expression in the prefrontal cortex, the hippocampus, and hypothalamus than saline-treated mice (MD = 2.15, SE = 0.81, $p < 0.05$; MD = 2.08, SE = 0.77, $p < 0.05$; MD = 2.79, SE = 0.4, $p < 0.01$, respectively). Regardless of sex, control-LPS mice showed greater TNFα mRNA expression in the prefrontal cortex and in the hippocampus than their saline counterparts (MD = 3.87, SE =1.2, $p < 0.01$; MD =3.74, SE =1.13, $p < 0.01$, respectively). Also, control-LPS mice showed greater TNFα mRNA expression than *R. badensis* subsp. *acadiensis* -LPS mice in the prefrontal cortex (MD = 2.6, SE = 1.17, $p < 0.05$). However, *R. badensis* subsp. *acadiensis* males expressed more TNFα mRNA in the hypothalamus than *R. badensis* subsp. *acadiensis* females (MD = 1.55, SE = 0.55, $p < 0.01$). Males and females treated with LPS showed more TNFα mRNA expression in the hippocampus than their saline injected counterparts (MD = 3.3, SE = 1.6, $p < 0.05$; MD = 4.18, SE = 1.5, $p < 0.05$). Also, *R. badensis* subsp. *acadiensis* -LPS males expressed greater TNFα mRNA expression in the hippocampus (MD = 3.58, SE = 1.5, $p < 0.05$) than their saline-treated counterparts. In contrast, *R. badensis* subsp. *acadiensis* -LPS females did not display higher expression of TNFα in the hippocampus when compared to their saline-treated counterparts. TNFα mRNA expression in the hippocampus of *R. badensis* subsp. *acadiensis* -LPS females was reduced compared to their control-LPS counterparts (MD =3.7, SE =1.5, $p < 0.05$).

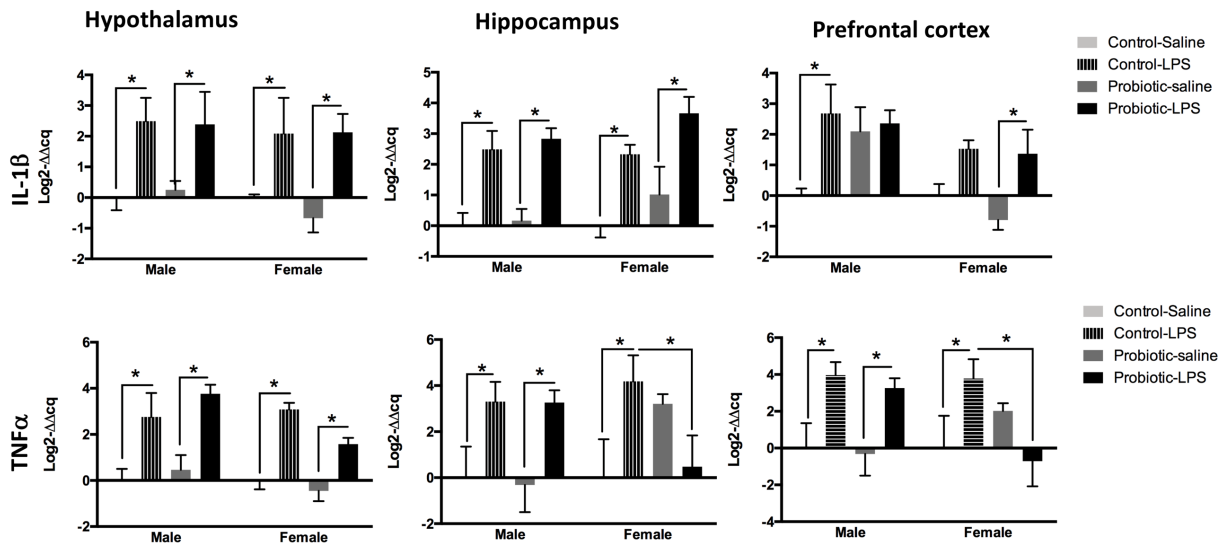

Figure 4.5. Pro-inflammatory cytokine IL-1β and TNFα mRNA expression in the hypothalamus, hippocampus and prefrontal cortex (PFC) in male and female mice treated with saline or LPS at 8 h post-injection and following one week of either probiotic (*Rouxiella badensis* subsp. *acadiensis*) or control treatment. Data represented as mean (±SEM); *p < 0.05, n = 5/group.

Assessment of the microbiota composition

The presence of *R. badensis* subsp. *acadiensis* within the 16S amplicon profiling results was assessed by conducting a blast search of the identified ASVs against the published *R. badensis* subsp. *acadiensis* rRNA sequences (Martin and Matar, 2005). Two ASVs with perfect matches to the two alleles present in the probiotic strain were identified (Supplemental Table S1). These two ASVs were essentially only present in the probiotic mouse groups (Figure 4.6), were present close to the expected allelic ratio (~8:1 vs 6:1) and were present at equal levels in both male and female mice. Notably, while saline injection did not affect *R. badensis* subsp. *acadiensis's* abundance, it was depleted after LPS injection.

Alpha diversity was determined through the calculation of the Shannon and Chao1 indices (Figure 4.7). There was no initial difference in alpha diversity between the saline/LPS groups prior to injection and administration of *R. badensis* subsp. *acadiensis* did not impact alpha diversity either. Although we expected that LPS injection could affect α-diversity, our results revealed no statistical difference in α-diversity in response to LPS injection. However, female mice did show a trend for reduced alpha diversity after LPS injection that did not reach significance.

When comparing all the mice prior to injection, principal coordinate analysis (PCoA) using the unweighted UniFrac distance did not reveal a significant clustering by *R. badensis* subsp. *acadiensis* administration, with the mice instead showing a tendency to cluster by sex along the first principal component (R^2=0.08, $p<0.01$, Figure 4.8A; Supplemental Table S2). To further probe whether these sex-specific differences could be obscuring *R. badensis* subsp. *acadiensis'* impact on the microbiome, we next analyzed the male and female mice separately. This analysis revealed that the male microbiomes were more impacted by *R. badensis* subsp.

acadiensis with a clear tendency to segregate along the second principal component ($R^2 = 0.05$, $p<0.05$, Figure 4.8B, Supplemental Table S2). In contrast, the female mice appeared to be less impacted by *R. badensis* subsp. *acadiensis* ($p=0.4$, Figure 4.8C, Supplemental Table S2). We were able to identify numerous taxa that were differentially abundant between male and female mice, but essentially only *R. badensis* subsp. *acadiensis* was identified as differentially abundant between the control and probiotic groups prior to injection (Supplemental Table S3). Interestingly, when the male and female mice were analyzed separately, *R. badensis* subsp. *acadiensis* was only identified as being differentially abundant (after FDR corrections) in the male mice, despite the obvious enrichment of the *R. badensis* subsp. *acadiensis* in both male and female mice (Figure 4.6). All-together, results suggest that the male microbiota compositions were more impacted as compared to the female mice and that *R. badensis* subsp. *acadiensis* may primarily impact rare taxa in male mice.

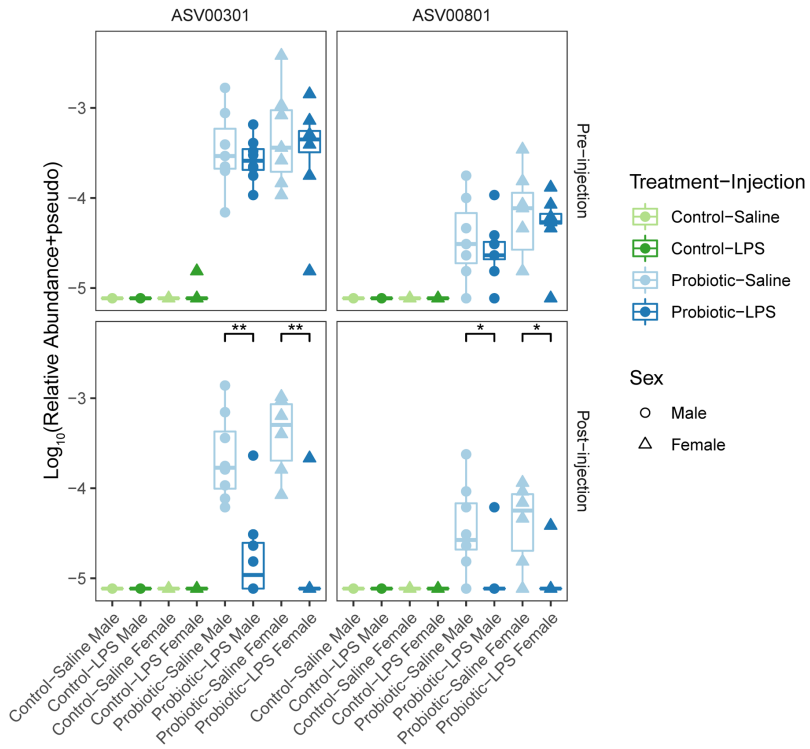

Figure 4.6. Log10 relative abundance of the two Amplicon Sequence Variants (ASVs) identified as perfectly matching *Rouxiella badensis* subsp. *acadiensis* V6-16S gene alleles (ASV00301 and ASV00801). To note, the ASV00301:ASV00801 abundance ratio is ~ 8:1 which closely matches the allelic frequency in *Rouxiella badensis* subsp. *acadiensis* (6:1). These ASvs are only present in the probiotic treated mice, there is no apparent difference between the *R. badensis* subsp. *acadiensis's* relative abundance in male vs female mice and there is a significant depletion of *R. badensis* subsp. *acadiensis* upon LPS and not saline injection. Where required, a pseudocount of 1 was added to allow for log scale plotting. *$p < 0.05$.

