TRYPTOPHAN-SYNTHESIZING BACTERIA ENHANCE INTESTINAL SEROTONIN SIGNALING AND MOTILITY

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ABSTRACT

A growing body of research on the gut microbiome highlights the pivotal role of enteric microbes as a dynamic interface with the host in healthy and pathological conditions. As such, the gut microbiome represents a promising novel target to treat a variety of clinical symptoms, including the alleviation of gastrointestinal (GI) symptoms. It is known that gut bacteria alter GI function through a variety of mechanisms including signaling through microbial metabolites. One notable metabolite is tryptophan (Trp), an essential amino acid. Trp can markedly impact signaling pathways in the gut through its role as the precursor to serotonin (5-hydroxytryptamine; 5-HT). 5-HT is a critical signaling molecule that regulates many important GI functions, including intestinal motility and secretion, and consequently has been implicated as a therapeutic target for the treatment of constipation and diarrhea.

An emerging, transient approach to manipulate the gut microbiota for therapeutic outcomes is through the oral administration of isolated bacterial strains. However, the underlying mechanisms of action of these bacteria are not well established, making it challenging to ascertain predictive treatment strategies. Here, we elucidate a novel strategy to manipulate 5-HT signaling and 5-HT-mediated functions by exploiting the biochemical capacity of specific bacteria to alter luminal Trp availability. We utilized the isolated bacterial strain *Bacillus (B.) subtilis R0179,* which expresses the key enzyme in Trp synthesis from indole, tryptophan synthase.

To test our hypothesis that oral administration of the Trp-synthesizing strain *B. subtilis R0179* alters intestinal 5-HT signaling, we treated C57BL/6J mice daily via oral gavage with *B. subtilis R0179* spores dissolved in phosphate buffered saline at a dose of 10^9 CFU/ml per day. We assessed colonic tissue levels of Trp, 5-HT, and the 5-HT break down product 5-hydroxyindoleacetic acid (5-HIAA) using high performance liquid chromatography. Following one week of treatment, mice that received *B. subtilis R0179* exhibited greater levels of Trp and increased 5-HT signaling, as indicated by an elevated 5-HIAA/5-HT ratio, compared to vehicle-treated mice. We further demonstrate that these effects are dependent on spore viability as well as the ability to synthesize Trp. Mice treated with either heat inactivated *B. subtilis R0179* or with a *B. subtilis* strain that does not express tryptophan synthase did not display altered colonic Trp or 5-HT signaling.

To test our hypothesis that *B. subtilis R0179* treatment alters 5-HT-mediated gut function, we assessed changes in intestinal function in response to bacteria treatment using two validated motility assays: whole gut transit and colonic motility. We found that one week of treatment with *B. subtilis R0179* accelerated colonic motility in healthy mice. Additionally, using a mouse model of constipation, we found that *B. subtilis R0179* treatment restores deficits in colonic motility in constipated animals. Lastly, we found that the prokinetic effects of *B. subtilis R0179* were blocked by co-administration of a 5-HT_4 receptor (5-HT_4R) antagonist and were absent in 5-HT_4R knockout mice, demonstrating the involvement of the 5-HT_4R in mediating the bacteria's prokinetic actions.

Taken together, these data demonstrate that serotonergic signaling and resulting intestinal motility can be augmented by treatment with bacteria that synthesize Trp. Our findings provide mechanistic insight into a transient and predictable strategy to alter microbiota-gut dynamics with the goal of promoting GI motility.

CITATIONS

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TABLE OF CONTENTS

Page

CITATIONS ii

ACKNOWLEDGEMENTS iii

LIST OF FIGURES viii

CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW, PART ONE 1

I. THE GASTROINTESTINAL TRACT 1

Longitudinal organization 1
Cross sectional organization 3
Intestinal motility 6

II. MUCOSAL SEROTONIN SIGNALING IN THE GUT 7

Synthesis of epithelial serotonin 7
Termination of serotonin signaling 7
Functions of mucosal serotonin in the intestines 10
5-HT_3 and 5-HT_4 Receptors 11

III. THE GUT MICROBIOME 13

Bacterial biogeography 13
Microbiome composition across the lifespan 16

IV. SPECIFIC AIMS 18

CHAPTER 1 REFERENCES 20

CHAPTER 2: DIRECT AND INDIRECT MECHANISMS BY WHICH THE GUT MICROBIOTA INFLUENCE HOST SEROTONIN SYSTEMS 23

Abstract 23

I. INTRODUCTION AND BACKGROUND 24

The gut microbiota 24
Serotonin in the gut 25

II. DIRECT MECHANISMS 27

III. INDIRECT MECHANISMS 31

Short chain fatty acids 31
Tryptophan 32
Tryptamine 34
Bile acids / secondary bile acids 35

IV. BENEFICIAL BACTERIA APPLICATIONS 38

V. IMPLICATIONS FOR 5-HT IN THE MICROBIOTA-GUT-BRAIN AXIS 41

Overview of the microbiota-gut-brain axis 41
Serotonin in the brain 42
Tryptophan as a potential microbiota-gut-brain target 43

VI. CONCLUSION 46

CHAPTER 2 REFERENCES 47

CHAPTER 3: TRYPTOPHAN-SYNTHESIZING BACTERIA ENHANCE COLONIC SEROTONIN SIGNALING AND GASTROINTESTINAL MOTILITY 57

ABSTRACT 57

INTRODUCTION 59

METHODS 61

RESULTS 67

DISCUSSION 82

CHAPTER 3 REFERENCES 91

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS 96

I. PROKINETIC EFFECTS OF *B. SUBTILIS R0179* 97

A. Summary and conclusions 97

B. Alternative approaches and future directions 98

Bacterial administration method 98
Strain incongruence between Trp+ and Trp- strains 99
Compensatory changes in 5-HT-related genes 100
Trp absorption along the GI tract 102
Colonization of B. subtilis R0179 103
Implications for the microbiota-gut-brain axis 104

II. INVOLVEMENT OF THE 5-HT_4 RECEPTOR 112

A. Summary and conclusions 112

B. Alternative approaches and future directions 113

Tissue specificity of 5-HT_4Rs 113
Possible role of tryptamine 114
Potential involvement of the 5-HT_3 receptor 115

III. THERAPEUTIC APPLICATIONS AND CONSIDERATIONS 117

A. Summary and conclusions 117

B. Alternative approaches and future directions 118

Other *in vivo* models of constipation 118
Trp-catabolizing strains of bacteria 120

IV. Concluding remarks: The Future of "Precision Probiotics" 123

CHAPTER 4 REFERENCES 124

COMPREHENSIVE REFERENCE LIST 131

LIST OF FIGURES

Figure Page

CHAPTER ONE

Figure 1……5
Figure 2……9
Figure 3……15

CHAPTER TWO

Figure 1……37

CHAPTER THREE

Figure 1……73
Figure 2……74
Figure 3……75
Figure 4……76
Figure 5……77
Figure 6……79
Figure 7……81

CHAPTER FOUR

Figure 1……107
Figure 2……108
Figure 3……109
Figure 4……111
Figure 5……122

CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW, PART ONE

I. THE GASTROINTESTINAL TRACT

The following information presented in Chapter 1, Section I can be found in *Physiology of the Gastrointestinal Tract, Sixth Edition* (Said, 2018).

Longitudinal organization

The gastrointestinal (GI) system collectively functions in the digestion of food and excretion of waste. The GI tract is a continuous passageway composed of a series of tube-like organs beginning at the mouth and spanning to the anus. This system also includes accessory organs, such as the pancreas, liver, and gallbladder, that assist in digestive processes. The organs of the GI tract are responsible for extracting and absorbing nutrients, energy, and water from ingested food, as well as expelling indigestible waste in the form of feces.

The longitudinal organization of the GI tract comprises a series of functionally distinct regions between the mouth and the anus. The digestion of food and fluids begins at the mouth, where food is mechanically broken down by chewing. Secreted saliva helps to soften the food and break down carbohydrates through the release of an enzyme called amylase. Following the initial step of digestion by the mouth, the food is then pushed into the esophagus by the collective actions of the tongue and muscles of the pharynx. The esophagus acts as a hollow channel from the throat to the stomach. Two esophageal sphincters facilitate the passage of food into the stomach through a wave of muscle

contractions and relaxations. The upper esophageal sphincter prevents air from entering the GI tract, and the lower esophageal sphincter prevents gastric reflux.

Once food reaches the stomach, it undergoes the gastric phase of digestion in which it is subjected to chemical breakdown by hydrochloric acid and digestive enzymes such as pepsin. This partially digested food, called chyme, then progresses to the small intestine by mechanical actions of the pyloric sphincter. The small intestine is structurally divided into three parts: the duodenum, jejunum, and ileum. These regions function collectively to absorb nutrients and water, and to mix and propagate the intestinal contents along the tract into the large intestine. A characteristic feature of the mucosa of the small intestine is the presence of specialized finger-like projections into the lumen called villi. These villi play a key role in absorption, as they increase the surface area to promote extensive contact with nutrients in the chyme.

Unlike the small intestine, which acts primarily to absorb nutrients and water, the large intestine functions mainly to absorb remaining water and propel undigestible luminal contents along the tract and into the rectum for removal by defecation. The large intestine consists of the cecum, colon, and rectum. Given that the primary purpose of the colon is water absorption, the colonic mucosa is characterized by crypt structures lined with goblet cells that secrete mucus to facilitate the passage of solid waste. The colon also harbors the greatest bacterial density of the gut microbiota, which act to ferment unabsorbed material. Waste is propelled anally through the colon by a process known as propulsive motility, where it is then stored in the rectum in the form of feces. The final step of the digestive process, defecation, occurs following the voluntary relaxation of the external anal sphincter muscle.

Cross sectional organization

The structural shape of the GI tract resembles that of a circular tube. The wall of the gut is composed of four concentric layers that are organized radially outward from the center, which is the lumen. The layers, described in further detail below, are the mucosa, submucosa, muscularis propria (externa), and serosa.

The innermost layer of the gut is the mucosa. The mucosa acts as a semipermeable barrier between the external environment and the internal body, and thus plays a vital role in the maintenance of our health. It is characterized by three layers: the epithelium, lamina propria, and muscularis mucosa. The mucosal epithelial layer is composed primarily of simple columnar epithelium that functions as both a physical and chemical barrier in interactions with luminal contents. There is a diversity of cell types present in the mucosal epithelium, the most prevalent of which is the enterocyte which plays an important role in absorption and secretion. Other classes of intestinal epithelial cells include enteroendocrine cells that secrete hormones, goblet cells that secrete mucus, and Paneth cells that produce antimicrobial molecules. These specialized cells are distributed differentially throughout the functionally distinct regions of the GI tract. The next layer of the mucosa is the lamina propria, a layer of loose connective tissue that contains blood vessels, lymphatic vessels, and nerves to support the avascular epithelial layer. The outermost layer of the mucosa is the muscularis mucosa, a thin layer of smooth muscle that coordinates contractions to facilitate local movement.

The next concentric layer is the submucosa. Similar to the lamina propria, the submucosa is a layer of connective tissue composed of rich vascular and lymphatic

networks. The submucosa also contains one of two major ganglionated plexuses that form the enteric nervous system (ENS). The submucosal plexus, also known as Meissner's plexus, is characterized by neurons that function in regulating epithelial secretion and vasodilation.

External to the submucosa is the muscularis propria. This muscular layer contains two perpendicular layers of smooth muscle, the inner circular layer and the outer longitudinal layer, that play integral roles in regulating GI motility. Between the two muscle layers lies the second major ganglionated plexus of the ENS, the myenteric (Auerbach's) plexus. Together, these neuromuscular networks generate a series of coordinated contractions and relaxations to mix and move luminal contents along through the GI tract.

The outermost layer of the gut wall is the serosa (adventitia). The serosa is an epithelial layer comprised of simple squamous epithelial cells that lubricate the wall and reduce external frictional contact.

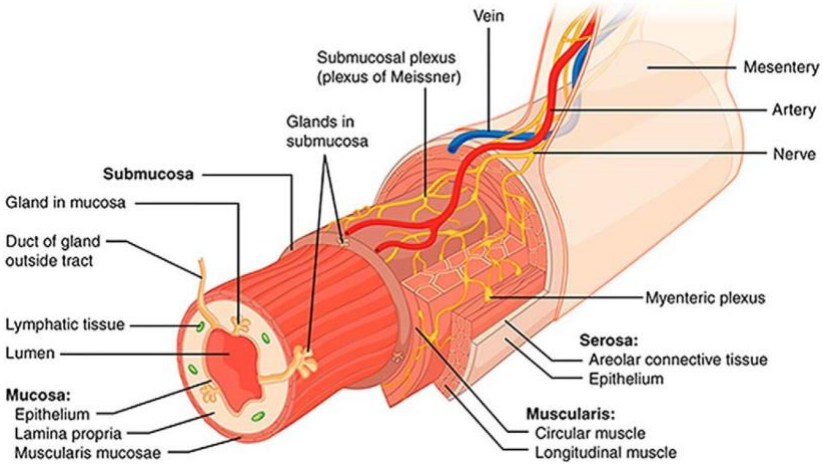

Figure 1. Cross sectional organization of the GI tract. The wall of the gut is organized into four concentric layers proceeding outward from the lumen. Innermost is the mucosa, which functions as a semipermeable barrier and plays an important role in absorption and secretion. Next is the submucosa, which is a connective tissue layer that is rich in vasculature and contains the submucosal nerve plexus. Further external is the muscularis propria, a muscular layer comprised of smooth muscle that contains the myenteric nerve plexus. The outermost layer is the serosa. Image obtained from Wikimedia and reproduced by (Kulkarni et al., 2018) under a Creative Commons Attribution-Share Alike 3.0 Unported license.

