LACTOBACILLUS REUTERI REDUCES VISCERAL HYPERSENSITIVITY AND ALTERS GUT MOTILITY WITHOUT CHANGING GUT SEROTONIN AVAILABILITY IN AN ANIMAL MODEL OF THE IRRITABLE BOWEL SYNDROME

By

Jasmina Jakupovic

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Neuroscience – Doctor of Philosophy

2013

ABSTRACT

LACTOBACILLUS REUTERI REDUCES VISCERAL HYPERSENSITIVITY AND ALTERS GUT MOTILITY WITHOUT CHANGING GUT SEROTONIN AVAILABILITY IN AN ANIMAL MODEL OF THE IRRITABLE BOWEL SYNDROME

By

Jasmina Jakupovic

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) tract disorder characterized by recurrent abdominal pain and changes in stool frequency and form, which are often comorbid with anxiety and depression. It affects up to 14% of the middle-aged population, is twice as prevalent in women as men and places a large cost on both the individual and the healthcare system. IBS results from a combination of genetic, psychosocial and physiological factors that make treatment difficult, creating a need for novel therapies that address the whole spectrum of symptoms. Probiotics have recently emerged as a new treatment option for people suffering from gastrointestinal diseases and numerous studies have shown that probiotics can help alleviate symptoms in IBS patients.

Currently, very little is known about the probiotic mechanism of action. Alterations in gut serotonin signaling have been implicated in abnormalities of gut motility and visceral sensitivity seen in IBS patients. In the gut, serotonin (5-hydroxytryptamine, 5-HT) is an important paracrine signaling molecule released by enterochromaffin cells in the mucosa. This 5-HT regulates gut motility, secretion and visceral sensation (pain). Probiotics are ingested with food and directly interact with the gut mucosa so we explored the interactions between a common probiotic and the 5-HT system in the gut to help elucidate possible mechanisms of action.

In this work, we studied the beneficial effects of a probiotic, *Lactobacillus reuteri* 6475, in an animal model of IBS: the serotonin transporter knockout (SERT KO) rat. We showed that SERT KO rats recapitulate some of the human symptoms of IBS such as increased visceral hypersensitivity and decreased fecal output. Alterations in gut 5-HT availability did not fully account for the increased visceral hypersensitivity seen in SERT KO rats nor did SERT KO rats exhibit a sub-clinical level of inflammation. *L.reuteri* treatment decreased visceral hypersensitivity in both male and female SERT KO rats and increased fecal output in male SERT KO rats only, but did not alter gut 5-HT availability. *L.reuteri* shows promise as a probiotic that may help alleviate symptoms of IBS.

ACKNOWLEDGMENTS

I have been lucky to have two advisors, Drs. James Galligan and Greg Swain, experts in their respective fields, who have taught me to be an effective researcher and collaborator. I would like to thank them for taking me into their labs at the end of my second year and providing me with a project that was challenging and that helped me mature into a well-rounded scientist. I would also like to thank Dr. Mark Kadrofske for being an “adjunct” advisor, allowing me to infiltrate his lab during the last year of my PhD and spending a significant amount of time teaching me molecular techniques, as well as how to analyze the data and make sense of it. I am grateful to Drs. Colleen Hegg and Robert Britton, my committee members, for their guidance and support through my PhD.

I would like extend a special thanks to Hong Zhao for teaching me the techniques of cyclic voltammetry and continuous amperometry and for spending countless hours helping me troubleshoot my experiments. I would also like to thank Rana Kabeer and Melissa Chavez who helped me with data collection and analysis for a portion of my study. I would like to thank Xiaochun Bian, Marion France, Roxanne Fernandes and Beth Lockwood for helping me learn techniques, collect data, and order supplies. They are selfless individuals who have made my work easier and more fun.

Lastly, I am thankful to have been a part of the Neuroscience Program at MSU. The Neuroscience program is unique in that is provides students not only with research experience but with service and teaching activities as well as tools to prepare them for their careers following the completion of their degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ACh	Acetylcholine
APS	Ammonium persulfate
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BDD	Boron-doped diamond electrode
BSA	Bovine serum albumin
cfu	Colony-forming units
CGRP	Calcitonin-gene related peptide
C-IBS	Constipation-predominant IBS
CNS	Central nervous system
CRD	Colorectal distention
DA	Dopamine
DAT	Dopamine transporter
D-IBS	Diarrhea-predominant IBS
DRG	Dorsal root ganglion
DRN	Dorsal raphe nucleus
E_2	17β-estradiol
EC cell	Enterochromaffin cell
EFS	Electrical field stimulation
EMG	Electromyographic

ENS	Enteric nervous system
ER	Estrogen receptor
GI tract	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
IBD	Inflammatory bowel disorder
IBS	Irritable Bowel Syndrome
IL	Interleukin
IK_{Ca}	Calcium-activated potassium channel
INFɣ	Interferon ɣ
IPAN	Intrinsic primary afferent neuron
L-AADC	L-Amino acid decarboxylase
MAO_A	Monoamine oxidase A
NE	Norepinephrine
NET	Norepinephrine transporter
NO	Nitric oxide
OCT	Organic cation transporter
$P2X_3$	Purinergic receptor subtype X_3
PI-IBS	Post-infectious IBS

PNS	Peripheral nervous system
PSNS	Parasympathetic nervous system
PTSD	Post-traumatic stress disorder
PVDF	Polyvinylidene difluoride membrane
SDS	Sodium dodecyl sulfate
SERT	Serotonin reuptake transporter
SERT KO	Serotonin transporter knockout
SNS	Sympathetic nervous system
SSRI	Selective serotonin reuptake inhibitor
Sub P	Substance P
TM	Trans-membrane
TNFα	Tumor necrosis factor α
TPH-1	Tryptophan hydroxylase 1
TTX	Tetrodotoxin
VIP	Vasoactive intestinal polypeptide
VMAT-1	Vesicular monoamine transporter 1
VMR	Visceromotor response
VR-1	Vanilloid receptor 1
WT	Wild-type

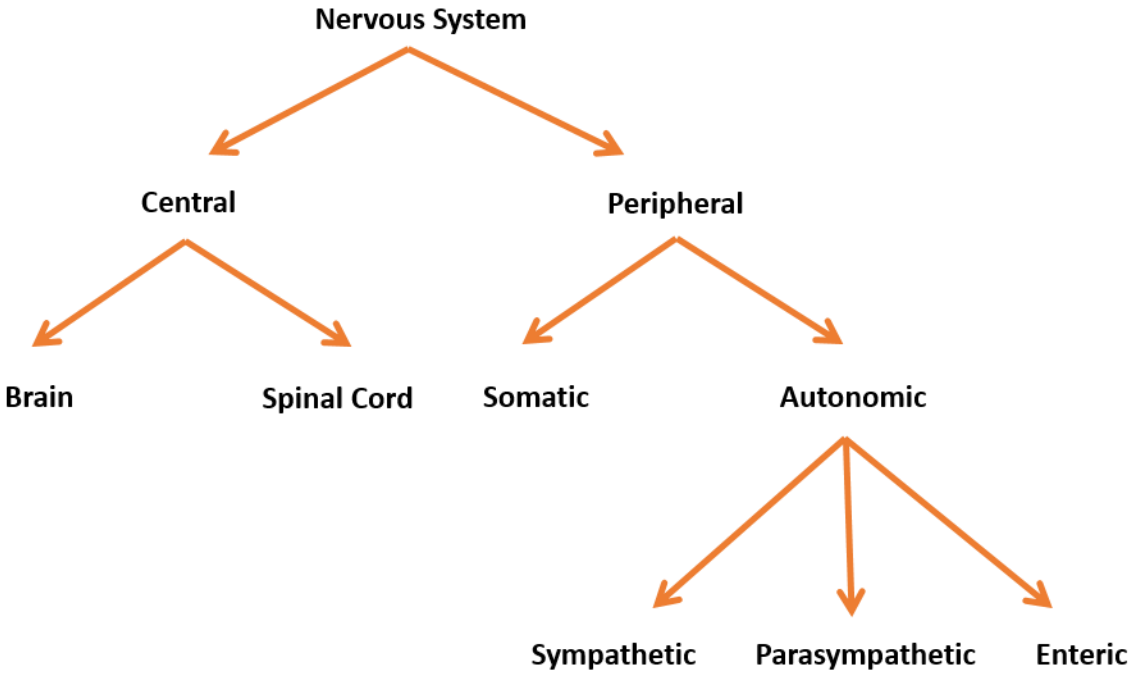
CHAPTER 1

GENERAL INTRODUCTION

1.1 Organization of the nervous system

The nervous system is divided into the central (CNS) and peripheral (PNS) branches (Kandel, 2000). The CNS consists of the brain and the spinal cord. The PNS is subdivided into the somatic and the autonomic nervous systems. The somatic subdivision is composed of neurons that innervate skeletal muscle to control voluntary movements such as walking. The autonomic subdivision is composed of neurons that innervate smooth muscle, cardiac muscle and glands to control involuntary movements such as breathing and digestion. The autonomic nervous system can further be subdivided into the sympathetic (SNS), parasympathetic (PSNS) and the enteric nervous systems (ENS) (Figure 1.1).

Figure 1.1 Organization of the nervous system. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

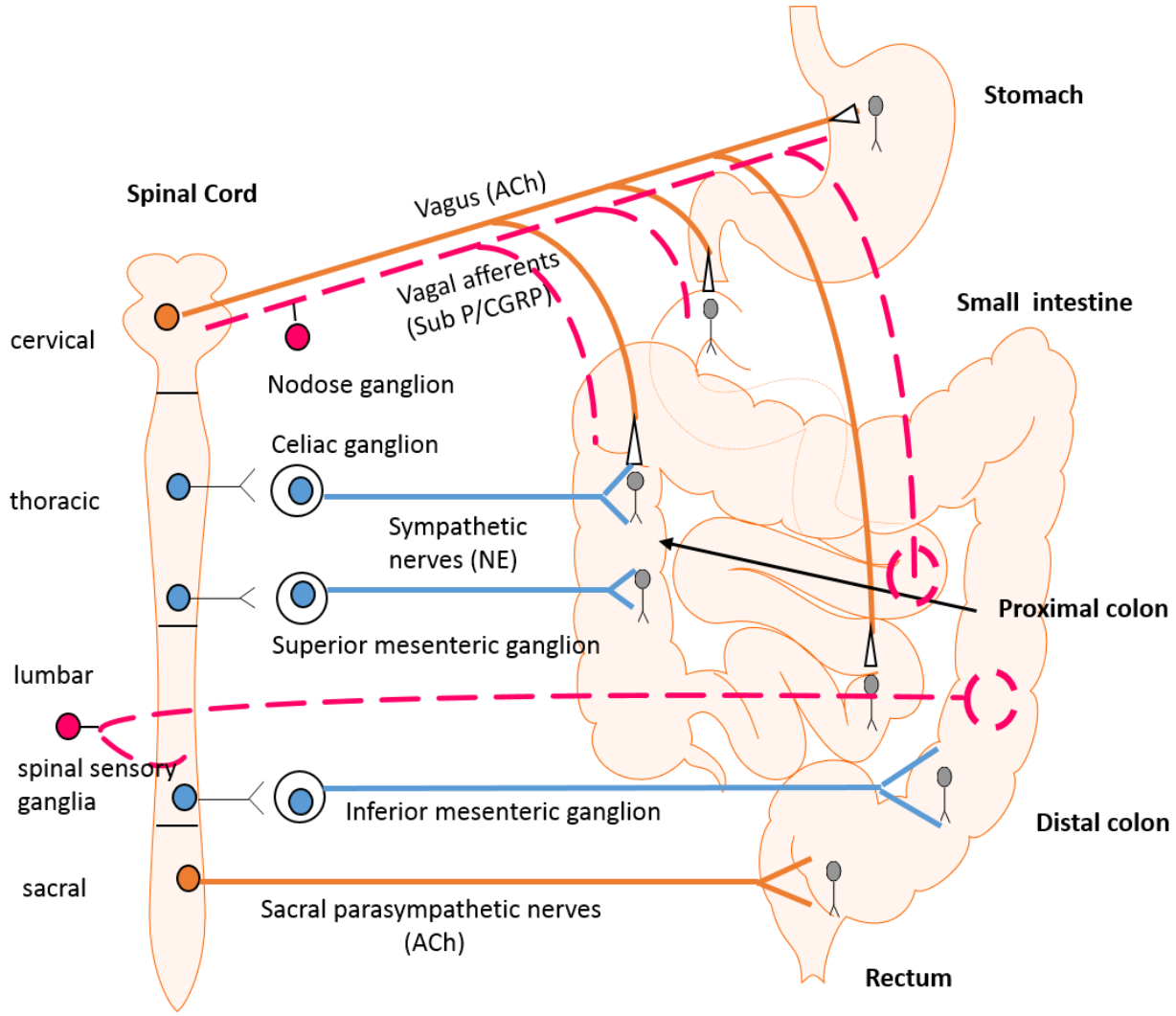


1.1.1 The brain-gut axis

The SNS and PSNS provide extrinsic innervation of the gut (Figure 1.2). The SNS reacts during stressful circumstances ("fight or flight") whereas the PSNS reacts during feeding, breeding and resting ("rest of digest") (Kandel, 2000). Both consist of a system of two neurons: pre- and post-ganglionic neurons. In the SNS, the cell bodies of pre-ganglionic neurons are located in the spinal cord and have very short axons that extend to the paravertebral ganglia. The post-ganglionic neurons have long axons that extend to the organs they innervate. Sympathetic fibers from the thoracic region extend to the celiac and superior mesenteric ganglion to innervate the proximal colon. Sympathetic fibers from the lumbar region extend to the inferior mesenteric ganglion to innervate the distal colon. The pre-ganglionic neurons use acetylcholine (ACh) as a neurotransmitter, whereas the post-ganglionic neurons use norepinephrine (NE). In the PSNS, the cell bodies of pre-ganglionic neurons are also located in the spinal cord and they send out long axons to ganglia that are very near or embedded in the organs. The vagus nerve provides parasympathetic innervation of the stomach, small intestine and proximal colon. The pelvic nerve provides parasympathetic innervation of the distal colon. Both the pre- and post-ganglionic neurons of the PSNS use ACh as a neurotransmitter.

The SNS and PSNS provide inputs from the CNS to the gastrointestinal (GI) tract (Kandel, 2000). There are also vagal and spinal sensory afferents that send information from the gut back to the brain. Vagal afferents transmit physiological information about the luminal content and motility whereas spinal afferents transmit information about noxious stimuli. This bidirectional exchange of information is termed the brain-gut axis and its primary function is to maintain homeostasis.

Figure 1.2 Extrinsic innervation of the gut. Sympathetic innervation is shown in blue, parasympathetic in orange and afferent in red dashes. ACh – acetylcholine, Sub P – substance P, CGRP – calcitonin-gene related peptide, NE – norepinephrine.



1.1.2 The enteric nervous system (ENS)

The ENS provides intrinsic innervation of the gut (Furness, 2006). Located in the gut wall, it lines the GI tract from the esophagus to the rectum. Unlike sympathetic and parasympathetic ganglia, which serve as relay-distribution centers for signaling between the CNS and the gut, neurons in the enteric ganglia can integrate and process information like the brain (Wood, 2012). Hence why the ENS is referred to as the brain in the gut or the "little brain." It "thinks on its own"

to control and regulate the organs of the GI tract. In fact, when all CNS connections are severed (both sympathetic and parasympathetic inputs) the gut can fully function on its own.

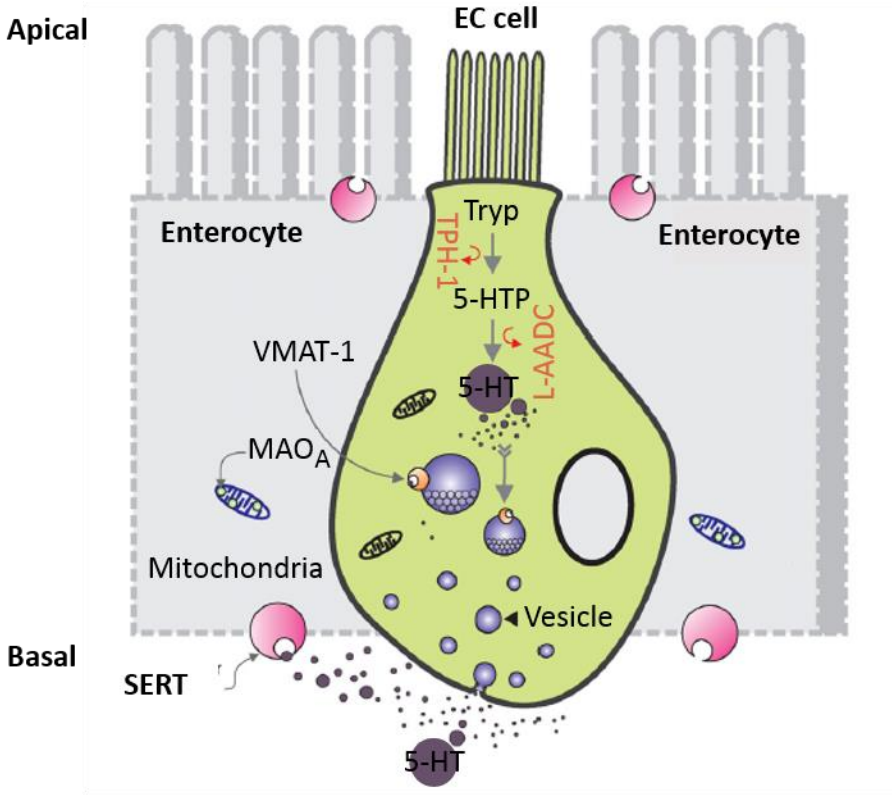
The ENS contains two nerve plexuses: the submucosal plexus that controls gut secretions and the myenteric plexus that controls gut motility (Furness, 2006). The submucosal plexus is located directly under the mucosa whereas the myenteric plexus is sandwiched between two muscle layers: the circular muscle and the longitudinal muscle. Each nerve plexus contains afferent neurons, interneurons, and motor neurons.

1.2 Serotonin signaling in the gut

Serotonin (5-hydroxytryptamine, 5-HT) is both an important neurotransmitter, released by enteric neurons, and a paracrine signaling molecule, released by enterochromaffin (EC) cells in the gut mucosa, that regulates gut motility, secretions and sensations (Bertrand and Bertrand, 2010). EC cells make up only about 1-3% of the cells in the gut mucosa and they release 90% of the body's 5-HT (Gershon and Tack, 2007).

As food passes through the intestinal lumen, it chemically, mechanically and/or by increasing intraluminal pressure stimulates the EC cell to release 5-HT (Gershon and Tack, 2007; Racké *et al*., 1996). Within the EC cell, 5-HT is made from a dietary amino acid L-tryptophan via the action of a rate-limiting enzyme, tryptophan hydroxylase 1 (TPH1; EC cell specific) (Yu *et al*., 1999). TPH1 converts L-tryptophan into 5-hydroxytryptophan (5-HTP). L-amino acid decarboxylase (L-AADC) then converts 5-HTP into 5-hydroxytryptamine (5-HT). Once produced, 5-HT is packaged into vesicles via a vesicular monoamine transporter 1 (VMAT1) and released mostly near the basal side (Schäfermeyer *et al*., 2004). Some is also released near the apical side (*i.e*., into the lumen) (Ahlman *et al*., 1981). 5-HT binds to receptors on the nerve endings in the gut mucosa. It also interacts with immune cells in the lamina propria and is taken up into blood flow through capillaries. Gut 5-HT is the only source of 5-HT in the blood. 5-HT action is terminated by uptake into the surrounding enterocytes via a high-affinity serotonin reuptake transporter (SERT) (Martel *et al*., 2003). It is then degraded by monoamine oxidase A (MAO_A) into 5-hydroxyindoleacetic acid (5-HIAA) (Figure 1.3) (Rodríguez *et al*., 2001).

Figure 1.3 Production and release of 5-HT from EC cells. EC – enterochromaffin cell, Tryp – L-tryptophan, TPH-1 – tryptophan hydroxylase 1, 5-HTP – 5-hydroxytryptophan, L-AADC – L-amino acid decarboxylase, 5-HT – 5-hydroxytryptamine, VMAT-1 – vesicular monoamine transporter 1, SERT – serotonin reuptake transporter, MAO_A - monoamine oxidase A. Reprinted from Autonomic Neuroscience: Basic and Clinical, volume 153, Paul P. Bertrand and Rebecca L. Bertrand, Serotonin release and uptake in the gastrointestinal tract, p49, Copyright (2010), with permission from Elsevier.



1.2.1 Serotonergic control of gut motility

Serotonin released from EC cells in the gut mucosa binds to 5-HT_3 receptors (5-HT_3Rs), on intrinsic primary afferent neurons (IPANs) (Bertrand *et al*., 2000). There are submucosal and myenteric IPANs. Myenteric IPANs (whose cell bodies are in the myenteric plexus) synapse with interneurons that then synapse with motor neurons. There are two types of motor neurons: excitatory and inhibitory. Excitatory motor neurons send fibers to innervate the surrounding

muscle and release ACh and substance P (Sub P) to produce muscle contractions. Inhibitory motor neurons send fibers to innervate the surrounding muscle and release nitric oxide (NO), vasoactive intestinal polypeptide (VIP) and adenosine triphosphate (ATP) to produce muscle relaxation. Food moves through the gut in a series of muscle contractions and relaxations; a set of movements known as peristalsis (Figure 1.4) (Sikander *et al*., 2009).

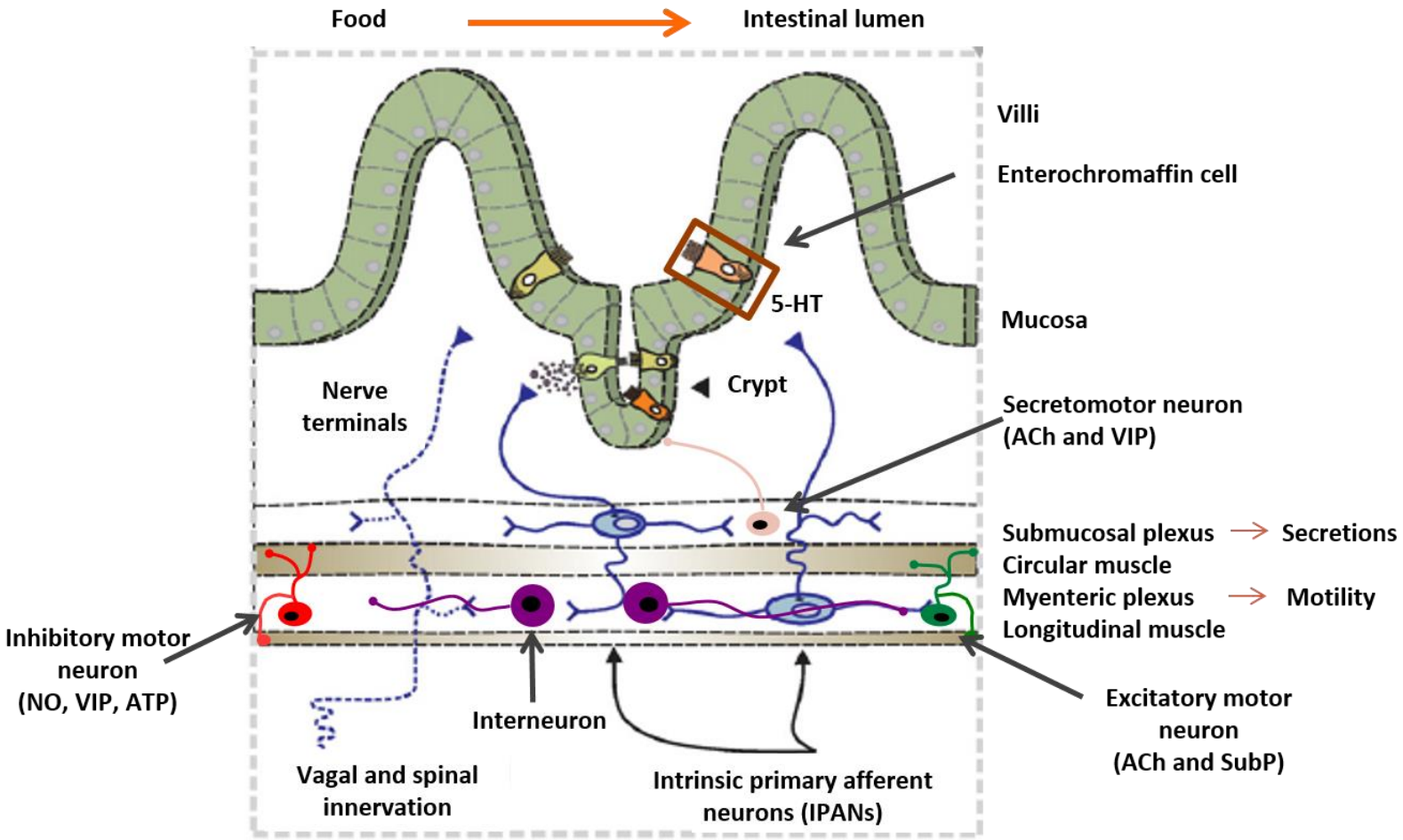
1.2.2 Serotonergic control of secretion

5-HT released from EC cells also binds to 5-HT_{1P}Rs and 5-HT_4Rs on submucosal IPANs (whose cell bodies are in the submucosal plexus) (Gershon, 2004). Submucosal IPANs synapse with secretomotor neurons. The secretomotor neurons send out a single axon that extends to the intestinal crypt and releases ACh and VIP to stimulate the secretory gland to produce water, mucus and electrolytes (Figure 1.4).