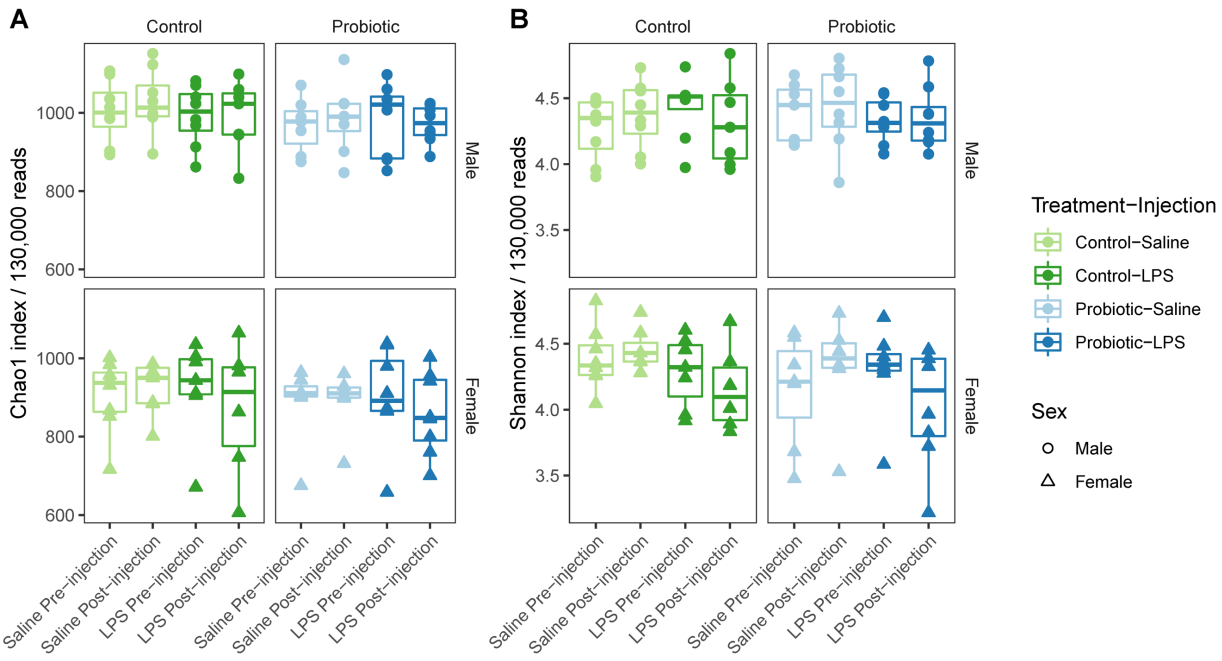

Figure 4.7. Chao1 index (A) and Shannon index (B) of fecal microbiotas of control (green) or probiotic (blue) mice treated at 6 weeks of age after one week of *Rouxiella badensis* subsp. *acadiensis* treatment (the day before saline/LPS injection) (Pre-injection) and at 8 h following the saline/LPS injection (Post-injection). Male (circle) and female (triangle) mice are plotted separately, with saline/LPS differentiated by light or dark colors respectively. The boxplots show 25th and 75th percentiles with a line at the median. No significant differences between the groups (same sex/treatment) pre/post injection were noted.

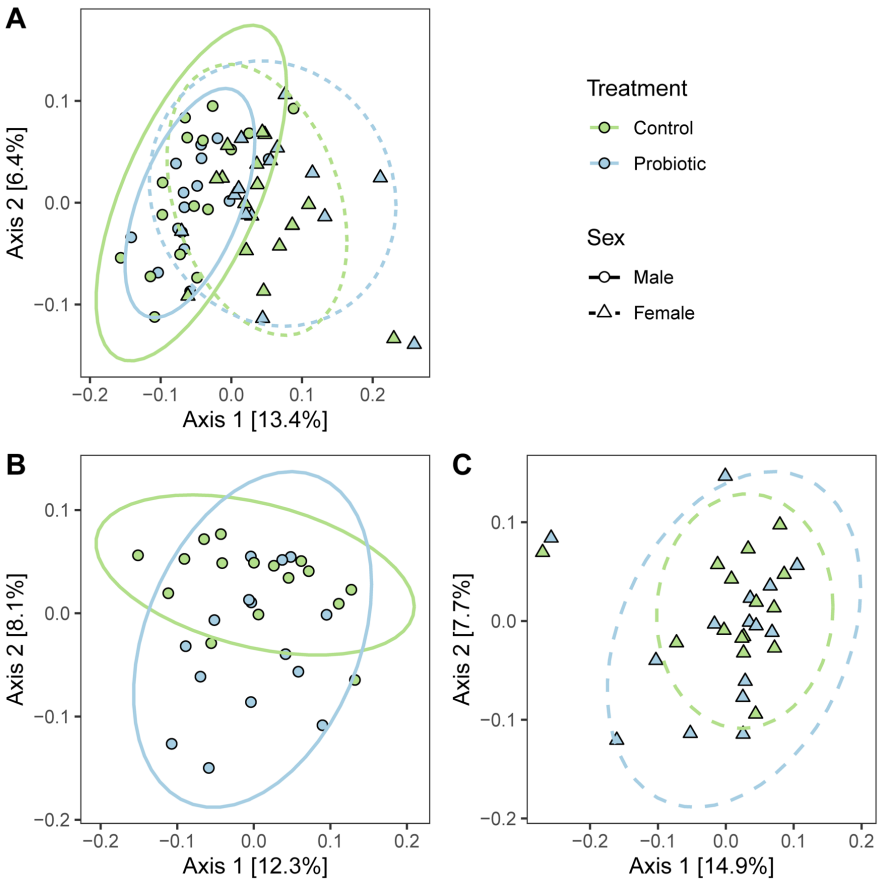

Figure 4.8. Principal coordinate analysis of pre-injected mice microbial β-diversity using the unweighted Unifrac distance of fecal microbiotas at 6 weeks of age after one week of *Rouxiella badensis* subsp. *acadiensis* treatment the day before saline/LPS injection. Panel A represents all mice while panels B and C display the male and female mice separately. Treatment groups (control = green, probiotic = blue) are colored and mouse sexes indicated by shape (male = circle, female = triangle). The ellipses show 95 % confidence intervals for male/female (solid/dashed lines) and control/probiotic (green/blue) groups (See Supplemental Table S2 for more details).

We next repeated this analysis with the mice after saline/LPS injection. When comparing all the mice together, there was a tendency for female LPS injected mice to segregate from the rest of the samples along the first component (R^2=0.06, p<0.01, Figure 4.9A, Supplemental Table S2). When analyzing the sexes separately, several interesting sex-dependent effects were noted. As seen in the pre-injected samples, the male mice continued to show separation principally by *R. badensis* subsp. *acadiensis* administration as compared to LPS injection (Figure 4.9B). This clustering was no longer significant (p=0.25), likely due to decreased sample numbers (Supplemental Table S2). In contrast, female mice showed more of an LPS-induced impact with most of the LPS-injected mice separating from their saline counterparts regardless of *R. badensis* subsp. *acadiensis* administration (R^2=0.07, p<0.01, Figure 4.9C, Supplemental Table S2). Female mouse microbiotas in the control group also appeared to be more impacted by LPS injection as compared female mice receiving *R. badensis* subsp. *acadiensis* (Figure 4.10). Interestingly, no taxa were identified as being differentially abundant between the saline and LPS treated male mice regardless of *R. badensis* subsp. *acadiensis* administration. However, the female mice showed numerous taxa differentially abundant upon LPS administration with an overall enrichment of *Escherichia/Shigella* genera with depletions in various Lachnospiraceae and Ruminococcaceae families (Supplemental Table S4).