Intestinal motility

A primary digestive function of the GI system is the regulation of intestinal motility, which involves the movement of luminal contents through the GI tract in the oral to aboral direction. This is accomplished by two neurally-mediated coordinated patterns of contraction and relaxation by enteric smooth muscle known as peristalsis and segmentation.

The peristaltic reflex begins with a luminal chemical or mechanical stimulus that activates sensory neurons known as intrinsic primary afferent neurons (IPANs). In turn, IPANs activate excitatory and inhibitory neurons of the myenteric plexus, which induces an upstream contraction of circular muscle, a downstream contraction of longitudinal muscle, and a descending relaxation of circular muscle below. The net effect of these muscular coordinations is a pattern referred to as propulsive motility, which is characterized by the anterograde propulsion of luminal contents in the anal direction.

The other component of intestinal motility is segmentation. This refers to another coordinated pattern that is characterized by localized contractions of circular muscle. Occurring primarily in the small intestine, segmentation acts to move luminal contents back and forth to mix the contents in with digestive enzymes, thereby facilitating the digestion and absorption of nutrients (Lentle & de Loubens, 2015).

II. MUCOSAL SEROTONIN SIGNALING IN THE GUT

Synthesis of epithelial serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is an important signaling molecule in many physiological systems throughout the body. In the gut, 5-HT is well-known for its role in regulating a variety of GI functions, including stimulation of motility reflexes, secretion, and vasodilation (Mawe & Hoffman, 2013). The primary sources of 5-HT synthesis, storage, and release are enterochromaffin (EC) cells, which are a subset of enteroendocrine cells located in the intestinal mucosa (Gershon & Tack, 2007). EC cells act as sensory transducers to sense and respond to luminal stimuli, and initiate reflexes for intrinsic and extrinsic afferent nerves, which do not reach the lumen (Raybould et al., 2004).

EC cells synthesize 5-HT using the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1). The metabolic precursor of 5-HT is tryptophan (Trp), an essential amino acid obtained through the diet. Once 5-HT is synthesized, it is packaged into vesicles and released predominantly at the basal surface of EC cells into the interstitial space of the lamina propria.

Termination of serotonin signaling

Following release, inactivation of 5-HT is a timely and essential step in the signaling process due to receptor desensitization. The termination of 5-HT signaling utilizes transporter-mediated uptake, followed by enzyme-mediated catabolism. Previously released 5-HT is transported from the interstitial space into epithelial cells by the serotonin-selective reuptake transporter (SERT). SERT, which is expressed by all

mucosal epithelial cells, acts as a key regulator of local 5-HT availability (Wade et al., 1996). Excess 5-HT that is not transported into epithelial cells gets taken up into platelets, which also express SERT.

5-HT degradation involves catabolism by intracellular enzymes. One particular enzyme that catabolizes 5-HT is monoamine oxidase (MAO), of which the A type (MAO-A) is preferentially expressed within intestinal cells (Rodríguez et al., 2001) and has a high affinity for 5-HT (Ostadkarampour & Putnins, 2021). It then undergoes oxidation by aldehyde dehydrogenase to form the metabolite 5-hydroxyindoleacetic acid (5-HIAA). 5-HIAA is often used as a proxy to measure the concentrations of 5-HT within the human body.

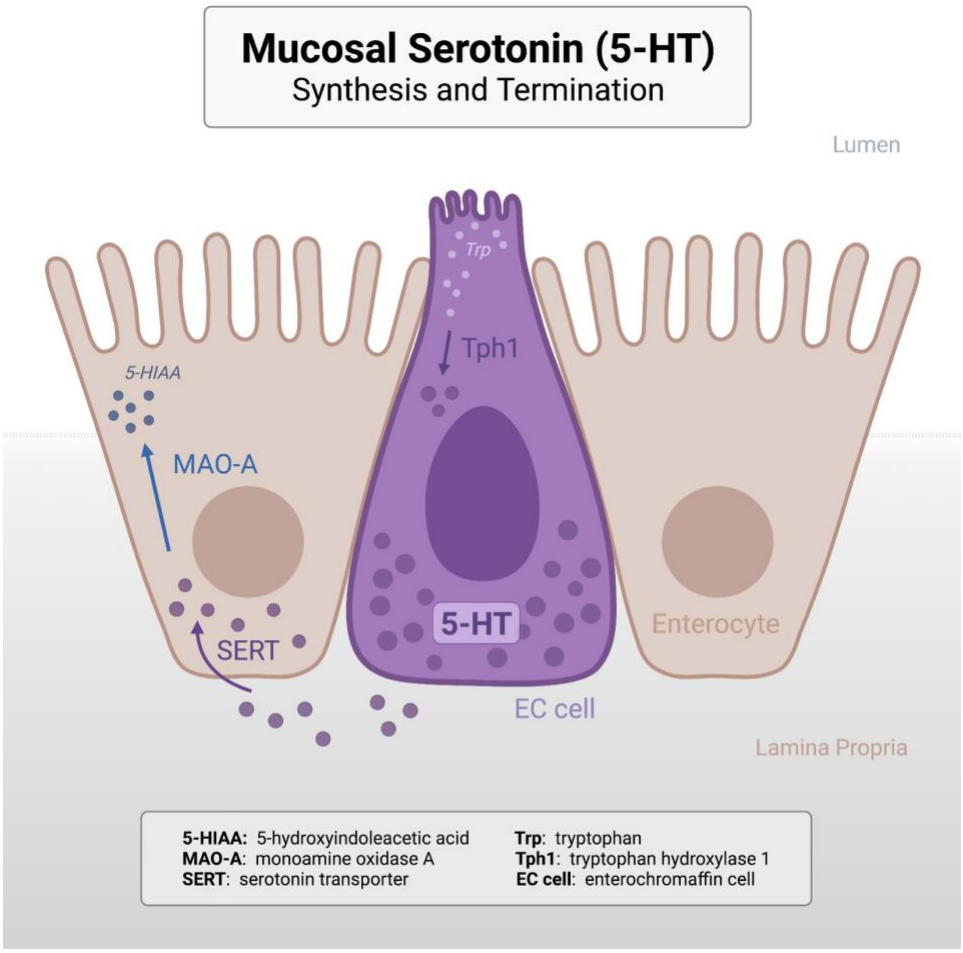

Figure 2. Mucosal serotonin signaling. Serotonin (5-HT) is synthesized within specialized enterocytes called enterochromaffin (EC) cells. 5-HT synthesis occurs from the precursor tryptophan (Trp) and utilizes the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1). Once 5-HT is released into the lamina propria, its termination necessitates the serotonin transporter (SERT) for reuptake into neighboring epithelial cells, where it can then be degraded by monoamine oxidase A (MAO-A) to produce the metabolite 5-hydroxyindoleacetic acid (5-HIAA). Created with BioRender.com.

Functions of mucosal serotonin in the intestines

Motility

Early investigations into the role of mucosal 5-HT synthesis and release in intestinal motility dates back to the 1950s when Edith Bülbring and colleagues made two key discoveries. They identified a mechanism of 5-HT release by EC cells in a pressure-dependent manner (Bulbring & Crema, 1959), and they found that 5-HT facilitates peristalsis when it is infused intraluminally (Bulbring & Crema, 1958). These insights laid the foundation for a body of literature that has since further demonstrated how mucosal 5-HT regulates intestinal motility. Notably, numerous studies have demonstrated that mechanical stimulation of EC cells leads to 5-HT release, which then initiates the peristaltic reflex through activation of 5-HT receptors located on enteric nerves (Foxx-Orenstein et al., 1996; Linden et al., 2003). Additionally, 5-HT is known to play a role in segmentation, as blocking 5-HT receptors inhibits segmenting contractions in isolated guinea pig small intestine (Ellis et al., 2013).

Secretion

Intestinal epithelial cells secrete Cl^- and bicarbonate that facilitate lubrication, dilution, and propulsion of luminal contents through the GI tract. This secretion is neurally-mediated by intrinsic enteric reflexes and involves the release of neurotransmitters such as acetylcholine and vasoactive intestinal peptide to activate these secretory responses. It has been shown that 5-HT released from EC cells also activates these reflexes. 5-HT-initiated secretion appears to involve multiple 5-HT receptors,

including the 5-HT_3 and 5-HT_4 receptors, as well as paracrine-mediated secretion through 5-HT_2 receptors (Mawe & Hoffman, 2013).

5-HT_3 and 5-HT_4 Receptors

The actions of gut 5-HT involve a host of different receptors expressed widely throughout the GI tract. Two receptors in particular, the 5-HT_3 receptor (5-HT_3R) and 5-HT_4R, play imperative roles in the regulation of intestinal motility and secretion, and consequently are prominent clinical targets in the treatment of GI dysfunction.

5-HT_3Rs

5-HT_3Rs are expressed mucosally by enterocytes and EC cells, and neuronally by intrinsic and extrinsic afferent nerves, interneurons, and motor neurons (Mawe & Hoffman, 2013). Activation of 5-HT_3Rs by 5-HT released from EC cells contributes to propulsive motility initiation and secretory responses in the gut. Conversely, inhibition of these 5-HT_3Rs decreases motility and secretion. The 5-HT_3R is targeted clinically in the treatments of both constipation and diarrhea symptoms. 5-HT_3R agonists are used in the treatment of constipation-predominant irritable bowel syndrome (IBS) for their ability to activate propulsive motility and secretion (Evangelista, 2007). These compounds are typically partial agonists, however, as the 5-HT_3 receptors exhibit rapid desensitization. On the other hand, 5-HT_3R antagonists, used commonly for anti-nausea and anti-emetic actions, are effective in slowing motility in the treatment of diarrhea symptoms in diarrhea-predominant IBS (Talley et al., 1990).

5-HT$_4$Rs

The 5-HT$_4$R is most known for its function as a prokinetic receptor in the gut. 5-HT$_4$Rs are extensively expressed in the intestinal mucosa by a variety of epithelial cells, such as EC cells, enterocytes, and goblet cells, with the greatest expression levels of 5-HT$_4$Rs seen in the distal colon (Hoffman et al., 2012). Activation of mucosal 5-HT$_4$Rs induces 5-HT release by EC cells as well as mucus release by goblet cells, and accelerates the peristaltic reflex (Hoffman et al., 2012). 5-HT$_4$Rs are also expressed on nerve terminals throughout the enteric neural circuitry, and stimulation of these receptors promotes synaptic activity through a presynaptic faciliatory mechanism. Transgenic mice with knockouts of the 5-HT$_4$R display slowed colonic motility (Liu et al., 2009). Given the prokinetic characteristics of the 5-HT$_4$R, pharmacological agents target this receptor in the treatment of constipation. Agonists of the 5-HT$_4$R have been developed to alleviate constipation symptoms in constipation-predominant IBS. For example, previous work in our lab demonstrates that the luminally acting modification of the 5-HT$_4$R agonist prucalopride accelerates whole gut transit and promotes colonic propulsive motility (Konen et al., 2021).

III. THE GUT MICROBIOME

Bacterial biogeography

The gut microbiome is a collective term that refers to the tens of trillions of microorganisms that reside in the lumen of the GI tract. It is widely known that these microbes interact dynamically with each other and with the host to regulate physiological functions. As such, a healthy gut microbiome is essential to our health and well-being. Extensive efforts by researchers have sought to characterize the intestinal microbiota in terms of identification, numbers, diversity, and functions (Donaldson et al., 2016). These efforts have focused primarily on bacteria, which encompass the majority of the microbiome. It is important to note that fungi and viruses are also known to influence this ecosystem and play a role in interacting with the host, however these will not be further discussed here.

The GI tract consists of physically and chemically diverse regions that give rise to a heterogeneity of bacterial compositions. The upper portions of the GI tract, including the oral cavity, esophagus, and stomach, exhibit the lowest bacterial numbers. This is due to a low pH and a short transit time of luminal contents (Berg, 1996). The small intestine, while also characterized by relatively lower abundance and diversity of bacteria, harbors a microbial population that is well suited to tolerate the exposures to bile acids and antimicrobial peptides (Donaldson et al., 2016). Firmicutes and Proteobacteria are the most dominant phyla in this small intestinal environment.

The colon harbors the greatest numbers and diversity of bacteria in the GI tract. Indeed, it is estimated that 70% of all bacteria within the body are located in the colon

(Hillman et al., 2017). The colon plays a key role in absorbing any remaining nutrients of food before excretion. As such, the colonic bacteria facilitate this process by degrading and fermenting indigestible food components. The most dominant gut phyla in the colon are Bacteroidetes and Firmicutes.