1.2.3 Serotonergic control of sensation

5-HT regulates sensation by binding to 5-HT_3Rs on nerve terminals of vagal and spinal sensory afferents. Vagal afferents send information to regulate the sensations of nausea, vomiting, and satiety, whereas spinal sensory afferents send information to regulate abdominal pain and discomfort (Figure 1.4) (Gershon, 1999).

Figure 1.4 Serotonin signaling controls gut motility, secretions and sensations. 5-HT – 5-hydroxytryptamine, ACh – acetylcholine, VIP – vasoactive intestinal polypeptide, Sub P – substance P, NO – nitric oxide, ATP – adenosine triphosphate. Reprinted from Autonomic Neuroscience: Basic and Clinical, volume 153, Paul P. Bertrand and Rebecca L. Bertrand, Serotonin release and uptake in the gastrointestinal tract, p48, Copyright (2010), with permission from Elsevier.



1.3 Irritable Bowel Syndrome (IBS)

IBS is a common and complex, functional GI tract disorder characterized by changes in gut motility, secretion and visceral sensation. It affects 7-14% of the middle-aged population, and is more prevalent in women than men (Barbara *et al*., 2011; Sikander *et al*., 2009). A combination of genetic, physiological and psychosocial factors play a role in the development of clinical IBS symptoms, which include changes in stool frequency and form, abdominal pain and bloating and are often accompanied by anxiety and depression leading to a decrease in the patient's quality of life (Hahn *et al*., 1999). IBS is divided into three subtypes depending on the nature of the symptoms: 1) Diarrhea-predominant IBS (D-IBS), 2) Constipation-predominant IBS (C-IBS), and 3) Alternating IBS. IBS places a large economic burden on the individual as well as the healthcare system in number of missed days from work, prescriptions, ambulatory visits and hospitalizations. In the US alone that cost is ~$30 billion/year (Lembo, 2007; Maxion-Bergemann *et al*., 2006). Due to the complex and multifactorial nature of the disorder, treatment is scarce and there is need for novel therapies.

In IBS, 5-HT metabolism is altered. C-IBS patients tend to exhibit decreased 5-HT signaling and decreased EC cell numbers, whereas D-IBS patients tend to exhibit increased 5-HT signaling associated with decreased SERT expression (Atkinson *et al*., 2006; Coates *et al*., 2004; Dunlop *et al*., 2005; El-Salhy *et al*., 1999). The reports are conflicting depending on the methodology, patient population and part of the GI tract studied; nevertheless all are consistent in that in IBS 5-HT signaling is altered.

As a result, current treatments available for IBS target 5-HT receptors on IPANs to alleviate symptoms. Tegaserod, a partial 5-HT_4R agonist is used for treatment of C-IBS, whereas Alosetron,

a 5-HT_3R antagonist, is used for treatment of D-IBS (Ford *et al*., 2009; Rahimi *et al*., 2008). However, both drugs come with harmful side effects. Alosetron currently has an FDA restricted use for women with severe D-IBS that show no response to anti-diarrheal agents within 6 months of treatment. It presents the side effects of ischemia, constipation, and ischemic colitis. Tegaserod comes with a side effect of cardiovascular ischemia, so it is currently prescribed only to women under the age of 55 with no cardiovascular problems. Clearly, better treatment regimens are needed.

1.3.1 Visceral hypersensitivity

Visceral hypersensitivity is a hallmark symptom of IBS, especially in women. It is characterized by two components: hyperalgesia – an enhanced response to a painful stimulus and allodynia – a painful response to an innocuous stimulus. To measure visceral hypersensitivity in humans, a balloon is inserted into the rectum and slowly inflated in set pressure increments. The patients are then asked to rate their pain on a scale. IBS patients experience pain and discomfort in the rectal area at lower levels of pressure compared to healthy controls (Bouin, 2002).

Visceral hypersensitivity occurs when there is abnormal processing of information at any stage in the pain transduction pathway from the gut to the brain (Figure 1.5) (Holzer *et al.*, 2001). At the level of the colon, excessive muscle contraction and distention lead to visceral hypersensitivity (Wood, 2012). Gut muscle contains mechanosensors. When the muscle contracts or stretches the mechanosensors get activated and lead to an increase in the firing of spinal sensory afferents that send information to the spinal cord and the brain. In IBS patients, those sensors are hypersensitive and that hypersensitivity is attributable to neurogenic factors

such as overactive nerves rather than muscle tension and compliance (Munakata *et al*., 1997; Ritchie, 1973). In fact, when electrical stimulation is applied to their colon, IBS patients report similar levels of pain as they do during balloon distention (Drewes *et al*., 2001). IBS patients have decreased sensory thresholds as a result of one or all of the following factors: 1) Sensitization of afferent nerve endings in the gut mucosa, 2) Sensitization of neurotransmission at the level of the spinal cord, 3) Abnormal processing of information in the brain and 4) Decreased descending inhibitory inputs (Wood, 2012) (Figure 1.6).

Figure 1.5 Visceral pain pathway. DRG – dorsal root ganglion.

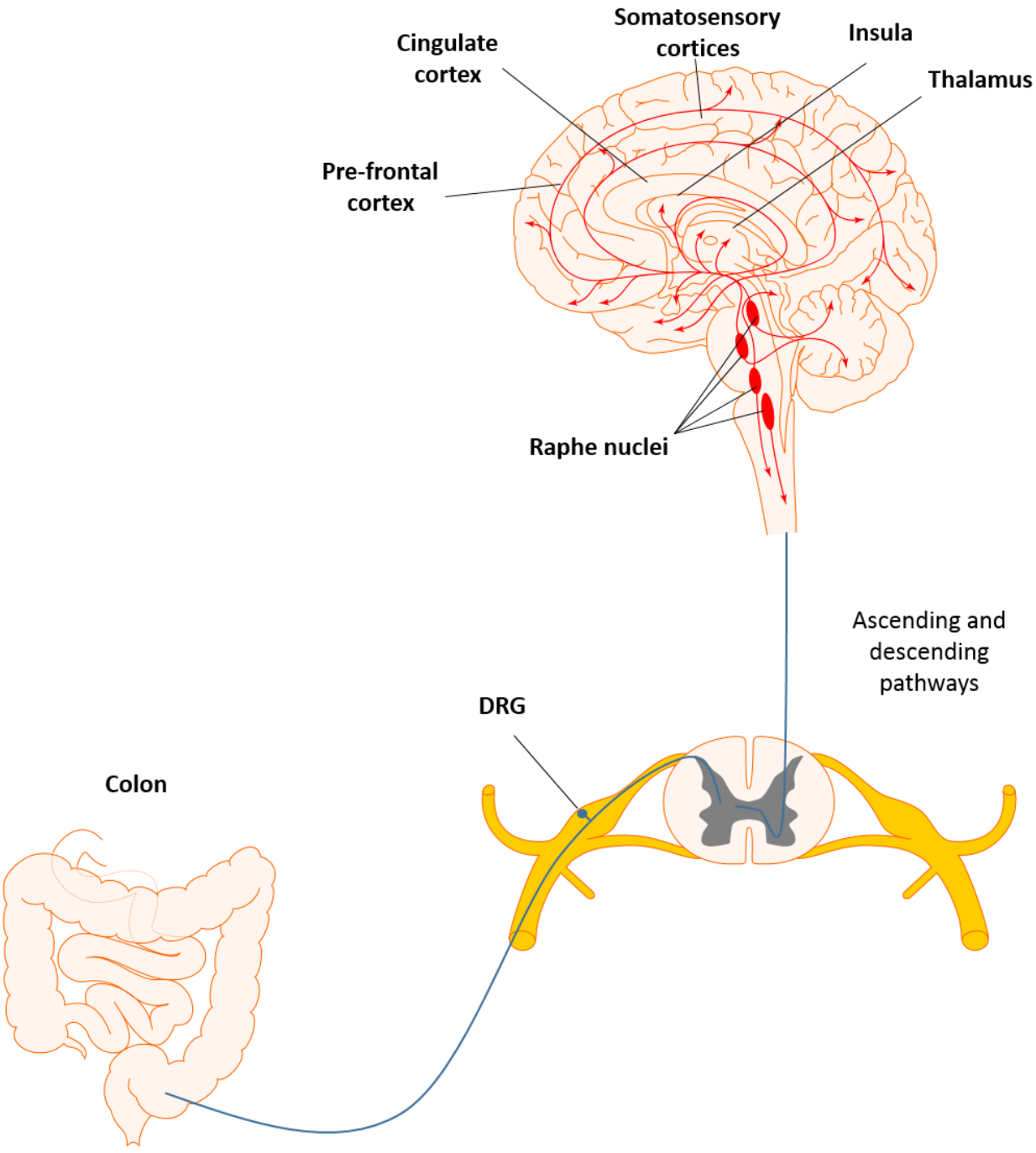
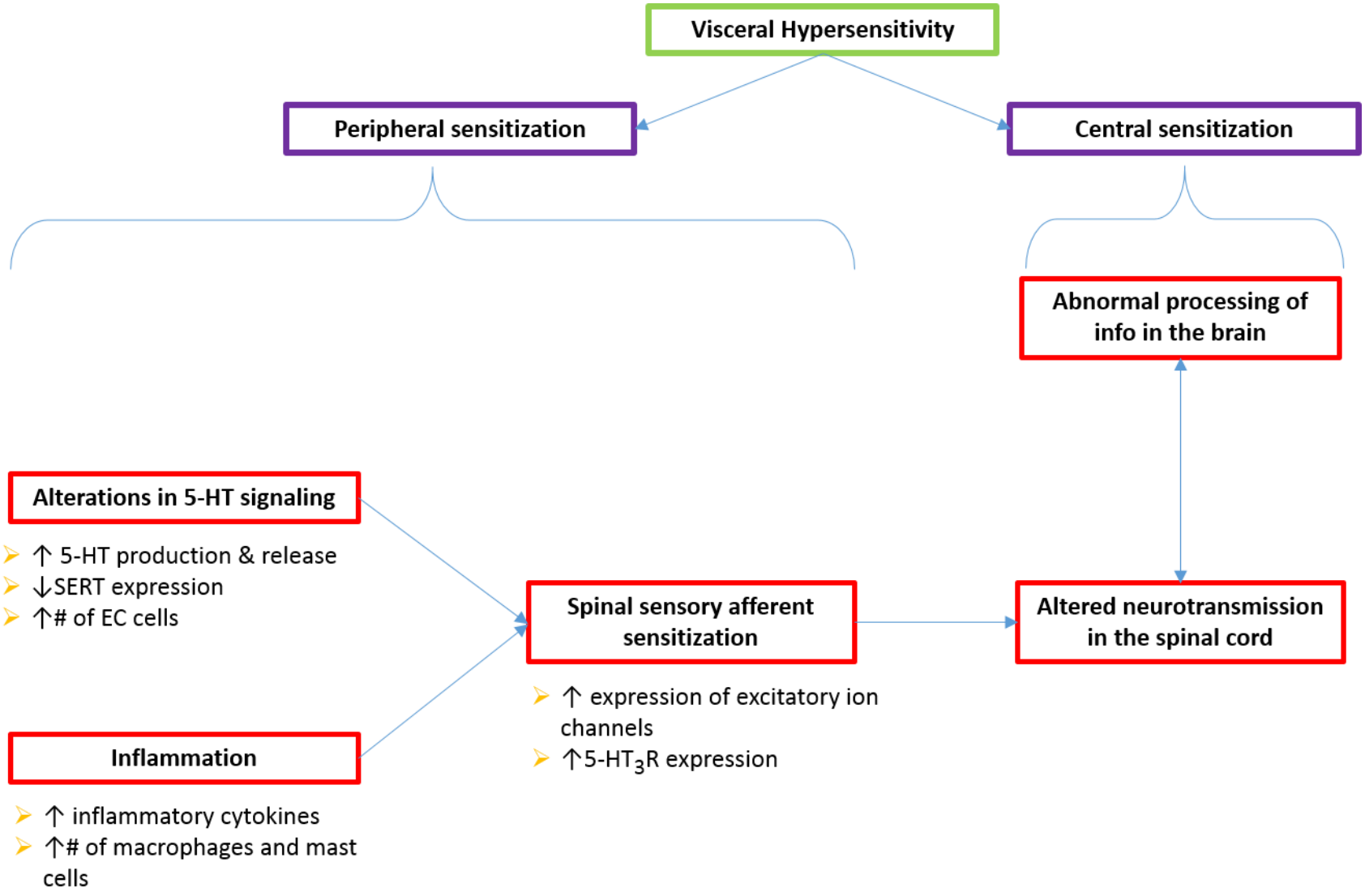


Figure 1.6 Possible mechanisms leading to visceral hypersensitivity. 5-HT – 5-hydroxytryptamine, SERT – serotonin reuptake transporter, EC – enterochromaffin cell.



1.3.2 Role of 5-HT in visceral hypersensitivity

Spinal sensory afferents transmit information about noxious stimuli from the colon to the brain (Foreman, 2004). They are mostly unmyelinated C fibers and some thinly-myelinated Aδ fibers and are divided into: low-threshold, high-threshold and silent afferents. Low and high-threshold afferents respond to, as their name indicates, small and large changes in gut wall tension. Silent afferents only get activated during tissue injury and inflammation. Silent afferents lack spontaneous activity and display no sensitivity to mechanical, thermal or chemical stimuli.

Sensory nerve endings in the colonic mucosa express excitatory ion channels, such as the vanilloid receptor 1 (VR-1), acid-sensing ion channels (ASICs), purinergic receptors ($P2X_3R$) and tetrodotoxin (TTX)-resistant voltage-gated sodium channels. In functional bowel disorders, alterations in the expression and functioning of these channels have been implicated in visceral hypersensitivity (Holzer *et al*., 2001).

Afferent nerve endings also express 5-HT_3Rs (Sikander *et al*., 2009). Increased 5-HT levels, decreased SERT expression and/or activity and overstimulation of the 5-HTRs in IBS have been implicated in visceral hypersensitivity (Figure 1.6). Administration of Alosetron, a 5-HT_3R antagonist, lowers pain in women suffering from D-IBS (Camilleri *et al*., 2001). Colonic mucosal biopsies from IBS patients show a downregulation in SERT mRNA expression (Coates, 2004). Furthermore, some studies have looked at a link between SERT polymorphisms and a genetic predisposition to IBS. The best-characterized polymorphism in the human SERT gene occurs in the 5' flanking promoter region, the serotonin transporter length polymorphic region (5-HTTLPR), leading to a 43 base pair deletion or insertion and resulting in a short (s) or long (l) allele. The s/s,

s/l or l/l genotypes are believed to affect the transcriptional rate of SERT such that the s allele is associated with lower transcriptional activity, decreased expression of SERT, lower affinity for 5-HT and vice versa for the l allele (Heils *et al*., 1996). The s/s and s/l genotypes were found in a subset of IBS patients possibly indicating a genetic predisposition to IBS, but the reports are conflicting (Saito *et al*., 2005; Van Kerkhoven *et al*., 2007).

Cell bodies of spinal sensory afferents that innervate the colon are located in the dorsal root ganglia (DRG) and they go on to synapse with second-order neurons in the dorsal horn of the spinal cord. These neurons ascend to the brain via the spinothalamic, spinoreticular, spinoparabrachial, spinohypothalamic, spinosolitary, and the dorsal column pathways (Van Oudenhove *et al*., 2004). At the level of the medulla, third-order projections are sent to the thalamus and the cortex. Three main areas of the brain are involved in the processing of visceral pain information: the somatosensory cortices, cingulate cortex and the insula (Almeida *et al*., 2004; Van Oudenhove *et al*., 2004). The primary and secondary somatosensory cortex process information about the intensity and localization of the noxious stimulus, the cingulate cortex processes pain affect and the insula integrates the two. The descending pathways involve both adrenergic and serotonergic projections from the periaqueductal gray, dorsal raphe nuclei (DRN), and nucleus magnocellularis and project to the dorsal horn neurons to modulate pain (Figure 1.5) (Millan, 2002).

1.3.3 Inflammation and visceral hypersensitivity

There is evidence to indicate a sub-clinical level of inflammation in IBS (Barbara *et al*., 2002; Ohman and Simrén, 2010). By definition, sub-clinical inflammation is asymptomatic and not easily detectable but can still disrupt normal functioning. A subset of patients develop post-

infectious IBS (PI-IBS) following an acute enteric infection (Collins and Barbara, 2004; Spiller, 2007). Psychogenic stress and a weakened immune system increase the risk for development of PI-IBS following gastroenteritis (Gwee *et al*., 1999). Furthermore, studies report that patients in remission from inflammatory bowel disorders (IBD), such as ulcerative colitis exhibit IBS-like symptoms (Isgar *et al*., 1983; Simrén *et al*., 2002). In addition, 50% of patients with microscopic colitis and about 30% of patients with celiac disease show symptom overlap with IBS (Abboud *et al*., 2013; Sainsbury *et al*., 2013). These lines of evidence support a role of inflammation in IBS.

Immune activation in IBS, as a result of sub-clinical inflammation, can lead to sensitization of afferent nerve endings in the gut mucosa thus contributing to visceral hypersensitivity (Bueno *et al*., 1997; Collins, 1996; Mayer and Gebhart, 1994). Enteric mast cells are located in the lamina propria of the gut mucosa and their primary role is to provide inflammatory surveillance. When activated, they release a number of inflammatory mediators. Some of the main ones include: 5-HT, histamine, adenosine, nitric oxide, interleukin 6 (IL-6), platelet-activating factor, IL-1β, leukotrienes, mast cell proteases and prostaglandins (Wood, 2012). Matricon *et al*., 2012 did a meta-analysis of studies looking at inflammatory markers in IBS patients. They found that most IBS patients have an increase in the number of mast cells. 80% of the studies that looked at colonic mast cell density found a 50-100% increase in IBS patients compared to healthy controls.

Sensory never endings in the gut mucosa contain receptors for inflammatory mediators such as ATP, bradykinin, adonesine, prostaglandins, leukotrienes and mast cell proteases (Coelho *et al*., 1998). Studies have shown that treatment with a mast cell stabilizer, ketotifen, decreases visceral hypersensitivity in IBS patients (Klooker *et al*., 2010; Siddiqui and Miner, 2004).

1.4 Serotonin transporter knockout (SERT KO) rat

Due to the multifactorial etiology and pathophysiology of IBS in humans, animal models are scarce. SERT KO mice have been used to study alterations in 5-HT signaling underlying abnormalities in gut motility seen in IBS patients (Chen *et al*., 2001). SERT KO mice display increased colonic motility and water in their stool (diarrhea). Sometimes, they exhibit episodes of decreased colonic motility (constipation). However, studies looking at SERT KO mice employ indirect methods for assessing gut motility, such as placing a glass bead into the colon and measuring time to expulsion, and have been highly variable (Bischoff *et al*., 2009). Furthermore, there are limited data on visceral hypersensitivity in mice; their size presenting a challenge (Annahazi *et al*., 2009; Larsson *et al*., 2003).

IBS is an integrative disorder and as such there is a need for animal models that allow for integrative physiological studies. We propose the SERT KO rat is a good animal model for studying aspects of the disruptions in 5-HT signaling that underlie visceral hypersensitivity and abnormal gut motility seen in IBS patients. In SERT KO rats, a chemically-induced mutation causes a transversion of a single amino acid from a cysteine to an adenine in the sequence encoding the second extracellular loop of the SERT protein and results in a premature stop codon. (Homberg *et al*., 2007). As a result, the SERT KO rat does not express SERT at all. It is a total body knockout.

SERT KO rats exhibit increased anxiety and depressive behaviors, also seen in IBS patients (Olivier *et al*., 2008). Furthermore, SERT KO rats have elevated extracellular 5-HT levels in the brain, but no differences in the number of serotonergic neurons in the dorsal raphe nuclei (DRN). DRN is the largest serotonergic nucleus and it provides innervation to the forebrain areas.

Likewise, in the gut, 3-4 month old SERT KO rats have the same whole-tissue levels of 5-HT as do WT rats, but they have much higher extracellular 5-HT concentrations near the EC cells because of decreased reuptake (Galligan *et al*., 2013). Furthermore, 3-4 month old female SERT KO rats exhibit increased visceral hypersensitivity compared to WT females. This effect occurs at the level of the colon projecting spinal sensory afferents which exhibit increased excitability in female SERT KO rats. This also nicely mimics what is seen in female patients suffering from IBS (Chang *et al*., 2006).

1.5 Probiotics

Probiotics are viable, non-pathogenic bacteria that are ingested and are known to improve digestive tract function. Two of the most common genera with the greatest potential benefit in IBS are *Bifidobacteria* and *Lactobacilli*, and each has many strains. Even though probiotics have been used for decades and their beneficial effects on the digestive system are widespread, very little is still known about their interaction with the ENS and their mechanism of action.

1.5.1 Beneficial effects of probiotics

Probiotics live in a symbiotic relationship with the host (Walter *et al*., 2011). They help break down otherwise indigestible polysaccharides in our diets to extract energy and nutrients, defend against harmful bacteria and help maintain a healthy immune system. In turn, they have access to an unlimited supply of nutrients (Bixquert Jiménez, 2009).

Studies in IBS patients have shown that treatment with either *Lactobacillus* or *Bifidobacteria* results in a significant improvement of symptoms, such as abdominal pain, bloating, irregularity in bowel movements, flatulence, as well as quality of life (Niedzielin *et al*., 2001; Nobaek *et al*., 2000; Sinn *et al*., 2008).

1.5.2 Probiotic mechanism of action

Probiotics enter the gut lumen with food and interact with the gut mucosa to exert a number of beneficial effects, most important of which include: 1) prevention of toxins and pathogens from adherence to the mucosa, 2) decrease in inflammation, 3) maintenance of the intestinal barrier function, and 4) regulation of the immune system (Vanderpool *et al*., 2008). Probiotics compete with harmful bacteria for binding to the epithelial cell layer and some can

even displace them (Candela *et al*., 2005; Collado *et al*., 2007). Furthermore, they produce antibacterial substances, such as bacteriocins and acids, to inhibit pathogen growth (Cotter *et al*., 2005; Servin, 2004). *Lactobacilli* produce small antimicrobial peptides that can destroy pathogenic bacteria by disrupting their membranes and enzyme activity. They also produce short-chain fatty acids to lower the local pH and inhibit pathogen growth (De Keersmaecker *et al*., 2006; Makras *et al*., 2006). Probiotics decrease inflammation by up-regulating anti-inflammatory cytokines and down-regulating pro-inflammatory cytokines. *L.reuteri* and *L.casei* can induce dendritic cells to release an anti-inflammatory cytokine interleukin 10 (IL-10) (Smits *et al*., 2005). Various strains have also been shown to inhibit production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-6 and interferon ɣ (INFɣ) (Matsumoto *et al*., 2005; Peña and Versalovic, 2003).

Despite the growing evidence of the beneficial effects of probiotics, very little is known about their interaction with the serotonergic system in the gut, alterations of which have been implicated in abnormalities of gut motility and visceral sensitivity seen in IBS patients.

1.5.3 *Lactobacillus reuteri*

Lactobacillus reuteri is a prominent member of the *Lactobacillus* population in rodent GI tract, but it is only found occasionally in humans (Brooks *et al*., 2003; Salzman *et al*., 2002; Walter, 2008). It has been shown to survive in both the human stomach and small intestine and it has never been implicated in human disease. As a probiotic, different strains of *L.reuteri* have been used to: 1) decrease abdominal pain, 2) prevent diarrhea, 3) reduce infant colic, and 4) activate the immune system (Indrio *et al*., 2008; Romano *et al*., 2010; Savino *et al*., 2007; Shornikova *et*

al, 1997; Valeur *et al*., 2004). In our studies, we used *L.reuteri* strain 6475, a novel probiotic strain not yet studied clinically.

1.6 Hypothesis and specific aims

The overall aim of this research was to identify the beneficial effects *Lactobacillus reuteri* has on visceral hypersensitivity and gut motility in WT and SERT KO rats, and to determine the mechanisms of action. Our overarching hypothesis is that probiotics produce their beneficial effects by modulating serotonin signaling, which leads to decreased visceral hypersensitivity and restoration of normal motility. The study was divided into two specific aims.

Specific aim 1: To determine the beneficial effects of *Lactobacillus reuteri* by studying the effects of chronic *L.reuteri* treatment on 1) visceral hypersensitivity and 2) gut motility in WT and SERT KO rats.

Specific aim 2: To determine the mechanism of *Lactobacillus reuteri* action by studying the effects of chronic *L.reuteri* treatment on 1) gut serotonin signaling and 2) gut inflammation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abboud R, Pardi DS, Tremaine WJ, Kammer PP, Sandborn WJ, Loftus EV Jr. Symptomatic overlap between microscopic colitis and irritable bowel syndrome: a prospective study. Inflammatory bowel diseases. (2013) 19: 550-3.

Ahlman H, Bhargava HN, Dahlström A, Larsson I, Newson B, Pettersson G. On the presence of serotonin in the gut lumen and possible release mechanisms. Acta physiologica Scandinavica. (1981) 112: 263-9.

Almeida TF, Roizenblatt S, Tufik S. Afferent pain pathways: a neuroanatomical review. Brain research. (2004) 1000: 40-56.

Annaházi A, Gecse K, Dabek M, Ait-Belgnaoui A, Rosztóczy A, Róka R, Molnár T, Theodorou V, Wittmann T, Bueno L, Eutamene H. Fecal proteases from diarrheic-IBS and ulcerative colitis patients exert opposite effect on visceral sensitivity in mice. Pain. (2009) 144: 209-17.