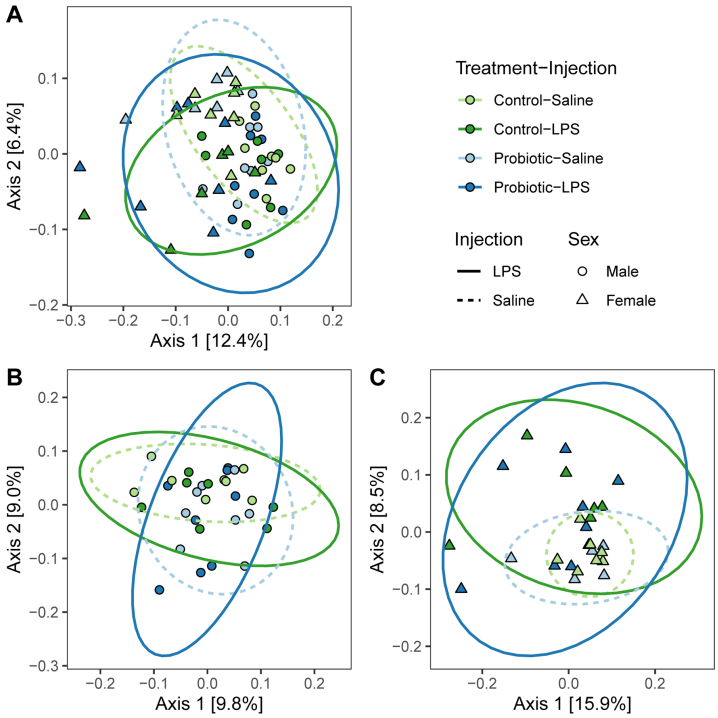

Figure 4.9. Principal coordinate analysis of post-injected mice microbial β-diversity using the unweighted Unifrac distance of fecal microbiotas at 6 weeks of age after one week of *Rouxiella badensis* subsp. *acadiensis* treatment 8 hrs after saline/LPS injection. Panel A represents all mice while panels B and C display the male and female mice separately. Treatment groups (control = green, probiotic = blue) are colored, saline/LPS injection indicated by shade (saline = light, LPS = dark), and mouse sexes indicated by shape (male = circle, female = triangle). The ellipses show 95 % confidence intervals for LPS/saline (solid/dashed lines) and control/probiotic (green/blue) groups. (See Supplemental Table S2 for more details).

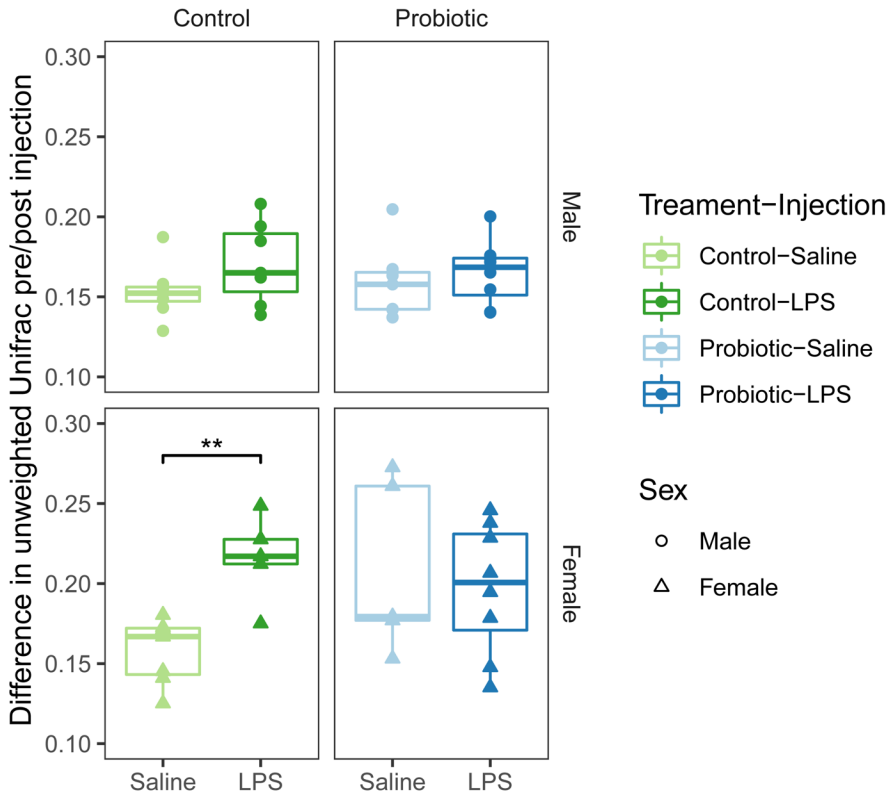

Figure 4.10. Difference in the unweighted Unifrac distance between matched mice pre/post injection. Treatment groups (control = green, probiotic = blue) are colored, saline/LPS indicated by shade (saline = light, LPS = dark) and mouse sexes indicated by shape (male = circle, female = triangle). The boxplots show 25th and 75th percentiles with a line at the median. **represents $p < 0.01$.

Discussion

Puberty is a critical period of development characterized by significant brain remodeling and reorganizing (Levitt, 2003; Sisk and Foster, 2004). Exposure to stress, including an immune challenge, during puberty can cause enduring changes in brain functioning (Kane and Ismail, 2017; Yahfoufi, Matar and Ismail, 2020) and behavior, and lead to adverse mental health outcomes later in life (Laroche *et al.*, 2009a; Ismail, Garas and Blaustein, 2011; Olesen *et al.*, 2011). Moreover, exposure to stress during puberty can cause inflammation which may lead to gut dysbiosis, altered brain functioning, and increased vulnerability to psychiatric illnesses later in life (Desbonnet *et al.*, 2015; Golubeva *et al.*, 2015). Recent findings from our laboratory revealed that *R. badensis* subsp. *acadiensis* can prevent LPS-induced depression-like behavior and alterations in 5HT1A receptor expression in females (Yahfoufi *et al.*, 2021). However, the mechanisms underlying these effects remained unclear. One possibility could be that *R. badensis* subsp. *acadiensis* consumption is mitigating the effects of LPS treatment on the immune system and/or the gut microbiome. Therefore, the current study was designed to gain mechanistic insight into the sex-dependent effects of *R. badensis* subsp. *acadiensis* consumption during puberty on LPS-induced inflammatory outcomes and changes to the gut microbiome.

We first hypothesized that *R. badensis* subsp. *acadiensis* may mitigate LPS-induced sickness behaviors in a sex-specific manner. As expected, LPS-induced more sickness behaviors in all mice compared to saline with LPS-inducing greater and more prolonged sickness behaviors in males compared to females as previously reported (Cai *et al.*, 2016; Murray *et al.*, 2019). Consumption of *R. badensis* subsp. *acadiensis* mitigated LPS-induced sickness behaviors in a sex-dependent manner, with greater reductions in sickness behaviors in females than in males. These results suggest a sex-dependent immune modulatory effect of *R. badensis* subsp.

acadiensis and are consistent with our previous work suggesting that *R. badensis* subsp. *acadiensis* is effective at mitigating LPS-induced sickness behavior in females but has limited effect in males (Yahfoufi *et al.*, 2021). LPS treatment is known to increase pro-inflammatory cytokine concentrations, including IL-6, TNFα, IL-1β, IFNγ and IL-12 in the blood. An increase in the concentration of these cytokines has been associated with increased sickness behaviors (Correa, S.G. Maccioni, M., Rivero, V.E., Iribarren P. , Sotomayor, C.E., 2007; Cai *et al.*, 2016).

As expected, our results also showed that LPS treatment increased plasma IL-6, TNFα, IL-1β, IFNγ, IL-10, and IL-12 concentrations. While these results are consistent with previous studies (Cai *et al.*, 2016; Sharma *et al.*, 2018; Murray *et al.*, 2019), we also found that LPS treatment induces greater concentrations of the anti-inflammatory cytokines IL-10 and IL-4 and of the pro-inflammatory cytokines IFNγ and IL-1β in females than in males. Anti-inflammatory cytokines are known to reduce the intensity and duration of sickness behaviour by either inhibiting the production or attenuating the signalling of pro-inflammatory cytokines and antagonizing their effects (Zhang, J., & An, 2007; Dantzer *et al.*, 2008). Thus, increased plasma concentrations of IL-10 and IL-4 in control-LPS females could explain the reduced sickness behaviors and faster recovery in females compared to males. The sex-dependent increase in anti- and pro-inflammatory cytokines is in line with previous findings (Cai *et al.*, 2016; Sharma *et al.*, 2018; Murray *et al.*, 2019; Murray, Smith, Stoby, Thomas, Swenson, Arber, Frenette, *et al.*, 2020) and could be caused by differences in sex hormones concentrations between males and females (Bhatia, Sekhon and Kaur, 2014). Testosterone is an immune suppressor (Wichmann *et al.*, 1997), while estradiol is an immune enhancer (Klein, 2000). Given that females have more estradiol, they tend to mount more robust cellular and humoral immune responses and recover

faster from infections than males (Bouman, Heineman and Faas, 2005; Klein and Flanagan, 2016). Our results also showed that *R. badensis* subsp. *acadiensis* mitigated LPS-induced increases in plasma of IL-1β and IL-12 concentrations in females, but not in males.