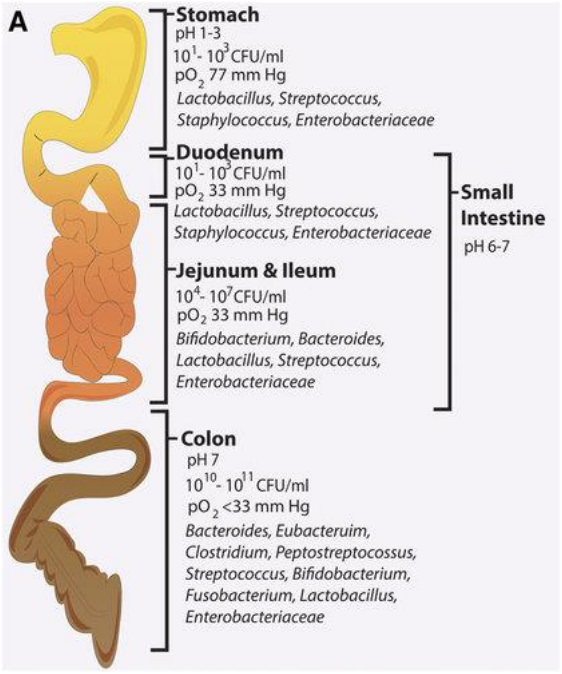

Figure 3. Bacterial niches of the gut. The GI tract is characterized by a diversity of physical and chemical habitats leading to heterogeneous bacterial colonization from the oral to distal end. Bacteria are present in fewer numbers and diversity in the upper portions of the GI tract as a result of the low pH and short transit time. The colon is the primary site of bacterial fermentation and is home to the greatest numbers and diversity of bacteria. Image reproduced from (Clarke et al., 2019) under a Creative Commons BY Attribution 4.0 International license.

Microbiome composition across the lifespan

The composition of the microbiome is originally impacted by early life factors such as mode of delivery and early feeding patterns. Notably, vaginal births result in a neonatal microbial composition that initially resembles that of the mother's vaginal composition. This is thought to positively shape the microbiome during this sensitive developmental window path towards a path of healthy microbial composition and diversity later in life (Bokulich et al., 2016). A Cesarean section mode of delivery, on the other hand, is associated with greater risk of adverse developmental impacts on the immune system (Cho & Norman, 2013). After birth, early life feeding has a substantial impact on the development of the microbiome as well. For example, breastfeeding is thought to promote enriched colonization and diversity of healthy bacteria (Clarke et al., 2014) in contrast to formula solutions.

Both the composition and functions of the microbiome are influenced by a variety of factors throughout the lifespan. This highly dynamic nature is of particular relevance in the role of these bacteria in health and pathophysiology. For example, dietary factors are known to transiently alter gut bacterial compositions. Increased intake of fiber, such as "prebiotics", promotes greater bacterial diversity and stimulates the growth of beneficial bacteria. Furthermore, microbiome shifts are observed in drastic changes to diet, such as in a complete switch from a meat-based to plant-based diet (David et al., 2014). In addition to diet, other lifestyle factors including exercise positively promote microbiome stability.

Numerous factors throughout life also induce adverse effects on microbial composition and function. One well-documented factor is stress. Mounting evidence suggests that psychological and/or physical stress can lead to a compromised intestinal epithelial barrier, which in turn increases intestinal permeability and exposes the host to pathogenic threats (Moloney et al., 2014). Additionally, many factors are associated with a detrimental narrowing of gut bacterial diversity, including the excessive use of antibiotics and a variety of disease states. Taken together, it is clear that the gut microbiome represents a highly active system that is susceptible to alterations, both positive and negative, throughout the lifespan.

IV. SPECIFIC AIMS

The gut microbiome, composed of tens of trillions of microorganisms within the GI tract, represents a promising target for novel treatments of GI dysmotility. A growing body of research demonstrates that gut microbes alter GI function primarily by signaling through microbial metabolites. One notable microbial metabolite is Trp, an essential amino acid and metabolic precursor to several bioactive neurochemicals, including the critical gut signaling molecule 5-HT. It is known that 5-HT regulates many important GI functions, notably intestinal motility and secretion. Consequently, the enteric 5-HT system is often targeted therapeutically in the treatment of constipation and diarrhea symptoms.

Emerging interventions seek to target the microbiome to alter pathological conditions and dysbiosis either by manipulating existing gut microbiota or by delivering exogenous bacteria. In this study, we elucidate a novel strategy to manipulate 5-HT signaling and function by administering the exogenous bacterial strain *Bacillus (B.) subtilis R0179*, which expresses the key enzyme in Trp synthesis from indole. Our central hypothesis is that oral administration of Trp-synthesizing bacteria can regulate intestinal 5-HT signaling and motility.

Aim 1. To test the hypothesis that administration of *B. subtilis R0179* alters intestinal Trp and 5-HT levels. We used high performance liquid chromatography to assess colonic tissue levels of Trp, 5-HT, and the 5-HT break down product 5-HIAA following one week of *B. subtilis R0179* administration.

Aim 2. To test the hypothesis that administration of *B. subtilis R0179* induces functional changes in intestinal motility, in both healthy mice and in a mouse model of constipation. We assessed changes in gut function following one week of bacteria treatment using two *in vivo* motility assays, whole gut transit and colonic motility.

Aim 3. To test the involvement of the 5-HT_4R in mediating the prokinetic actions of *B. subtilis R0179*. We targeted the 5-HT_4R pharmacologically by co-administering *B. subtilis R0179* and a selective 5-HT_4R antagonist, and genetically by administering *B. subtilis R0179* to 5-HT_4R knockout mice. With both approaches, we used *in vivo* motility assays to examine changes in intestinal motility following one week of treatment.

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CHAPTER 2: DIRECT AND INDIRECT MECHANISMS BY WHICH THE GUT MICROBIOTA INFLUENCE HOST SEROTONIN SYSTEMS

Legan, T. B., Lavoie, B. & Mawe, G. M. Direct and indirect mechanisms by which the gut microbiota influence host serotonin systems. *Neurogastroenterology & Motility*, e14346, doi:https://doi.org/10.1111/nmo.14346 (2022).

ABSTRACT

Mounting evidence highlights the pivotal role of enteric microbes as a dynamic interface with the host. Indeed, the gut microbiota, located in the lumen of the gastrointestinal (GI) tract, influence many essential physiological processes that are evident in both healthy and pathological states. A key signaling molecule throughout the body is serotonin (5-hydroxytryptamine; 5-HT), which acts in the GI tract to regulate numerous gut functions including intestinal motility and secretion. The gut microbiota can modulate host 5-HT systems both directly and indirectly. Direct actions of gut microbes, evidenced by studies using germ-free animals or antibiotic administration, alter the expression of key 5-HT-related genes to promote 5-HT biosynthesis. Indirectly, the gut microbiota produce numerous microbial metabolites, whose actions can influence host serotonergic systems in a variety of ways. This review summarizes the current knowledge regarding mechanisms by which gut bacteria act to regulate host 5-HT and 5-HT-mediated gut functions, as well as implications for 5-HT in the microbiota-gut-brain axis.

Keywords: bacteria, enteric nervous system, gut–brain axis, microbial metabolites, tryptophan, tryptophan synthase

I. INTRODUCTION AND BACKGROUND

The gut microbiota

Despite decades of research, we are only beginning to fully appreciate the immense complexity in which microbes colonize the gut and interact dynamically with the host in healthy and pathological conditions. Located in the lumen of the gastrointestinal (GI) tract on the order of tens of trillions of microorganisms, the gut microbiota demonstrate remarkable capacity to influence nearly every physiological system in the body (Cryan et al., 2019; Mayer, 2011).

Research into the ecology of the microbiome demonstrates that bacteria colonization is diverse and varies along the GI tract (Hillman et al., 2017). Notably, the microbial communities become increasingly heterogeneous progressing from the oral to distal ends of the gut due to the diverse microhabitats associated with each region of the GI tract. For example, fewer bacteria are present in the small intestine of the gut where transit time is short and pH is low, in comparison to the colon, which harbors the highest numbers and biodiversity of bacteria and is the primary site for bacterial fermentation.

Pivotal early insights into host-microbiota interactions stem largely from germ-free (GF) and fecal microbiota transplantation (FMT) studies (Cryan et al., 2019). GF studies exploit the postnatal timing of microbiota colonization of the GI tract, such that rodents born and raised in a sterile environment develop without a microbiome. Studies employing this strategy demonstrate that GF mice exhibit abnormal physiology, such as slowed colonic motility (Vincent et al., 2018) and total intestinal transit time (Yano et al.,

2015). Notably, colonization with either normal mouse-derived or human-derived microbiota can restore these GF-induced deficits in motility (Kashyap et al., 2013).

FMT is the process by which stool harvested from a donor is transplanted into the intestines of a recipient in order to alter the microbiota composition (Gupta et al., 2016). Preclinical FMT studies demonstrate remarkable transfers of microbiota-driven disease symptomologies in both rodent models and humans, including transfers of slow transit constipation (Ge et al., 2017), rapid transit diarrhea (De Palma et al., 2017), and intestinal barrier dysfunction (De Palma et al., 2017). While GF and FMT approaches certainly have clinically translatable limitations, they nevertheless reliably demonstrate that healthy gut function is microbiota-dependent.

Serotonin in the gut

Serotonin (5-hydroxytryptamine; 5-HT) is a prominent signaling molecule throughout the body. The major source of 5-HT is from the enterochromaffin (EC) cells in the epithelium of the GI tract (Mawe & Hoffman, 2013). EC cells are a subset of enteroendocrine cells that synthesize, store, and release 5-HT in a regulated manner (Mawe & Hoffman, 2013). EC cells synthesize 5-HT from tryptophan (Trp) using the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1), and since Tph1 is not saturated under baseline Trp concentrations, it is likely that elevated Trp could increase metabolic 5-HT output (O'Mahony et al., 2015). Myenteric neurons also express 5-HT, though at much lower levels than in the mucosa.

Termination of 5-HT signaling involves a serotonin-selective reuptake transporter (SERT) to remove 5-HT from the interstitial space. 5-HT is then degraded intracellularly

by monoamine oxidase A (MAO-A) to form 5-hydroxyindoleacetic acid (5-HIAA). SERT is expressed by all mucosal epithelial cells in the intestines and functions as an important regulator of interstitial 5-HT availability (Gershon & Tack, 2007).

In the gut, 5-HT released from EC cells in response to luminal chemical and mechanical stimuli functions as a critical activator of many GI reflexes by signaling through a variety of receptors located on intrinsic and extrinsic afferent nerve fibers (Mawe & Hoffman, 2013). Mucosal 5-HT can initiate the peristaltic reflex, a component of propulsive motility (Mawe & Hoffman, 2013). In the intestinal epithelium, 5-HT stimulates secretory responses acting primarily on the 5-HT_3 receptor (5-HT_3R) and the 5-HT_4R, with evidence for paracrine-mediated secretion through the 5-HT_2R as well (Gershon & Tack, 2007). 5-HT release and activation of 5-HT_3R located on vagal afferent neurons can result in various reflex responses including pancreatic secretion, gallbladder contraction, and inhibition of gastric emptying when released as part of the normal digestive response, as well as nausea and vomiting when released at higher concentrations in pathological conditions.

Numerous approaches to treat clinical GI disorders target the enteric serotonergic system with the goal of promoting healthy gut function. For example, in patients with irritable bowel syndrome, 5-HT_3R antagonists have been used to treat diarrhea (Andresen et al., 2008), and 5-HT_4R agonists are used to treat constipation (Hoffman et al., 2012; Mawe & Hoffman, 2013). Taken together, 5-HT is a critical mediator of many important gut functions and consequently is an important target in the context of GI dysfunction.

II. DIRECT MECHANISMS

5-HT's prominent role in the regulation of GI function is well documented (Mawe & Hoffman, 2013). However, the contribution of gut microbes in impacting host gut-derived 5-HT signaling is a burgeoning field that may offer key insights into the link between the microbiota and GI function.

Early studies examining the role of the microbiota in gut-derived 5-HT regulation demonstrated that GF mice have significantly lower serum 5-HT levels (Sjögren et al., 2012; Wikoff et al., 2009), decreased colonic Tph1 mRNA expression (Sjögren et al., 2012), and increased colonic SERT mRNA expression (Sjögren et al., 2012) as compared to control mice.

More recently, two landmark studies provided further strength toward the notion that the microbiota can regulate gut-derived 5-HT levels (Reigstad et al., 2015; Yano et al., 2015). A study by Kashyap and colleagues examined the impact of the gut microbiota on host colonic 5-HT production (Reigstad et al., 2015). The authors utilized three groups of mice with differing microbiotas: GF mice, GF mice colonized with human gut microbiota (HM), and conventionally raised (CR) mice with normal mouse microbiomes. HM and CR mice exhibit significantly higher colonic Tph1 mRNA and protein levels, as well as higher colonic 5-HT concentrations, compared to GF mice. Notably, there is no difference in EC cell density in the proximal colon between the groups, suggesting a microbiome-induced effect of increased Tph1 transcription.

Along similar lines, Hsiao and colleagues demonstrated that the gut microbiota promote host 5-HT biosynthesis (Yano et al., 2015). Consistent with the findings

described above, adult GF mice exhibit decreased colonic 5-HT levels as well as decreased colonic mRNA expression of Tph1, while also exhibiting significantly higher concentrations of Trp in feces. Colonization of GF mice with spore-forming bacteria from healthy mice restores colonic 5-HT levels and elevates colonic Tph1 expression. Notably, the restorative increases in 5-HT are blocked by the administration of the Tph inhibitor para-chlorophenylalanine (PCPA), suggesting that host Tph activity is necessary for promoting 5-HT biosynthesis.