Atkinson W, Lockhart S, Whorwell PJ, Keevil B, Houghton LA. Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. Gastroenterology (2006) 130: 34-43.

Barbara G, Cremon C, De Giorgio R, Dothel G, Zecchi L, Bellacosa L, Carini G, Stanghellini V, Corinaldesi R. Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. Current gastoenterology reports. (2011) 13: 308-15.

Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? Gut. (2002) 51: i41-4.

Bertrand PP, Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. Autonomic neuroscience: basic and clinical. (2010) 153: 47-57.

Bertrand PP, Kunze WA, Furness JB, Bornstein JC. The terminals of myenteric intrinsic primary afferent neurons of the guinea-pig ileum are excited by 5-hydroxytryptamine acting at 5-hydroxytryptamine-3 receptors. Neuroscience. (2000) 101: 459-69.

Bischoff SC, Mailer R, Pabst O, Weier G, Sedlik W, Li Z, Chen JJ, Murphy DL, Gershon MD. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. American journal of physiology. Gastrointestinal and liver physiology. (2009) 296: G685-95.

Bixquert Jiménez M. Treatment of irritable bowel syndrome with probiotics. An etiopathogenic approach at last? Revista espanola de enfermedades digestivaa. (2009) 101: 553-64.

Bouin M, Plourde V, Boivin M, Riberdy M, Lupien F, Laganière M, Verrier P, Poitras P. Rectal distention testing in patients with irritable bowel syndrome: sensitivity, specificity, and predictive values of pain sensory thresholds. Gastroenterology. (2002) 122: 1771-7.

Brooks SP, McAllister M, Sandoz M, Kalmokoff ML. Culture-independent phylogenetic analysis of the faecal flora of the rat. Canadian journal of microbiology. (2003) 49: 589-601.

Bueno L, Fioramonti J, Delvaux M, Frexinos J. Mediators and pharmacology of visceral sensitivity: from basic to clinical investigations. Gastroenterology. (1997) 112: 1714-43.

Camilleri M, Chey WY, Mayer EA, Northcutt AR, Heath A, Dukes GE, McSorley D, Mangel AM. A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome. Archives of internal medicine. (2001) 161: 1733-40.

Candela M, Seibold G, Vitali B, Lachenmaier S, Eikmanns BJ, Brigidi P. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. Research in microbiology. (2005) 156: 887-95.

Chang L, Mayer EA, Labus JS, Schmulson M, Lee OY, Olivas TI, Stains J, Naliboff BD. Effect of sex on perception of rectosigmoid stimuli in irritable bowel syndrome. The American journal of physiology. Regulatory, integrative and comparative physiology. (2006) 291: R277-84.

Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. The Journal of Neuroscience. (2001) 21: 6348-61.

Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology. (2004) 126: 1657-64.

Coelho AM, Fioramonti J, Bueno L. Mast cell degranulation induces delayed rectal allodynia in rats: role of histamine and 5-HT. Digestive diseases and sciences. (1998) 43: 727-37.

Collado MC, Meriluoto J, Salminen S. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in applied microbiology. (2007) 45: 454-60.

Collins SM. The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. Gastroenterology. (1996) 111: 1683-99.

Collins SM, Barbara G. East meets west: infection, nerves, and mast cells in the irritable bowel syndrome. Gut. (2004) 53: 1068-9.

Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nature reviews. Microbiology. (2005) 3: 777-88.

De Keersmaecker SC, Verhoeven TL, Desair J, Marchal K, Vanderleyden J, Nagy I. Strong antimicrobial activity of Lactobacillus rhamnosus GG against Salmonella typhimurium is due to accumulation of lactic acid. FEMS microbiology letters. (2006) 259: 89-96.

Drewes AM, Petersen P, Rössel P, Gao C, Hansen JB, Arendt-Nielsen L. Sensitivity and distensibility of the rectum and sigmoid colon in patients with irritable bowel syndrome. Scandinavian journal of gastroenterology. (2001) 36: 827-32.

Dunlop SP, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA, Spiller RC. Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. Clinical gastroenterology and hepatology. (2005) 3: 349-57.

El-Salhy M, Norrgard O, Spinnell S. Abnormal colonic endocrine cells in patients with chronic idiopathic slow-transit constipation. Scandinavian journal of gastroenterology. (1999) 34: 1007-11.

Ford AC, Brandt LJ, Young C, Chey WD, Foxx-Orenstein AE, Moayyedi P. Efficacy of 5-HT3 antagonists and 5-HT4 agonists in irritable bowel syndrome: systematic review and meta-analysis. The American journal of gastroenterology. (2009) 104: 1831-43.

Foreman RD. Mechanisms of visceral pain: from nociception to targets. Drug discovery today: disease mechanisms. (2004) 1: 457-63.

Furness JB. The enteric nervous system. (2006) Blackwell Publishing Inc.: Australia.

Galligan JJ, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM. Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. Neurogastroenterology and motility. (2013) 25: e373-81.

Gershon MD. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. Alimentary pharmacology and therapeutics. (1999) 13: 15-30.

Gershon MD. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. Alimentary pharmacology and therapeutics. (2004) 20: 3-14.

Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. (2007) 132: 397-414.

Gwee KA, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW. The role of psychological and biological factors in postinfective gut dysfunction. Gut. (1999) 44: 400-6.

Hahn BA, Yan S, Strassels S. Impact of irritable bowel syndrome on quality of life and resource use in the United States and United Kindgom. Digestion. (1999) 60: 77-81.

Heils A, Teufel A, Petri S, Stöber G, Riederer P, Bengel D, Lesch KP. Allelic variation of human serotonin transporter gene expression. Journal of neurochemistry. (1996) 66: 2621-4.

Holzer P, Michl T, Danzer M, Jocic M, Schicho R, Lippe IT. Surveillance of the gastrointestinal mucosa by sensory neurons. Journal of physiology and pharmacology. (2001) 52: 505-21.

Homberg JR, Olivier JD, Smits BM, Mul JD, Mudde J, Verheul M, Nieuwenhuizen OF, Cools AR, Ronken E, Cremers T, Schoffelmeer AN, Ellenbroek BA, Cuppen E. Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience. (2007) 146: 1662-76.

Indrio F, Riezzo G, Raimondi F, Bisceglia M, Cavallo L, Francavilla R. The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. The Journal of pediatrics. (2008) 152: 801-6.

Isgar B, Harman M, Kaye MD, Whorwell PJ. Symptoms of irritable bowel syndrome in ulcerative colitis in remission. Gut. (1983) 24: 190-2.

Kandel ER, Schwartz JH, Jessell TM. Principles of neural science. (2000) 4th edition. McGraw-Hill: New York.

Klooker TK, Braak B, Koopman KE, Welting O, Wouters MM, van der Heide S, Schemann M, Bischoff SC, van den Wijngaard RM, Boeckxstaens GE. The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome. Gut. (2010) 59: 1213-21.

Larsson M, Arvidsson S, Ekman C, Bayati A. A model for chronic quantitative studies of colorectal sensitivity using balloon distension in conscious mice -- effects of opioid receptor agonists. Neurogastroenterology and motility. (2003) 15: 371-81.

Lembo AJ. The clinical and economic burden of irritable bowel syndrome. Practical gastroenterology. (2007) 31: 3-9.

Makras L, Triantafyllou V, Fayol-Messaoudi D, Adriany T, Zoumpopoulou G, Tsakalidou E, Servin A, De Vuyst L. Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards Salmonella enterica serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. Research in microbiology. (2006) 157: 241-7.

Martel F, Monteiro R, Lemos C. Uptake of serotonin at the apical and basolateral membranes of human intestinal epithelial (Caco-2) cells occurs through the neuronal serotonin transporter (SERT). The Journal of pharmacology and experimental therapeutics. (2003) 306: 355-62.

Matricon J, Meleine M, Gelot A, Piche T, Dapoigny M, Muller E, Ardid D. Review article: Associations between immune activation, intestinal permeability and the irritable bowel syndrome. Alimentary pharmacology and therapeutics. (2012) 36: 1009-31.

Matsumoto S, Hara T, Hori T, Mitsuyama K, Nagaoka M, Tomiyasu N, Suzuki A, Sata M. Probiotic Lactobacillus-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. Clinical and experimental immunology. (2005) 140: 417-26.

Maxion-Bergemann S, Thielecke F, Abel F, Bergemann R. Costs of irritable bowel syndrome in the UK and US. PharmacoEconomics. (2006) 24: 21-37.

Mayer EA, Gebhart GF. Basic and clinical aspects of visceral hyperalgesia. Gastroenterology. (1994) 107: 271-93.

Millan MJ. Descending control of pain. Progress in neurobiology. (2002) 66: 355-474.

Munakata J, Naliboff B, Harraf F, Kodner A, Lembo T, Chang L, Silverman DH, Mayer EA. Repetitive sigmoid stimulation induces rectal hyperalgesia in patients with irritable bowel syndrome. Gastroenterology. (1997) 112: 55-63.

Niedzielin K, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. European journal of gastroenterology and hepatology. (2001) 13: 1143-7.

Nobaek S, Johansson ML, Molin G, Ahrné S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. The American journal of gastroenterology. (2000) 95: 1231-8.

Ohman L, Simrén M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. Nature reviews. Gastroenterology and hepatology. (2010) 7: 163-73.

Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, Deen PM, Cuppen E, Cools AR, Ellenbroek BA. A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience. (2008) 152: 573-84.

Peña JA, Versalovic J. Lactobacillus rhamnosus GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. Cellular microbiology. (2003) 5: 277-85.

Racké K, Reimann A, Schwörer H, Kilbinger H. Regulation of 5-HT release from enterochromaffin cells. Behavioural brain research. (1996) 73: 83-7.

Rahimi R, Nikfar S, Abdollahi M. Efficacy and tolerability of alosetron for the treatment of irritable bowel syndrome in women and men: a meta-analysis of eight randomized, placebo controlled, 12-week trials. Clinical therapeutics. (2008) 30: 884-901.

Ritchie J. Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. Gut. (1973) 14: 125-32.

Rodríguez MJ, Saura J, Billett EE, Finch CC, Mahy N. Cellular localization of monoamine oxidase A and B in human tissues outside of the central nervous system. Cell and tissue research. (2001) 304: 215-20.

Romano C, Ferrau' V, Cavataio F, Iacono G, Spina M, Lionetti E, Comisi F, Famiani A, Comito D. Lactobacillus reuteri in children with functional abdominal pain (FAP). Journal of peadiatrics and child health. (2010)

Sainsbury A, Sanders DS, Ford AC. Prevalence of irritable bowel syndrome-type symptoms in patients with celiac disease: a meta-analysis. Clinical gastroenterology and hepatology. (2013) 11: 359-65.

Saito YA, Petersen GM, Locke GR 3rd, Talley NJ. The genetics of irritable bowel syndrome. Clinical gastroenterology and hepatology. (2005) 3: 1057-65.

Salzman NH, de Jong H, Paterson Y, Harmsen HJ, Welling GW, Bos NA. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. (2002) Microbiology. 148: 3651-60.

Savino F, Pelle E, Palumeri E, Oggero R, Miniero R. Lactobacillus reuteri (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. Pediatrics. (2007) 119: e124-30.

Schäfermeyer A, Gratzl M, Rad R, Dossumbekova A, Sachs G, Prinz C. Isolation and receptor profiling of ileal enterochromaffin cells. Acta physiologica Scandinavica. (2004) 182: 53-62.

Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS microbiology reviews. (2004) 28: 405-40.

Shornikova AV, Casas IA, Isolauri E, Mykkänen H, Vesikari T. Lactobacillus reuteri as a therapeutic agent in acute diarrhea in young children. Journal of pediatric gastroenterology and nutrition. (1997) 24: 399-404.

Siddiqui AA, Miner PB Jr. The role of mast cells in common gastrointestinal diseases. Current allergy and asthma reports. (2004) 4: 47-54.

Sikander A, Rana SV, Prasad KK. Role of serotonin in gastrointestinal motility and irritable bowel syndrome. Clinica chimica acta. (2009) 403: 47-55.

Simrén M, Axelsson J, Gillberg R, Abrahamsson H, Svedlund J, Björnsson ES. Quality of life in inflammatory bowel disease in remission: the impact of IBS-like symptoms and associated psychological factors. The American journal of gastroenterology. (2002) 97: 389-96.

Sinn DH, Song JH, Kim HJ, Lee JH, Son HJ, Chang DK, Kim YH, Kim JJ, Rhee JC, Rhee PL. Therapeutic effect of Lactobacillus acidophilus-SDC 2012, 2013 in patients with irritable bowel syndrome. Digestive diseases and sciences. (2008) 53: 2714-8.

Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, Zaat BA, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. The Journal of allergy and clinical immunology. (2005) 115: 1260-7.

Spiller RC. Role of infection in irritable bowel syndrome. Journal of gastroenterology. (2007) 42: 41-7.

Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. Colonization and immunomodulation by Lactobacillus reuteri ATCC 55730 in the human gastrointestinal tract. Applied and environmental microbiology. (2004) 70: 1176-81.

Vanderpool C, Yan F, Polk DB. Mechanisms of probiotics action: implications for therapeutic applications in inflammatory bowel diseases. Inflammatory bowel diseases. (2008) 14: 1585-96.

Van Kerkhoven LA, Laheij RJ, Jansen JB. Meta-analysis: a functional polymorphism in the gene encoding for activity of the serotonin transporter protein is not associated with irritable bowel syndrome. Alimentary pharmacology and therapeutics. (2007) 26: 979-86.

Van Oudenhove L, Demyttenaere K, Tack J, Aziz Q. Central nervous system involvement in functional gastrointestinal disorders. Best practice and research. Clinical gastroenterology. (2004) 18: 663-80.

Walter J. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. Applied and environmental microbiology. (2008) 74: 4985-96.

Walter J, Britton RA, Roos S. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. Proceedings of the National Academy of Sciences of the United States of America. (2011) 108: 4645-52.

Wood JD. Pathophysiology underlying the irritable bowel syndrome. In: Johnson LR, ed. *Physiology of the gastrointestinal tract.* (5th ed., vol 1). London UK: Elsevier Inc.; 2012. p.2157-81.

Yu PL, Fujimura M, Okumiya K, Kinoshita M, Hasegawa H, Fujimiya M. Immunohistochemical localization of tryptophan hydroxylase in the human and rat gastrointestinal tracts. The Journal of comparative neurology. (1999) 411: 654-65.

CHAPTER 2

LACTOBACILLUS REUTERI REDUCES VISCERAL HYPERSENSITIVITY AND ALTERS GUT MOTILITY IN SEROTONIN TRANSPORTER KNOCKOUT (SERT KO) RATS

2.1 Abstract

IBS is a common and complex, functional GI tract disorder characterized by abnormal gut motility, secretion and visceral sensation. Altered serotonin signaling in the gut contributes to IBS symptoms. 5-HT is released by EC cells in the intestinal mucosa to regulate gut motility, secretions and sensations. We use the SERT KO rat for studying the relationship between 5-HT signaling, visceral sensation and gut motility. SERT KO rats do not express the SERT protein that is responsible for clearing 5-HT and, thus, have altered 5-HT signaling. Probiotics are viable, non-pathogenic bacteria that are known to improve IBS symptoms. In this study, we 1) characterized the SERT KO rat as an animal model of IBS and 2) determined the beneficial effects of chronic ingestion of a probiotic, *Lactobacillus reuteri,* on visceral hypersensitivity and gut motility in 7-8 month old male and female, WT and SERT KO rats. Visceromotor response (VMR) to colorectal balloon distention (CRD) was assessed following 14 days of treatment with either *L.reuteri* or bacteria-free medium (broth). Gut motility was assessed by measuring fecal output (*in vivo)* and neurogenic (evoked by transmural electrical stimulation) and acetylcholine-induced colonic longitudinal muscle contractility *in vitro*. Male and female SERT KO rats exhibited increased visceral hypersensitivity to CRD compared to WT rats. *L.reuteri* treatment eliminated that difference. Furthermore, male and female SERT KO rats exhibited decreased fecal output compared to WT rats. *L.reuteri* treatment increased fecal output for male SERT KO rats but had no effects on female SERT KO rats. There were no significant differences between all groups in longitudinal muscle contractions evoked by either acetylcholine or electrical field stimulation. The SERT KO rat is a good animal model for aspects of IBS. SERT KO rats exhibit increased visceral hypersensitivity and decreased gut motility, hallmark symptoms of IBS in humans. *L.reuteri*

reduces visceral hypersensitivity and alters fecal output in SERT KO rats. *L.reuteri* shows promise in the treatment of IBS symptoms.

2.2 Introduction

IBS is a functional GI tract disorder characterized by abdominal pain, bloating and alternating, irregular episodes of diarrhea and constipation. It is termed a functional disorder since the abnormalities in structure cannot fully account for the abnormalities in function. There is no single identifiable cause of IBS. Instead, a combination of genetic, psychosocial and physiological factors lead to the development of symptoms. At the physiological level, it is the alterations in serotonin signaling that are believed to be producing the symptoms (Cremon *et al*., 2011; Spiller, 2007).

In the gut, 5-HT is released by the EC cells in the mucosa and binds to nerve endings to regulate gut motility, secretions and sensations (Bertrand and Bertrand, 2010; Gershon and Tack, 2007). SERT ends its action by up-taking 5-HT into enterocytes. In our study, we used the SERT KO rat to investigate possible disruptions in 5-HT signaling that underlie the visceral hypersensitivity and altered gut motility characteristic of IBS patients. The SERT KO rat does not express SERT. It is a total-body knockout (Homberg *et al*., 2007). A previous study in our lab showed that 3-4 month old female SERT KO rats exhibit increased visceral hypersensitivity, a hallmark symptom of IBS in women (Galligan *et al*., 2013). However, there were no significant differences in intestinal transit rates between SERT KO and WT rats (unpublished data). The first aim of these experiments was therefore to characterize the SERT KO rat as an animal model of IBS by assessing 1) visceral hypersensitivity and 2) gut motility in 7-8 month old male and female, SERT KO and WT rats.

Probiotics improve digestive tract function and have been used in clinical studies with IBS patients to alleviate abdominal pain, bloating, irregularity in bowel movements, and flatulence

(Niedzielin *et al*., 2001; Nobaek *et al*., 2000; Sinn *et al*., 2008). Studies in rats have shown the beneficial effects of probiotics in decreasing visceral hypersensitivity in animal models of stress-induced visceral hyperalgesia (Ait-Belgnaoui *et al*., 2006; McKernan *et al*., 2010). Furthermore, acute *L.reuteri* treatment has been shown to decrease mouse jejunal motility *in vitro* by altering enteric neuron excitability (Kunze *et al*., 2009; Wang *et al*., 2010a, b). The second aim of these experiments was to determine if there are therapeutically beneficial effects of *Lactobacillus reuteri* by studying the effects of chronic *L.reuteri* treatment on 1) visceral hypersensitivity and 2) gut motility in 7-8 month old male and female, SERT KO and WT rats.

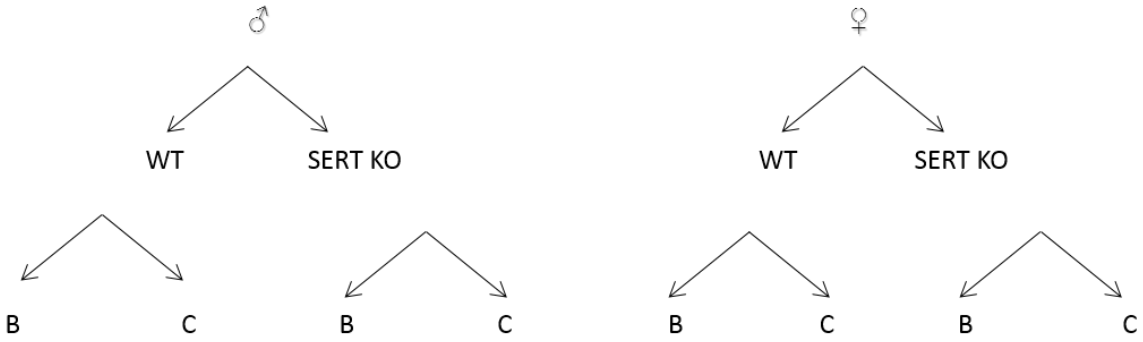
2.3 Methods

2.3.1 Animals

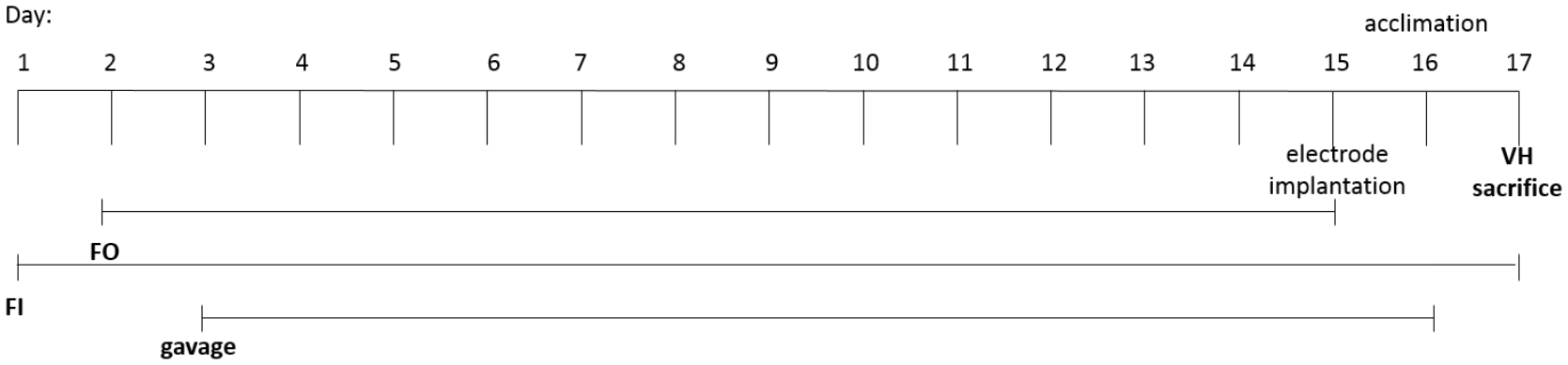
All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. SERT KO rats are maintained under a license from Genoway Inc. (http://www.genoway.com). Age matched (7-8 ½ month old), Wistar rats (n = 64), were divided into 8 groups (n = 8 per group) based on sex (M vs. F), genotype (WT vs. SERT KO) and treatment (*L.reuteri* vs. broth). All rats were housed individually on a 12 h light/dark cycle with *ad libitum* access to food and water. All rats were administered either the *L.reuteri* 6475 (0.5 mL, $5x10^8$ colony-forming units (cfu)) or acidified MRS media (0.5 mL, with HCl, pH ~4.6, control broth) by gavage daily for 14 days. The control broth was acidified with HCl to match the pH of *L.reuteri*-containing media. Figure 2.1 lists the treatment groups and the timeline for the experiments carried out.

Figure 2.1 Treatment groups and timeline for the experiments. (A) Treatment groups (n = 8 per group). (B) Timeline for the experiments. Abbreviations: B – experimental group, administered *L.reuteri* by gavage daily for 14 days; C – control group, administered acidified MRS media (broth) by gavage daily for 14 days. FO – fecal output; FI – food intake; VH – visceral hypersensitivity.

A. Treatment Groups



B. Timeline



2.3.1.1 Animals weight and food intake

The animal weight was tracked at three time points: the day they entered the experiment (day 1), the day of the surgery (day 15) and the day they were sacrificed (day 17) (Figure 2.1). The animal food intake was measured daily.

2.3.2 Visceral hypersensitivity

To assess visceral hypersensitivity, on day 15 of the experiment, rats underwent surgery to implant electrodes onto their abdominal oblique muscles. Rats were anesthetized with isoflurane gas (4% induction, 2% maintenance, 1.8 L O_2) and kept on a heating pad to maintain their body temperature. Teflon-coated stainless steel wires were implanted into the external oblique muscles, passed subcutaneously to the back of the neck and placed through a harness tether to keep them from retracting under the skin. Following surgery, rats were injected with Timentin (antibiotic, 60 mg/kg, intramuscularly) and Ketoprofen (analgesic, 5 mg/kg, subcutaneously). The following day (day 16), rats were acclimated to a Plexiglass restrainer for 30 min. On the day of testing (day 17), rats were placed into and allowed to acclimate to the Plexiglass restrainer for 30 min. A latex balloon (2 cm in length) attached to a piece of tubing that went into a pressure transducer, was lubricated with glycerin, inserted into the colon and secured with tape to the rat's tail. The latex balloon was also attached to a syringe that was used to inflate it. The pressure transducer and the electrode leads were connected to an amplifier, while the pressure and the electromyographic (EMG) outputs were connected to an analog-digital converter. Signals were recorded using Axoscope 9 software. After acclimation, two measurements were collected: 1) Visceromotor response (VMR) to colorectal distention (CRD) and 2) Colonic compliance.

Visceral hypersensitivity was assessed by inflating the balloon in set pressure increments of 0, 10, 20, 40, 60 and 80 mmHg and measuring the EMG response of the abdominal muscles. Recording episodes consisted of a 10 s baseline, 10 s of CRD to a fixed pressure, and 10 s of recovery, for a total of 30 s, and were separated by 5 min intervals. EMGs were analyzed using Clampfit 9.0 software by rectifying the area under the curve during the 10 s of CRD and normalizing it to the baseline response during the initial 10 s.

Colonic compliance was measured by inflating the balloon, using a 1 mL syringe, in set volume increments, of 0.1 mL every 5 seconds, from 0.1 to 0.8 mL, and measuring pressure at each step. The volume inflations were small enough as to not induce distention-evoked contractions of the colon. Colonic compliance was measured in order to eliminate it as a factor contributing to differences in visceral hypersensitivity.