Our results also showed that LPS treatment increases IL-1β and TNFα mRNA expressions in the PFC, hypothalamus, and hippocampus in a sex-dependent manner. More specifically, we observed that LPS treatment induced greater IL-1β and TNFα mRNA expressions in the PFC, hypothalamus, and hippocampus in males than in females. These results are consistent with previous findings from our laboratory (Murray *et al.*, 2019). Interestingly, regardless of sex, *R. badensis* subsp. *acadiensis* mitigated LPS-induced increases in TNFα mRNA expression in the PFC. However, in the hippocampus, the mitigating effects of *R. badensis* subsp. *acadiensis* on TNFα mRNA expression were limited to female mice only. Our findings are consistent with those of previous studies showing that central inflammatory responses may differ from peripheral inflammatory responses (Sharma *et al.*, 2018; Murray *et al.*, 2019). In the brain, LPS treatment induces prostaglandins and cytokine release either from microglia and endothelial cells or from the circumventricular organ (Qin *et al.*, 2010; Lopes, 2016). LPS treatment can also induce cytokine production within the brain by activating neural afferent signaling (Watkins, Maierl and Goehlerlt, 1995). Gonadal hormones are known to modulate central immune response (Kentner and Pittman, 2010; Bhatia, Sekhon and Kaur, 2014). Testosterone increases cerebrovascular inflammation by augmenting the expression of cyclooxygenase-2 and inducible nitric oxide synthase (Razmara *et al.*, 2022), while 17β-estradiol suppresses IL-1β-mediated induction of COX-2 pathway in cerebral blood vessels (Ospina *et al.*, 2024) and prevents LPS-induced inflammatory response in microglia (Vegeto *et al.*, 2001).

Moreover, dihydrotestosterone increases the expression of inflammatory mediators via the NF-κB pathway (Death *et al.*, 2004), while 17β-estradiol mitigates LPS-induced cytokine production and NF-κB pathway activation (Deshpande *et al.*, 1997).

Previous studies have found that probiotic consumption can reduce LPS-induced production of pro-inflammatory cytokines and the associated sickness behaviors in mice (Mahony *et al.*, 2005; D'Mello *et al.*, 2015; Murray *et al.*, 2019). Moreover, probiotics can reduce cerebral LPS concentration; this effect has been associated with their ability to maintain the integrity of the intestinal barrier and to prevent the disruption of the blood-brain barrier (Yang *et al.*, 2020).

In this study, *R. badensis* subsp. *acadiensis* was more effective at reducing LPS-induced sickness in female than in male mice, which is consistent with other reports suggesting that certain probiotics have the ability to reduce inflammation in a sex-dependent manner (Lee *et al.*, 2017). Previous studies have also noted age and sex-dependant gut dysbiosis following LPS treatment (Murray, Smith, Stoby, Thomas, Swenson, Arber, Frenette, et al., 2020). There were obvious differences between the male and female mouse microbiotas in our study that were maintained regardless of *R. badensis* subsp. *acadiensis* treatment and/or LPS/saline injection (Figure 4.6). Male mice appeared to show greater shifts in their microbiota composition upon *R. badensis* subsp. *acadiensis* administration, despite the probiotic being present at similar levels in both sexes (Figure 4.6). In contrast, female mice microbiotas were more greatly impacted by LPS as compared to males (Figures 4.9 & 4.10) with enrichment of potential pathobionts such as *Escherichia/Shigella* (Supplemental Table S4).

At first glance, these results are counterintuitive as *R. badensis* subsp. *acadiensis* was more beneficial for female than male mice. However, protective microbes are often more sensitive to inflammatory stimuli as compared to potential pathobionts such as *E. coli* (Lopez., et al. 2022). Indeed, we noted a dramatic decrease of *R. badensis* subsp. *acadiensis* upon LPS injection (Figure 4.6). These findings suggest that female mouse microbiotas may be over-represented with commensal microbes that are better able to mitigate the impact of LPS injection as compared to male mouse microbiotas. Therefore, *R. badensis* subsp. *acadiensis* may therefore be able to promote more beneficial functional outputs from the resident microbes in female mice as compared to male mice following exposure to LPS. Changes in microbial functionality can not be captured using techniques such as 16S amplicon profiling and would require alternate approaches such as shotgun metagenomics, metatranscriptomics, metaproteomics, and metabolomics (Zhang et al., 2019). It is also possible that these sex differences are due to an interplay between the microbiotas of male and female mice, circulating steroid hormones, *R. badensis* subsp. *acadiensis* and/or LPS treatment (He et al., 2021).

Further studies delineating the microbial mechanisms and explaining the sex differences that we observed in this study would provide additional insight into the precise mechanisms used by *R. badensis* subsp. *acadiensis* and other probiotics to impart beneficial impacts to the host. In particular, there is growing interest in using probiotics to improve human health. Understanding how these products may differentially impact human male/females due to their potentially distinct microbiotas will be key for their true benefits to be realized.

Conclusion

This study investigated the sex-dependent effects of pubertal LPS treatment and *R. badensis* subsp. *acadiensis* consumption on the acute immune response and the gut microbial composition in male and female mice. Our results show that *R. badensis* subspecies *acadiensis* reduces LPS-induced peripheral and central inflammation without overtly impacting overall microbial composition in female, but not male mice 8h post challenge. This research highlights the critical role of gut microbial alterations during adolescence on the immunomodulatory and neurochemical outcomes. It also provides further evidence that probiotics can have sex-specific effects on the immune system and on the gut microbiota, and it also provides new insights into the potential for probiotic use during puberty as a preventive measure to improve resiliency against infections.

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Declaration of Interests

AS is a co-founder of MedBiome, a clinical microbiomics company.

Rouxiella badensis subsp. *acadiensis* has been filed in a U.S. Provisional Application No. 62/916,921 titled “Probiotics Composition and Methods” for its potential probiotic effects (Matar et al. 2020).

Supplemental Table S1: BLASTn results for top ASVs matching *Rouxiella badensis* strain C173 chromosome

Query	Subject	Percent ID	Alignment Length	Mismatches	Gaps	Alignment Start Query	Alignment End Query	Alignment Start Subject	Alignment End Subject	Expect Value	Bitscore	Query Sequence
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	1393511	1393385	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	1763557	1763683	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	1898675	1898801	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	2025301	2025427	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	2064102	2064228	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	100	127	0	0	1	127	2587189	2587315	2.41E-61	230	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
ASV00301	NZ_CP060592.1	99.213	127	1	0	1	127	810103	809977	1.03E-59	225	GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCG

												GGAACTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	100	127	0	0	1	127	810103	809977	2.41E-61	230	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	1393511	1393385	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	1763557	1763683	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	1898675	1898801	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	2025301	2025427	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	2064102	2064228	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG
ASV00801	NZ_CP060592.1	99.213	127	1	0	1	127	2587189	2587315	1.03E-59	225	GGGCCCGCACAAGCG GTGGAGCATGTGGTT TAATTCGATGCAACG CGAAGAACCTTACCT ACTCTTGACATCCAG AGAATTTGCTAGAGA TAGCTTAGTGCCTTCG GGAGCTCTGAGACAG GTGCTG

Supplemental Table S2: Results from PERMANOVA testing of various mouse groups

Comparison	Figure Reference	Model Term	Values	Degrees of Freedom	Sums Of Squares	Mean Squares
Pre-injection; all Mice	Figure 8A	Sex	Male/Female	1	0.25	0.25
Pre-injection; all Mice	Figure 8A	Probiotic.adm	Control/Probiotic	1	0.06	0.06
Pre-injection; all Mice	Figure 8A	Sex:Probiotic.adm	Interaction term	1	0.06	0.06
Pre-injection; all Mice	Figure 8A	Residuals	NA	57	2.87	0.05
Pre-injection; only Male Mice	Figure 8B	Probiotic.adm	Control/Probiotic	1	0.07	0.07
Pre-injection; only Male Mice	Figure 8B	Residuals	NA	29	1.30	0.04
Pre-injection; only Female Mice	Figure 8C	Probiotic.adm	Control/Probiotic	1	0.06	0.06
Pre-injection; only Female Mice	Figure 8C	Residuals	NA	28	1.57	0.06
Post-injection; all Mice	Figure 9A	Sex	Male/Female	1	0.21	0.21
Post-injection; all Mice	Figure 9A	Probiotic.adm	Control/Probiotic	1	0.05	0.05
Post-injection; all Mice	Figure 9A	Injection	Saline/LPS	1	0.10	0.10
Post-injection; all Mice	Figure 9A	Sex:Probiotic.adm	Interaction term	1	0.06	0.06
Post-injection; all Mice	Figure 9A	Sex:Injection	Interaction term	1	0.06	0.06
Post-injection; all Mice	Figure 9A	Probiotic.adm:Injection	Interaction term	1	0.05	0.05
Post-injection; all Mice	Figure 9A	Sex:Probiotic.adm:Injection	Interaction term	1	0.04	0.04
Post-injection; all Mice	Figure 9A	Residuals	NA	50	2.62	0.05
Post-injection; only Male Mice	Figure 9B	Probiotic.adm	Interaction term	1	0.05	0.05
Post-injection; only Male Mice	Figure 9B	Injection	Saline/LPS	1	0.05	0.05
Post-injection; only Male Mice	Figure 9B	Probiotic.adm:Injection	Interaction term	1	0.04	0.04
Post-injection; only Male Mice	Figure 9B	Residuals	NA	27	1.23	0.05
Post-injection; only Female Mice	Figure 9C	Probiotic.adm	Interaction term	1	0.06	0.06
Post-injection; only Female Mice	Figure 9C	Injection	Saline/LPS	1	0.11	0.11
Post-injection; only Female Mice	Figure 9C	Probiotic.adm:Injection	Interaction term	1	0.05	0.05
Post-injection; only Female Mice	Figure 9C	Residuals	NA	23	1.39	0.06