Taken together, it is now generally accepted that the gut microbiota play a key role in regulating the serotonergic system of the host primarily through altering the expression of 5-HT-related genes. Specifically, intestinal microbiota influence Tph1 transcription, thereby promoting 5-HT biosynthesis (Fig 1A). While the microbiota have also been shown to influence SERT expression (Sjögren et al., 2012; Yano et al., 2015), this likely represents an indirect, compensatory effect to deficient 5-HT biosynthesis, rather than a direct effect by the microbiota. Inhibition of Tph via PCPA administration modulates SERT expression (Yano et al., 2015), suggesting that changes in SERT expression occur independently of the intestinal microbiota. Furthermore, the strong positive correlations between colonic mRNA expressions of SERT and other key 5-HT-related genes such as $5\text{-HT}_4\text{R}$ does not depend on microbiota colonization status (Reigstad et al., 2016), providing further support that SERT is not directly mediated by the gut microbiota. Additionally, GF mice do not exhibit differences in mRNA expression of enzymes that release, package, or break down 5-HT (Yano et al., 2015). Therefore, it is likely that the microbiota influence enteric 5-HT biosynthesis primarily through the elevation of Tph1 expression.

Gut microbiota may also alter expression levels of a key 5-HT receptor, the 5-HT_3R, known to be involved in intestinal secretion and peristalsis. Indeed, Bhattarai et al. found a microbiota-mediated modulation of host colonic secretion through altered epithelial 5-HT_3R expression (Bhattarai et al., 2017). GF mice display elevated 5-HT_3R expression levels compared to HM mice, which correspond with increased colonic secretory responses. Notably, there was no microbiota-mediated effects on 5-HT_4R expression, or on 5-HT_4R-dependent colonic secretory responses.

Further support for the notion that the microbiota influence the enteric serotonergic system comes from antibiotic studies. Administration of broad-spectrum antibiotics can deplete luminal bacteria in an inducible and temporally controlled manner. Unlike GF studies, in which microbiota alterations are persistent throughout development, antibiotics provide an alternative strategy to alter microbiota composition following normal development, and to assess microbiota-dependent physiological processes and behavior.

Perturbation of the gut microbiota using antibiotic administration alters host 5-HT, 5-HT-mediated gut functions, and enteric nervous system (ENS) neuroanatomy. Mice treated with antibiotics exhibit lower levels of colonic 5-HT and decreased Tph1 expression (Ge et al., 2017; Yano et al., 2015), which parallel the findings of GF-associated deficits to the gut 5-HT system. Furthermore, these alterations in 5-HT levels result in functional changes to intestinal transit, as antibiotic-treated mice display slower whole gut transit (Caputi et al., 2017; Ge et al., 2017; Grasa et al., 2015; Vicentini et al., 2021) and slower colonic motility times (Ge et al., 2017; Grasa et al., 2015) compared to controls. In addition, these functional deficits are accompanied by loss of enteric neurons

(Vicentini et al., 2021). Interestingly, antibiotic-induced changes are reversible. Following reconstitution of the microbiota, mice display restored GI motility and enteric neurogenesis (Vicentini et al., 2021).

The evidence for whether specific commensal bacteria found in the human gut synthesize 5-HT *de novo* in physiologically relevant concentrations remains unclear. Indeed, certain bacteria have been shown to synthesize 5-HT in vitro (Roshchina, 2010; Shishov et al., 2009). However, this 5-HT synthesis may occur through indirect means and independent of Tph (Williams et al., 2014). Thus far, no human commensal bacteria that synthesize 5-HT have been identified.

III. INDIRECT MECHANISMS

Insights into host-microbiota dynamics further demonstrate that gut bacteria actively produce microbial metabolites capable of impacting the host. Emerging evidence suggests that these metabolites are key aspects of communication between the microbiota and the host to regulate physiological function in health and disease states. In the context of the enteric 5-HT system, microbial metabolites can stimulate 5-HT biosynthesis and release, leading to alterations in gut functions. This section highlights four key categories of microbial metabolites and summarizes the evidence of how they influence gut-derived 5-HT.

Short chain fatty acids

A notable group of microbial metabolites known to influence host physiology are short chain fatty acids (SCFAs). SCFAs are the products of bacterial fermentation of dietary fiber in the colon (Miller & Wolin, 1996). The most abundant SCFAs are acetate, propionate, and butyrate, which are present in an approximate molar ratio of 60:20:20, respectively. These dietary-derived microbial metabolites play a prominent role in the regulation of a variety of host physiological processes (Tan et al., 2014).

SCFAs influence the enteric 5-HT system by stimulating the release of 5-HT by intestinal EC cells, which express SCFA receptors (Lund et al., 2018). An early study by Fukumoto et al. demonstrated that intraluminal perfusion of SCFAs into the proximal colon of rats induced release of 5-HT, and consequently accelerated colonic transit and motility (Fukumoto et al., 2003).

SCFAs can also promote 5-HT biosynthesis. Stimulatory actions of acetate and butyrate on EC cells promote colonic Tph1 expression and 5-HT production in an in vitro human EC cell model (Reigstad et al., 2015). Furthermore, application of butyrate or propionate on RIN14B chromaffin cell culture leads to an elevation of 5-HT release and increase in Tph1 expression (Yano et al., 2015). In addition to regulating Tph1 expression, SCFAs can also impact expression levels of key 5-HT receptors. Indeed, acetate has been shown to decrease 5-HT_3R expression in vitro in GF mouse-derived colonoids (Bhattarai et al., 2017). Taken together, SCFAs are microbial metabolites that play an important role in the stimulation of enteric 5-HT biosynthesis (Fig 1B).

Tryptophan

A critical process that is influenced by gut microbes is Trp metabolism (Bosi et al., 2020). Trp is an essential amino acid, indicating that it cannot be synthesized de novo and therefore must be supplied through dietary intake. The recommended daily allowance of dietary Trp for adults is 4 mg/kg/day, or roughly 250-450 mg/day. Considering that the average intake for most adults is approximately 800-1000 mg/day (Richard et al., 2009), most individuals achieve this recommended daily intake of dietary Trp readily to meet their protein metabolic demands.

Once ingested, Trp is absorbed nearly completely in the small intestine of the GI tract, specifically within intestinal epithelial cells known as enterocytes (Keszthelyi et al., 2009). Two transporters facilitate this absorptive process. On the apical membrane of enterocytes, Trp absorption is mediated by the epithelial amino acid transporter B^0AT1 (Slc6a19) (Keszthelyi et al., 2009), along with all other neutral amino acids. On the basal

membrane of enterocytes, Trp transport occurs via the basolateral aromatic amino acid transporter TAT1 (Slc16a10) (Mariotta et al., 2012). Internalized Trp is then distributed to tissues through the body via the circulatory system.

Trp functions primarily as a component of protein synthesis. In the GI tract, Trp undergoes three different avenues of metabolism: 5-HT synthesis, kynurenine synthesis, and production of metabolites that act as ligands of the aryl hydrocarbon receptor (AhR). Though it is estimated that the kynurenine synthesis pathway accounts for over 90% of Trp metabolism, the significance of Trp metabolism in the kynurenine and AhR pathways is outside the scope of this manuscript, but this information can be found in recent reviews of the topic (Agus et al., 2018; Gao et al., 2018).

The gut microbiota play an active role in Trp availability, although the mechanisms and functional implications of this remain to be elucidated. While the majority of Trp is absorbed in the small intestine, it is possible some Trp can also reach the colon where it is subject to catabolism by the gut bacteria. GF mice exhibit 40% greater levels of plasma Trp than their conventional counterparts (Wikoff et al., 2009). These increased Trp levels could result from a variety of factors, such as GF-induced deficits in host Tph1 activity (Reigstad et al., 2015; Yano et al., 2015), lack of tryptophanase activity by enteric bacteria to metabolize dietary Trp (Wikoff et al., 2009), or altered metabolism down other pathways. Consistent with GF mice, antibiotic-treated mice also display elevated levels of Trp, in both the colonic mucosa (Hung et al., 2020) and cecal contents (Ogawa et al., 2020).

Microbially-mediated changes in Trp availability may have an impact on gut 5-HT availability and 5-HT-mediated intestinal functions (Fig 1C), though more research is

needed to characterize these effects. Some bacteria are known to possess the enzymatic capabilities to metabolize Trp directly using key enzymes in its catabolic and anabolic pathways. Specifically, Trp-catabolizing bacteria produce tryptophanase, the enzyme responsible for metabolizing Trp into indole, pyruvate, and ammonia (Agus et al., 2018). Conversely, Trp-synthesizing bacteria produce tryptophan synthase, an enzyme that catalyzes the biosynthesis of Trp from the metabolite indole (Agus et al., 2018). However, it remains to be determined whether these bacteria are present as human gut commensals.

Tryptamine

Another key metabolite indirectly influenced by gut microbes is tryptamine. Tryptamine is an indoleamine that is formed by the decarboxylation of L-tryptophan. Though it is present at relatively low concentrations in mammalian tissue, it is known to have a physiologically significant role in interacting with the host.

The characterization of tryptamine's potential functions in the ENS was brought about by an early study by Takaki et al. demonstrating that tryptamine stimulates the release of endogenous 5-HT within the GI systems of guinea pigs (Takaki et al., 1985). More recent work indicates that tryptamine acts as a 5-HT_4R ligand (Bhattarai et al., 2018). 5-HT_4Rs are G-protein-coupled receptors that are expressed in the epithelium of the GI tract, with the greatest level of expression in the distal colon (Hoffman et al., 2012). Stimulation of 5-HT_4Rs produces prokinetic effects (Mawe & Hoffman, 2013), and 5-HT_4R agonists promote colonic propulsive motility (Hoffman et al., 2012; Konen et al., 2021).

Tryptamine-induced activation of 5-HT_4Rs accelerates gut transit through increased secretion by intestinal epithelial cells (Bhattarai et al., 2018). Tryptamine can also, acting as a 5-HT_4R agonist, stimulate mucus release from goblet cells and prevent epithelial barrier disruption (Bhattarai et al., 2020), which has important implications for gut-derived inflammatory conditions such as irritable bowel syndrome.

The gut microbiota can play a role in tryptamine production (Fig 1D). For example, GF mice exhibit significantly lower levels of tryptamine in feces compared to HM mice (Marcobal et al., 2013). Furthermore, specific microbes present in the human intestinal microbiota possess the enzymatic capacity to decarboxylase Trp to produce tryptamine (Williams et al., 2014). It remains an open question whether, and to what degree, microbiota-mediated metabolism of Trp into tryptamine influences host physiology and 5-HT-related gut functions. Nonetheless, bacterially-mediated metabolism of Trp into tryptamine represents an exciting avenue for future investigation in the context of potential therapeutic applications.

Bile acids / secondary bile acids

Bile acids (BAs) are water-soluble, cholesterol-derived surfactants that are critical for digestive and absorptive processes. The effects of BAs in the lumen of the intestine have been shown to be region-specific. Colonic EC cells express the G protein-coupled bile acid receptor 1 (GPBAR1; also known as TGR5), for which BAs are endogenous ligands (Schaap et al., 2014). Notably, activation of GPBAR1 by intestinal BAs promotes 5-HT release and mediates prokinetic actions (Alemi et al., 2013; Li et al., 2021).

The gut bacteria play an active role in biotransforming primary BAs in the colon through deconjugation and dehydroxylation to produce the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA). The regulation of secondary BA metabolism by the gut microbiota is further supported by GF studies in which GF mice exhibit nonexistent levels of secondary BAs in intestinal tissue compared to conventional mice (Sayin et al., 2013; Selwyn et al., 2015). Similarly, antibiotic-treated mice display decreased levels of secondary BAs (Ge et al., 2017).

Secondary BAs are key microbial metabolites that can impact the enteric serotonergic system (Fig 1E). DCA, induced by spore-forming microbes, has been shown to elevate 5-HT levels and increase Tph1 expression, both in vitro in RIN14B chromaffin cells and in vivo in mice (Yano et al., 2015). Furthermore, reductions in relative abundances of bile-metabolizing bacteria is associated with impaired Trp metabolism, leading to reduced 5-HT bioavailability and delayed intestinal motility (Golubeva et al., 2017). Taken together, it is evident that microbially-regulated alterations in secondary BAs can influence 5-HT bioavailability and consequently induce changes in gut function.