2.3.3 Motility assays

2.3.3.1 Fecal output

The animal fecal output was recorded daily. To quantify fecal output, an animal was placed into a clean cage for 2 h at the same time each day (8 am - 10 am) with *ad libitum* access to food and water. At the end of the period, the total fecal pellet number and wet weight were measured. The animal was then returned to its home cage.

2.3.3.2 Neurogenic and drug-induced longitudinal muscle contractility *in vitro*

On day 17 of the experiment, rats were euthanized with sodium pentobarbital (50 mg/kg, intraperitonealy) and a piece of distal colon (~1.5 to 2 cm in length) was harvested. The organ bath protocol was followed to measure the strength of the longitudinal muscle contraction. Plexiglass tissue holders containing two parallel platinum foil electrodes were attached to each

end of the tissue which was then suspended in a small reservoir (20 mL) filled with oxygenated (95% O_2 + 5% CO_2) Krebs buffer solution (in mM: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose; pH 7.4) at 37^{O}C. One of the holders was attached to an isometric force transducer which applies a tension of ~1 g (0.27 V). After allowing it to acclimate for 30 min, the tissue was first stimulated with 10 µM acetylcholine (ACh) to induce a maximum contraction and the rest of the contractions were all normalized to it. The tissue was then stimulated with increasing doses of 0.1, 0.3, 1, 3, 10, and 30 µM ACh for 1 min and the strength of the contraction recorded. Between consecutive ACh concentrations, the tissue was washed twice with Krebs and allowed to recover for 10 min. Following that, electrical field stimulation (EFS, 20 V) was applied at increasing frequencies of 1, 2, 5, 10, and 20 Hz and the strength of the muscle contraction was recorded. Between consecutive stimulations, the tissue was washed twice with Krebs solution and allowed to recover for 10 min. Data was collected using the Axoscope 9 software and analyzed by taking the peak amplitude of each contraction, normalizing it against the amplitude of the maximum contraction (10 µM ACh) and plotting it against increasing stimulation (ACh or EFS).

2.3.4 Statistics

All data are presented as mean ± standard error of the mean (S.E.M). Two groups were analyzed using an unpaired student's *t* test. Multiple groups were compared using a Two-way ANOVA with or without repeated measures (as experimental design dictated) and Bonferroni *post-hoc* tests to assess statistical significance. When standard deviations of the groups were significantly different from each other, either a log transformation of the data was used or a non-

parametric test such as a Kruskall-Wallis ANOVA. Data were analyzed using InStat 3 and Prism 5. Statistical significance was assigned at a p value less than 0.05.

2.4 Results

At the start of the experiment, male SERT KO rats weighed significantly less than male WT rats. There were no differences in body weight across the female groups (Table 2.1).Overall, female rats weighed half as much as male rats. On average, all groups lost 1-3% of their body weight during the course of treatment and there were no significant differences in % change of weight between the groups. Furthermore, all groups lost 5-8% of their body weight following surgery to implant electrodes onto their abdominal muscles.

Table 2.1 Animal weights (g) at the start of the study. Data are mean ± S.E.M. n = 8 per group, *p<0.05 compared to WT broth.

	WT Broth	**WT *L.reuteri***	**SERT KO Broth**	**SERT KO *L.reuteri***
Males	515 ± 22	532 ± 21	453 ± 12*	459 ± 10*
Females	266 ± 7	285 ± 12	263 ± 4	264 ± 7

During the course of treatment, male SERT KO rats consumed significantly less food compared to male WT rats, regardless of treatment. There were no differences in food consumption between the male broth and *L.reuteri*-treated groups within each genotype. Female SERT KO broth-treated group consumed significantly less food than either of the female WT rat groups or the female SERT KO *L.reuteri*-treated group (Table 2.2).

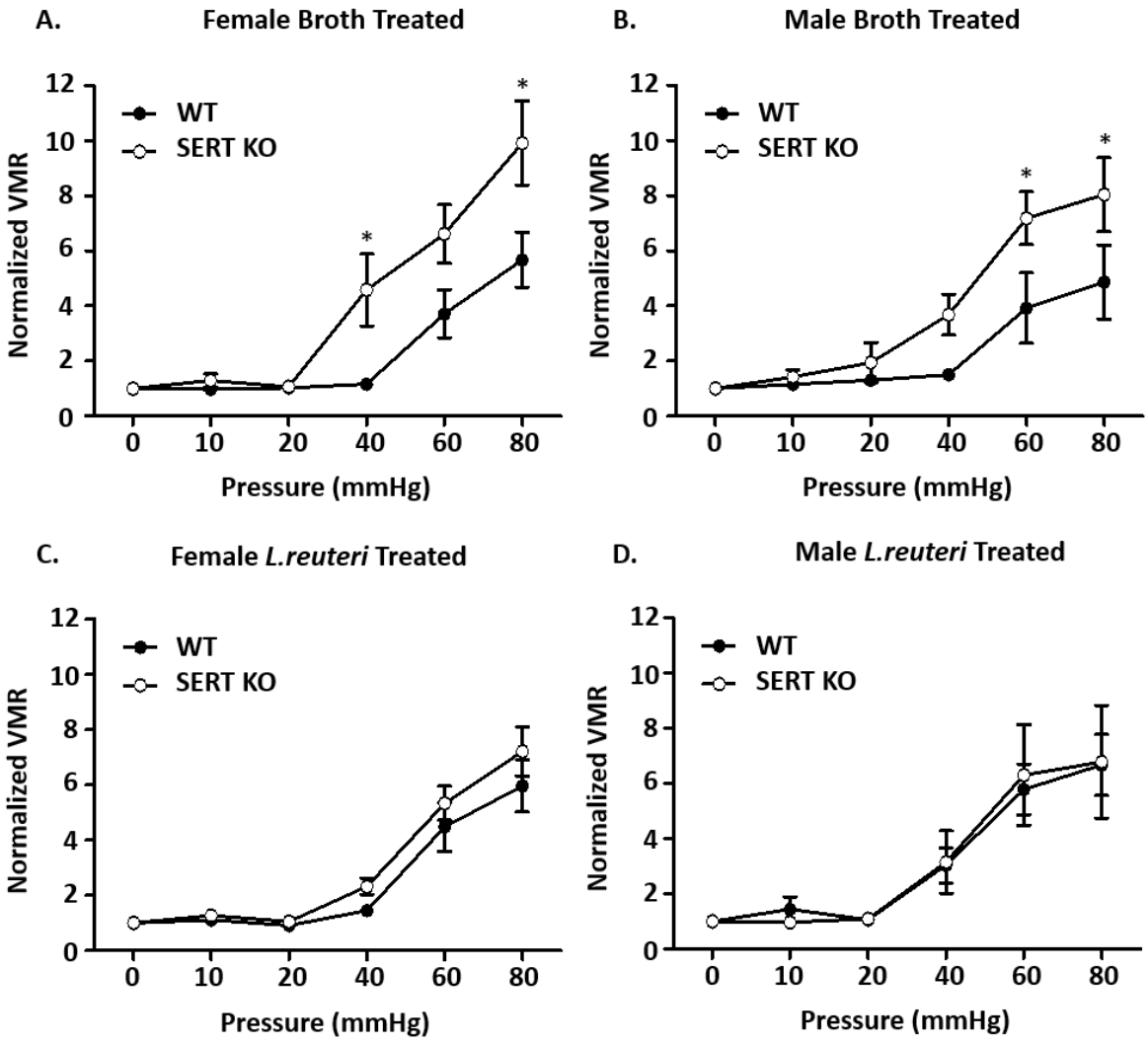
Table 2.2 Animal daily food intake (g) for the duration of the study. Data are mean ± S.E.M. n = 8 per group, *p<0.05 **p<0.01 compared to WT broth; ##p<0.01 compared to SERT KO broth.

	WT Broth	**WT *L.reuteri***	**SERT KO Broth**	**SERT KO *L.reuteri***
Males	20.9 ± 0.9	19.0 ± 1.3	17.5 ± 0.5**	17.2 ± 0.4**
Females	13.5 ± 0.5	12.8 ± 0.3	11.1 ± 0.4*	12.7 ± 0.4##

Both male and female SERT KO rats (broth-treated) exhibited allodynia – a painful response to an innocuous stimulus at a pressure of 40 mmHg as indicated by an increase in the normalized VMR compared to WT rats. Likewise, both male and female SERT KO rats (broth-treated) exhibited visceral hypersensitivity – an exaggerated response to a painful stimulus at pressures of 60 and 80 mmHg as indicated by an increase in the normalized VMR compared to WT rats. The magnitude of the visceral hypersensitivity response was similar in males and females (Figure 2.2A and B).

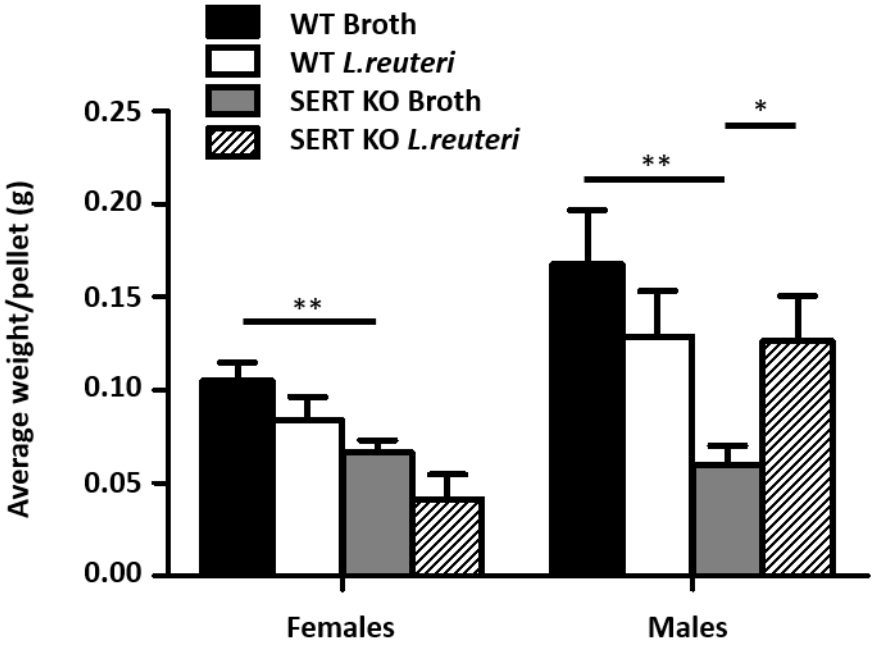
L.reuteri treatment eliminated the difference in visceral hypersensitivity between WT and SERT KO rats, males and females (Figure 2.2C and D). *L.reuteri* treatment lowered visceral hypersensitivity in female SERT KO rats without changing visceral hypersensitivity in female WT rats. *L.reuteri* treatment lowered visceral hypersensitivity in male SERT KO rats but it also slightly raised visceral hypersensitivity in male WT rats thus shifting the two curves towards each other. There were no differences in colonic compliance between the groups.

Figure 2.2 Visceromotor response (VMR) to colorectal balloon distension (CRD). Female WT and SERT KO rats (A) broth and (C) *L.reuteri*-treated. Male WT and SERT KO rats (B) broth and (D) *L.reuteri*-treated. There is an upward shift in the curve in SERT KO females and males compared to their WT counterparts. *L.reuteri* treatment eliminates that difference. Data are mean ± S.E.M. n = 8 per group, *p<0.05.



Male SERT KO rats (broth-treated) had a significantly lower number of fecal pellets per day, fecal wet weight per day and weight/pellet compared to WT rats. *L.reuteri* treatment increased all three variables. There were no differences in fecal output between the WT broth and *L.reuteri*-treated rats. Female SERT KO rats (broth-treated) had significantly lower fecal pellet wet weight per day and weight/pellet compared to WT rats with no differences in fecal pellet number per day. *L.reuteri* treatment had no effects on female SERT KO rats' fecal output. There were no differences in fecal output between the WT broth and *L.reuteri*-treated rats (Figure 2.3).

Figure 2.3 Fecal output: average wet weight per fecal pellet. Both male and female SERT KO rats broth-treated exhibit significantly lower fecal output compared to WT rats. *L.reuteri* treatment increases fecal output in male SERT KO rats with no effects in female SERT KO rats. Data are mean ± S.E.M. n = 8 per group, *p<0.05 **p<0.01.



Overall, there were no significant differences in the strength of the longitudinal muscle contraction in intact pieces of colon induced by either electrical field stimulation or acetylcholine across genotypes (SERT KO vs. WT) and treatment groups (*L.reuteri* vs. broth) within the same sex (M or F) (Figures 2.4 and 2.5). The strength of the muscle contraction induced by EFS at frequencies of 5, 10 and 20 Hz was higher in females compared to males (broth-treated) (Figure 2.4A and B). Female SERT KO *L.reuteri*-treated group had a significantly higher strength of the muscle contraction induced by EFS at frequencies of 10 and 20 Hz compared to WT rats (Figure 2.4C). The longitudinal muscle responses induced by ACh were similar in males and females (Figure 2.5A and B). Female SERT KO *L.reuteri*-treated group had a significantly higher strength of the muscle contraction induced by 30 µM ACh compared to the WT group (Figure 2.5C). 30 µM is the highest concentration on the dose-response curve and it is well outside the physiological range therefore a response at such a dose is not reliable.

Figure 2.4 Electrical field stimulation (EFS) of distal colon. Female WT and SERT KO rats (A) broth and (C) *L.reuteri*-treated. Male WT and SERT KO rats (B) broth and (D) *L.reuteri*-treated. Overall, there are no significant differences between the groups. Data are mean ± S.E.M. n = 8 per group, *p<0.05 **p<0.01.

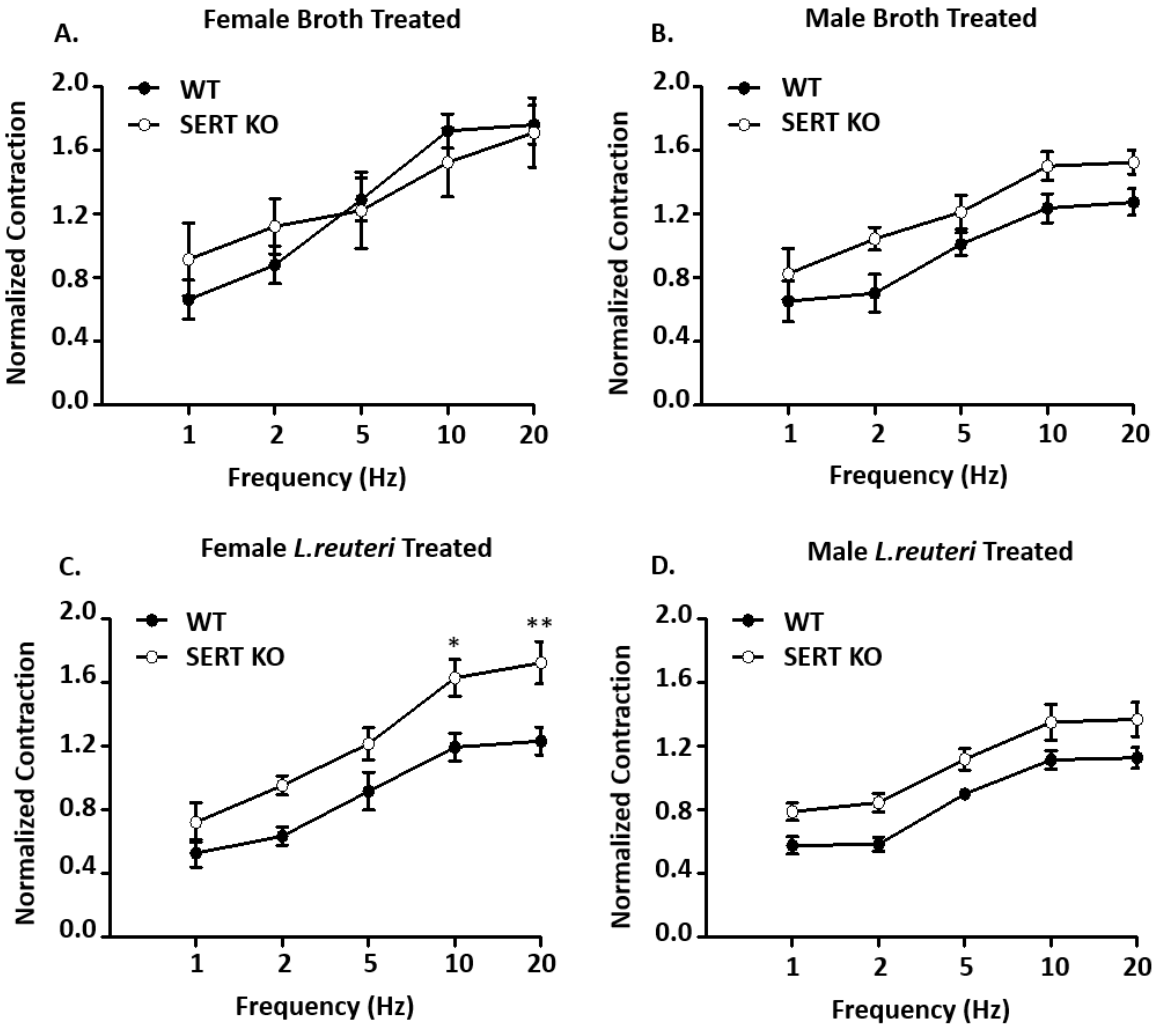
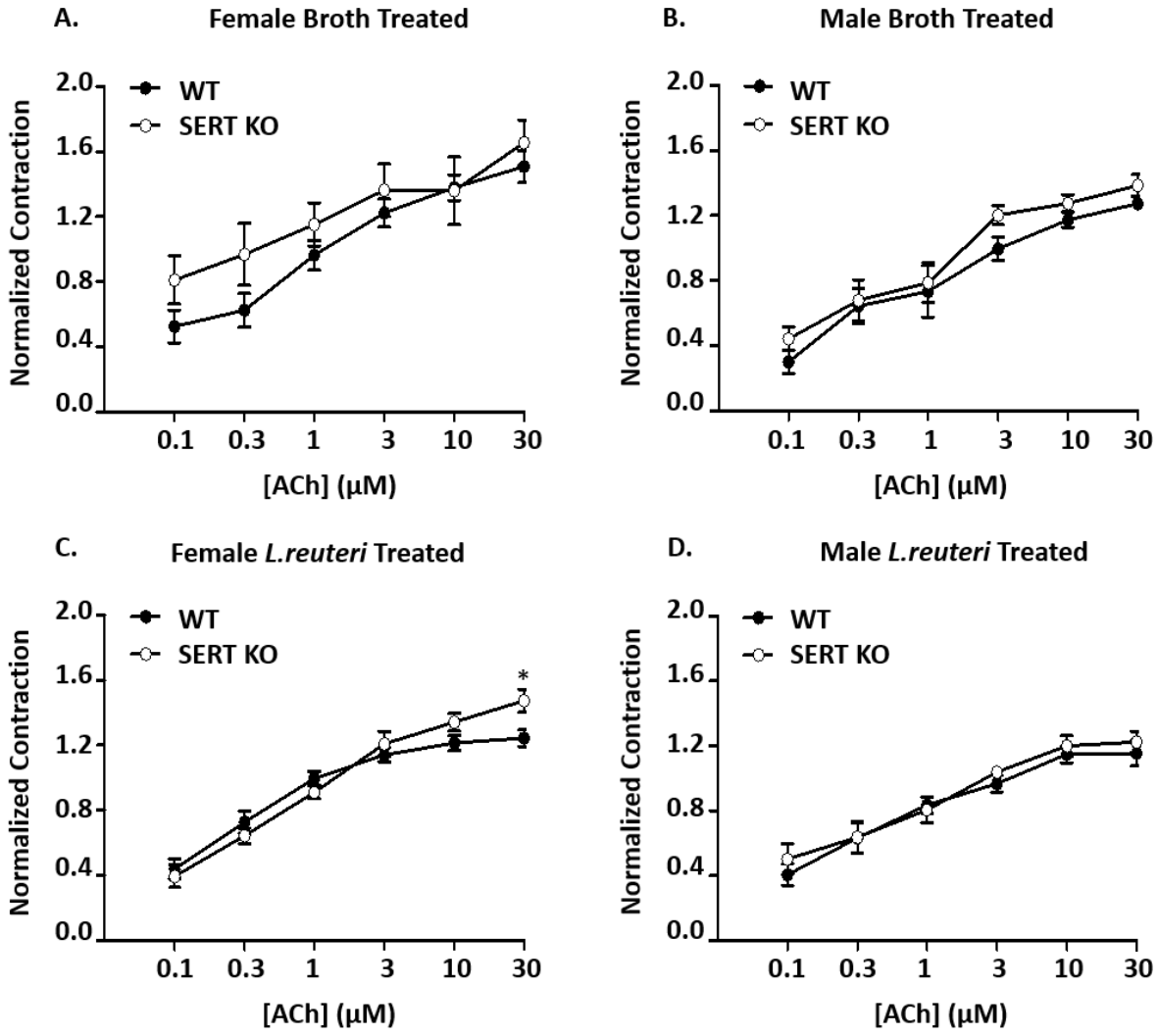


Figure 2.5 Acetylcholine (ACh) stimulation of distal colon. Female WT and SERT KO rats (A) broth and (C) *L.reuteri*-treated. Male WT and SERT KO rats (B) broth and (D) *L.reuteri*-treated. Overall, there are no significant differences between the groups. Data are mean ± S.E.M. n = 8 per group, *p<0.05.



2.5 Discussion

Gavage is an efficient method for treatment delivery because it allows us to administer a fixed amount of *L.reuteri* or broth, at a fixed time each day, while imparting little stress on the animal. On average, all groups lost 1-3% of their body weight during the course of treatment and 5-8% of their body weight following surgery to implant electrodes onto their abdominal muscles. While the small weight decrease during the course of treatment is not significant, the weight decrease following surgery is significant and expected, since following surgery the animals' abdominal muscles are sore and eating exacerbates pain.

Male SERT KO rats had significantly less food intake and lower body weight compared to WT rats, regardless of treatment. This indicates that, in males: 1) *L.reuteri* treatment has no effects on food intake and 2) SERT KO rats weigh less because they consume less food. Male SERT KO broth-treated rats also had decreased fecal output, as measured by fecal pellet number and wet weight, compared to WT rats. *L.reuteri* treatment increased fecal output in male SERT KO rats without affecting their food intake or weight compared to broth-treated SERT KO rats thus indicating that *L.reuteri* increased motility in male SERT KO rats. We also looked at the strength of the longitudinal muscle contraction and found no differences across any of the groups. Other factors such as an increase in the number of contractions or circular muscle contractility could account for increased motility following *L.reuteri* treatment in male rats.

On the other hand, there were no differences in body weight between the female groups and only the SERT KO broth-treated group had significantly decreased food intake. In females, *L.reuteri* treatment had no effects on food intake in the WT groups. However, female *L.reuteri*-treated SERT KO rats consumed significantly more food than broth-treated SERT KO rats even

though their weight did not change. Also, female SERT KO broth-treated rats had decreased fecal output compared to WT rats. *L.reuteri* treatment had no effects on fecal output in females. Since the female SERT KO *L.reuteri*-treated rats had increased food intake compared to SERT KO broth-treated rats, yet their weight and fecal output were the same, this indicates that female SERT KO rats have decreased motility and *L.reuteri* treatment does not affect gut motility in female rats. There were no differences in longitudinal muscle contractility between any of the groups.

Other factors that play a role in the energy balance that we did not measure are energy expenditure (exercise) and gut microbiota composition. While the animals are bred and housed in the same facility as well as fed the same diet, inherent differences in their genetics could contribute to differences in their microbiota composition. Also, SERT KO rats exhibit anxiety and depressive-like states which affects their energy expenditure and their responses to treatment (Olivier *et al*., 2008). Hormones could play a role in the differential effects of *L.reuteri* on gut motility in male and female rats (Chang and Heitkemper, 2002).

A study in SERT KO mice by Chen *et al*., 2001 also showed altered gut motility. In their study, however, SERT KO mice alternated irregularly between episodes of diarrhea and constipation as assessed by the intestinal transit rate and water in their stool. Chen *et al*., 2001, employed an indirect method of assessing gut motility by placing a pellet into the rectum of lightly anesthetized mice and measuring time to expulsion. They also measured fecal output over a period of 24 h. In our study, we found decreased fecal output in SERT KO rats with no changes in longitudinal muscle contractility. To assess motility, we measured fecal output over a period of 2 weeks for 2 h each day. We also used an organ bath setup to measure the contractility of

colonic longitudinal muscle. Our differential results could be produced by differences in the generation of the two knockout models or by differences in methodology.

A study by Wang *et al*., 2010a showed that *L.reuteri* decreases jejunal motility in an *in vitro* model within minutes of application. Our results indicate that chronic ingestion of *L.reuteri* has no effects on colonic longitudinal muscle contractility. Another study by Wang *et al*., 2010b showed that chronic ingestion of *L.reuteri* inhibits colonic circular muscle contractions in male Sprague-Dawley rats *in vitro*. We can conclude that *L.reuteri* exerts differential effects in acute vs. chronic models and on different parts of the GI tract. Also, different strains of *L.reuteri* can produce differential effects.