Supplemental Table S3: MaAsLin2 analysis of pre-injected mice

Analysis	Group	Taxa Taxonomy	Value	coef	stderr	N	N.not.0
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-013_g	Male	0.000662	0.000124	61	59
All mice; preinjection	Probiotic.adm	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o	Water	-0.00017	3.39E-05	61	60
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Clostridiales_vadinBB60_group_f	Male	-0.00296	0.000615	61	61
All mice; preinjection	Sex	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Betaproteobacteriales_o.Burkholderiaceae_f.Mitsuaria_g	Male	2.83E-06	5.79E-07	61	18
All mice; preinjection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o.Coriobacteriales_Incertae_Sedis_f	Male	6.08E-05	1.35E-05	61	52
All mice; preinjection	Probiotic.adm	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Yersiniaceae_f.Rouxiella_g	Water	-0.00014	3.03E-05	61	32
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_g	Male	-0.0031	0.000702	61	61
All mice; preinjection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o	Male	0.000148	3.53E-05	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcus_2_g	Male	0.000175	4.16E-05	61	55
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-014_g	Male	0.002942	0.000686	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Oscillibacter_g	Male	-0.00029	6.91E-05	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Christensenellaceae_f.Christensenellaceae_R-7_group_g	Male	3.42E-05	8.25E-06	61	55
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcus_2_g.Undefined_s	Male	0.000171	4.15E-05	61	53
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Family_XIII_f.Family_XIII_AD3011_group_g	Male	2.31E-05	5.82E-06	61	59
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Peptococcaceae_f.Peptococcus_g	Male	-2.3E-05	5.93E-06	61	42
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-010_g	Male	8.08E-05	2.16E-05	61	60
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcus_1_g	Male	0.000197	5.57E-05	61	55
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.ASF356_g	Male	-0.00013	3.98E-05	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-002_g.bacterium_s	Male	-5.4E-05	1.64E-05	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-002_g	Male	-6E-05	1.87E-05	61	61
All mice; preinjection	Sex	Bacteria_k.Bacteroidetes_p.Bacteroidia_c.Bacteroidales_o.Undefined_f	Male	2.72E-05	8.96E-06	61	58
All mice; preinjection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o.Undefined_f	Male	1.65E-06	5.36E-07	61	28
All mice; preinjection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o.Eggerthellaceae_f	Male	8.59E-05	2.8E-05	61	61

All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Erysipelotrichia_c.Erysipelotrichales_o.Erysipelotrichaceae_f.Erysipelatoclostridium_g	Male	0.000467	0.000154	61	61
All mice; preinjection	Sex	Bacteria_k.Deferribacteres_p.Deferribacteres_c.Deferribacterales_o.Deferribacteraceae_f.Mucispirillum_g	Male	-0.00586	0.001928	61	61
All mice; preinjection	Sex	Bacteria_k.Bacteroidetes_p.Bacteroidia_c.Bacteroidales_o.Bacteroidaceae_f.Bacteroides_g.ovatus_s	Male	5.76E-05	1.9E-05	61	51
All mice; preinjection	Sex	Bacteria_k.Deferribacteres_p.Deferribacteres_c.Deferribacterales_o.Deferribacteraceae_f.Mucispirillum_g.schaedleri_s	Male	-0.00583	0.001915	61	61
All mice; preinjection	Sex	Bacteria_k.Firmicutes_p.Erysipelotrichia_c.Erysipelotrichales_o.Erysipelotrichaceae_f	Male	0.000611	0.000204	61	61
Only Male mice; preinjection	Probiotic.adm	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o	Water	-0.00013	2.55E-05	31	31
Only Male mice; preinjection	Probiotic.adm	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Yersiniaceae_f.Rouxiella_g	Water	-0.0001	2.42E-05	31	16

Note: No taxa were significant in the Only Female mice; preinjection analysis

Supplemental Table S4: MaAsLin2 analysis of post-injected mice

Analysis	Group	Taxa Taxonomy	Value	coef	stderr	N	N
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p	Saline	0.02373	0.005703	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c	Saline	0.022444	0.00556	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o	Saline	0.022405	0.005554	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Intestinimonas_g	Saline	0.000526	0.000122	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_6_g	Saline	0.001744	0.000432	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.ASF356_g	Saline	-0.00031	7.16E-05	58	
All mice; post-injection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_UCG-013_g	Male	0.000405	9.23E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Negativibacillus_g	Saline	0.000128	3.12E-05	58	
All mice; post-injection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Family_XIII_f.Family_XIII_AD3011_group_g	Male	2.54E-05	6.46E-06	58	
All mice; post-injection	Probiotic.adm	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Yersiniaceae_f.Rouxiella_g	Water	-6E-05	1.55E-05	58	
All mice; post-injection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o	Male	6.87E-05	1.77E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f	Saline	0.006492	0.0017	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.Acetatifactor_g.Undefined_s	Saline	7.37E-05	1.95E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_g	Saline	0.002498	0.000716	58	
All mice; post-injection	Sex	Bacteria_k.Actinobacteria_p.Coriobacteriia_c.Coriobacteriales_o.Eggerthellaceae_f	Male	4.71E-05	1.38E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcaceae_NK4A214_group_g	Saline	6.16E-06	1.78E-06	58	
All mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Yersiniaceae_f.Rouxiella_g	Saline	5.29E-05	1.55E-05	58	
All mice; post-injection	Injection	Bacteria_k.Bacteroidetes_p.Bacteroidia_c	Saline	-0.02548	0.007648	58	
All mice; post-injection	Injection	Bacteria_k.Bacteroidetes_p.Bacteroidia_c.Bacteroidales_o	Saline	-0.02548	0.007648	58	
All mice; post-injection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminococcus_1_g	Male	0.000295	8.86E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.Undefined_g	Saline	0.012533	0.003793	58	
All mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f	Saline	-0.00189	0.000589	58	
All mice; post-injection	Sex	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_g	Male	-0.00231	0.000716	58	
All mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f.Escherichia/Shigella_g	Saline	-0.00189	0.000587	58	
All mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f.Escherichia/Shigella_g.Undefined_s	Saline	-0.00188	0.000586	58	
All mice; post-injection	Injection	Bacteria_k.Bacteroidetes_p.Bacteroidia_c.Bacteroidales_o.Bacteroidaceae_f.Bacteroides_g	Saline	-0.01927	0.006029	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Undefined_f	Saline	0.006978	0.002205	58	
All mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o	Saline	-0.00184	0.00059	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.Acetatifactor_g	Saline	0.000219	6.98E-05	58	
All mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Anaerotruncus_g.colihominis_s	Saline	4.03E-06	1.29E-06	58	

Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p	Saline	-0.00571	0.001129	27	
Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c	Saline	-0.00476	0.001022	27	
Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f	Saline	-0.00431	0.00103	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.ASF356_g	Saline	-0.00044	0.0001	27	
Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f.Escherichia/Shigella_g	Saline	-0.0043	0.001026	27	
Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o.Enterobacteriaceae_f.Escherichia/Shigella_g.Undefined_s	Saline	-0.00429	0.001023	27	
Only Female mice; post-injection	Injection	Bacteria_k.Proteobacteria_p.Gammaproteobacteria_c.Enterobacteriales_o	Saline	-0.00425	0.001034	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f	Saline	0.010649	0.002629	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p	Saline	0.0376	0.009553	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c	Saline	0.035623	0.009147	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o	Saline	0.035544	0.009138	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Lachnospiraceae_f.Acetatifactor_g	Saline	0.000355	9.45E-05	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_g	Saline	0.004824	0.001298	27	
Only Female mice; post-injection	Injection	Bacteria_k.Firmicutes_p.Clostridia_c.Clostridiales_o.Ruminococcaceae_f.Ruminiclostridium_6_g	Saline	0.00193	0.000545	27	

Note: No taxa were significant in the Only Male mice; post-injection analysis

Chapter 5: General Discussion

General Discussion

The gut microbiome is considered central in the maintenance of dynamic host–microbiota interactions (Collins and Bercik 2013). In the last decades, the gut microbiota has emerged as an important regulator of the gut-brain axis (Carabotti et al. 2015; Bailey and Cryan 2017). Gut microbiota plays a key role in maintaining intestinal homeostasis via an intricate crosstalk between the immune, endocrine, as well as the central and enteric nervous systems (Carabotti et al. 2015; Bravo et al. 2012). Also, the gut-brain axis is gaining more importance in research studies investigating the biological and physiological basis of neurodegenerative, psychiatric, behavioral, neurodevelopmental and cognitive disorders(Montiel-Castro et al. 2013; O'Mahony et al. 2009; Borre et al. 2014).