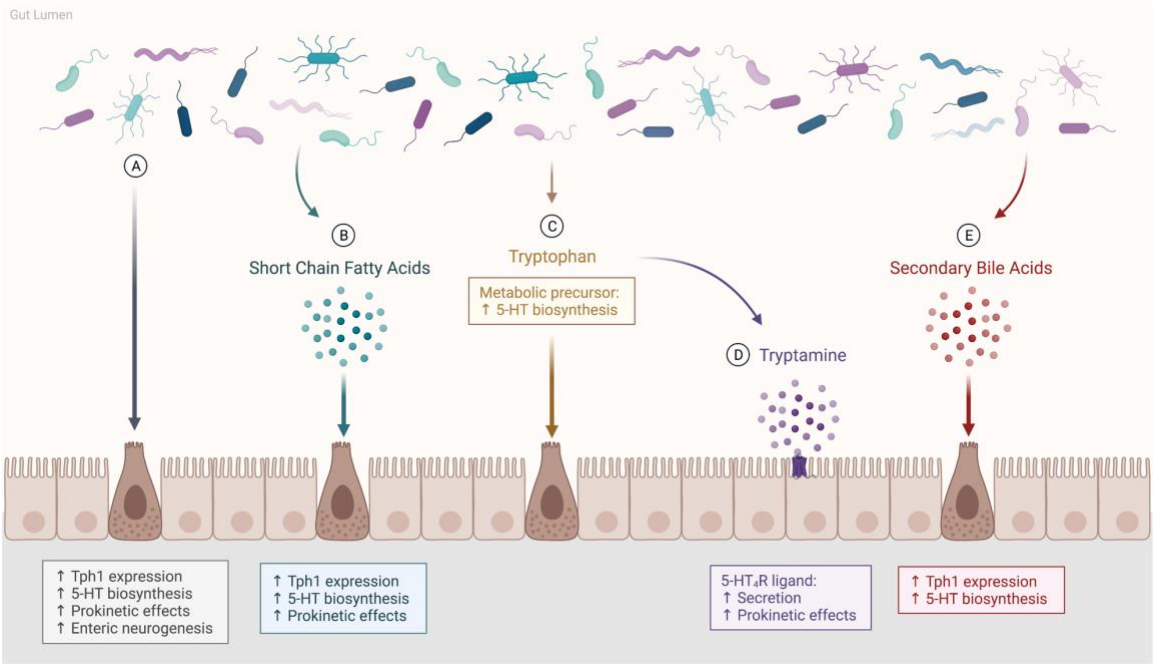

Figure 1. The gut microbiota mediate host serotonin (5-HT) through direct and indirect means. (A) Gut bacteria act directly on enterochromaffin (EC) cells to increase colonic tryptophan hydroxylase 1 (Tph1) expression and promote 5-HT synthesis. (B-E) Enteric bacteria alter host 5-HT indirectly through microbial metabolites, including short chain fatty acids, tryptophan, tryptamine, and secondary bile acids. (B) Short chain fatty acids are dietary-derived metabolites produced by bacterial fermentation that stimulate 5-HT synthesis and release by EC cells, and induce prokinetic effects on intestinal motility. (C) Tryptophan is an essential amino acid and its metabolism is under control of the gut microbiota. It functions as the metabolic precursor to 5-HT as well as tryptamine. (D) Tryptamine acts as a ligand for the 5-HT_4 receptor ($5\text{-HT}_4\text{R}$) to stimulate secretion by intestinal epithelial cells, thereby inducing prokinetic actions. (E) Secondary bile acids, formed by the gut microbiota from primary bile acids, promote Tph1 expression and stimulate 5-HT synthesis. Created with BioRender.com.

IV. BENEFICIAL BACTERIA APPLICATIONS

A transient approach to manipulate the gut microbiota is through oral administration of isolated bacterial strains such as those included in non-colonizing "probiotic" formulations. Numerous studies report beneficial effects on host physiological processes of orally-delivered bacteria in both animal models and humans. In the context of GI function, for example, administration of certain bacteria can prevent stress-induced intestinal barrier dysfunction (Fukui et al., 2018; Zareie et al., 2006) and colonic dysfunction (Gareau et al., 2007), as well as reduce whole gut transit time (Dimidi et al., 2014; Krammer et al., 2011; Marteau et al., 2002).

Despite evident impacts of bacteria administration on many functions, the underlying mechanisms of action of such bacteria are not well elucidated. Along with the need for more mechanistic-based investigations, it is becoming clear that there is a need for a deeper understanding of bacterial species and strain-specific effects. Certainly, these investigations are underway and will yield stimulating findings in the coming years.

A developing avenue of exploration and potential therapeutic exploitation is the use of specific microbial strains that can alter the production and regulation of key bioactive metabolites. Indeed, certain strains have been shown to change the bioavailability of enteric 5-HT. A study by Nzakizwanayo et al. (2015) demonstrated that *Escherichia coli Nissle 1917*, a bacteria found in "probiotics" currently available on the market, enhances 5-HT bioavailability in a dose-dependent manner in an *ex-vivo* model (Nzakizwanayo et al., 2015). Furthermore, this strain increases intracellular 5-hydroxytryptophan (5-HTP)

levels and decreases 5-HIAA levels, supporting a hypothesis that *Escherichia coli Nissle 1917* alters 5-HT through synthesis and clearance, respectively.

Spore-forming bacteria can promote 5-HT biosynthesis through the elevation of host colonic Tph1 expression (Yano et al., 2015). Furthermore, monoassociation of GF mice with the strain *Clostridium ramosum* increases host 5-HT bioavailability (Mandić et al., 2019). Expression levels of ileal and colonic Tph1 and MAO-A are significantly greater in *Clostridium ramosum* monocolonized mice compared to GF controls. In addition, in vitro stimulation with *Clostridium ramosum* provokes an increased 5-HT release in RIN14B chromaffin cells as well as organoids from murine small intestine and colon.

Commensal lactic acid bacteria represent another class of "beneficial" bacteria known to exert effects on the enteric serotonergic system. For example, studies report that increased intestinal SERT concentrations are associated with *Bifidobacterium (B.) dentium* (Engevik et al., 2021), *B. longum* (Cao et al., 2018), *Lacticaseibacillus* (formerly classified as *Lactobacillus*) rhamnosus (Cao et al., 2018; Wang et al., 2015), *Lactobacillus acidophilus* (Cao et al., 2018), and *Limosilactobacillus* (formerly classified as *Lactobacillus*) *reuteri* (Engevik et al., 2021). Additionally, monoassociation with the strain *B. dentium* promotes the secretion of microbial metabolites, including the SCFA acetate, that act to modulate the host serotonergic system (Engevik et al., 2021). Specifically, mice monoassociated with *B. dentium* displayed increased intestinal 5-HT levels and upregulated colonic 5-HT_4R and SERT mRNA expression.

Taken together, mounting evidence suggests that the administration of beneficial isolated bacteria can alter host gut-derived 5-HT and influence 5-HT-mediated gut functions in a transient, inducible, and reversible way. Further resolution of the

underlying mechanisms of actions of specific bacteria will move the field forward to encourage investigations into potential therapeutic applications.

V. IMPLICATIONS FOR 5-HT IN THE MICROBIOTA-GUT-BRAIN AXIS

Overview of the microbiota-gut-brain axis

The gut-brain axis refers to the bidirectional line of communication between the gut and the brain through neural, immune, and endocrine means. Dating back to the foundational theoretical work of William James and Carl Lange in the 1800s, a wealth of research has since demonstrated top-down contributions of psychological stress and emotions on GI function (Mayer, 2011). Recently, a growing body of research on the microbiome highlights its remarkable capacity for bottom-up modulation, leading many in the field to expand the concept into the "microbiome-gut-brain axis".

The brain communicates with the GI tract in a top-down fashion primarily through parallel efferent pathways originating in the hypothalamus, amygdala, and cortical regions. Such pathways include the sympathetic and parasympathetic components of the autonomic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis, and descending monoaminergic pathways. Sympathetic effects on the gut are inhibitory and act to slow intestinal transit and secretion (Mayer, 2011). Parasympathetic effects, on the other hand, act in an excitatory manner to stimulate motility, secretion, and release of key signaling molecules such as serotonin, gastrin, and somatostatin (Mayer, 2011). The HPA axis coordinates adaptive stress responses and regulates numerous homeostatic systems, including digestion and metabolism (Farzi et al., 2018).

The gut communicates with the brain primarily through neural and endocrine means. The vagus nerve acts as a bidirectional conduit for information passing between the GI

tract and the brain (Martin et al., 2018; Mayer, 2011). Moreover, gut peptides and neuropeptides, released in response to luminal stimuli, can act in a paracrine manner to activate receptors located on adjacent vagal afferents. These peptides can also act in an endocrine manner when released into the circulation to signal to various brain regions, particularly circumventricular organs such as the hypothalamus and area postrema (Martin et al., 2018).

Serotonin in the brain

In the central nervous system (CNS), the primary sites of 5-HT synthesis and storage are clusters of neurons in midbrain and pontine regions, with the raphe nuclei accounting for the majority of serotonergic cell bodies and projection fibers (Mohammad-Zadeh et al., 2008). The rate limiting enzyme of neuronal 5-HT synthesis is the isoform tryptophan hydroxylase 2 (Tph2) (Walther et al., 2003). Notably, gut 5-HT and CNS 5-HT are considered separate pools, since 5-HT does not cross the blood-brain barrier (BBB).

5-HT activates a variety of receptors located pre- and post-synaptically, with the 5-HT_1, 5-HT_2, and 5-HT_4 families being most common in the CNS. The diverse array of receptor subtypes offers an expansive range of 5-HT-mediated actions in the brain (Uphouse, 1997). In the CNS, 5-HT availability, in addition to serotonergic neurons expressing SERT, is also controlled by presynaptic autoreceptors that act as negative feedback mechanisms to inhibit further 5-HT release into the synaptic cleft (Cerrito & Raiteri, 1979). While 5-HT's functions in the gut are fairly well characterized, its actions in the brain, though extensively studied, remain more elusive. Serotonin has been implicated in a myriad of functions, including but not limited to mood (Kandel et al.,

2012), anxiety (Lowry et al., 2005), food consumption (Simansky, 1996), reward (Li et al., 2016), and sleep (Chowdhury & Yamanaka, 2016; Muraki et al., 2004).

Converging lines of evidence indicate that dysfunctions in microbiota-gut-brain communication can have salient pathophysiological consequences, and at least some of these appear to involve 5-HT. Indeed, it is estimated that nearly 60% of individuals diagnosed with anxiety or depression also report symptoms of intestinal dysfunction (Liu & Zhu, 2018). Clinically, pharmacological treatments for anxiety and depression target the 5-HT system and act to increase 5-HT levels by a variety of mechanisms, including selectively inhibiting or impairing 5-HT reuptake, inhibiting 5-HT metabolism, or increasing 5-HT release (Mohammad-Zadeh et al., 2008). 5-HT's enigmatic role in mood is of particular relevance to gut-brain communication given the high comorbidities of anxiety and depressive symptoms and GI dysfunction. However, such treatments pose distinct disadvantages. Notably, the delayed onset of action of selective serotonin reuptake inhibitors (SSRIs) as well as mixed efficacies (Mohammad-Zadeh et al., 2008) suggest alternative or adjuvant treatment options are worth pursuing. The microbiome is one such promising target.

Tryptophan as a potential microbiota-gut-brain target

Given that 5-HT does not cross the BBB, changes in circulating 5-HT levels are unlikely to influence 5-HT signaling in the CNS. However, intervention in microbiota-gut-brain 5-HT signaling may be possible through Trp acting as a precursor-mediated signal. Given that Trp metabolism is decisively regulated by the gut microbiota, it is possible that Trp is a critical point of intersection within the microbiota-gut-brain axis.

Early studies demonstrate that the relationship between brain Trp concentrations and 5-HT synthesis is directly proportional, such that an injection of Trp produces a rapid elevation of brain Trp concentrations, and consequently a rapid rise in 5-HT synthesis (Carlsson & Lindqvist, 1978; Moir & Eccleston, 1968).

Subsequent work to elucidate this relationship reveals that the ability of Trp to influence brain 5-HT synthesis depends principally on three factors. The first factor is the competitive nature by which Trp crosses the BBB. Upon absorption in the gut, circulating Trp must compete with other large neutral amino acids (LNAAs) to enter the brain through the amino acid transporter. The bioavailability of Trp to cross the BBB therefore depends on the sum of the competing amino acids, often expressed as the "tryptophan ratio" (Fernstrom & Wurtman, 1972; Richard et al., 2009). Altering the tryptophan ratio by either raising plasma Trp levels or lowering concentrations of other LNAAs can thus maximize the amount of Trp available for brain 5-HT synthesis.

The competitive transport mechanism for Trp is particularly relevant during food consumption. Studies in rats suggest the composition of a meal can indirectly determine brain Trp uptake and subsequent 5-HT synthesis due to the meal's effects on the Trp ratio. A protein-rich meal, for instance, raises both plasma Trp and other LNAA levels to a similar degree, thus the net effect on brain Trp and 5-HT is negligible (Fernstrom et al., 1985). On the other hand, a carbohydrate-rich meal increases plasma Trp concentrations and decreases concentrations of the other LNAAs through insulin activation, which therefore favors Trp transport into the brain to increase precursor availability and 5-HT synthesis (Caballero et al., 1988; Richard et al., 2009).

The second main factor depends on the enzyme kinetics of Tph2, which like Tph1, is only partially saturated under normal conditions. Thus, in theory, raising or lowering brain Trp concentrations could respectively increase or decrease the rate of brain 5-HT synthesis (Carlsson & Lindqvist, 1978; Fernstrom, 2013). Further investigation is warranted to determine the characteristics by which this occurs.