A previous study in our lab showed that 3-4 month old female, but not male SERT KO rats, exhibit increased visceral hypersensitivity compared to their WT counterparts (Galligan *et al*., 2013). We confirmed that finding in 7-8 month old females only. The normalized VMR in 3-4 month old and 7-8 month old female rats was similar. Both SERT KO groups exhibited allodynia at 40 mmHg followed by visceral hypersensitivity at 60 and 80 mmHg. Colorectal balloon distention at low levels of pressure activates Aβ fibers that transmit mechanosensitivity. Allodynia occurs when Aβ fibers activate dorsal horn neurons in the spinal cord that are part of the pain pathway (Costigan and Woolf, 2000). Once that occurs, non-noxious stimuli get misinterpreted as noxious. Hyperalgesia, on the other hand, occurs when the pain signal, transmitted by Aδ and C fibers is amplified as a result of sensitization in the pain pathway.

We also showed an age-dependent change in the sex differential. Unlike the 3-4 month old male SERT KO rats, 7-8 month old male SERT KO rats showed increased visceral hypersensitivity compared to WT males. This is a novel finding indicating that while female SERT

KO rats exhibit increased visceral hypersensitivity from early on, that symptom only develops in males with increasing age.

These results mimic what is seen in humans suffering from IBS. In humans, visceral hypersensitivity is measured in a similar way as in rodents. A balloon is inserted into the rectum, inflated in set pressure increments, and patients are asked to rate their pain on a scale. IBS patients experience pain and discomfort in the rectal area at lower levels of pressure and at a higher intensity compared to healthy controls (Bouin *et al*., 2002). Furthermore, female IBS patients exhibit lower pain thresholds compared to male IBS patients and healthy controls (Chang *et al.,* 2006).

IBS is twice as prevalent in women as men and estrogen plays a role in it. Women tend to develop it with the onset of menstruation and the symptoms go away following menopause. Also, during menses, women with IBS have more exacerbated symptomology (Houghton *et al*, 2002). Lu *et al*., 2007 showed that in rats, ovariectomy lowers inflammation-induced visceral hypersensitivity and a single injection of estrogen restores it within 2 h. Furthermore, a previous study from our lab showed that visceral hypersensitivity in female SERT KO rats varies with the estrous cycle (Galligan *et al*., 2013). 3-4 month old female SERT KO rats had the highest VMR during proestrous when estrogen levels peak. Combined with elevated extracellular 5-HT levels in SERT KO rats, this indicates that a combination of estrogen and altered serotonin signaling plays a role in the early development of visceral hypersensitivity in females. 3-4 month old male SERT KO rats also had elevated extracellular 5-HT levels, but did not exhibit visceral hypersensitivity. Furthermore, studies have shown expression of estrogen receptors α (ERα) and β (ERβ) in dorsal root ganglia (DRG) neurons which transmit visceral pain information to the brain

(Chaban *et al*., 2003). In addition, 17β-estradiol (E_2) has been shown to alter ion channel opening, G-protein coupling and signal transduction pathways leading to the conclusion that estrogen plays an important role in modulating pain sensitivity in females (Levin, 2002).

On the other hand, testosterone has been shown to have a protective effect on nociception in males (Craft, 2007). As testosterone decreases with age, so could its protective effects. Another factor contributing to the age-related changes in visceral hypersensitivity in males could be changes in gut microbiota. Studies have shown that aging leads to a change in the number and composition of intestinal bacteria but the results seem to vary from study to study (Tiihonen *et al*., 2010). Aging also leads to a weakened immune system (Gill *et al.,* 2001).

L.reuteri exerted an anti-nociceptive effect in both male and female SERT KO rats. *L.reuteri* eliminated the difference in visceral hypersensitivity between SERT KO and WT males and females. Likewise, probiotics have been shown to decrease abdominal pain and bloating in IBS patients (Brenner *et al*., 2009).

L.reuteri decreased visceral hypersensitivity in female SERT KO rats to the level of WT rats. Interestingly, the effect was different in males. *L.reuteri* decreased visceral hypersensitivity in SERT KO males but it also slightly increased visceral sensitivity in WT males thus shifting the two curves towards each other. It is possible that two mechanisms are at play. *L.reuteri* could lower visceral hypersensitivity in female SERT KO rats by acting at sensory nerve endings in the gut mucosa and diminishing their sensitivity with no effects on motility. On the other hand, *L.reuteri* could be lowering visceral hypersensitivity in male SERT KO rats by increasing motility and relieving constipation.

Furthermore, there were no changes in colonic compliance between genotypes (SERT KO and WT) within the same treatment group (*L.reuteri* or broth) and across sexes (M or F) indicating that differences in visceral hypersensitivity are not due to differences in colonic compliance. Colonic compliance refers to the ability of the gut wall muscle to yield to changes in pressure. A lack of differences in colonic compliance across groups indicates that visceral hypersensitivity is a result of abnormal sensory neurophysiology not a mechanical factor. Previous studies have shown that patients with IBS exhibit similar colonic compliance as healthy controls (Bradette *et al*., 1994).

Overall, we can conclude that the SERT KO rat is a good animal model of IBS. Both male and female SERT KO rats exhibit increased visceral hypersensitivity and decreased gut motility, symptoms characteristic of IBS patients. Furthermore, SERT KO rats exhibit anxiety and depressive-like behaviors, two conditions that share comorbidity with IBS in humans. *L.reuteri* decreases visceral hypersensitivity in both male and female SERT KO rats and increases gut motility in male SERT KO rats. These results show promise for the use of *L.reuteri* 6475 in alleviating IBS symptoms.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ait-Belgnaoui A, Han W, Lamine F, Eutamene H, Fioramonti J, Bueno L, Theodorou V. Lactobacillus farciminis treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. Gut. (2006) 55: 1090-4.

Bertrand PP, Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. Autonomic Neuroscience: basic and clinical. (2010) 153: 47-57.

Bouin M, Plourde V, Boivin M, Riberdy M, Lupien F, Laganière M, Verrier P, Poitras P. Rectal distention testing in patients with irritable bowel syndrome: sensitivity, specificity, and predictive values of pain sensory thresholds. Gastroenterology. (2002) 122: 1771-7.

Bradette M, Delvaux M, Staumont G, Fioramonti J, Bueno L, Frexinos J. Evaluation of colonic sensory thresholds in IBS patients using a barostat. Definition of optimal conditions and comparison with healthy subjects. Digestive diseases and sciences. (1994) 39: 449-57.

Brenner DM, Moeller MJ, Chey WD, Schoenfeld PS. The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. The American journal of gastroenterology. (2009) 104: 1033-49.

Chaban VV, Mayer EA, Ennes HS, Micevych PE. Estradiol inhibits atp-induced intracellular calcium concentration increase in dorsal root ganglia neurons. Neuroscience. (2003) 118: 941-8.

Chang L, Heitkemper MM. Gender differences in irritable bowel syndrome. Gastroenterology. (2002) 123: 1686-1701.

Chang L, Mayer EA, Labus JS, Schmulson M, Lee OY, Olivas TI, Stains J, Naliboff BD. Effect of sex on perception of rectosigmoid stimuli in irritable bowel syndrome. American journal of physiology. Regulatory, integrative and comparative physiology. (2006) 291: R277-84.

Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. The Journal of neuroscience. (2001) 21: 6348-61.

Costigan M, Woolf CJ. Pain: molecular mechanisms. The journal of pain. (2000) 1: 35-44.

Craft RM. Modulation of pain by estrogens. Pain. (2007) 132: S3-12.

Cremon C, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, Corinaldesi R, Barbara G. Intestinal serotonin release, sensory neuron

activation, and abdominal pain in irritable bowel syndrome. The American journal of gastroenterology. (2011) 106: 1290-8.

Galligan JJ, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM. Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. Neurogastroenterology and motility. (2013) 25: e373-81.

Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. (2007) 132: 397-414.

Gill HS, Darragh AJ, Cross ML. Optimizing immunity and gut function in the elderly. The journal of nutrition, health and aging. (2001) 5: 80-91.

Homberg JR, Olivier JD, Smits BM, Mul JD, Mudde J, Verheul M, Nieuwenhuizen OF, Cools AR, Ronken E, Cremers T, Schoffelmeer AN, Ellenbroek BA, Cuppen E. Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience. (2007) 146: 1662-76.

Houghton LA, Lea R, Jackson N, Whorwell PJ. The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers. Gut. (2002) 50: 471-4.

Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, Bienenstock J. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. Journal of cellular and molecular medicine. (2009) 13: 2261-70.

Levin ER. Cellular functions of plasma membrane estrogen receptors. Steroids. (2002) 67: 471-5.

Lu CL, Hsieh JC, Tsaur ML, Huang YH, Wang PS, Wu LL, Liu PY, Chang FY, Lee SD. Estrogen rapidly modulates mustard oil-induced visceral hypersensitivity in conscious female rats: A role of CREB phosphorylation in spinal dorsal horn neurons. American journal of physiology. Gastrointestinal and liver physiology. (2007) 292: G438-46.

McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. Neurogastroenterology and motility. (2010) 22: 1029-35.

Niedzielin K, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. European journal of gastroenterology and hepatology. (2001) 13: 1143-7.

Nobaek S, Johansson ML, Molin G, Ahrné S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. The American journal of gastroenterology. (2000) 95: 1231-8.

Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, Deen PM, Cuppen E, Cools AR, Ellenbroek BA. A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience. (2008) 152: 573-84.

Sinn DH, Song JH, Kim HJ, Lee JH, Son HJ, Chang DK, Kim YH, Kim JJ, Rhee JC, Rhee PL. Therapeutic effect of Lactobacillus acidophilus-SDC 2012, 2013 in patients with irritable bowel syndrome. Digestive diseases and sciences. (2008) 53: 2714-8.

Spiller RC. Role of infection in irritable bowel syndrome. Journal of gastroenterology. (2007) 42: 41-7.

Tiihonen K, Ouwehand AC, Rautonen N. Human intestinal microbiota and healthy ageing. Ageing research reviews. (2010) 9: 107-16.

Wang B, Mao YK, Diorio C, Pasyk M, Wu RY, Bienenstock J, Kunze WA. Luminal administration ex vivo of a live Lactobacillus species moderates mouse jejunal motility within minutes. FASEB journal. (2010a) 24: 4078-88.

Wang B, Mao YK, Diorio C, Wang L, Huizinga JD, Bienenstock J, Kunze W. Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurons. Neurogastroenterology and motility. (2010b) 22: 98-107.

CHAPTER 3

LACTOBACILLUS REUTERI DOES NOT ALTER GUT SEROTONIN AVAILABILITY IN SEROTONIN TRANSPORTER KNOCKOUT (SERT KO) RATS

3.1 Abstract

Gut mucosal serotonin signaling plays an important role in the regulation of intestinal motility, secretions and sensations. When disrupted, it could lead to visceral hypersensitivity and altered gut motility as seen in the SERT KO rat. Inflammation is another factor that leads to visceral hypersensitivity and can disrupt normal gut motility. Probiotics have been shown to decrease inflammation but their effects on gut serotonergic signaling have not been studied. In this study, we determined the effects of chronic ingestion of a probiotic, *Lactobacillus reuteri,* on 5-HT availability and inflammation in the colon of 7-8 month old female, WT and SERT KO rats. Extracellular recordings of local 5-HT concentrations in colonic mucosa preparations maintained *in vitro* were measured as an oxidation current using continuous amperometry with a boron-doped diamond microelectrode. SERT function was assessed by measuring oxidation currents with and without fluoxetine (SERT blocker, 1 μM). Whole-tissue levels of 5-HT and its metabolite, 5-HIAA, were measured using HPLC. Protein expression of SERT, norepinephrine transporter (NET), dopamine transporter (DAT) and organic cation transporter 3 (OCT 3) was quantified using Western Blots. EC and mast cell counts were carried out using immunohistochemistry. A multiplex kit was used to measure levels of pro-inflammatory cytokines: GM-CSF, IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-12 and an anti-inflammatory cytokine: IL-10. There were no significant differences in EC cell number, extracellular 5-HT concentration or whole-tissue levels of 5-HT and 5-HIAA in colonic mucosa of WT and SERT KO rats, *L.reuteri* or broth-treated. Likewise, there were no significant differences in protein expression of NET, DAT and OCT 3 across groups. There were no significant differences in mast cell numbers across groups and both WT and SERT KO rats had low levels of inflammatory cytokines in the colonic mucosa before and

after *L.reuteri* treatment. Alterations in 5-HT availability at the colonic mucosa level do not account for the differences in visceral hypersensitivity seen in WT and SERT KO rats. More likely, there are modulations in serotonin receptor expression on nerve endings in the gut mucosa or serotonin signaling at upstream targets in the pain pathway, at the level of the dorsal root ganglion neurons (DRG, spinal cord) or the brain. *L.reuteri* does not alter 5-HT availability at the colonic mucosa level. SERT KO rats do not exhibit a sub-clinical level of inflammation.

3.2 Introduction

5-HT availability is a term that encompasses the production, storage, release and reuptake of this neurotransmitter. 5-HT production and storage primarily contribute to the whole-tissue levels of 5-HT, which can be measured by HPLC, whereas a balance between release and reuptake contributes to the luminal levels of 5-HT, assessed by continuous amperometry. Continuous amperometry is an electrochemical technique that directly measures changes in extracellular 5-HT concentration near EC cells. Continuous amperometry relies on the principle that electro-active molecules get oxidized at the electrode surface and that the oxidation current is directly proportional to the concentration of those molecules (Bertrand *et al*., 2008; Patel *et al*., 2008).

Changes in 5-HT availability, such as the ones that occur in functional gastrointestinal tract disorders, can lead to visceral hypersensitivity and abnormal gut motility. Coates *et al*., 2004 showed decreased mucosal 5-HT levels as well as SERT and TPH1 mRNA, indicating decreased 5-HT production, release and reuptake in rectal biopsy specimens from C-IBS and D-IBS patients. On the other hand, Dunlop et *al*., 2005 reported higher 5-HT levels as well as lower 5-HT turnover in C-IBS patients indicating increased release and impaired reuptake compared to healthy controls. These and other reports demonstrate the importance of serotonin signaling in control of gut functions.

Inflammation of the gut mucosa can also lead to altered gut physiology. Wang *et al*., 2007 reported increased mast cell numbers in the ileum of C-IBS and D-IBS patients compared to healthy controls. Mast cells provide inflammatory surveillance of the gut mucosa and are the first sign of inflammation. Furthermore, the post-infectious IBS subset of patients, who develop IBS

symptoms following infectious gastroenteritis, showed elevated peripheral levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 compared to healthy controls (Liebregts *et al*., 2007). While IBS is not an inflammatory bowel disorder, a sub-clinical level of inflammation may be contributing to symptom development.

Probiotics decrease inflammation. Different strains of *Lactobacillus reuteri* have been specifically shown to up-regulate the production of anti-inflammatory cytokine IL-10 and down-regulate the production of pro-inflammatory cytokine TNFα (Ma *et al*., 2004). However, the probiotic effects on gut serotonin availability are unknown.

The aim of these experiments was to determine the mechanism for the beneficial effects of *Lactobacillus reuteri* on visceral hypersensitivity and gut motility in SERT KO rats by studying the effects of chronic *L.reuteri* treatment on 1) gut serotonin availability and 2) gut inflammation.

3.3 Methods

3.3.1 Animals

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. SERT KO rats are maintained under a license from Genoway Inc. (http://www.genoway.com). Age matched (7-8 ½ month old), female Wistar rats were divided into 4 groups (n = 5-8 per group) based on genotype (WT vs. SERT KO) and treatment (*L.reuteri* vs. broth). All rats were housed individually on a 12 h light/dark cycle with *ad libitum* access to food and water. All rats were administered either *L.reuteri* 6475 (0.5 mL, $5x10^8$ cfu) or acidified MRS media (0.5 mL, with HCl, pH ~4.6, control broth) by gavage daily for 14 days. The control broth was acidified with HCl to match the pH of *L.reuteri*-containing media.

3.3.2 Continuous amperometry

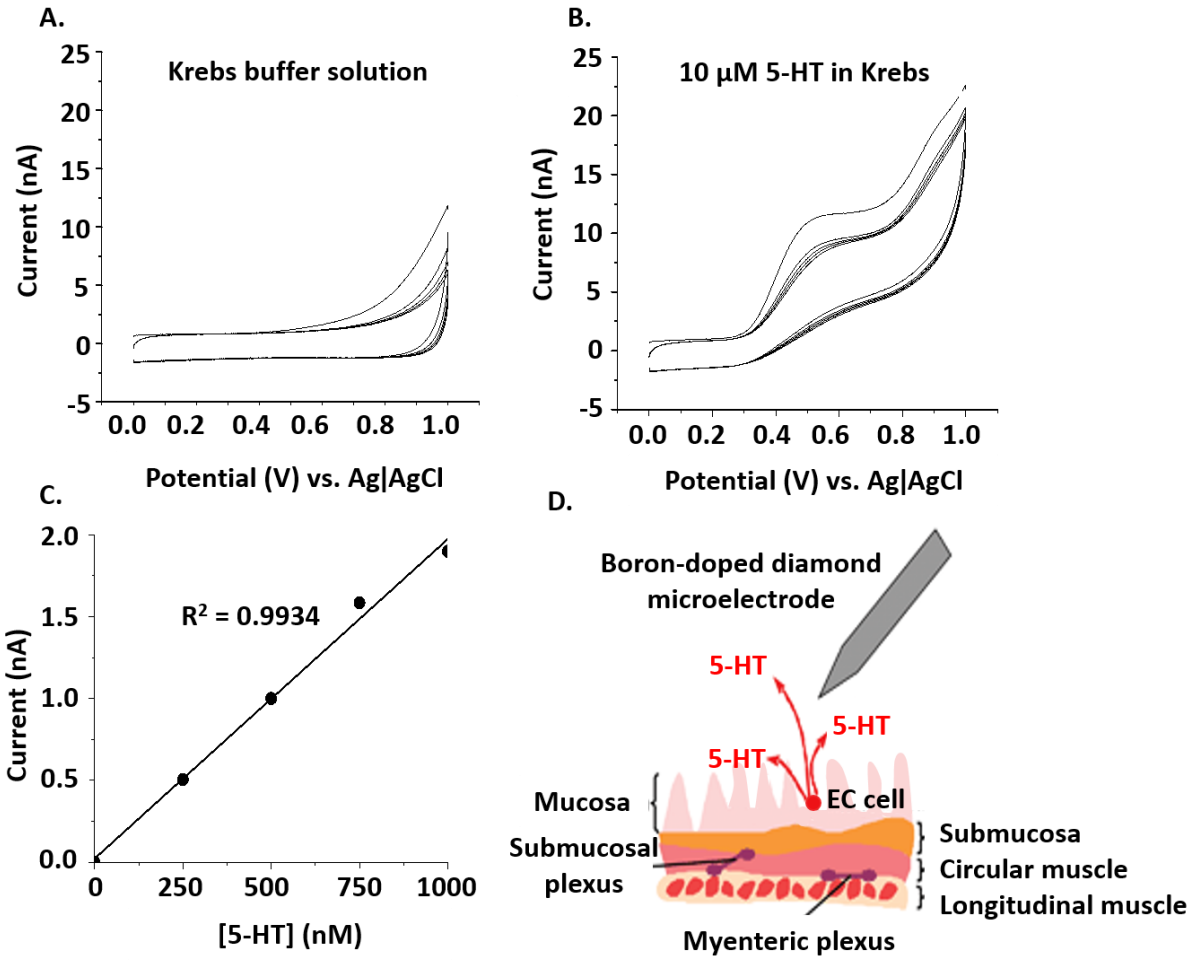
To assess changes in extracellular 5-HT levels in the gut mucosa, at the completion of treatment, rats were euthanized with sodium pentobarbital (50 mg/kg, intraperitonealy). A piece of colon (~1 cm in length) was removed and placed into Krebs buffer solution (in mM: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose; pH 7.4). Continuous amperometry was used to apply a potential of 600-700 mV (vs. a Ag|AgCl reference electrode, 3 M KCl) and measure 5-HT oxidation current. Prior to each tissue experiment, cyclic voltammetry was used to determine the potential at which 5-HT is oxidized for each electrode using Krebs buffer solution (Figure 3.1A) and a standard 10 µM 5-HT solution (Figure 3.1B). The peak current in Figure 3.1B indicates that 5-HT, for that particular electrode, is oxidized at a potential of 600 mV.

Continuous amperometry with boron-doped diamond (BDD) microelectrodes allowed us to directly record local, transient changes in 5-HT concentration near EC cells from *in vitro* preparations of rat colon (Figure 3.1D). Boron-doping imparts electrical conductivity to the diamond microelectrode. Diamond microelectrodes were used because they offer better sensitivity, selectivity and stability over carbon fiber microelectrodes in tissue recordings (Patel *et al*., 2008; Sarada *et al*., 2000).

The setup consisted of a recording chamber positioned on an inverted microscope stage, at one end connected to a gravity flow and at the other end to a suction pump. Oxygenated (95% O_2 + 5% CO_2) Krebs buffer solution, kept at room temperature, flowed through the recording chamber at a rate of 1 mL per min. A piece of colon was pinned down at the bottom of the chamber, mucosal surface up (Figure 3.1D). A BDD microelectrode was placed directly over the tissue with a Ag|AgCl reference electrode and a Pt counter electrode contacting the bath to complete the electrochemical circuit. A constant potential of 600-700 mV versus the Ag|AgCl reference was applied. This potential was selected because it gave the maximum current; a current limited by the mass transport of 5-HT to the electrode surface. The measured current-time profile is a measure of the transient concentration of 5-HT in the extracellular solution surrounding the electrode. 5-HT concentration was sampled at different distances from the mucosa via approach curves. The electrode was first brought in to touch the top of the mucosa, and then retracted to a distance of 2 mm. The electrode was then moved using a micromanipulator in increments of 250 µm per min until it touched the mucosa at which point it was retracted again. This current approach curve was repeated 2 times before the electrode was positioned at a distance of 250 µm and 1 µM fluoxetine was added. Fluoxetine is a selective

serotonin reuptake inhibitor (SSRI) used to study SERT function. A 5-HT calibration curve, constructed from a series of standard 5-HT solutions, was used to convert the recorded 5-HT oxidation current into concentrations (Figure 3.1C). Data were collected and analyzed using a Biostat.

Figure 3.1 Electrode calibration for real-time detection of 5-HT from colonic mucosa of rats. Cyclic voltammetric curves for a diamond microelectrode in (A) Krebs buffer only and (B) 10 µM 5-HT in Krebs buffer. (C) A current-concentration response curve for a series of standard 5-HT solutions. (D) A cartoon depicting how the locally released 5-HT is detected by the diamond microelectrode. EC – enterochromaffin cell.



3.3.3 Western blots for SERT, NET, DAT and OCT 3

3.3.3.1 Tissue processing

Segments of distal colon were removed, pinned out and washed with warm 0.9% NaCl. Mucosal scrapes were collected and snap-frozen in liquid nitrogen. At the time of analysis, the samples were ground-up using a mortar and pestle in 400 µL of Lysis buffer (62.5 mM Tris pH 6.8, 20% SDS, 5 mM EDTA) containing 10 mM NaF, 1 mM Na_3VO_4, and a commercially available protease inhibitor cocktail (Sigma-Aldrich). Tissue was sonicated (3 x 10 s) to break up the cells and then centrifuged at 14,000 rpm for 12 min at room temperature to remove debris. An aliquot of the supernatant was removed for protein analysis. The rest of the sample was added to equal volume of Lamemli buffer with 5% β-mercaptoethanol, boiled for 5 min, aliquoted and frozen at -20^{O}C. These samples were later used for gel electrophoresis.

For protein analysis, a set of protein standards of bovine serum albumin (BSA) in lysis buffer with protease inhibitors was used to create a standard curve. A colorimetric, DC Protein Assay Kit (Bio-Rad) and a spectrophotometer (UV-1201S) were used to measure absorbance and calculate protein levels for each sample.

3.3.3.2 Gel electrophoresis

Samples were loaded into wells and resolved on a 10% Tris/Glycine SDS-PAGE running gel (30% Acrylamide mix, 1.5 M Tris pH 8.8, 10% sodium dodecyl sulfate (SDS), 10% ammonium persulfate (APS), TEMED) with a 4% Tris/Glycine SDS-PAGE stacking gel (30% Acrylamide mix, 1.5 M Tris pH 8.8, 10% SDS, 10% APS, TEMED). The gels were placed into running buffer (Tris-Glycine, 10% SDS). Samples were first run at 50 V for 30 min through the stacking gel, followed by 110 V

for 1 ½ to 2 h through the running gel. Running time depended on the molecular weight of the protein. Samples were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane overnight at 30 V in transfer buffer (Tris-Glycine, MeOH, 10% SDS). Prior to transfer, the PVDF membrane was activated in MeOH for 15 s followed by a rinse in dH_2O. The following day, the membrane was blocked with 10% w/v non-fat dry milk in T-TBS (1% Tween-20, 20 mM Tris pH 7.5, 200 mM NaCl) for 1 h at room temperature. The membrane was then incubated with a primary antibody (for either SERT, NET, DAT or OCT 3) for 2 h at room temperature, washed with T-TBS, and incubated with a horseradish-peroxidase conjugated secondary antibody for 1 h at room temperature. The wash phase consisted of 3 quick washes in distilled water, followed by one 10 min wash and two 5 min washes in T-TBS on the orbital shaker. Following another T-TBS wash, the blot was developed using an ECL Western Blotting Detection Reagent (GE Healthcare). The membrane was incubated for 5 min, placed under a transparency and exposed to film. The film was developed, fixed and rinsed. Different exposure times were used to achieve good band resolution. Densitometry was used to measure relative amounts of membrane proteins, which were normalized to the amounts of a housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The bands were quantified using ImageJ. Table 3.1 lists the primary and secondary antibodies that were used as well as the molecular weight at which the band for each protein was detected.