Microbiota disturbances or dysbiosis is sufficient to drive immune and neuroendocrine alterations as well as perturbation of developmental programing at distant sites from the gut (Hemarajata and Versalovic 2013; Rinninella et al. 2019). Therefore, gut dysbiosis has been linked to many diseases while probiotics have been found to be effective dietary agents to treat and prevent gut dysbiosis (Hemarajata and Versalovic 2013). Manipulating gut microbiome with probiotics created new avenues for intensive research in identification and studying of novel probiotic microbes. The knowledge of the composition and function of the human gut microbiome with the development of genome and metagenome sequencing and tools to edit bacterial genome have extended the diversity of organisms known as Next-Generation Probiotics (NGPs) that constitute a range of live microorganisms with potential health benefits (Martín and Langella 2019). Undoubtedly, genome sequencing and analysis of potential probiotic candidates have become compulsory in the last years, to determine information on their safety features and functional properties (Reid et al. 2019). *Rouxiella badensis* subsp. *Acadiensis*, a bacterium

isolated from the microbiota of the wild blueberry fruit, has been found to enhance the anti-inflammatory properties of polyphenol-enriched blueberry preparation that has been found to be beneficial for numerous diseases, like neurodegeneration, diabetes, and cancer (Nachar et al. 2017; Vuong et al. 2016, 2007; Sánchez-villavicencio et al. 2017). The whole-genome shotgun sequencing of *Rouxiella badensis* subsp. *Acadiensis* revealed no pathogenicity genes (PCT Patent Application No. PCT/CA2020/05138). The bacterium was previously called *Serratia vaccinii* (Martin and Matar 2005) and was isolated from the surface of lowbush blueberries (*Vaccinium angustifolium Aiton*) by inoculation of a Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA)(Martin and Matar 2005). It is a gram negative, catalase positive, facultative anaerobic, coccobacillus, and has fermentative ability for D-glucose, D-fructose, D-mannose, arbutin, esculin, salicin, saccharose and D-raffinose. Under specific conditions, the bacterium can also ferment mannitol, lactose, and trehalose (Martin and Matar 2005; Martin 2003). The bacterium has been deposited under Accession Number 160 103, at the International Depositary Authority of Canada. They referred to it as *Serratia* based on its 16S rRNA gene sequence, biochemical profile, and physical properties (Martin and Matar 2005). As the 16S rRNA gene sequence analysis was shown to be insufficiently robust to distinguish closely related species and new advanced techniques have emerged, a whole-genome shotgun sequencing and assembly of bacterial strain were performed to determine the phylogenetic position of the isolate. These analyses led to the description of a novel *R. badensis* subspecies following which the name *R. badensis* subsp. *Acadiensis* was proposed to also highlight the region where it was firstly isolated. The strain type is *Canan SV-53.* A U.S. Provisional Application No. 62/916,921 entitled “Probiotics Composition and Methods” has been filed related to the bacterium. Unpublished study revealed that heat killed bacterium was unable to induce significant intestinal

immunomoduation in IgG+ cells and IgA+ cells at the lamina propria of the ileum neither in the levels of cytokines (IL-6 and IL-10) in the intestinal fluid. The safety of the alive bacterium was evaluated in this study by *in vitro* and *in vivo* analysis. The immunomodulatory effects as well as the impact on intestinal homeostasis and mucosal immunity was also examined. The gut microbiota plays a key role in the gut-brain interactions (Borre et al. 2014). Recently, studies have focused on the promising role and potential of probiotics in various mental disorders by restoring normal microbial balance, and improving mental health through the gut-brain axis (Bravo et al. 2012; Murray *et al.*, 2020; Bailey and Cryan 2017). Using probiotics to modulate the gut microbiome composition can enhance the integrity of the gastro-intestinal barrier and mitigate inflammation (Kelly et al. 2015).

Although several studies have reported the ability of probiotics to relieve stress response and to contribute to anti-depressive and anti-anxiolytic mechanisms (Park et al. 2018; Murray, et al. 2020; Murray et al. 2019), there is limiting knowledge about its effects on the developing brain and on the immune responses during puberty. CD 1 mice were used in LPS, microbiome, depression and anxiety studies. The effect of LPS on anxiety and depression was validated in CD 1 mice (Murray et al. 2019; Murray et al. 2020). Also, CD 1 mice are outbred mouse populations that provide a powerful experimental platform and an ecologically valid model for appropriately accounting for the background genetic variation.

Thus, the aim of this thesis was to investigate the potential probiotic effects of the bacterium *R. badensis* subsp. *acadiensis,* to study its impact on stress-induced depression, immune response, and the brain during puberty, and to validate its immunomodulatory, anti-depressive and anxiolytic effect through the gut-brain axis.

R. badensis subsp. *acadiensis* survived gastro-intestinal conditions and maintained the intestinal barrier integrity

The first study aimed to investigate the safety and resistance of the bacterium in conditions mimicking low pH and bile acid present in the stomach and small intestine. The ability to persist in the intestine and adhere to the gut epithelial cells and the interaction with the gut associated immune cells were also investigated. The results showed that there is no disruption of the intestinal barrier and no changes in the gut architecture. In the same line of investigation, immunohistochemical analyses showed that the bacterium administered orally to mice did not affect the structure or the morphology of epithelial cell or mucosal linings. Moreover, no translocation was observed to organs such as the liver or the spleen. In fact, bacterial translocation or passage of viable bacteria from the gastrointestinal tract to other sites such as the mesenteric lymph nodes, spleen and liver is an indicator of potential infectivity (Berg 1995). In order to better decipher the probiotic potential of the bacterium, antibiotic resistance studies were also carried out. Antibiotic resistance is a concern when novel bacterial strains are isolated as they may pass it to pathogenic bacteria through horizontal gene transfer which can present a challenge to their safe usage (Imperial and Ibana 2016). *R. badensis* subsp. *acadiensis* showed sensitivity to the various examined antibiotics.

Probiotic properties entail bacteria survival and adhesion to intestinal tract. The ileum and the large intestine are the sites of action of most probiotic bacteria; thus to be effective, probiotic bacteria should survive the passage in the upper intestinal tract (Goldin et al. 1992; Argyri et al. 2013). The stomach acidic environment and the presence of bile in the duodenum are major factors reducing the viability of probiotic bacteria (Goldin et al. 1992; Argyri et al. 2013). The studies in our lab demonstrated the ability of the bacterium to survive in the intestine in *in vitro*

conditions. However, the bacterium was more vulnerable when exposed to low pH simulated gastric juice and subsequent intestinal. Therefore, it is important to continuously ingest the exogenous to maintain their protective effects, as it was reported by probiotic clinical pharmacokinetic studies (Bezkorovainy 2001). It is believed that the optimal probiotic effect is attained if the microorganisms adhere to intestinal mucosal cells which may provide a mean by which the probiotic can modulate the immune system (Helgeland and Brandtzaeg 2000) and allow competitive exclusion of other microorganisms (Alderberth et al. 2000; Fons et al. 2000). Although, *R. badensis* subsp. *acadiensis* demonstrated *in vitro* adhesion capability when tested on Caco-2 and HT-29 cells, it should be taking into consideration that models assessing the adhesion of probiotics *in vitro* have many limitations. Noteworthy, the correlation between *in vitro* and *in vivo* adhesion to intestinal epithelium was identified with several strains of microorganisms (Zárate et al. 2002).

***R. badensis* subsp. *acadiensis* contributes to mucosal tolerance and may have anti-inflammatory properties**

In this work, we also demonstrated that the oral administration of *R. badensis* subsp. *acadiensis* plays multiple protective roles related to non-specific innate immune mechanisms by inducing an increase in the number of IgA+ cells in the ileum lamina propria associated with an increase in the secretory IgA content in the intestinal lumen without modification of the number of IgG+ cells. Secretory Ig A is known to entrap dietary antigens and microorganisms in the mucin and contribute to the maintenance of convenient bacterial communities within the intestine. These immunoglobulin protective functions are related to non-specific innate immune mechanisms (Vinderola et al. 2006; Cerutti and Rescigno 2008). Furthermore, *R. badensis* subsp.

acadiensis is thought to contribute to mucosal tolerance and may have anti-inflammatory properties (Lee and Mazmanian 2010) as the increase in the number of the regulatory IL-10+ cells at day 7 after the bacterial use can be involved in the control of the intestinal homeostasis after the administration of a dietary antigen. This was supported by the increase of the anti-inflammatory cytokine interleukin 10 (IL-10) in the intestinal fluid. Il-10is implicated in reduced mucosal immune responses, immunopathology and inflammation in the gut associated lymphoid tissue (Roers et al. 2004; Rubtsov et al. 2008; Glocker et al. 2009; Moran et al. 2013; Lee and Mazmanian 2010).