Lastly, in order to influence CNS 5-HT-mediated behaviors, changes in Trp levels must ultimately affect neuronal 5-HT release, following which 5-HT can interact with various receptors to influence behavior. In support of this concept, Sharp et al. used in vivo microdialysis in rats to demonstrate that pre-treatment with Trp significantly enhanced 5-HT release upon electrical stimulation of serotonergic dorsal raphe nucleus neurons (Sharp et al., 1992).

Though it is thought that less than 5% of dietary Trp is used for 5-HT synthesis throughout the body, the serotonergic impacts on the CNS are demonstrated in experiments involving manipulations of dietary Trp availability. Indeed, acute Trp depletion via consumption of a Trp-free diet produces striking reductions in brain Trp and 5-HT levels in both animal models and humans (Biskup et al., 2012; Fadda, 2000; Gessa et al., 1974; Nishizawa et al., 1997). Conversely, Trp loading via ingestion of pure Trp, α-lactalbumin, or carbohydrates induces robust increases in brain 5-HT levels (Fernstrom & Wurtman, 1972; Silber & Schmitt, 2010). Furthermore, such modulation of 5-HT levels may have impacts at the behavioral level, as several rodent studies report Trp manipulations impact anxiety- and depressive-like behavior (Biskup et al., 2012; Jans et al., 2010).

Certain gut bacteria may also have the ability to alter Trp levels as well. A novel, microbiome-targeted strategy to manipulate Trp availability and subsequent 5-HT synthesis could exploit certain bacteria's biochemical capacity to manipulate Trp. By possessing key enzymes in Trp catabolism or anabolism, such bacteria may be able to alter availability of the amino acid for absorption. Oral administration of these bacteria may impact microbiota-gut-brain signaling by altering Trp levels, and ultimately modulate 5-HT-mediated physiological and behavioral effects. Further investigation is necessary to better understand how to utilize orally administered bacteria as a potential therapeutic interface between microbiota-gut-brain communication and function.

VI. CONCLUSION

The dynamic interactions between the host and the gut microbiota underlie many physiological processes. It is evident that a deeper understanding of how gut microbes act to mediate the enteric serotonergic system will drive the field forward towards clinical applications for the targeted use of bacteria to enhance intestinal function and behavior.

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CHAPTER 3: TRYPTOPHAN-SYNTHESIZING BACTERIA ENHANCE COLONIC SEROTONIN SIGNALING AND GASTROINTESTINAL MOTILITY

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Legan, T. B., Lavoie, B., Norberg, E., Schaer, H. H., Tack, S., Parker, K., Tompkins, T., Wargo, M., Mawe, G. M. Tryptophan-synthesizing bacteria enhance colonic serotonin signaling and gastrointestinal motility. *Gut Microbes*.

ABSTRACT

An emerging strategy to treat symptoms of gastrointestinal (GI) dysmotility utilizes the oral administration of isolated bacteria. However, the underlying mechanisms of action of these bacterial agents are not well established. Here, we elucidate a novel strategy to promote serotonin (5-HT) signaling and 5-HT-mediated gut functions by exploiting the biochemical capability of specific bacteria to alter luminal availability of the essential amino acid and 5-HT precursor tryptophan (Trp). We utilized the isolated bacterial strain *Bacillus (B.) subtilis R0179*, which expresses tryptophan synthase, a key enzyme in Trp synthesis from indole. To test our hypothesis that oral administration of the Trp-synthesizing strain *B. subtilis R0179* alters intestinal 5-HT signaling and motility, we treated C57BL/6J mice daily via oral gavage with *B. subtilis R0179* spores. Mice that received one week of *B. subtilis R0179* treatment exhibited greater colonic tissue levels of Trp and increased 5-HT signaling, as indicated by an elevated 5-HIAA/5-HT ratio, compared to vehicle-treated mice. Furthermore, treatment with *B. subtilis R0179* accelerated colonic motility in both healthy mice as well as in a mouse model of constipation. Lastly, we found that the prokinetic effects of *B. subtilis R0179* were

blocked by co-administration of a 5-HT_4 receptor (5-HT_4R) antagonist and were absent in 5-HT_4R knockout mice, demonstrating the involvement of the 5-HT_4R in mediating the bacteria's prokinetic actions. Taken together, these data demonstrate that serotonergic signaling and intestinal motility can be augmented by treatment with bacteria that synthesize Trp. Our findings provide mechanistic insight into a transient and predictable bacterial strategy to promote GI motility.

Keywords: bacteria, enteric nervous system, intestinal motility, serotonin signaling, microbial metabolites, tryptophan, tryptophan synthase

INTRODUCTION

It is widely accepted that the gut microbiome plays an active role in contributing to our health and well-being. Indeed, the tens of trillions of microorganisms lining the gastrointestinal (GI) tract interact dynamically to influence the physiological homeostasis of the host (Cryan et al., 2019). As such, an emerging field of research identifies interventions to manipulate the existing gut microbiota or additions of exogenous bacteria to affect pathologic conditions and dysbiosis.

Healthy GI function necessitates a series of coordinated neural reflexes and signaling molecules. Serotonin (5-hydroxytryptamine; 5-HT) is known to play an integral role in regulating intestinal functions including motility, secretion, and blood flow, as well as delivering signals to the central nervous system (CNS) (Mawe & Hoffman, 2013). The majority of the body's 5-HT is synthesized in the epithelium of the gut by enterochromaffin (EC) cells from the precursor tryptophan (Trp) using the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1). Once synthesized, 5-HT is released in response to luminal chemical and mechanical stimuli to activate a host of GI reflexes, including peristalsis and secretion. It is known that 5-HT exerts its effects on GI function through the activation of a variety of receptors, including the 5-HT_3 and 5-HT_4 receptors (Cryan et al., 2019; Hoffman et al., 2012).

Mounting evidence points to a key role of gut microbes in influencing the host enteric 5-HT system as well as 5-HT-mediated gut functions (Legan et al., 2022). Both germ-free mice and antibiotic-treated animals exhibit decreased levels of colonic 5-HT (Ge et al., 2017; Reigstad et al., 2015; Yano et al., 2015) and decreased colonic mRNA expression

of Tph1 (Ge et al., 2017; Reigstad et al., 2015; Yano et al., 2015), which are accompanied by functional deficits in GI motility (Ge et al., 2017; Grasa et al., 2015; Vicentini et al., 2021; Yano et al., 2015). Moreover, gut bacteria produce microbial metabolites that can stimulate 5-HT biosynthesis and release, and induce prokinetic effects on GI motility (Legan et al., 2022). One critical microbially-mediated process is the regulation of Trp metabolism (Agus et al., 2018). Trp, as an essential amino acid, cannot be synthesized de novo by the body and instead is acquired through dietary intake in sufficient quantities to meet metabolic demands. Notably, some bacteria are enzymatically capable of degrading Trp directly using tryptophanase or synthesizing Trp using tryptophan synthase (Agus et al., 2018).

The administration of isolated bacterial strains, commonly referred to as “probiotics”, is an emerging field of research that seeks to beneficially alter host physiology for therapeutic ends. These live microorganisms, given commonly at doses of billions of colony forming units per day, are thought to exert pleiotropic effects on the host without inducing dysbiosis or major shifts in the microbiota composition (Kristensen et al., 2016). Orally delivered bacteria can influence mucosal serotonergic signaling by promoting the bioavailability of host enteric 5-HT (Mandić et al., 2019; Nzakizwanayo et al., 2015; Yano et al., 2015), or through indirect means such as stimulating the secretion of microbial metabolites that act on the serotonergic system (Engevik et al., 2021). While targeted bacterial treatments offer exciting potential to alter gut function in a transient, inducible, and reversible manner, there is a growing need for the elucidation of mechanisms underlying the effects of specific bacterial species and strains. This will facilitate optimization of desired outcomes.

In this study, we investigated a novel strategy to promote enteric 5-HT signaling and subsequent 5-HT-mediated functions by modulating Trp availability using the biochemical properties of specific bacteria. We utilized the bacterial strain *Bacillus (B.) subtilis R0179* that is known to express tryptophan synthase, a key enzyme that synthesizes Trp from indole. We tested the hypothesis that oral administration of these Trp-synthesizing bacteria alters the availability of the amino acid for absorption in the intestinal lumen to promote 5-HT biosynthesis and induce prokinetic effects on GI function.

METHODS

Animals

This study used male C57BL/6J mice (Jackson Labs; Bar Harbor, ME), and male and female 5-HT_4RKO mice and their WT littermates aged 8-9 weeks and bred in-house on an SV129 background (Dr. Valerie Compané, Université Montpellier, via Dr. David Linden, Mayo Clinic). All animals were housed at the University of Vermont's Given Animal Facility and cared for in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* as well as the Office of Animal Care Management of the University of Vermont. Mice were weighed and monitored daily, although no adverse effects on health due to bacterial treatment were expected. All experimental protocols were approved by the University of Vermont Institutional Animal Care and Use Committee (Protocols# PROTO202000040; PROTO202100028).

Bacteria

These experiments primarily utilized *Bacillus (B.) subtilis* strain *R0179*, provided by Lallemand Health Solutions© (Montreal, QC), which has been used in probiotic formulations and is known to be safe in humans.

Freeze-dried spores of *B. subtilis R0179* are stored at 4°C. Each day of treatment, the bacteria were suspended at a concentration of 10^9 CFU/ml in phosphate buffered saline (PBS) and administered by oral gavage with a 100µl dose per mouse per day. Mice in the vehicle control group received a daily oral gavage of 100µl PBS.

To validate our experimental approach, we tested for the presence of the R0179 strain of *B. subtilis* in fecal samples collected from experimental and control mice. A QIAamp PowerFecal DNA Kit (Qiagen; Germantown, MD) was used to extract DNA from stool samples collected on the last day of treatment. PCR assays, using primers designed and tested by Lallemand to be specific to the R0179 strain of *B. subtilis*, confirmed the presence of the experimental strain in bacteria-treated mice. No bacterial cross-contamination was present in stool samples from mice in the control group.

In addition to the vehicle group, the bacterial experiments for this study utilized two additional control treatments. Heat-inactivated *B. subtilis R0179* was generated by autoclaving *B. subtilis R0179* spores for 40 minutes. The heat-killed spores were then combined with PBS and incubated for 30 minutes in a 56ºC water bath, plated on LB agar plates, and incubated in a 37ºC warm room for 24 hours to confirm cell death.

The *B. subtilis* strain *1A2*, which is a non-revertible loss of function mutant in the tryptophan synthase gene, was obtained as spore-containing discs from the Bacillus

Genetic Stock Center (Ohio State University, Columbus, OH). The spore-containing discs were plated on LB agar, covered with 1 mL LB liquid medium, and incubated at 37ºC overnight. Growth from this plate was streaked for isolation on another LB agar plate for 37ºC overnight incubation. One colony was then isolated and transferred to a 3 mL aliquot of LB liquid medium for repeat incubation at 37ºC overnight. 50% glycerol stock was then created using 500 µL glycerol and 500 µL water before being stored at -80ºC. For daily gavage, one LB agar plate was inoculated with the bacterial glycerol stock and incubated at 37ºC overnight. One single colony was then incubated at 37ºC overnight with 3 mL LB liquid medium. The *1A2* gavage solution was created by combining 10 µL fresh bacterial culture with 3 mL PBS each day for a concentration of 10^9 CFUs/mL.

Incubation times for the desired bacterial concentration of 10^9 CFUs/mL were determined using a 96-well plate serial dilution procedure. The optical density of the liquid cultures was measured daily at 600 nm to ensure the desired bacterial concentration was being reached daily.

High performance liquid chromatography with electrochemical detection

Measurements of Trp, 5-HT, and 5-HIAA in intestinal tissue were obtained using high performance liquid chromatography (HPLC) with electrochemical detection. Upon euthanasia, 0.5cm of distal colon tissue was collected from experimental mice, flash frozen in liquid nitrogen, and stored at -80ºC.

For processing, tissue samples were thawed, homogenized in 800µl 0.1 M perchloric acid (Sigma Aldrich), and centrifuged for 15 minutes at 13,000 x g at 4ºC. The

supernatant was collected and centrifuged once more for 15 minutes at 13,000 x g at 4ºC. The resultant supernatant was processed through a series of filtration steps using 17mm Nylon 0.45micron and 0.2micron syringe filters (Thermo Scientific). The final retention volume was 400-500µl per sample, which was aliquoted and stored at -80ºC until analysis by HPLC.

For analysis, 20µl of each sample was injected into the HPLC system. The system includes a Shimadzu LC-10ADvp pump (Shimadzu Corp.; Kyoto, Japan) and a Coulochem II electrochemical detector (ESA Inc.; Chelmsford, MA). The mobile phase was comprised of 90mM dihydrogen phosphate, 50mM citric acid, 4% acetonitrile, and 50µM EDTA at a pH of 3, and was pumped through a Microsorb 100-3 C18 100 x 4.6mm column (Agilent Technologies, Inc.; Santa Clara, CA) at a flow rate of 0.4 mL/min.

Calibration curves were generated for each metabolite based on injections of known standard concentrations (Sigma Aldrich). Trp, 5-HT, and 5-HIAA were identified by their expected retention times in chromatographs generated using PC software (Chromperfect; Denville, NJ). Concentrations of Trp, 5-HT, and 5-HIAA were quantified by calculating the area under the curve in the chromatographs for each injected sample, and then were normalized to tissue weight.