Table 3.1 List of primary and secondary antibodies used in the Western blots.

Protein	Primary antibody	Secondary antibody	Molecular weight of the band
Serotonin reuptake transporter (SERT)	Goat anti-SERT 1:1000 Santa Cruz, SC1458	Donkey anti-goat 1:2000 Santa Cruz, SC2056	100 kDa
Norepinephrine transporter (NET)	Rabbit anti-NET 1:4000 Alpha Diagnostic, NET11	Donkey anti-rabbit 1:8000 Santa Cruz, SC2313	54 kDa
Dopamine transporter (DAT)	Rabbit anti-DAT 1:4000 Millipore, AB2231	Donkey anti-rabbit 1:8000 Santa Cruz, SC2313	60 kDa
Organic cation transporter 3 (OCT 3)	Rabbit anti-OCT 3 1:1000 Sigma-Aldrich, AV44026	Donkey anti-rabbit 1:8000 Santa Cruz, SC2313	70 kDa
GAPDH	Mouse anti-GAPDH 1:16,000 Santa Cruz, SC32233	Goat anti-mouse 1:24,000 Santa Cruz, SC2005	37 kDa

3.3.4 HPLC for 5-HT and 5-HIAA

3.3.4.1 Tissue processing

Segments of distal colon were removed, pinned out and washed with warm 0.9% NaCl. Mucosal scrapes were collected, snap-frozen in liquid nitrogen and stored at -80^{O}C until analysis time. Tissue was thawed, weighed, and 500 µL of ice-cold 0.1 M perchloric acid was added to each sample. Perchloric acid denatures and precipitates proteins. Tissue was then sonicated for 5 s, centrifuged at 14,000 rpm for 10 min at 4^{O}C, and the supernatant removed for HPLC analysis. The remaining pellet was used for measurement of the protein concentration.

The pellet was dissolved in 500 µL of Lysis buffer (62.5 mM Tris pH 6.8, 20% SDS, 5 mM EDTA) containing 10 mM NaF, 1 mM Na_3VO_4, and a commercially available protease inhibitor

cocktail (Sigma-Aldrich). It was then sonicated (3 x 10 s), centrifuged at 14,000 rpm for 12 min at room temperature, and an aliquot was used for protein measurement.

For protein analysis, a set of protein standards of BSA in lysis buffer with protease inhibitors was used to create a standard curve. A colorimetric, DC Protein Assay Kit (Bio-Rad) and a spectrophotometer (UV-1201S) were used to measure absorbance and calculate protein levels for each sample.

3.3.4.2 HPLC

HPLC was carried out using a commercial system (ESA Biosciences, Chelmsford, MA, now Thermo-Fisher) that consisted of a solvent delivery module (model 584), an autosampler (model 542) with sample cooling to 4^{O}C and a coulometric detector (CoulArray detector, ESA Biosciences, now Dionex). Detection was performed at 200 mV relative to in internal Pd quasi-reference electrode. An MD-150 reverse phase HPLC column was used for the separation. The mobile phase consisted of 90 mM phosphate buffer, 50 mM citrate, 50 µM EDTA and 1.9 mM sodium octyl sulfate with 9.2% acetonitrile. A set of 5-HT and 5-HIAA standards were run daily for standard curve generation.

3.3.5 EC and mast cell counts

3.3.5.1 Immunohistochemistry

Segments of distal colon were removed, pinned out, and fixed in 10% neutral buffered formalin overnight at 4^{O}C. The following day, samples were placed in cassettes, soaked in 40-70% EtOH, and sent to the Investigative Histopathology Laboratory (Michigan State University) for processing. Samples were embedded in paraffin, cross-sections were cut on a rotary microtome

at 4-5 µm, and placed on slides. The slides were coated with 2% 3-Aminopropyltriethoxysilane and dried at 56^{O}C overnight. Sections were deparaffinized in Xylene followed by hydration with graded ethyl alcohol and distilled water. pH adjustment was done by placing the slides in Tris Buffered Saline pH 7.4 (Scytek Labs, Logan, UT) for 5 minutes. Heat pretreatment was used for antigen retrieval. Samples were incubated with Citrate Plus pH 6.0 (Scytek) for 30 minutes in a vegetable steamer at 100^{O}C followed by 10 min at room temperature and then several changes of distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in MeOH (1:4 ratio) for 30 min followed by tap and distilled water rinses. Incubation in normal goat serum (Vector Labs, Burlingame, CA) for 30 min was used to block non-specific staining. Sections were then incubated with Avidin/Biotin blocking system for 15 minutes each (Avidin D, Vector Labs/Biotin D, Sigma, St. Louis, MO). Sections were stained for 1 h with rabbit anti-serotonin primary antibody (S5545, Sigma-Aldrich) in Normal Antibody Diluent (NAD, Scytek) at 1:40,000. Then they were stained for 30 min with biotinylated goat anti-rabbit secondary antibody conjugated to HRP diluted in NAD at 1:136. Nova red (Vector Laboratories) was used to visualize the staining by incubation for 15 min. A light hematoxylin (Thermo Fisher, Kalamazoo, MI) stain was used for background. 15 s of staining were followed by differentiation, dehydration, clearing and mounting with synthetic mounting media.

3.3.5.2 EC and mast cell counts

5-HT containing EC and mast cells were differentiated based on shape and localization in the rat colonic mucosa. Mast cells are located in the lamina propria while EC cells are located in the epithelial layer. EC cells were further classified based on shape as: 1) wineglass – single

process that opens to the lumen, 2) round/closed, 3) multiple – multiple processes, and 4) undefined – cannot be classified into other categories. Pictures were taken at 4x and cells were counted at 40x in single cross-sections for each rat. Data are presented as the number of cells per mm length.

3.3.6 Inflammatory cytokine assay

3.3.6.1 Tissue processing

Segments of distal colon were removed, pinned out and washed with warm 0.9% NaCl. Mucosal scrapes were collected, snap-frozen in liquid nitrogen and stored at -80^{O}C. At the time of analysis, tissues were thawed, weighed, and immersed in 10 µL of NP40 Cell Lysis Buffer (Invitrogen) with 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich) per mg of tissue. Tissue was then sonicated for 5 s, centrifuged at 14,000 rpm for 10 min at 4^{O}C, and the supernatant removed for inflammatory cytokine analysis. An aliquot was separated for measurement of protein concentration.

For protein analysis, a set of protein standards of BSA in lysis buffer with protease inhibitors was used to create a standard curve. A colorimetric, DC Protein Assay Kit (Bio-Rad) and a spectrophotometer (UV-1201S) were used to measure absorbance and calculate protein levels for each sample.

3.3.6.2 Inflammatory cytokine kit

Levels of inflammatory cytokines were measured using a commercially available Rat Cytokine 10-Plex Panel (Invitrogen) and a Luminex 100/200 instrument. The kit uses polystyrene microspheres (beads) to allow for simultaneous, quantitative measurements of the following

inflammatory cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon ɣ (IFN-ɣ), interleukin 1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40/p70), and tumor necrosis factor α (TNF-α).

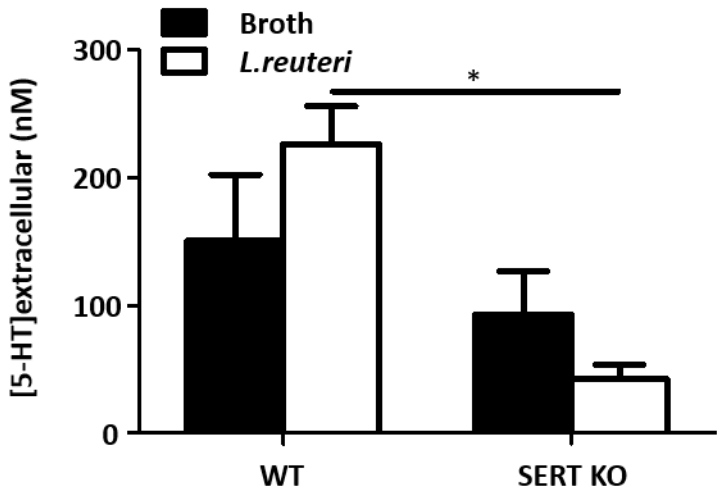
3.3.7 Statistics

All data are presented as mean ± standard error of the mean (S.E.M). Multiple groups were compared using a Two-way ANOVA with or without repeated measures (as experimental design dictated) and Bonferroni *post-hoc* tests to assess statistical significance. When standard deviations of the groups were significantly different from each other, either a log transformation of the data was used or a non-parametric test such as a Kruskall-Wallis ANOVA. Data were analyzed using InStat 3 and Prism 5. Statistical significance was assigned at a p value less than 0.05.

3.4 Results

There were no significant differences in extracellular 5-HT levels, as measured by continuous amperometry, between the WT and SERT KO rats (broth-treated). Likewise, there were no significant differences in extracellular 5-HT levels between the *L.reuteri* and broth-treated groups within the same genotype (SERT KO or WT). However, the WT *L.reuteri*-treated group had a significantly higher extracellular 5-HT level compared to the SERT KO *L.reuteri*-treated group (Figure 3.2).

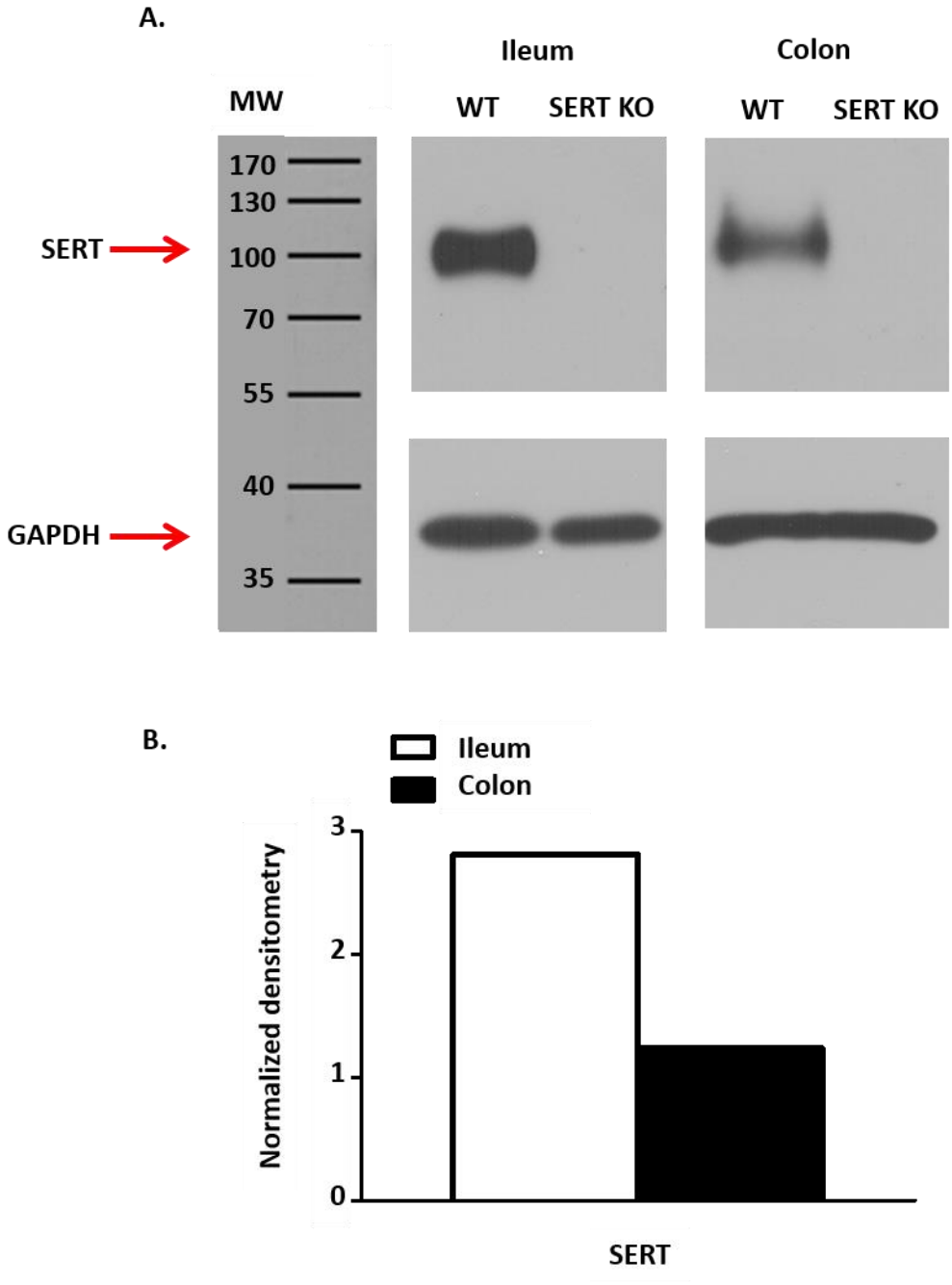
Figure 3.2 Extracellular 5-HT levels in colonic mucosa of female rats. WT *L.reuteri*-treated group has significantly higher extracellular 5-HT level compared to SERT KO *L.reuteri*-treated group. There are no significant differences in extracellular 5-HT levels across any of the other groups. Data are mean ± S.E.M. n = 8 for all groups, *p<0.05.



Using Western blots, we detected SERT as a single band at 100 kDa that is present in the ileum and colon of WT rats and absent in the ileum and colon of SERT KO rats (Figure 3.3)(Chamba *et al*., 2008). We used GAPDH as a housekeeping protein and we detected it as a single band at 37 kDa in both WT and SERT KO rat, ileum and colon. SERT expression in the WT colon was

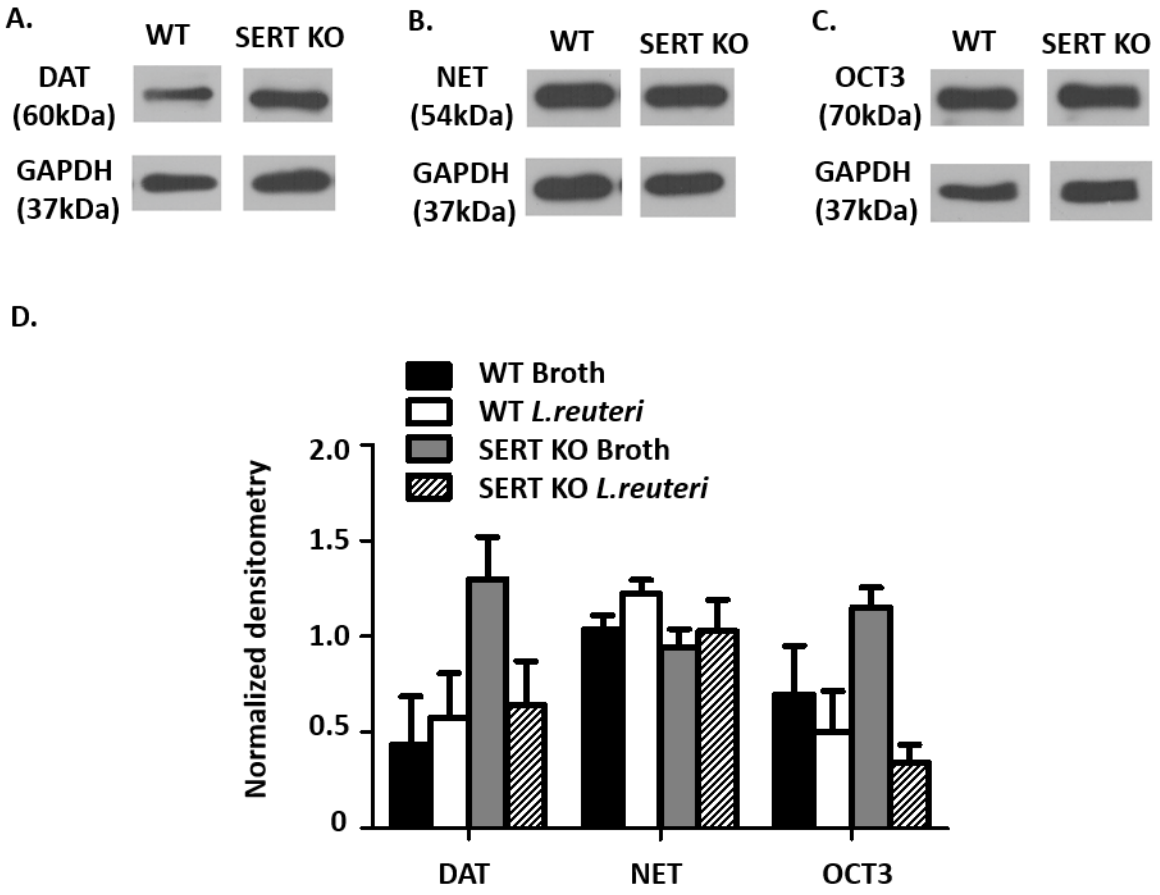
significantly lower than SERT expression in the WT ileum. We loaded 4 times as much protein in the colon just to be able to detect SERT.

Figure 3.3 Western blot for SERT in the ileum and colon of WT and SERT KO rats. (A) SERT is present as a single band at 100 kDa in the ileum and colon of WT rats. SERT is absent in SERT KO rats. (B) SERT expression in the WT colon is significantly lower compared to the WT ileum. GAPDH is used as a housekeeping protein. MW – molecular weight marker.



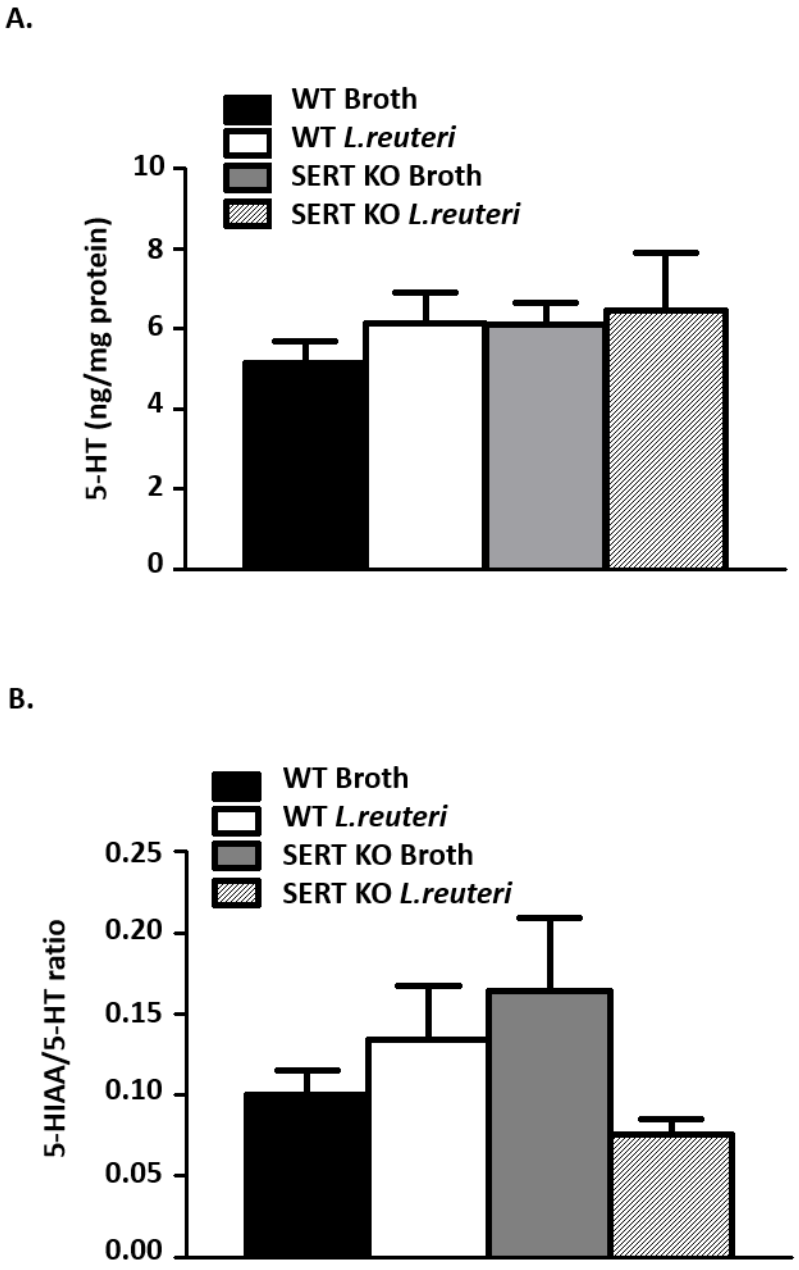
Using Western blots, we detected DAT, NET, and OCT 3 in the colon of both WT and SERT KO rats as bands at 60, 54 and 70 kDa, respectively. We used GAPDH as a housekeeping protein. When quantified using densitometry, there were no significant differences in protein levels of the uptake transporters DAT, NET and OCT 3 across genotypes (SERT KO vs. WT) within the same treatment group or across treatments (*L.reuteri* vs. broth) within the same genotype (Figure 3.4).

Figure 3.4 Protein expression and quantification of DAT, NET and OCT 3 in colonic mucosa of female rats. Representative blots for (A) DAT, (B) NET and (C) OCT 3 in broth-treated WT and SERT KO rats. (D) There are no significant differences in protein expression of DAT, NET and OCT 3 in WT and SERT KO rats, *L.reuteri* and broth-treated. Protein expression of uptake transporters is normalized to a housekeeping protein, GAPDH. Data are mean ± S.E.M. n = 6-8 for all groups.



Likewise, there were no significant differences in whole-tissue levels of 5-HT or the 5-HIAA/5-HT turnover ratio as measured by HPLC (Figure 3.5).

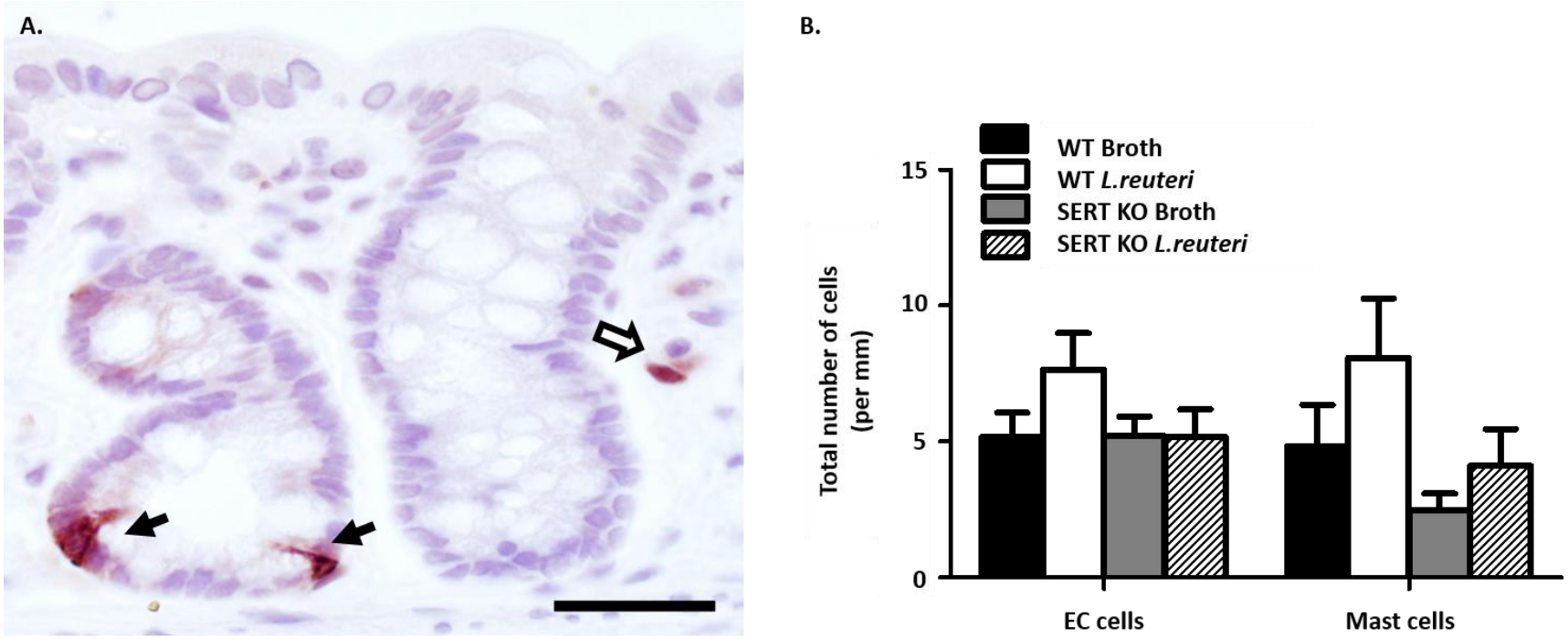
Figure 3.5 Serotonin levels and 5-HIAA/5-HT ratio in colonic mucosa of female rats. (A) There are no significant differences in 5-HT levels in WT and SERT KO rats, *L.reuteri* and broth-treated. (B) There are no significant differences in the 5-HIAA/5-HT ratio in WT and SERT KO rats, *L.reuteri* and broth-treated. Data are mean ± S.E.M. n = 5 for all groups.



We used immunohistochemistry to label and quantify EC and mast cells in rat colonic mucosa. While both cell types contain and release 5-HT and are labelled by the same antibody, they are located in two separate and distinct locations in the colonic mucosa. EC cells are located in the epithelial cell layer while mast cells populate the lamina propria, thus allowing for easy distinction between them. There were no significant differences in the total number of EC or mast cells across any of the groups (Figure 3.6).