***R. badensis* subsp. *acadiensis* modulates the expression of miRNAs in the ileum and the brain**

Small non-coding RNAs regulate gene expression post-transcriptionally (Zhou, Zhou, and Chen 2017). MicroRNAs are emerging as a key player in host-gut microbiome interactions and symbiosis (Zhou, Zhou, and Chen 2017) as they facilitate the relationship between the microbiome and the gut-brain axis (Moloney et al. 2019). In fact, the gut microbiota influences the host microRNAs expression which can shape on their turn the gut microbiota structure (Zhou, Zhou, and Chen 2017; Moloney et al. 2019). They have been shown to regulate various biological processes involved in inflammation, immune homeostasis and intestinal integrity (Gaudet et al. 2017) and are now subject to intense investigations. Certain miRNAs could initiate an anti-inflammatory response such as miR145 and miR146a, a proinflammatory response such as miR155 or mixed immunomodulatory effects (Bermudez-Humaran and Langella 2017). The expression of IL-10 is stimulated by miR146a overexpression which results in reduction of inflammatory response (Dharap et al. 2009; H. J. Liu et al. 2016) .

Therefore, the overexpression of miR146a in the ileum and in the brain may further explain the anti-inflammatory properties of the bacterium. Our data revealed a slight increase in miR155 to the opposite to the robust increase observed after LPS injections (data not shown) which can be associated with basal physiological immune response. miRNAs have been found able to regulate various biological processes related to inflammation, immune homeostasis and intestinal integrity (Gaudet et al. 2017). Certain miRNAs could initiate a pro-inflammatory response or anti-inflammatory response (Li et al. 2016). Probiotics as well as the gut microbiota can influence the host miRNAs expression (Fonken et al. 2016b). During neuro-inflammation, pro-inflammatory microglia demonstrate increased miR155 and decreased miR146a levels that promote the expression of proinflammatory molecules. After induction of pro-inflammatory mediators, miRNA expression shifts to downregulation of miR155 and upregulation of miR146a which stimulates the expression of anti-inflammatory molecules (IL-10, TGF-β) leading to a reduction of the inflammation and neuronal tissue repair (Dharap et al. 2009; Liu et al. 2016). Therefore, we decided to investigate the effect of the bacterium on brain neuroinflammation.

R. badensis subsp. *acadiensis* reduced LPS-induced sickness behaviour in a sex and time-specific manner

Neuroendocrine alterations and neuroinflammation are particularly damaging, if kept untreated, during rapid development period and reorganizing the brain in the adolescence. Heightened vulnerability of adolescent brain to stressors and immune challenges related to the significant neuronal reorganization and remodeling of neuronal circuits during this period may lead to enduring changes in behaviors and adverse mental health outcomes observed later in life (Burnett et al. 2011; Spear 2013). Given the consequences of pubertal stress and inflammation

on mental illnesses, interventions with probiotics during such a critical period of development may contribute to the prevention of brain diseases in adulthood (Yahfoufi, Matar, and Ismail 2020; Cai et al. 2016; Kane and Ismail 2017). Also, recent findings from our laboratory showed that modulation of the gut microbiota using probiotics from kefir during adolescence can mitigate LPS-induced depression-like behaviour in females and anxiety-like behaviours in males while pubertal consumption of the probiotic *Lactobacillus reuteri* mitigates LPS-induced enduring effects on memory dysfunction, anxiety-like behaviour and stress reactivity in adulthood (Murray et al. 2020; Murray et al. 2019). Thus, we decided to investigate the sex-specific effects of pubertal *R. badensis* subsp. *acadiensis* consumption on LPS induced enduring anxiety- and depression- like behaviors and the associated changes in the serotonergic system. We also investigated the sex-specific effect of *R. badensis* subsp. *acadiensis* on the inflammatory markers in the blood and brain and changes in the gut microbiota. We hypothesized that *R. badensis* subsp. *acadiensis* would reduce sickness behavior caused by LPS treatment. All mice treated with LPS showed more sickness behaviors than saline controls with greater and more prolonged symptoms in males when compared to females. These results are consistent with previous work from our laboratory (Cai et al. 2016; Murray et al. 2019). *R. badensis* subsp. *acadiensis* also decreased LPS-induced sickness behavior in female mice while males displayed limited improvement in sickness scores that started 6h after the injection in mice provided the bacterium by gavage and was observed only at 2h post-injection when the bacterium was provided in water bottle. These results support our hypotheses and are in line with previous studies showing reduced sickness behavior in mice following probiotic consumption, with the exception of males displaying limited improvement in sickness scores (Cai et al. 2016; Murray et al. 2019). Greater sickness behaviors were observed in probiotic-LPS treated males, compared to

their female counterparts, at 2h, 4h and 24h post-LPS treatment in the chronic study and at 6h and 8h in the acute study. These results suggest a sex-dependent immune response to the bacterium and a stronger pro-inflammatory cytokines production in males. Probiotic-LPS treated males had greater plasma cytokines concentration of IL-6, IL-12 and TNFα compared to females of the same treatment group. The bacterium failed to mitigate LPS-induced weight loss at 24h and at 48h; but it significantly reduced LPS-induced weight loss 1 week following treatment in both sexes.

***R. badensis* subsp. *acadiensis* treatment during puberty prevents LPS-induced depression-like behaviour in female mice during adulthood by inducing changes in 5HT1A receptors expression in in the hippocampus and the dorsal raphé nuclei**

We also hypothesized that LPS injection at 6 weeks old induces depression-like behaviors in adult female CD1 mice and anxiety-like behaviors in adult CD1 males (Murray and Frenette 2020; Murray et al. 2019) that can be prevented by pubertal use of *R. badensis* subsp. *acadiensis*. As expected for depression-like behaviors, our findings showed that depression-like behaviors were observed in adult female CD1 mice after pubertal LPS injection. *R. badensis* subsp. *acadiensis* treatment during puberty eliminated differences in immobility duration in FST and TST tests between LPS- and saline- injected female mice. The LPS-induced depression-like behavior in females was accompanied by an increase in the expression of 5HT1A receptors in the DRN and a reduction of 5HT1A receptors' expression in the CA1 region of the hippocampus. We found that *R. badensis* subsp. *acadiensis* (Canan SV-53) mitigated the LPS-induced decrease in 5HT1A expression in CA1 region of the hippocampus as well as the LPS-induced increase in 5HT1A expression in the raphe nuclei of female mice. These results revealed the

ability of *R. badensis* subsp. *acadiensis* to block the effects of LPS treatment on 5HT1A expression in the DRN and CA1 region in LPS-treated females. Thus, *R. badensis* subsp. *acadiensis* may act as an anti-depressant by decreasing serotonin reuptake in the DRN and increasing serotonin release in projection areas. Our data highlight the protective enduring effects of *R. badensis* subsp. *acadiensis* during puberty against depression-like behavior in females.

These findings are in line with several studies that proposed beneficial effects of probiotics on mental health at different ages(Bercik et al. 2011; Bravo et al. 2011; Bercik et al. 2010; Messaoudi et al. 2011; Rao et al. 2009). In order to identify the mechanisms underlying these effects we studied the ability of *R. badensis* subsp. *acadiensis* consumption to mitigate the effects of LPS treatment on the immune system and the gut microbiome.

However, we could not replicate anxiety-like behaviors in adult males following LPS treatment. *R. badensis* subsp. *acadiensis* did not induce anxiogenic effects. The lack of significant difference in anxiety-like behaviors between saline- and LPS-treated males could be caused by an increase of anxiety-like behavior in saline-treated males due to chronic stress (gavage). Chronic stress such as social isolation or daily cage change during adolescence causes significant increase in anxiety-like behaviour in adulthood(Bourke and Neigh 2011; Mccormick, Smith, and Mathews 2008; McCormick and Mathews 2007). These findings support that possibility that the daily gavage may have been a chronic stress that could have modulated LPS-induced anxiety-like behavior in males.