Whole Gut Transit Time

Whole gut transit time is a net measure of intestinal function that encompasses gastric emptying, small bowel transit, and colonic transit times. Unfasted mice were orally gavaged with 300µL non-nutrient solution made of 6% carmine red, 0.5%

methylcellulose, and tap water. Carmine red is nonabsorbable in the gut lumen and therefore provides a visual indication of transit time through the entire GI tract. Following gavage, mice were placed individually in empty cages with access to food and Napa Nectar *ad libitum*. The whole gut transit time was calculated by the amount of time elapsed from time of gavage to time of expulsion of the first carmine red-positive fecal pellet.

Colonic Motility

Colonic motility was assessed using the bead expulsion assay. Following an acclimation period during which mice were placed in separate cages for one hour, unfasted mice were then lightly anesthetized with 3% isoflurane and a size 10 bead was inserted 2 cm into the distal colon using a blunt gavage needle. Colonic motility time was calculated by the amount of time elapsed from insertion of bead to its expulsion.

Experimental autoimmune encephalomyelitis (EAE) induction

Mice were immunized at 8 weeks old with mouse spinal cord homogenate (MSCH) using the complete Freund's adjuvant (CFA) plus pertussis toxin (PTX) single-inoculation protocol (Teuscher et al., 2006). Mice received three posterior subcutaneous injections (50µl each) of an emulsion of CFA containing 200µg/mL *Mycobacterium tuberculosis* H37RA (Becton Dickinson & Co.) and 25mg/mL MSCH. These injections were followed by intraperitoneal injection of 200 ng PTX on day 0 and again on day 2 of induction.

Mice were weighed and monitored daily beginning at 10 days post-induction. Cumulative clinical disease scores were recorded daily as previously described (Spear et

al., 2018). Constipation, the hallmark symptom of GI dysmotility in this model, was assessed on day 21 post-induction. Following the assessment, mice displaying constipation were treated for one week with *B. subtilis R0179* or vehicle control.

Tryptophan diets

Mice were fed a control normal diet (Baker Amino Acid Diet 5CC7, TestDiet; St. Louis, MO) with 0.20% Trp for 10 days prior to the start of the experiment. Baseline motility was assessed following this period. Mice were then placed on diets that had been modified based on the control diet to consist of no added Trp (0.0%, 575J) or twice the amount of Trp (0.45%, LT570) for 10 days. All diets contained the same nutritional profiles except for these modifications in Trp content. Diets were fed ad libitum in irradiated pellet form in cage hoppers with full access to fresh water at all times. Motility was assessed following 10 days of modified diet access.

Statistics

All statistical analyses were performed using GraphPad Prism (v.9, GraphPad Software; San Diego, CA). Data are presented as individual values or as mean ± standard error of the mean (SEM). Statistical differences were determined by analysis using unpaired Student's *t*-test, paired Student's *t*-test, or one-way ANOVA with Tukey's post-hoc multiple comparisons test. Outliers were determined using a Q test, and values that were greater than 2 standard deviations from the mean were excluded. Statistical significance is considered as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

RESULTS

While Trp synthesis is a well-recognized feature of *B. subtilis*, we confirmed that the *R0179* strain produces Trp by comparing Trp levels in growth medium at the beginning and end of a 48 hour incubation period. In this experiment, an increase in Trp of 75 µg/mL was detected, confirming that this *R0179* strain of *B. subtilis* is a Trp synthesizer.

B. subtilis R0179 treatment alters colonic Trp levels and 5-HT turnover

To determine whether *B. subtilis R0179* treatment alters intestinal 5-HT signaling, concentrations of Trp, 5-HT, and 5-HIAA in distal colon tissue were measured by HPLC. Mice treated with *B. subtilis R0179* exhibited greater levels of Trp in the distal colon compared to vehicle-treated mice (Fig. 1a). While there was no significant difference in colonic 5-HT levels (Fig. 1b), bacteria-treated mice displayed greater levels of the 5-HT breakdown product, 5-HIAA (Fig. 1c), as well as a significantly elevated 5-HIAA/5-HT ratio (Fig. 1d), which indicates an increase in 5-HT release and breakdown.

To examine whether these *B. subtilis R0179*-induced changes were due to the bacteria's live nature, we analyzed distal colon samples from mice treated with autoclaved *B. subtilis R0179*. Treatment with heat-inactivated *B. subtilis R0179* did not alter levels of Trp, 5-HT, or 5-HIAA in distal colon tissue compared to vehicle treatment (Fig. 2a), demonstrating that administration of viable *B. subtilis R0179* spores is required to induce alterations in metabolite levels.

This *R0179* strain of *B. subtilis* expresses the enzyme tryptophan synthase, which synthesizes Trp from indole. To determine whether these altered colonic metabolite levels from *B. subtilis R0179* treatment were due to this strain's Trp-synthesizing

property, we analyzed distal colon tissue of mice treated with a *B. subtilis* strain (*B. subtilis 1A2*) that is a tryptophan auxotroph due to mutation of the tryptophan synthase gene. Mice treated with *B. subtilis 1A2* exhibited no differences in colonic Trp, 5-HT, or 5-HIAA levels compared to vehicle-treated mice (Fig. 2b).

B. subtilis R0179 treatment induces prokinetic effects on colonic motility

The findings described above demonstrate that *B. subtilis R0179* increases intestinal Trp and 5-HT signaling. We therefore sought to examine whether bacteria treatment consequently alters 5-HT-mediated gut function. To assess changes in intestinal function in response to *R0179* treatment, we performed validated motility assays at the beginning and end of a 7 day daily treatment period.

Whole gut transit time is a non-localized measure of intestinal function comprised of the net motility times of gastric emptying, small bowel transit, and colonic transit. Following a week of treatment with *B. subtilis R0179*, there was no statistically significant change in whole gut transit time in either vehicle-treated mice (Fig. 3a) or bacteria-treated mice (Fig. 3b). However, the two treatment groups were significantly different in percent change from baseline (Fig. 3c).

To evaluate colonic motility, we employed the bead expulsion assay. Mice that received *B. subtilis R0179* exhibited significantly faster colonic motility on day 7 compared to baseline (Fig. 3e), whereas vehicle-treated mice displayed no change in colonic motility (Fig. 3d). Furthermore, the treatment groups were significantly different when the findings were evaluated as percent change from baseline (Fig. 3f). The data

from these functional assays indicate that *B. subtilis R0179* produces prokinetic effects on intestinal motility that are localized to the colon.

To further elucidate that these prokinetic effects were produced only by the viable, Trp-synthesizing strain *B. subtilis R0179*, we assessed changes in intestinal function in mice treated with heat-inactivated *B. subtilis R0179* spores as well as mice treated with the Trp synthase-null strain *B. subtilis 1A2*. There were no significant changes in either whole gut transit times (Fig. 4a-c) or colonic motility times (Fig. 4d-f) in mice receiving either of these treatments. Taken together, these results indicate that the prokinetic effects of *B. subtilis R0179* are due to viable bacteria and the presence of a functional tryptophan synthase gene.

B. subtilis R0179 restores colonic motility in a model of constipation

Since we detected prokinetic effects of *B. subtilis R0179* treatment in healthy mice, we next sought to investigate whether bacteria treatment could promote motility in the context of GI dysfunction. Similar to patients with multiple sclerosis, mice with EAE exhibit features of constipation, including slower whole gut transit and slower colonic motility (Spear et al., 2018). We therefore used this model to test whether *B. subtilis R0179* treatment could restore motility in mice with constipation.

We assessed intestinal motility in mice at day 0 and at day 21 following the development of constipation for three weeks post-induction. We treated mice displaying constipation with either *B. subtilis R0179* or vehicle for one week to evaluate changes in motility due to bacteria treatment in constipated animals (Fig. 5a). Bacteria-treated mice exhibited faster whole gut transit times following a week of treatment with *B. subtilis*

R0179 (Fig. 5c). This recovery was seen in vehicle-treated mice as well (Fig. 5b), and there was no significant difference between groups in the percentage change from baseline (Fig. 5c), suggesting that there is a possibility for natural recovery from EAE-induced dysmotility in the net motility measure of whole gut transit.

To examine whether *B. subtilis R0179* induces prokinetic effects localized to the colon in constipated animals, as was the case in healthy mice (Fig. 3), we performed the bead expulsion assay following a week of bacteria treatment. We found that *B. subtilis R0179*-treated mice displayed significantly faster colonic motility times after the treatment course (Fig. 5f). Notably, there was no significant change in colonic motility time in vehicle-treated mice (Fig. 5e). Taken together, these findings demonstrate that *B. subtilis R0179* treatment restores colonic motility function in a mouse model of constipation.

B. subtilis R0179 enhances intestinal motility through 5-HT$_4$Rs

5-HT signals through a variety of receptors to regulate intestinal motility (Mawe & Hoffman, 2013). The 5-HT$_4$R, which is located on enteric neurons as well as on intestinal epithelial cells (Hoffman et al., 2012), is known to play an important role in enhancing intestinal motility and secretion. Notably, 5-HT$_4$R stimulation produces prokinetic effects (Mawe & Hoffman, 2013), and agonists at the 5-HT$_4$R promote colonic propulsive motility (Hoffman et al., 2012; Konen et al., 2021). Thus, we sought to further elucidate the mechanisms by which *B. subtilis R0179* exerts prokinetic effects and investigate whether the 5-HT$_4$R plays a role.

To test the involvement of 5-HT$_4$Rs in the prokinetic actions of *B. subtilis R0179*, we first employed a pharmacological approach to block the 5-HT$_4$R with a selective antagonist. We treated mice daily with *B. subtilis R0179* + GR113808 (1 mg/kg; Tocris Bioscience, Minneapolis, MN) via oral gavage for one week. Treatment with the 5-HT$_4$R antagonist GR113808 blocked the prokinetic effects of *B. subtilis R0179* alone in whole gut transit (Fig. 6a) and colonic motility (Fig. 6b).

We also utilized a genetic approach to target the 5-HT$_4$R by administering *B. subtilis R0179* to 5-HT$_4$R knockout mice and their wild type littermate controls. Following a week of *B. subtilis R0179* treatment, wild type mice exhibited significantly faster whole gut transit times (Fig. 6c) and colonic motility times (Fig. 6f), which is consistent with bacteria-induced prokinetic actions. However, *B. subtilis R0179* treatment did not elicit any changes to intestinal motility in 5-HT$_4$R KO mice (Fig. 6d,g). Taken together, these findings demonstrate that the 5-HT$_4$R plays a pivotal role in the prokinetic actions of *B. subtilis R0179*.

Dietary increases in Trp do not enhance intestinal motility

The traditional approach to modifying Trp availability is to alter the concentration of this essential amino acid in the diet. To determine whether dietary manipulations of Trp alter colonic 5-HT signaling and intestinal motility, we fed mice chow that contained no Trp (Trp-) or twice as much Trp (Trp2x) compared to a control chow that had normal levels of Trp. Mice on the Trp2x diet exhibited significantly elevated levels of colonic 5-HT compared to mice on both the control and Trp- diets (Fig. 7a). There was no difference in colonic 5-HT levels between the Trp- and control-diet fed mice.

Furthermore, there was a trend towards a reduction of the 5-HIAA/5-HT ratio in the Trp2x diet group compared to the Trp- diet group, suggesting that there may be reduced colonic 5-HT turnover, however this effect did not reach statistical significance.

Assessments of intestinal function indicated that mice on the Trp2x diet displayed significantly slower whole gut transit times after consuming a Trp2x diet for 10 days (Fig. 7c), whereas there was no change in whole gut transit time in mice that consumed the Trp- diet (Fig. 7b). Furthermore, there was no significant effect of either the Trp2x or Trp- diets on colonic motility. Taken together, these findings demonstrate that dietary increases in Trp do not induce prokinetic actions in the gut.

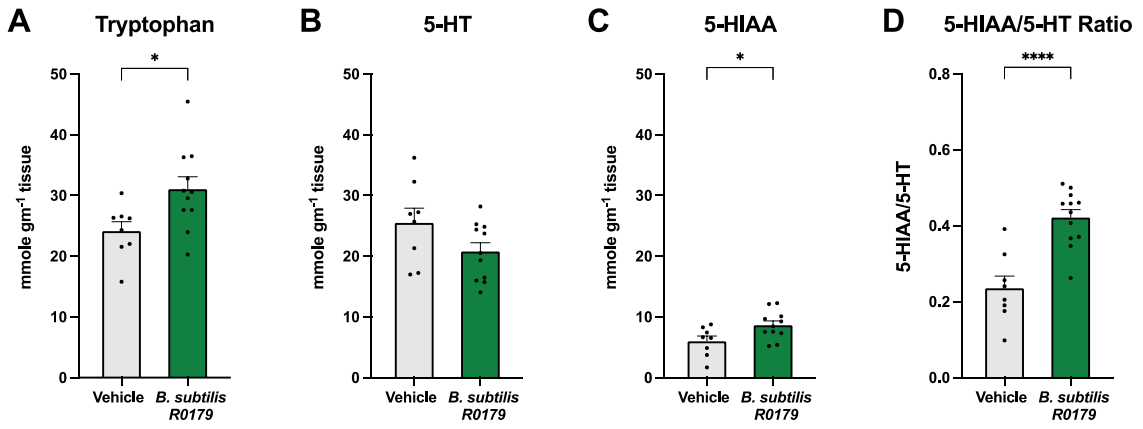

Figure 1. *B. subtilis R0179* treatment increases colonic Trp, 5-HIAA, and 5-HIAA/5-HT ratio. To determine whether one week of treatment with the Trp-synthesizing strain *B. subtilis R0179* alters colonic metabolite levels in mice, distal colon tissue was collected at day 7 upon euthanasia and analyzed by HPLC. Mice that received *B. subtilis R0179* (n=11) displayed significantly higher levels of colonic Trp and 5-HIAA, as well as an elevated 5-HIAA/5-HT ratio, compared to vehicle control mice (n=8). Data are shown as mean ± SEM. Unpaired Student's *t*-test. * $p < 0.05$, **** $p < 0.0001$.