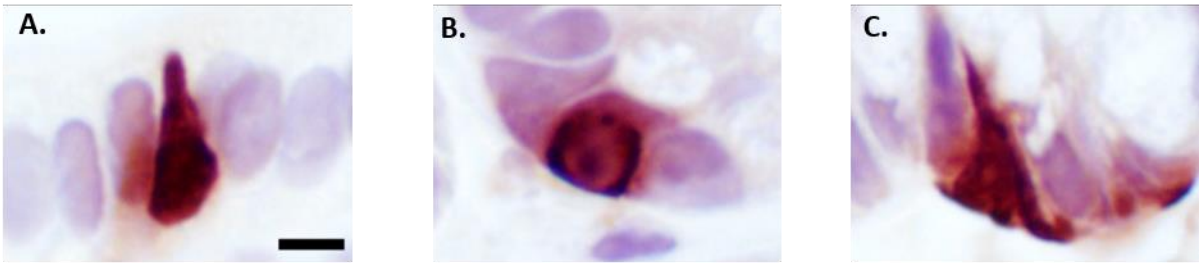
Overall, there were no significant differences in the EC cell subtypes classified based on morphology (Figure 3.7). The only significant difference was in the number of undefined cells between the WT broth and *L.reuteri*-treated groups. Any EC cell that could not be classified based on morphology into the other three categories was classified as undefined. Therefore, any differences across groups in that category do not provide much meaning. The undefined category was created to decrease error in the other groups by trying to classify cells that do not have a distinct shape and to contribute to an accurate total EC cell count.

Figure 3.6 5-HT containing cells in colonic mucosa of female rats. (A) Localization of EC and mast cells. Filled arrow – EC cells; open arrow – mast cell. (B) There are no significant differences in the total number of EC and mast cells in WT and SERT KO rats, *L.reuteri* and broth-treated. Data are mean ± S.E.M. n = 8 for all groups. Scale bar 30µm.



Finally, we used a multiplex kit to measure pro-inflammatory cytokines: GM-CSF, IFN-ɣ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-12 and an anti-inflammatory cytokine: IL-10 in female rat colonic mucosa. Inflammatory cytokine levels were very low in the colonic mucosa and there were no significant differences across groups (Figure 3.8).

Figure 3.7 Morphology and quantification of EC cells in colonic mucosa of female rats. Representative morphologies: (A) Wineglass, (B) Round, (C) Multiple. (D) Overall, there are no significant differences in the number of EC cells based on morphology in WT and SERT KO rats, *L.reuteri* and broth-treated. WT *L.reuteri*-treated group has a significantly higher number of undefined EC cells compared to WT broth-treated group. Data are mean ± S.E.M. n = 8 for all groups. Scale bar 5µm. EC – Enterochromaffin cell. *p<0.05



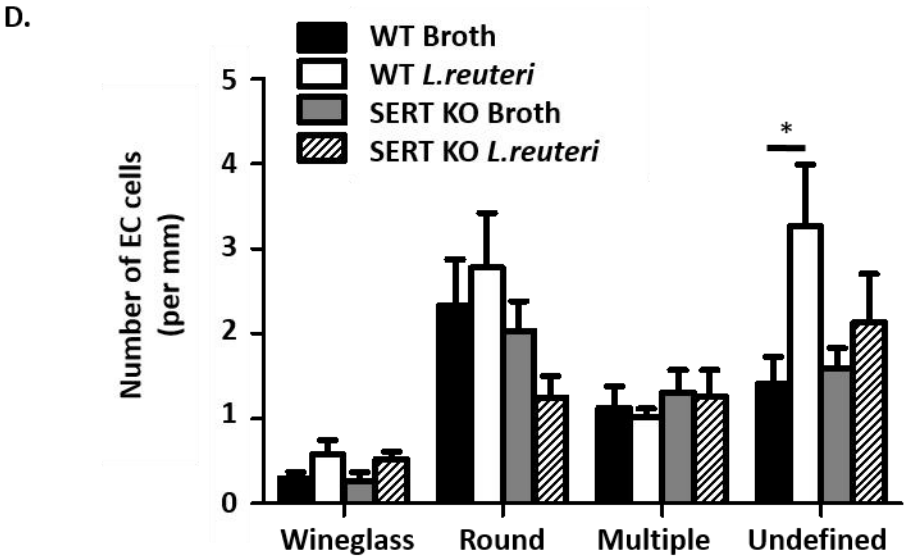
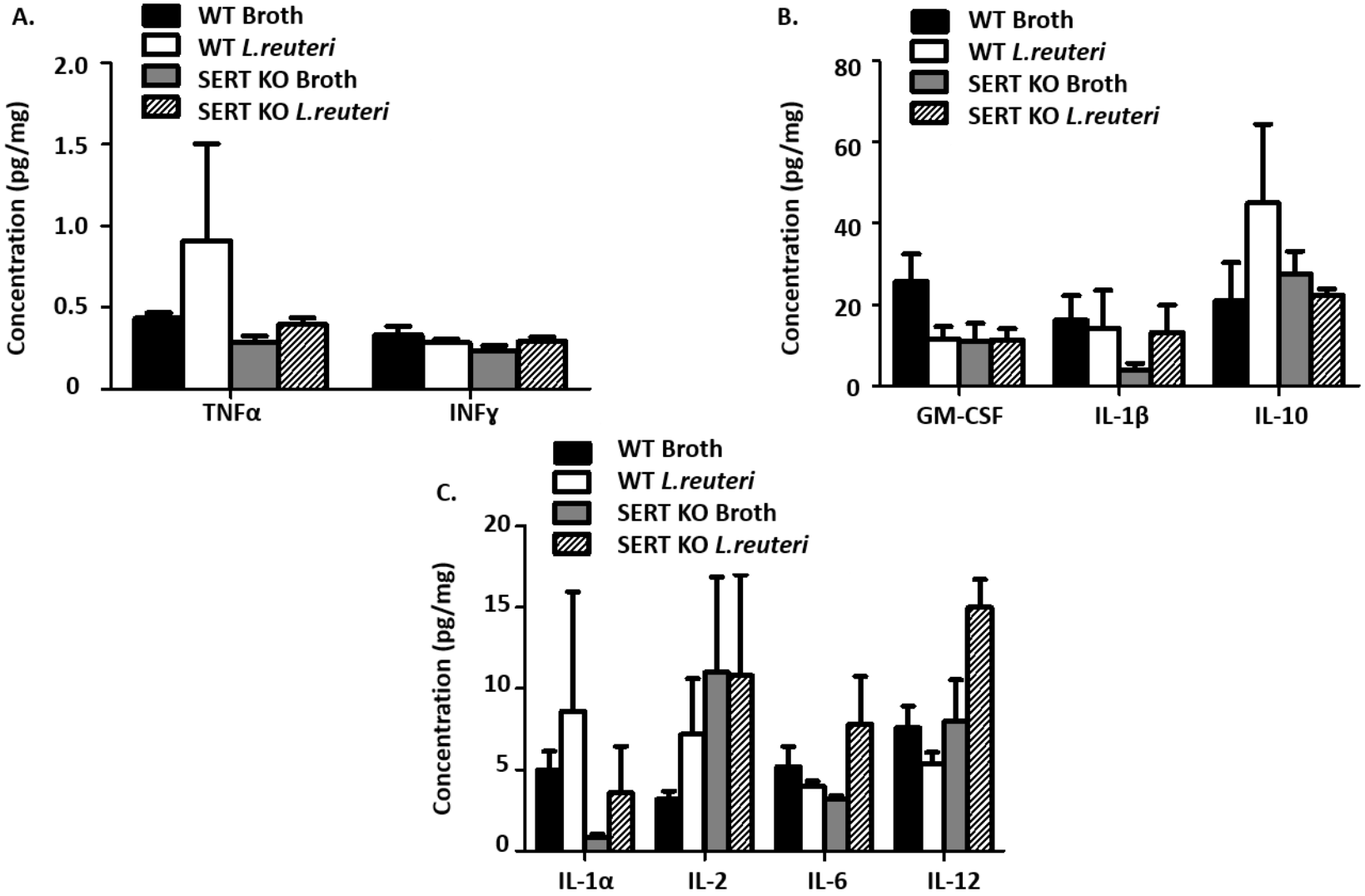


Figure 3.8 Inflammatory cytokine levels in colonic mucosa of female rats. (A) TNFα and INFɣ, (B) GM-CSF, IL-1β and IL-10 and (C) IL-1α, IL-2, IL-6, and IL-12. There are no significant differences in inflammatory cytokine levels in WT and SERT KO rats, *L.reuteri* and broth-treated. IL-4 was not plotted since its levels were very close to zero. Data are mean ± S.E.M. n = 5 for all groups.



3.5 Discussion

5-HT is produced and released by EC cells. Release occurs mainly on the basal side, where 5-HT can bind to nearby sensory nerve endings via 5-HTRs. However, there is also some release on the apical side where 5-HT diffuses into the lumen (Ahlman *et al*., 1981). 5-HT action is terminated by reuptake into the surrounding enterocytes via SERT (Martel *et al*., 2003). SERT has a high-affinity for 5-HT and it can transport it at high-capacity. Within the enterocyte, 5-HT is broken down into 5-HIAA via MAO_A (Rodríguez *et al*., 2001).

We classified EC cells into 3 different types based on morphology: wineglass, round, and multiple. A subset of EC cells that could not be easily classified as either of those categories was classified as undefined. The presence of distinct EC cell types in the rat colon has been previously described and it has been suggested that different types may have different functions but there is no hard evidence to date to support this (Gustafsson *et al*., 2006; Kuramoto *et al*., 2007). Gustaffson *et al*., 2006 developed a novel technique that allowed them to isolate and visualize individual EC cells with their processes still attached. They classified rat colonic EC cells into two distinct subtypes: 1) one with multiple short processes of which one extends to the lumen and is located near glands and 2) one with one or two very long thin basal extensions that contain 5-HT granules. Kuramoto *et al*., 2007 used immunohistochemistry with electron microscopy to label and visualize EC cells in 60-80 µm thick sections of rat distal colon. They confirmed that majority (80%) of colonic EC cells were the "open" type with processes extending into the lumen. Two-thirds of these cells were located in crypts. In our study, we only visualized colonic EC cells with short processes. We used immunohistochemistry on 4-5 µm thick paraffin-embedded sections,

which could account for loss of processes. We did see some 5-HT containing fibers in the lamina propria usually located near the epithelial layer.

Bertrand *et al*., 2012, looked at the morphology and number of EC cells in rat colon using immunohistochemistry on 10-14 µm thick frozen cross-sections. Based on morphology, they classified EC cells into 1) wineglass (single process open to the lumen), 2) single process (closed to the lumen), and 3) multiple processes. They found that only about 10% of colonic EC cells are wineglass-shaped whereas the rest are equally divided between single and multiple types. We also found that wineglass-shaped EC cells comprise the smallest number in the colon of WT and SERT KO rats. EC cells with multiple processes were mostly found in the crypts with one process open to the lumen and the others circling the surrounding enterocytes. A significant proportion of EC cells had a round shape with no apparent processes and a portion of EC cells could not be clearly classified. Overall, there were no differences in the total number of EC cells between WT and SERT KO rats and both groups had the same proportion of cells based on morphology.

Our data indicate that there are no alterations in 5-HT steady-state and luminal levels in 7-8 month old SERT KO rats. We found no differences in whole-tissue or extracellular levels of 5-HT, as measured by HPLC and continuous amperometry, respectively, between WT and SERT KO rats. Lack of differences in whole-tissue levels of 5-HT likely indicates that 5-HT production and storage by EC cells is not altered between the two genotypes. Furthermore, there were no differences in 5-HT turnover as measured by the 5-HIAA/5-HT ratio.

We confirmed a finding from a previous study in our lab that revealed no differences in whole-tissue 5-HT levels or the 5-HT turnover in 3-4 month old SERT KO rats compared to WT rats (Galligan *et al*., 2013). However, in that study, SERT KO rats had significantly higher

extracellular levels of 5-HT compared to WT rats whereas in our study that difference was eliminated. While extracellular 5-HT levels were measured employing the same technique of continuous amperometry in both studies, there was an age difference between the animals. The rats in the Galligan *et al*., 2013 study were 3-4 months old, while the rats in our study were 7-8 months old. It is possible that there is a compensatory mechanism in SERT KO rats that helps clear excess 5-HT and as the animals age, compensatory action plays a bigger role in re-establishing homeostatic levels of 5-HT.

There are numerous examples of compensatory mechanisms in place when the primary mechanism fails. They provide partial to full compensation of function to restore the organisms' viability. Serotonin, dopamine and norepinephrine are monoamine neurotransmitters that have a wide variety of functions in the central and peripheral nervous systems. Their transporters, SERT, DAT and NET have similar structure that allows them to adapt depending on the circumstances (Torres *et al*., 2003). While they each transport their respective neurotransmitter at high-affinity and high-capacity, when concentrations of one of the other neurotransmitters are really high they can pick up the slack by transporting them at low-affinity and low-capacity to restore homeostasis. Not only is there an increase in their function, often times there is up-regulation of expression too.

SERT, NET, DAT, and OCTs are all members of the large solute carrier superfamily of transporters and share similarities in structure and size. They consist of 12 trans-membrane (TM) domains, have intracellular –COOH and $–NH_2$ termini with multiple phosphorylation sites for protein modulation, and have a large hydrophilic extracellular loop between TM 3 and 4 with 3 N-linked glycosylation sites for 1) protein stability, 2) intracellular routing to the plasma

membrane and 3) protection from extracellular proteases (Colucci *et al*., 2008; Eisenhofer, 2001). Similarities in their structure also lead to similarities in their function. SERT, NET, and DAT all have a high-affinity for their respective neurotransmitters; however, they are all capable of transporting other molecules as well. Organic cation transporters mediate non-specific, bi-directional transport of organic molecules (60-350 Da in size) (Jonker and Schinkel, 2004; Koepsell *et al*., 2007). OCT 1 mRNA is expressed in the liver, kidney and intestine. OCT 2 mRNA is predominantly expressed in the kidney and various brain regions that are dopamine (DA) rich. OCT 3 mRNA is highest in the intestine and placenta and intermediate in heart and liver (Sata *et al*., 2005).

A study in SERT KO mice showed the presence of other uptake transporters in the gut, specifically DAT and OCTs, all capable of clearing 5-HT in the absence of SERT (Chen *et al*., 2001). Their study showed DAT immunoreactivity in the submucosal and the myenteric plexus, but not in the mucosa of SERT KO mice. They also demonstrated the presence of OCT 1 and OCT 3 mRNA in the colon of SERT KO mice using RT-PCR. However, DAT, OCT 1 and OCT 3 were also present in the gut of WT mice and without a quantitative technique the study could not determine if these transporters were up-regulated in the absence of SERT.

Furthermore, other studies in the brain have demonstrated increased function of DAT, NET and OCT 3 in the absence of SERT. A study by Zhou *et al*., 2002 showed uptake of 5-HT by dopaminergic neurons of the substantia nigra and ventral tegmental areas in SERT KO mice using immunocytochemistry. This uptake was blocked by a DAT specific antagonist GBR-12935. Furthermore, administration of fluoxetine, a SERT blocker, to WT mice, lead to 5-HT uptake by dopaminergic neurons in the striatum (Zhou *et al*., 2005). Another study by Daws *et al*., 2005

showed uptake of exogenously applied excess 5-HT by neurons in the dorsal raphe nuclei and the hippocampus via both NET and SERT indicating that in special circumstances NET can transport 5-HT at low-affinity but high-capacity. Finally, a study by Baganz *et al*., 2008, showed both an up-regulation of OCT 3 protein expression and an increase in function in the brain of SERT KO mice. The study used chronoamperometry to measure clearance of 5-HT injected into the CA3 region of the hippocampus of WT, heterozygous and homozygous SERT KO mice in the presence and absence of OCT 3 receptor antagonist D22. In WT mice, the clearance of 5-HT was identical before and after D22 injection, indicating that OCT 3 does not contribute to it. In heterozygous and homozygous SERT KO mice, on the other hand, the clearance of 5-HT was prolonged in the presence of D22 indicating that OCT 3 contributes to it. These studies led to two crucial conclusions: in the absence of or decreased functional SERT: 1) a compensatory mechanism tries to re-establish homeostasis by clearing excess 5-HT into surrounding neurons and 2) DAT, NET and OCT 3 can transport 5-HT at low-affinity, yet high-capacity in special circumstances.

In our study, we used Western Blots to determine protein expression levels of SERT, NET, DAT and OCT 3 in the colonic mucosa of SERT KO rats and compare them to WT rats. Their calculated molecular weights are as follows: SERT 70 kDa, NET 70 kDa, DAT 68.8 kDa, and OCT 3 61 kDa. For SERT, we obtained a single band at 100 kDa in WT rats that was absent in SERT KO rats. Numerous studies reported SERT protein expression at a range of molecular weights depending on the species and tissue/cell studied, likely due to post-translational modifications of the protein (Chamba *et al*., 2008). NET is reported in the literature at two molecular weights: 80 kDa, as highly glycosylated and 58 kDa. The 58 kDa NET is a functional, glycosylated form of NET, not a fragment (Brüss *et al*., 1995). We obtained a single band for NET at 54 kDa in both WT

and SERT KO rats. We also obtained a band for DAT at 60 kDa and OCT 3 at 70 kDa, both of which were very close to their calculated molecular weights. We did not look at the expression of OCT 1, also capable of transporting 5-HT in special circumstances and it would be worth to do so in the future (Schmitt *et al*., 2003).

NET, DAT and OCT 3 were all present and robustly expressed in both WT and SERT KO rats. There were no differences in their expression levels between the groups. These data indicate that there is no up-regulation in protein levels of these other reuptake transporters in the absence of SERT. It is still likely thought in SERT KO rats that they are providing a compensatory mechanism by clearing 5-HT. While their expression levels are not up-regulated, their function could be altered. This would account for the same levels of 5-HIAA seen in both WT and SERT KO rats. One way to test that would be to measure ^{3}H-5-HT uptake into enterocytes in the presence of various transporter blockers.

To summarize, in the absence of SERT, 5-HT production and storage remain the same as in WT rats, but extracellular 5-HT levels are initially higher because of decreased uptake. Over time, increase in function of NET, DAT and OCT 3 provides the necessary compensation for 5-HT reuptake that leads to a re-establishment of extracellular 5-HT levels in SERT KO rats to those seen in WT rats. This is indicated by the equivalent 5-HIAA/5-HT ratios across all groups.

In Chapter 2, we showed that 7-8 month old male and female SERT KO rats (broth-treated) exhibit increased visceral hypersensitivity compared to WT rats. We next looked at the effects of *L.reuteri* on gut 5-HT availability and inflammatory markers in SERT KO rats in order to link probiotic effects to possible mechanisms of action.

L.reuteri could affect 5-HT signaling at the colonic mucosa level by 1) altering EC cell number, 2) altering 5-HT production and/or release, and 3) altering reuptake. Our data indicate that *L.reuteri* does not alter serotonin availability in the colonic mucosa since we found no significant differences in EC cell number, extracellular 5-HT from continuous amperometric currents, whole-tissue levels of 5-HT or 5-HIAA nor the protein expression of NET, DAT and OCT 3 between *L.reuteri* and broth-treated groups within the same genotype.

Therefore, alterations in 5-HT availability in the gut do not appear to be linked with the differences in visceral hypersensitivity seen between 7-8 month old WT and SERT KO rats. In other words, *L.reuteri* does not alter serotonin availability in the gut. Alterations in 5-HT signaling at other targets in the pain pathway could account for differences in visceral hypersensitivity. This remains to be investigated. Galligan *et al*., 2013 showed that 3-4 month old female SERT KO rats have increased excitability of colon projecting spinal sensory neurons as measured by whole-cell patch clamp at the level of the dorsal root ganglia. *L.reuteri* could dampen this effect by altering ion channel expression and opening on sensory nerve endings. Other studies have shown that *L.reuteri* ingestion inhibits colorectal distention induced hyper-excitability of colon projecting spinal sensory afferents in healthy rats (Kamiya *et al*., 2006; Ma *et al*., 2009).

Finally, the SERT KO rat does not appear to exhibit a sub-clinical level of inflammation based on the measurement of inflammatory markers selected. Our data indicate there are no differences in mast cell number between WT and SERT KO rats (broth-treated) as well as no differences in inflammatory cytokine levels in both groups, at least the markers that were tested. A substantial body of data exists indicating a mechanism of probiotic action is a decrease in inflammation. For the SERT KO rats, *L.reuteri* treatment had no effect on the levels of the

inflammation markers probed, as the levels were apparently not abnormal. It would be worth investigating if *L.reuteri* treatment decreases inflammation in animal models of inflammatory bowel disease, such as ulcerative colitis. Animal models of chronic intestinal inflammation have been described that resemble aspects of the pathology found in patients with IBS.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ahlman H, Bhargava HN, Dahlström A, Larsson I, Newson B, Pettersson G. On the presence of serotonin in the gut lumen and possible release mechanisms. Acta physiologica Scandinavica. (1981) 112: 263-9.

Baganz NL, Horton RE, Calderon AS, Owens WA, Munn JL, Watts LT, Koldzic-Zivanovic N, Jeske NA, Koek W, Toney GM, Daws LC. Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proceedings of the National Academy of Sciences of the United States of America. (2008) 105: 18976-81.

Bertrand PP, Hu X, Mach J, Bertrand RL. Serotonin (5-HT) release and uptake measured by real-time electrochemical techniques in the rat ileum. American journal of physiology. Gastrointestinal and liver physiology. (2008) 295: G1228-36.

Bertrand RL, Senadheera S, Tanoto A, Tan KL, Howitt L, Chen H, Murphy TV, Sandow SL, Liu L, Bertrand PP. Serotonin availability in rat colon is reduced during a Western diet model of obesity. American journal of physiology. Gastrointestinal and Liver Physiology. (2012) 303: G424-34.

Brüss M, Hammermann R, Brimijoin S, Bönisch H. Antipeptide antibodies confirm the topology of the human norepinephrine transporter. The Journal of biological chemistry. (1995) 270: 9197-201.

Chamba A, Holder MJ, Barnes NM, Gordon J. Characterisation of the endogenous human peripheral serotonin transporter SLC6A4 reveals surface expression without N-glycosylation. Journal of neuroimmunology. (2008) 204: 75-84.

Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. The Journal of neuroscience. (2001) 21: 6348-61.

Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology. (2004) 126: 1657-64.

Colucci R, Blandizzi C, Bellini M, Ghisu N, Tonini M, Del Tacca M. The genetics of the serotonin transporter and irritable bowel syndrome. Trends in molecular medicine. (2008) 14: 295-304.

Daws LC, Montañez S, Owens WA, Gould GG, Frazer A, Toney GM, Gerhardt GA. Transport mechanisms governing serotonin clearance in vivo revealed by high-speed chronoamperometry. Journal of neuroscience methods. (2005) 143: 49-62.

Dunlop SP, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA, Spiller RC. Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. Clinical gastroenterology and hepatology. (2005) 3: 349-57.

Eisenhofer G. The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. Pharmacology and therapeutics. (2001) 91: 35-62.

Galligan JJ, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM. Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. Neurogastroenterology and motility. (2013) 25: e373-81.

Gustafsson BI, Bakke I, Tømmerås K, Waldum HL. A new method for visualization of gut mucosal cells, describing the enterochromaffin cell in the rat gastrointestinal tract. Scandinavian journal of gastroenterology. (2006) 41: 390-5.

Jonker JW, Schinkel AH. Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). The Journal of pharmacology and experimental therapeutics. (2004) 308: 2-9.

Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J. Inhibitory effects of Lactobacillus reuteri on visceral pain induced by colorectal distension in Sprague-Dawley rats. Gut. (2006) 55: 191-6.

Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharmaceutical research. (2007) 24: 1227-51.

Kuramoto H, Kadowaki M, Sakamoto H, Yuasa K, Todo A, Shirai R. Distinct morphology of serotonin-containing enterochromaffin (EC) cells in the rat distal colon. Archives of histology and cytology. (2007) 70: 235-41.

Liebregts T, Adam B, Bredack C, Röth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ, Holtmann G. Immune activation in patients with irritable bowel syndrome. Gastroenterology. (2007) 132: 913-20.

Ma X, Mao YK, Wang B, Huizinga JD, Bienenstock J, Kunze W. Lactobacillus reuteri ingestion prevents hyperexcitability of colonic DRG neurons induced by noxious stimuli. American journal of physiology. Gastrointestinal and liver physiology. (2009) 296: G868-75.

Martel F, Monteiro R, Lemos C. Uptake of serotonin at the apical and basolateral membranes of human intestinal epithelial (Caco-2) cells occurs through the neuronal serotonin transporter (SERT). The Journal of pharmacology and experimental therapeutics. (2003) 306: 355-62.

Patel BA, Galligan JJ, Swain GM, Bian X. Electrochemical monitoring of nitric oxide released by myenteric neurons of the guinea pig ileum. Neurogastroenterology and motility. (2008) 20: 1243-50.

Rodríguez MJ, Saura J, Billett EE, Finch CC, Mahy N. Cellular localization of monoamine oxidase A and B in human tissues outside of the central nervous system. Cell and tissue research. (2001) 304: 215-20.

Sarada BV, Rao TN, Tryk DA, Fujishima A. Electrochemical oxidation of histamine and serotonin at highly boron-doped diamond electrodes. Analytical chemistry. (2000) 72: 1632-8.

Sata R, Ohtani H, Tsujimoto M, Murakami H, Koyabu N, Nakamura T, Uchiumi T, Kuwano M, Nagata H, Tsukimori K, Nakano H, Sawada Y. Functional analysis of organic cation transporter 3 expressed in human placenta. The Journal of pharmacology and experimental therapeutics. (2005) 315: 888-95.