Pubertal *R. badensis* subsp. *acadiensis* treatment mitigates LPS-induced inflammatory markers in a sex- and time-specific manner

We further investigated the role of *R. badensis* subsp. *acadiensis* in modulating sex-specific immune responses and reducing acute immune responses induced by LPS. Our results showed that the exposure to *R. badensis* subsp. *acadiensis* during puberty mitigates LPS-induced pro-inflammatory cytokines and sickness behaviors at the periphery in LPS-treated female mice whereas no effect was observed in males. At the central level, it reduced the expression of central TNFα mRNA expression in the prefrontal cortex of probiotic treated mice and the hippocampus of probiotic treated females. Many studies suggest a potent immunomodulatory activity of certain probiotic strains in diverse disorders; however underlying mechanisms are still unclear (Isolauri et al. 2001; Galdeano et al. 2007; Yahfoufi et al. 2018). Also, efficacy of probiotic administration depends on the doses and types of strains (Kang and Im 2015). Probiotics can interact directly or via their metabolites with the immune cells (antigen representing cells and T cells) leading to immunoregulation (Kang and Im 2015). Moreover, they can improve the integrity of the intestinal barrier by competing with pathogens, modulating the intestinal immune homeostasis, inducing antimicrobial peptides and promoting mucin production (Yahfoufi et al. 2018; Lopetuzo et al. 2015; López-Varela, González-Gross and Marcos 2002; Isolauri, Salimen and Ouwehand 2004). Probiotics contribute to immune homeostasis by balancing pro-inflammatory and anti-inflammatory immune responses(Kang and Im 2015; Frei, Akdis, and O'mahony 2015). *R. badensis* subsp. *acadiensis* has been demonstrated to enhance intestinal barrier: it ameliorates mucin production, improves tight junction and modulate intestinal homeostasis (Yahfoufi et al. 2021; Matar et al. 2020). Sex differences in the immunological responses to immune challenges are observed between males and females that demonstrate distinctions in innate and adaptive immune responses Klein and Flanagan 2016). Immunological sex differences are either present throughout life, or appear after puberty suggesting a role of

genes and hormones (Kane and Ismail 2017; Klein and Flanagan 2016; Klein 2000). Environmental exposures change the microbiome and exert sex-dependent effects on immune function (Klein and Flanagan 2016). Recent study about *lactobacillus reuteri* DMSZ8533 has identified sex differences in the immune modulation after probiotic treatments (He et al. 2019). Sex dimorphism in the immune response after exposure to the probiotic can be related to alteration in the microbiota composition depending on the sex of the host (He et al. 2019).

Pubertal *R. badensis* subsp. *acadiensis* modulate the microbiota composition in a sex-specific manner and prevents LPS-induced microbial fluctuations in female mice

The gut microbiome can be altered by different environmental factors such as pathogenic bacteria, stress, infection, diet, antibiotic use and antibiotics (Galley et al. 2014; Marietta, Horwath, and V. 2018). During critical periods of development and maturation like adolescence, the gut microbiome is subject to significant shifts in composition and is vulnerable to environmental stressors (Jasarevic, Morrison and Bale 2016). Our findings revealed that α-diversity was not affected by probiotic neither by LPS. No sex differences were observed between males and females. Regarding β-diversity, clustering by sex was observed and especially low abundance taxa were affected. Pubertal exposure to *R. badensis* subsp. *acadiensis* affected rare taxa β-diversity in male mice while no effect was observed in females. LPS induced dysbiosis in female mice by affecting rare and abundant taxa while *R. badensis subspecies* revealed its ability to reverse the dysbiosis induced by LPS in female mice.

Sex differences in gut microbial composition can be induced by gonadal steroid hormones, while castration eliminates these differences (Yurkovetskiy et al. 2013). During puberty the gut

microbiome is subject to significant shifts in composition that occur simultaneously with dynamic brain development (Yahfoufi, Matar and Ismail 2020).

Stress exposure during puberty such as infection, poor diet, and inflammation can lead to dysbiosis and may predispose the subject to brain disorders and psychiatric illnesses later in life (Golubeva et al. 2015; Desbonnet et al. 2015). LPS, is a potent activator of innate immune signaling, recognized by intestinal epithelial cells in the gut after binding to toll-like receptor 4 (TLR4) (Bogunovic et al. 2011; Lotz, Menard and Hornef 2007). Study 3 demonstrated that LPS treatment significantly disrupted gut microbial composition of pubertal mice in a sex specific manner with females more affected than males. The mechanisms for these sex-specific microbial are still unclear. One might argue that males have a lower innate immune responses(Klein 2000; Cai et al. 2016) while females have more antibody production, greater antigen presenting activity and higher immunoglobulin levels (Bhatia, Sekhon and Kaur 2014; Bouman, Heineman and Faas 2005)

Critical Findings or Summary

Taken together, our findings revealed that *R. badensis* subsp. *acadiensis* is a non-invasive bacterium that can survive the gastrointestinal tract conditions and interact with the intestinal mucosa. The orally administered strain enhances the gut-associated non-specific immunity without inducing an inflammatory outcome in the intestinal mucosa. It modulates the mucosal barrier, the gut homeostasis, and the immune response at the intestinal level. *R. badensis* subsp. *acadiensis* may contribute to mucosal tolerance and has anti-inflammatory properties.

We also filled an important knowledge gap in our understanding of the pubertal exposure to stress and the molecular mechanisms contributing to the development of long-term mood disorders. Our studies improve our understanding of the influence of probiotic consumption on the developing pubertal brain and highlight the protective enduring effects of probiotics use during the pubertal period. Our findings add to our knowledge of sex-specific pubertal vulnerabilities to immune challenge. We demonstrated that the exposure to immune challenge during puberty causes enduring 5HT1A receptor expression alterations in a sex specific manner in association with enduring sex-specific behavioral alterations. However, probiotic use during puberty mitigates these LPS-induced behavioral and neurochemical changes. We also found that pubertal immune challenge induces sex-specific immune responses in the periphery while in the CNS, the immune response is region- and sex- specific. Probiotic use during puberty could modulate the immune responses in a sex-specific manner. Also, the current studies showed a tendency for female mice microbiome to be more impacted by LPS injection than males which could be reduced by probiotic administration. These findings highlight the importance of gut microbiome composition and sex differences at the peripheral and central inflammatory responses following an immune challenge. It is important to further study the link between the

composition of the gut microbiota and sex-specific development of mental disorders in order to develop new procedures that can alleviate the long-term neural and behavioral alterations induced by stress.

Future Directions

This thesis sets the stage for future investigations on the mechanism(s) by which *R. badensis* subsp. *acadiensis* influences the gut homeostasis and the potential health effects. As many studies propose that a primary deficiency of innate and adaptive immunity can be at the origin of intestinal inflammation (Korzenik and Dieckgraefe 2005), probiotics that reinforce the complex intestinal immune system emerge as promising tools of treatment. Further investigations should evaluate the ability of *R. badensis* subsp. *acadiensis* supplementation to reduce inflammatory gastrointestinal disorders, including ulcerative colitis and Crohn's disease as well as induced intestinal inflammation such as non-steroidal anti-inflammatory induced small intestine inflammation. Future research might investigate the optimal dose of the probiotic preparation and the comparative effectiveness of different probiotic interventions. Given the anti-depressive effect in females and despite the lack of effect on anxiety outcomes in the present study in males, future studies should seek to broaden the areas of investigation and should test the application of this probiotic in different psychotic conditions like (depression, anxiety, obsessive compulsive disorders, etc.). Although our studies focus on the importance of puberty, future directions are required to investigate whether these effects extend to adult group. We need to consider longer treatment periods with the probiotic using prepubertal and adult mice and collecting blood samples at different time points. For the anti-depressive effects, the strain should be compared with probiotics that have demonstrated efficacy in reducing depression

(Bailey and Cryan 2017) in animal models such as *Bifidobacterium bifidum*, *B. lactis*, *Lactobacillus acidophilus*, *L. brebis*, *L. casei*, *L. salivarius*, and *L. lactis* (Bravo et al. 2011).

This thesis sets the stage for future studies on the mechanism(s) by which pubertal LPS and probiotic treatments influence immune function, gut microbial composition and causes enduring effects on the CNS and behaviors in a sex-specific manner. Given the enduring behavioral effect of probiotic use during puberty (Murray et al. 2020; Murray et al. 2019), it is important to investigate for how long the inflammatory pathways are affected after treatment discontinuation.

Future studies are important to assess the use of *R. badensis* subsp. *acadiensis* at different doses and to study dose-related effects. Also, the length of treatment duration can be extended to evaluate brain adaptation to long-term ingestion. It is important to examine the effect of LPS and probiotics on modulating the permeability of the blood brain barrier. In order to better understand the ways in which *R. badensis* subsp. *acadiensis* interventions affect the host, examining the metabolome is important to be associated with the analysis of the microbial community. Therefore, it is important to identify bacterial derived metabolites in the blood and how they affect the central nervous system. Future studies may also consider *R. badensis* subsp. *acadiensis* as an adjuvant treatment for depression; trials can investigate its ability to enhance the treatment effects of existing psychological treatments rather than a stand-alone treatment. They can also investigate the role of probiotics in personalized medicine using integrative approaches about microbiome, epigenome, metabolome and behaviours and benefitting from artificial intelligence to better contribute to prevent depressive-like behaviors before their onset.

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