Schmitt A, Mössner R, Gossmann A, Fischer IG, Gorboulev V, Murphy DL, Koepsell H, Lesch KP. Organic cation transporter capable of transporting serotonin is up-regulated in serotonin transporter-deficient mice. Journal of neuroscience research. (2003) 71: 701-9.

Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. Nature reviews. Neuroscience. (2003) 4: 13-25.

Wang SH, Dong L, Luo JY, Gong J, Li L, Lu XL, Han SP. Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome. World journal of gastroenterology. (2007) 13: 6041-7.

Zhou FC, Lesch KP, Murphy DL. Serotonin uptake into dopamine neurons via dopamine transporters: a compensatory alternative. Brain research. (2002) 942: 109-19.

Zhou FM, Liang Y, Salas R, Zhang L, De Biasi M, Dani JA. Corelease of dopamine and serotonin from striatal dopamine terminals. Neuron. (2005) 46: 65-74.

CHAPTER 4

GENERAL DISCUSSION, PERSPECTIVES AND FUTURE DIRECTIONS

4.1 Results summary and importance

4.1.1 SERT KO rat: an animal model of IBS

Our research contributes to the field by characterizing a novel animal model of IBS that allows for studying the origins of visceral hypersensitivity and altered gut motility seen in IBS patients. Our results show that the SERT KO rat exhibits increased visceral hypersensitivity and decreased gut motility compared to the WT rat. Furthermore, we show that the SERT KO rat exhibits sex differences in the development of visceral hypersensitivity that resemble sex differences seen in IBS patients. A previous study in our lab showed that at 3-4 months of age, only female SERT KO rats exhibit increased visceral hypersensitivity compared to WT rats (Galligan *et al*., 2013). We found that at 7-8 months of age, both male and female SERT KO rats exhibit increased visceral hypersensitivity compared to WT rats. This finding resembles what occurs in humans. IBS has an earlier age of onset in women and estrogen contributes to it. In men, testosterone has a protective effect early on which disappears as its levels decrease with age (Craft, 2007).

We also found that the SERT KO rat does not express the SERT protein that is responsible for 5-HT clearance, thus allowing us to study the relationship between altered 5-HT signaling and functional abnormalities in gut motility and visceral sensitivity. A previous study in our lab showed that in 3-4 month old, male and female SERT KO rats, extracellular levels of 5-HT, measured by continuous amperometry, are higher than in WT rats (Galligan *et al*., 2013). However, increased visceral hypersensitivity was only seen in female SERT KO rats, suggesting that a combination of two factors: increased serotonin signaling and estrogen may play a role in the development of early visceral hypersensitivity. The same is true in humans. Women tend to

develop IBS with the onset of menstruation and the symptoms diminish following menopause (Heitkemper and Jarrett, 2008; Lee *et al*., 2001). Furthermore, studies have shown that in women, symptoms are exacerbated during menses when estrogen levels are highest (Houghton *et al*., 2002). Likewise, visceral hypersensitivity in female rats is highest during proestrous when estrogen levels are high (Galligan *et al*., 2013). Our results indicate that in 7-8 month old rats, a compensatory mechanism restores extracellular 5-HT levels in SERT KO rats to those of WT rats. However, both male and female SERT KO rats exhibit increased visceral hypersensitivity indicating that while alterations in serotonin availability in the gut may play a role in the early onset of visceral hypersensitivity, changes in ion channel expression and neuronal firing patterns may be the cause of long-lasting effects (Galligan *et al*., 2013; Holzer *et al*., 2001). Galligan *et al*., 2013 showed that in 3-4 month old female SERT KO rats colon projecting spinal sensory neurons exhibit increased excitability which could account for increased visceral hypersensitivity.

In our study, we explored two possible contributing factors to the development of visceral hypersensitivity and decreased gut motility in SERT KO rats: 1) gut serotonin availability and 2) gut inflammation. We showed that there are no differences in mucosal serotonin availability between WT and SERT KO rats and that SERT KO rats do not have an elevation in mast cell number or inflammatory cytokine levels in the gut mucosa. This however, does not preclude alterations in serotonin signaling as a contributing factor to aberrations of gut motility and visceral sensitivity.

Serotonin plays an important role in pain signaling from the gut to the brain. The SERT KO is a total-body knockout indicating that alterations in serotonin signaling at any stage in the pain transduction pathway could play a role in the establishment of visceral hypersensitivity (Homberg

et al., 2007). SERT KO rats exhibit anxiety and depressive-like states when tested with behavioral assessments indicating alterations in serotonin signaling in the brain (Olivier *et al*., 2008). Likewise, IBS patients develop anxiety and depression that lead to a significant decrease in their quality of life and likely contribute to an exacerbation of their symptoms (Blanchard *et al*., 1990). Alterations in 5-HTR expression on sensory nerve endings or IPANs in the gut mucosa could lead to visceral hypersensitivity and altered gut motility, respectively (Kirkup *et al*., 2001). In addition, there could be alterations in receptor expression, ion channel opening, G-protein coupling and pathway transduction machinery at the level of the spinal cord or the brain leading to amplification of the pain signal and/or altered perception of pain (Anand *et al*., 2007).

4.1.2 *L.reuteri* 6475 shows promise in the alleviation of IBS symptoms

According to definition, probiotics are viable, non-pathogenic bacteria that when ingested in adequate amount confer a health benefit on the host. *L.reuteri* fits that definition and various strains have been shown to 1) decrease abdominal pain in children, 2) reduce infant colic, 3) prevent diarrhea and 4) boost the immune system (Indrio *et al*., 2008; Romano *et al*., 2010; Savino *et al*., 2007; Shornikova *et al*, 1997; Valeur *et al*., 2004). Our data further contribute to the field by showing that *L.reuteri* 6475 reduces visceral hypersensitivity in an animal model of IBS, the SERT KO rat. Our results show that *L.reuteri* lowers visceral hypersensitivity in both male and female SERT KO rats following 14 days of treatment. *L.reuteri* also increases gut motility in male SERT KO rats but has no effects on gut motility in female SERT KO rats.

L.reuteri treatment does not alter gut serotonin availability, mast cell number or inflammatory cytokine levels in SERT KO rats. Most likely, it produces its anti-nociceptive effects

at the level of the colon projecting spinal sensory neurons that transmit pain information to the brain.

Inflammatory marker levels were not abnormal in this animal model so consequently, probiotic treatment had no effect on these levels. Inflammation is likely a contributor to altered 5-HT signaling that is characteristic of IBS. Our results suggest that the effects of probiotics might best be studied in another animal model that is characterized by inflammation.

4.2 General discussion

4.2.1 *L.reuteri* mechanism of action

Probiotics are currently considered dietary supplements. There are multiple genera, species and strains and it has been shown that both live and dead cultures exert beneficial effects (Adams, 2010). Chronic ingestion of probiotics leads to alterations in the intestinal microflora and in disease states can lead to improvement of symptoms. Clinical studies provide strong evidence that probiotics have beneficial effects on inflammatory bowel disorders, irritable bowel syndrome and enteric infections (Quigley, 2011).

In IBS, *Lactobacillus* and *Bifidobacteria* have been shown to help alleviate symptoms of bloating, flatulence, constipation and abdominal pain (Quigley, 2010). *L.reuteri*, in particular, has been shown to colonize the stomach and duodenum of healthy subjects following 28 days of treatment and lead to an increase in B-lymphocytes and CD4+ T lymphocytes (Valeur *et al*., 2004). In Sprague-Dawley rats, *L.reuteri* has been shown to decrease visceral hypersensitivity following colorectal distension (Kamiya, 2006). Results from our study, further contribute to the field by showing that *L.reuteri* decreases visceral hypersensitivity in an animal model of IBS.

We showed that *L.reuteri* treatment decreases visceral hypersensitivity in SERT KO rats to that of WT rats. The question that remains is: By what mechanism does *L.reuteri* produce these beneficial effects? In our study, we used two treatment groups: the *L.reuteri*-treated group was administered MRS media containing *L.reuteri* and whatever mediators it releases while growing in it, and the broth-treated group was administered MRS media only. Wang *et al*., 2010 looked at the effects of *L.reuteri* on mouse jejunal motility in an *in vitro* model. They administered live cultures, heat-killed *L.reuteri* and another probiotic, *Lactobacillus salivarious* and showed that

only live *L.reuteri* had an effect on gut motility. Other studies, however, have shown that dead (heat-killed) probiotics can stimulate the immune system. Heat killed *Lactobacilli* have been shown to increase the release of IL-10, IL-12p70, and INFɣ (Chuang *et al*., 2007). Furthermore, both live and heat-killed *Lactobacillus* GG have been shown to exert anti-inflammatory properties in a rat model of inflammatory arthritis (Baharav *et al*., 2004). A study by Kamiya *et al*., 2006 demonstrated that both live, heat-killed and irradiated *L.reuteri* decreased visceral hypersensitivity induced by colorectal distention in healthy rats. These studies indicate that both live and dead probiotics can exert beneficial effects. The particular strain of the probiotic, the mediators it releases and its delivery method all determine the beneficial effects it exerts (Forsythe *et al*., 2007; Ma *et al*., 2004).

We treated our rats with *L.reuteri* for 14 days by daily gavage. *L.reuteri* depends on the availability of sugars, amino acids, vitamins and nucleotides for survival, growth and colonization (Walter *et al*., 2011). While it is not likely that *L.reuteri* colonized the colon of rats in our study, it exerted its effects by using the available substrates to produce mediators that had an effect directly or indirectly on visceral hypersensitivity. Probiotics produce a number of compounds and most likely a combination of a few leads to the beneficial effects. For example, *L.reuteri* is unique in that it uses glycerol in the gut to produce large amounts of reuterin, an antimicrobial substance (Talarico and Dobrogosz, 1989). It has also been shown to produce histamine which suppresses TNFα production (Thomas *et al*., 2012). Since the levels of pro-inflammatory cytokines were not elevated in SERT KO rats, it is unlikely that *L.reuteri* modulates visceral hypersensitivity by decreasing inflammation in our animal model. It more likely, releases mediators that have effects on colon projecting spinal sensory neurons.

We showed that *L.reuteri* does not alter gut serotonin availability, a likely target of its action since 5-HT is involved in the regulation of gut sensations. Binding of 5-HT to 5-HT_3Rs on colon projecting spinal sensory afferents transmits pain information to the brain. However, 7-8 month old SERT KO rats do not have altered gut serotonin availability eliminating it as a factor contributing to increased visceral hypersensitivity. They may have increased 5-HT_3R expression on nerve endings in the gut mucosa and *L.reuteri* may decrease visceral hypersensitivity in SERT KO rats by altering the receptor expression or function. 5-HT_3R is a ligand-gated cation channel that is prone to rapid desensitization and that requires high (>1 μM) 5-HT concentrations for activation (Gershon, 2004). Kunze *et al*., 2009 showed that *L.reuteri* can inhibit calcium-dependent potassium channel (IK_{Ca}) opening on IPANs thus enhancing neuronal excitability and slowing gut motility. They used patch-clamp recordings of enteric neurons to study the effects of chronic (9 day) *L.reuteri* treatment in rats on neuron excitability and ion channel activity. This provides evidence that *L.reuteri* can directly modulate ion channels to alter pain perception.

Pain transmission is complex. There are tons of receptor mechanisms underlying activation and sensitization of colon projecting spinal sensory neurons (Kirkup *et al*., 2001). Stimuli such as prostaglandin E_2, adenosine, bradykinin, and histamine, among many, can sensitize visceral afferents. These are released by both other cells in the gut mucosa or come from external sources and can often modulate the response of the afferents to stimuli like 5-HT. *L.reuteri* could alter the availability or pathway mechanism of any of these mediators to alleviate visceral hypersensitivity.

Probiotics exert a slew of beneficial effects, have a great safety record, come at low cost and are easily available. On the hand, there are multiple genera, species and strains which might have different efficacy in different patient populations. More studies are needed to elucidate the mechanism of probiotic action.

4.3 Therapeutic significance

4.3.1 Animal models of IBS

IBS is a functional GI tract disorder diagnosed with a set of symptom criteria. It is challenging to study because the symptoms vary greatly from individual to individual and cannot be fully explained by the presence of structural and biochemical abnormalities. In IBS, a combination of genetic, psychosocial, and physiological factors leads to the development of altered gut motility and visceral hypersensitivity making it difficult to create an animal model that encompasses all those factors (Gwee *et al*., 1999; Mayer and Collins, 2002). Furthermore, there are many subtypes of IBS depending on the nature of the symptoms and their underlying cause. Likewise, there are a number of animal models that mimic clinical symptoms of IBS and allow for studying of different factors that lead to its development (Mayer and Collins, 2002).

Animal models of IBS can be divided into three categories: 1) stress – induced models, 2) inflammation models and 3) genetic models (Mayer and Collins, 2002). Stress – induced animal models of IBS tackle the psychosocial component and are used to study a sub-population of IBS patients that develop IBS symptoms either following adverse life events early on (neonatal-stress animal model) or a single life-threatening event (post-traumatic stress disorder (PTSD) animal model). The neonatal-stress animal model is created by separating the pup from the dam daily for set periods of time and disrupting the grooming/licking behavior that is essential for the pup's proper development (Caldje *et al*., 2000). The neonatal-stress animal model has a compromised hypothalamus-pituitary axis (HPA) response to stress and exhibits anxiety and depression, increased colonic motility and intestinal permeability and stress-induced visceral hypersensitivity (Coutinho *et al*., 2002; Soderholm *et al*., 2002). The PTSD animal model is created by exposing an

adult animal to an uncontrollable stressor such as electrical shock. The PTSD animal model experiences stress-induced visceral hypersensitivity and colonic motility (Stam *et al*., 1997, 2000).

Acute inflammation animal models of IBS are useful for studying two subsets of IBS patients: 1) post-infectious IBS (PI-IBS) patients that develop IBS symptoms following infectious gastroenteritis (Neal *et al*., 1997) and 2) patients that develop IBS-like symptoms while in remission from inflammatory bowel disorders (IBD) (Isgar *et al*., 1983). These patients have increased EC cell numbers, intestinal permeability and immune activation (Spiller *et al*., 2000). One common example of an animal model of acute intestinal inflammation is a mouse infection by a nematode parasite *Trichinella spiralis*. These mice develop hypercontractility of intestinal smooth muscle weeks after the parasite is removed and prostaglanding E_2 is involved in maintaining it (Barbara *et al*., 2001). Furthermore, they exhibit visceral hypersensitivity produced by an increase in substance P production in the colon and spinal cord (Bercík *et al*., 2004). The limitations of this model lie in the fact that these symptoms are strain-specific and reversible with corticosteroid treatment (Vallance *et al*., 1997). Also the model is developed using a parasitic infection rather than a bacterial one as occurs in PI-IBS patients.

A genetic model, the SERT KO mouse shows potential as an animal model for studying alterations in gut serotonin signaling that underlie abnormalities in gut motility seen in IBS patients (Chen *et al*., 2001). SERT KO mice alternate between episodes of diarrhea and constipation, symptoms of alternating IBS in humans. However, measuring visceral hypersensitivity in mice is challenging because of their size.

The SERT KO rat displays anxiety and depressive-like behaviors, disorders that show comorbidity with IBS in humans. Our study indicates that the SERT KO rat is a good animal model

of IBS because it has the following features: 1) SERT KO rats exhibit two key symptoms of IBS: visceral hypersensitivity and decreased gut motility, 2) visceral hypersensitivity manifests differently in male and female SERT KO rats, and 3) SERT KO rats respond to treatment with *L.reuteri* 6475.

4.4 Future directions

We showed that alterations in gut 5-HT availability do not fully account for increased visceral hypersensitivity seen in 7-8 month old SERT KO rats. Likewise, *L.reuteri* treatment decreases visceral hypersensitivity without altering gut 5-HT availability. It is important to further investigate the upstream targets in the visceral pain pathway *L.reuteri* likely exerts its anti-nociceptive effects by dampening the excitability of colon-projecting spinal sensory neurons (Galligan *et al*., 2013). Further studies are needed to: 1) demonstrate the effects of *L.reuteri* on colon projecting sensory neurons and 2) determine the mechanism by which *L.reuteri* decreases visceral hypersensitivity. The most likely mechanism of *L.reuteri* action is the alteration in ion channel expression on sensory nerve endings in the gut mucosa. Kunze *et al*., 2008 showed that *L.reuteri* modulates the activity of IPANs to affect gut motility. Chronic *L.reuteri* treatment led to a decrease in the excitability of IPANs by decreasing the calcium-activated potassium channel (IK_{Ca}) opening. Whole-cell patch clamp studies in DRG neurons with pharmacological blockers of ion channels and G-protein coupled receptors are needed to elucidate the mechanism of *L.reuteri* action.

Our results also showed that SERT KO rats have decreased gut motility, assessed as fecal output, compared to WT rats. *L.reuteri* increased gut motility in male but had no effects in female SERT KO rats. Furthermore, SERT KO rats did not exhibit a sub-clinical level of inflammation, assessed by mast cell number and inflammatory cytokine levels. To evaluate the effects of chronic *L.reuteri* treatment on gut motility and inflammation, further studies are needed in an animal model of altered gut motility and inflammation. Diet-induced obesity leads to alterations

in gut motility and inflammation and it would be interesting to study the effects of *L.reuteri* on such a model (Ley, 2010; Zhang *et al*., 2009).

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams CA. The probiotic paradox: live and dead cells are biological response modifiers. Nutrition research reviews. (2010) 23: 37-46.

Anand P, Aziz Q, Willert R, van Oudenhove L. Peripheral and central mechanisms of visceral sensitization in man. Neurogastroenterology and motility. (2007) 19: 29-46.

Baharav E, Mor F, Halpern M, Weinberger A. Lactobacillus GG bacteria ameliorate arthritis in Lewis rats. The Journal of nutrition. (2004) 134: 1964-9.

Barbara G, De Giorgio R, Deng Y, Vallance B, Blennerhassett P, Collins SM. Role of immunologic factors and cyclooxygenase 2 in persistent postinfective enteric muscle dysfunction in mice. Gastroenterology. (2001) 120: 1729-36.

Bercík P, Wang L, Verdú EF, Mao YK, Blennerhassett P, Khan WI, Kean I, Tougas G, Collins SM. Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. Gastroenterology. (2004) 127: 179-89.

Blanchard EB, Scharff L, Schwarz SP, Suls JM, Barlow DH. The role of anxiety and depression in the irritable bowel syndrome. Behaviour research and therapy. (1990) 28: 401-5.

Caldji C, Diorio J, Meaney MJ. Variations in maternal care in infancy regulate the development of stress reactivity. Biological psychiatry. (2000) 48: 1164-74.

Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. The Journal of neuroscience. (2001) 21: 6348-61.

Chuang L, Wu KG, Pai C, Hsieh PS, Tsai JJ, Yen JH, Lin MY. Heat-killed cells of lactobacilli skew the immune response toward T helper 1 polarization in mouse splenocytes and dendritic cell-treated T cells. Journal of agricultural and food chemistry. (2007) 55: 11080-6.

Coutinho SV, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, McRoberts JA, Mayer EA. Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. American journal of physiology. Gastrointestinal and liver physiology. (2002) 282: G307-16.

Craft RM. Modulation of pain by estrogens. Pain. (2007) 132: S3-12.

Forsythe P, Inman MD, Bienenstock J. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. American journal of respiratory and critical care medicine. (2007) 175: 561-9.

Galligan JJ, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM. Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. Neurogastroenterology and motility. (2013) 25: e373-81.

Gershon MD. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. Alimentary pharmacology and therapeutics. (2004) 20: 3-14.

Gwee KA, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW. The role of psychological and biological factors in postinfective gut dysfunction. Gut. (1999) 44: 400-6.

Heitkemper MM, Jarrett ME. Update on irritable bowel syndrome and gender differences. Nutrition in clinical practice. (2008) 23: 275-83.

Holzer P, Michl T, Danzer M, Jocic M, Schicho R, Lippe IT. Surveillance of the gastrointestinal mucosa by sensory neurons. Journal of physiology and pharmacology. (2001) 52: 505-21.

Homberg JR, Olivier JD, Smits BM, Mul JD, Mudde J, Verheul M, Nieuwenhuizen OF, Cools AR, Ronken E, Cremers T, Schoffelmeer AN, Ellenbroek BA, Cuppen E. Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience. (2007) 146: 1662-76.

Houghton LA, Lea R, Jackson N, Whorwell PJ. The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers. Gut. (2002) 50: 471-4.

Indrio F, Riezzo G, Raimondi F, Bisceglia M, Cavallo L, Francavilla R. The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. The Journal of pediatrics. (2008) 152: 801-6.

Isgar B, Harman M, Kaye MD, Whorwell PJ. Symptoms of irritable bowel syndrome in ulcerative colitis in remission. Gut (1983) 24: 190–2.

Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J. Inhibitory effects of Lactobacillus reuteri on visceral pain induced by colorectal distension in Sprague-Dawley rats. Gut. (2006) 55: 191-6.

Kirkup AJ, Brunsden AM, Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. American journal of physiology. Gastrointestinal and liver physiology. (2001) 280: G787-94.

Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, Bienenstock J. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. Journal of cellular and molecular medicine. (2009) 13: 2261-70.

Lee OY, Mayer EA, Schmulson M, Chang L, Naliboff B. Gender-related differences in IBS symptoms. The American journal of gastroenterology. (2001) 96: 2184-93.

Ley RE. Obesity and the human microbiome. Current opinion in gastroenterology. (2010) 26: 5-11.

Ma D, Forsythe P, Bienenstock J. Live Lactobacillus rhamnosus [corrected] is essential for the inhibitory effect on tumor necrosis factor alpha-induced interleukin-8 expression. Infection and immunity. (2004) 72: 5308-14.

Marcelli G, Patel BA. Understanding changes in uptake and release of serotonin from gastrointestinal tissue using a novel electroanalytical approach. Analyst. (2010) 135: 2340-7.

Mayer EA, Collins SM. Evolving pathophysiologic models of functional gastrointestinal disorders. Gastroenterology. (2002) 122: 2032-48.

Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. BMJ. (1997) 314: 779–82.

Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, Deen PM, Cuppen E, Cools AR, Ellenbroek BA. A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience. (2008) 152: 573-84.

Quigley EM. Prebiotics and probiotics; modifying and mining the microbiota. Pharmacological research. (2010) 61: 213-8.

Quigley EM. Gut microbiota and the role of probiotics in therapy. Current opinion in pharmacology. (2011) 11: 593-603.

Romano C, Ferrau' V, Cavataio F, Iacono G, Spina M, Lionetti E, Comisi F, Famiani A, Comito D. Lactobacillus reuteri in children with functional abdominal pain (FAP). Journal of peadiatrics and child health. (2010)

Sarada BV, Rao TN, Tryk DA, Fujishima A. Electrochemical oxidation of histamine and serotonin at highly boron-doped diamond electrodes. Analytical chemistry. (2000) 72: 1632-8.

Savino F, Pelle E, Palumeri E, Oggero R, Miniero R. Lactobacillus reuteri (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. Pediatrics. (2007) 119: e124-30.

Schmitt A, Mössner R, Gossmann A, Fischer IG, Gorboulev V, Murphy DL, Koepsell H, Lesch KP. Organic cation transporter capable of transporting serotonin is up-regulated in serotonin transporter-deficient mice. Journal of neuroscience research. (2003) 71: 701-9.

Shornikova AV, Casas IA, Isolauri E, Mykkänen H, Vesikari T. Lactobacillus reuteri as a therapeutic agent in acute diarrhea in young children. Journal of pediatric gastroenterology and nutrition. (1997) 24: 399-404.

Söderholm JD, Yates DA, Gareau MG, Yang PC, MacQueen G, Perdue MH. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. American journal of physiology. Gastrointestinal and liver physiology. (2002) 283: G1257-63.

Spiller RC, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. Gut. (2000) 47: 804-11.

Stam R, Akkermans LM, Wiegant VM. Trauma and the gut: interactions between stressful experience and intestinal function. Gut. (1997) 40: 704-9.

Stam R, Bruijnzeel AW, Wiegant VM. Long-lasting stress sensitisation. European journal of pharmacology. (2000) 405: 217-24.

Talarico TL, Dobrogosz WJ. Chemical characterization of an antimicrobial substance produced by Lactobacillus reuteri. Antimicrobial agents and chemotherapy. (1989) 33: 674-9.

Thomas CM, Hong T, van Pijkeren JP, Hemarajata P, Trinh DV, Hu W, Britton RA, Kalkum M, Versalovic J. Histamine derived from probiotic Lactobacillus reuteri suppresses TNF via modulation of PKA and ERK signaling. PLoS One. (2012) 7: e31951.

Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. Nature reviews. Neuroscience. (2003) 4: 13-25.

Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. Colonization and immunomodulation by Lactobacillus reuteri ATCC 55730 in the human gastrointestinal tract. Applied and environmental microbiology. (2004) 70: 1176-81.

Vallance BA, Blennerhassett PA, Collins SM. Increased intestinal muscle contractility and worm expulsion in nematode-infected mice. The American journal of physiology. (1997) 272: G321-7.

Walter J, Britton RA, Roos S. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. Proceedings of the National Academy of Sciences of the United States of America. (2011) 108: 4645-52.

Wang B, Mao YK, Diorio C, Pasyk M, Wu RY, Bienenstock J, Kunze WA. Luminal administration ex vivo of a live Lactobacillus species moderates mouse jejunal motility within minutes. FASEB journal. (2010) 24: 4078-88.

Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R. Human gut microbiota in obesity and after gastric bypass. Proceedings of the National Academy of Sciences of the United States of America. (2009) 106: 2365-70.