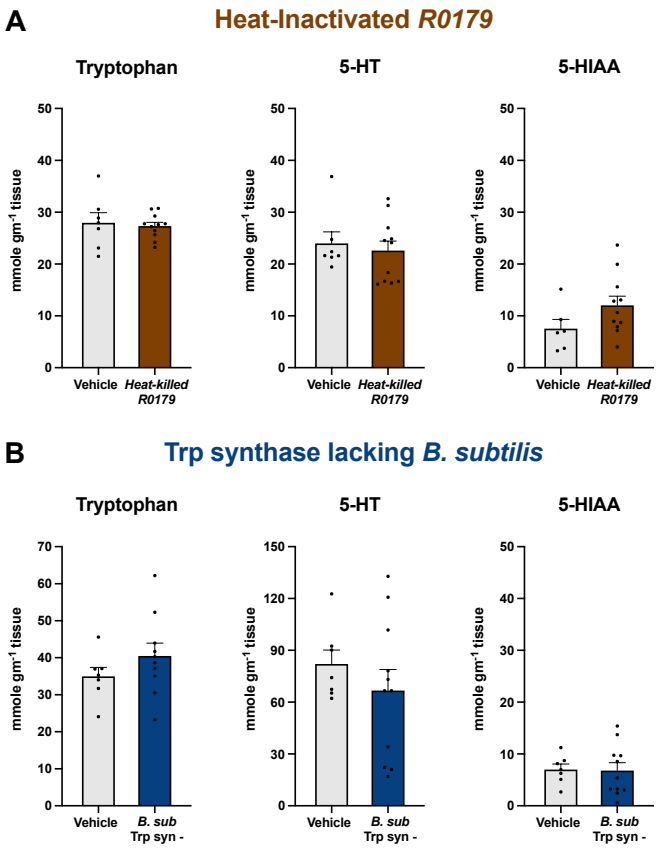

Figure 2. Treatment with either heat-inactivated *B. subtilis R0179* or with a non-Trp-synthesizing strain of *B. subtilis* does not alter colonic metabolite levels. (A) To determine whether the *B. subtilis R0179*-induced increases in colonic Trp and 5-HT signaling were due to the live nature of the administered bacteria, mice were treated for one week with heat-inactivated *B. subtilis R0179*. Analysis of distal colon tissue via HPLC revealed no significant differences in colonic Trp, 5-HT, or 5-HIAA between mice treated with heat-inactivated *B. subtilis R0179* (n=11) and vehicle control (n=7). (B) To examine whether the altered metabolite levels from *B. subtilis R0179* treatment were due to the Trp-synthesizing property of this bacterial strain, mice were treated for one week with *B. subtilis 1A2*, a strain that lacks tryptophan synthase, the key enzyme in the synthesis of Trp from indole. Mice that received this non-Trp-synthesizing strain (n=11) displayed no significant differences in colonic Trp, 5-HT, or 5-HIAA compared to vehicle-treated mice (n=7). Data are shown as mean ± SEM. Unpaired Student's *t*-test.

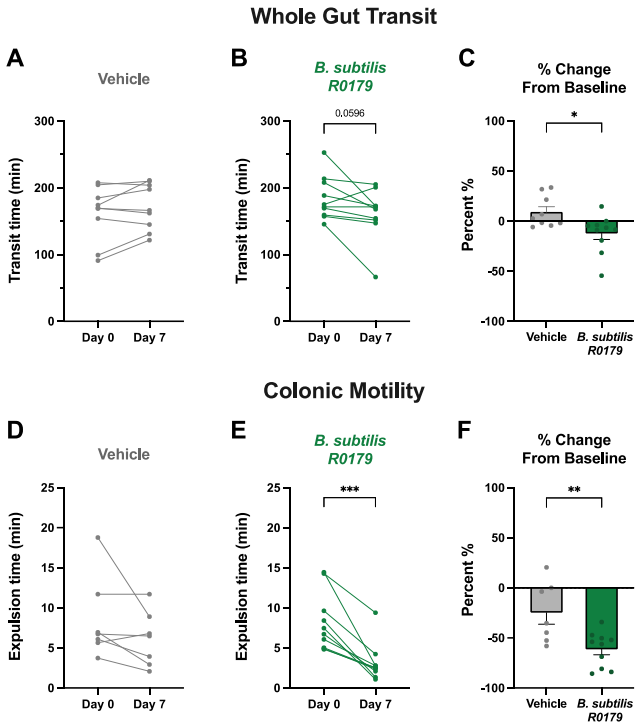

Figure 3. *B. subtilis R0179* treatment produces prokinetic actions on colonic motility. To investigate the functional effects of *B. subtilis R0179* treatment on intestinal motility, two different motility assays were performed at baseline and again after one week of treatment. (A-C) Whole gut transit time is a non-localized, net motility measure encompassing gastric emptying, small bowel transit, and colonic transit. Mice that received *B. subtilis R0179* (n=10) did not exhibit a significant change in whole gut transit time following a week of treatment. There was no change in whole gut transit time in vehicle-treated mice (n=9). (D-F) The bead expulsion assay was used to assess bacteria-induced changes in motility localized to the colon, the region of the GI tract that contains the highest numbers and biodiversity of bacteria. *B. subtilis R0179*-treated mice (n=10) exhibited significantly faster colonic motility times following a week of treatment. There was no change in colonic motility in vehicle-treated mice (n=7). Furthermore, there was a significantly different percent change from baseline between *B. subtilis R0179* and vehicle control treatment groups. Data are shown as individual animals (A,B,D,E), paired Student's *t*-test, or mean ± SEM (C,F), unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

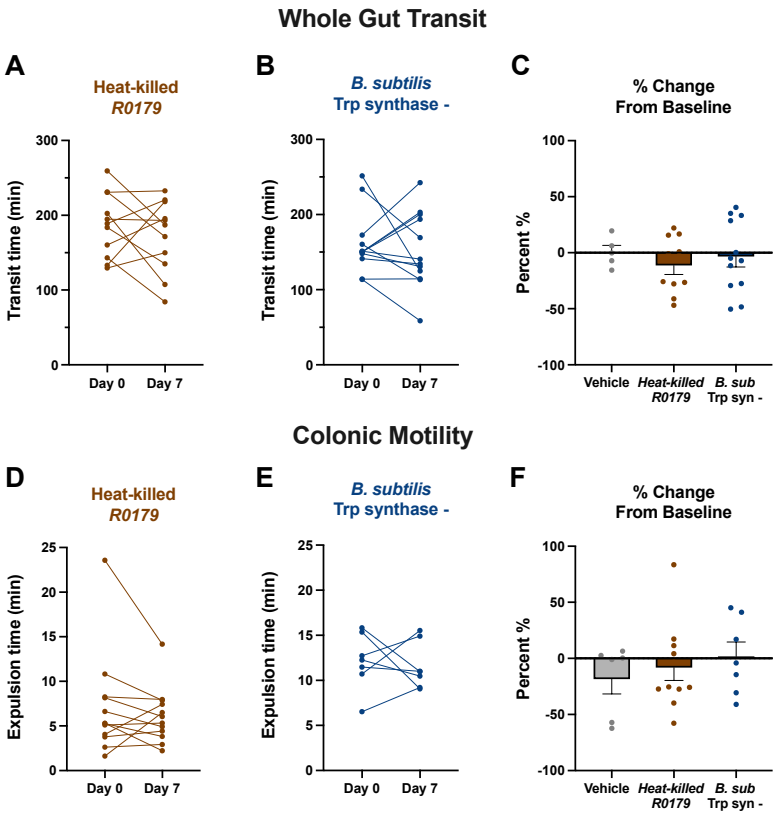

Figure 4. Heat-inactivated *B. subtilis R0179* and the non-Trp-synthesizing strain *B. subtilis 1A2* treatments do not induce prokinetic effects on GI motility. To assess whether the prokinetic effects of *B. subtilis R0179* were due to the bacteria's live nature as well as its Trp-synthesizing capability, motility was assessed following a week of treatment with either heat-inactivated *B. subtilis R0179* or with the tryptophan synthase lacking strain *B. subtilis 1A2*. (A-C) Mice that received heat-inactivated *B. subtilis R0179* (n=11) or *B. subtilis 1A2* (n=12) did not exhibit changes in whole gut transit time after a week of treatment. These treatment groups were not significantly different from each other or vehicle (n=5) in percent change from baseline. (D-F) In addition to whole gut transit, there was no significant change in colonic motility times in mice treated with either heat-inactivated *B. subtilis R0179* (n=12) or *B. subtilis 1A2* (n=7). Furthermore, there were no differences between both bacteria treatments or vehicle (n=6). Data are shown as individual animals (A,B,D,E), paired Student's *t*-test, or mean ± SEM (C,F), one-way ANOVA with Tukey's post-hoc multiple comparisons test.

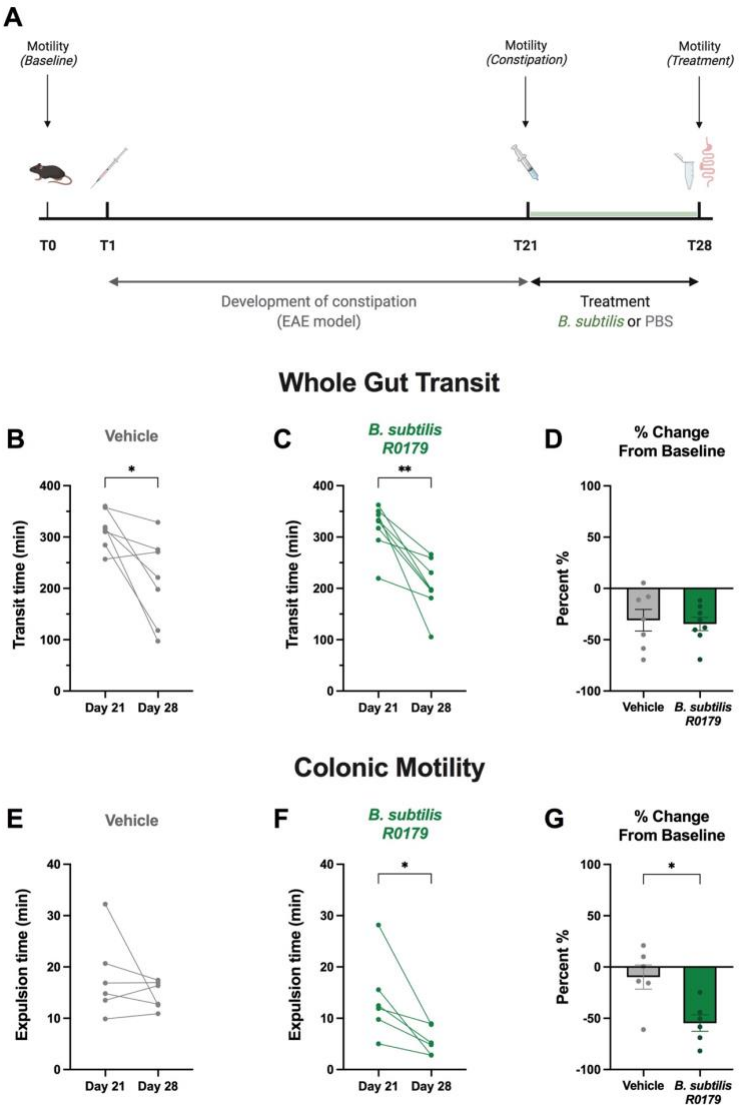

Figure 5. ***B. subtilis R0179*** **treatment restores colonic motility in constipated mice.** To investigate whether treatment with *B. subtilis R0179* could restore intestinal function in a model of constipation, mice were induced with experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis that exhibits features of constipation. (A) Schematic of experimental time course with development of constipation for three weeks beginning on day 1 post-induction, and treatment for one week beginning at the height of constipation on day 21. Only mice that were symptomatic for constipation received bacteria or vehicle treatment. (B-D) Following a week of treatment with *B. subtilis R0179*, constipated mice displayed faster whole gut transit times on day 28 compared to the height of constipation

on day 21 (n=8). This prokinetic effect on whole gut transit was seen in vehicle-treated mice as well (n=7). (E-G) *B. subtilis R0179*-treated mice (n=6) exhibited significantly faster colonic motility times as assessed by the bead expulsion assay, whereas there was no significant change in colonic motility in vehicle-treated mice (n=6). Moreover, there was a significant difference between treatment groups in percent change from baseline following one week of treatment. Data are shown as individual animals (B,C,E,F), paired Student's *t*-test, or mean ± SEM (D,G), unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

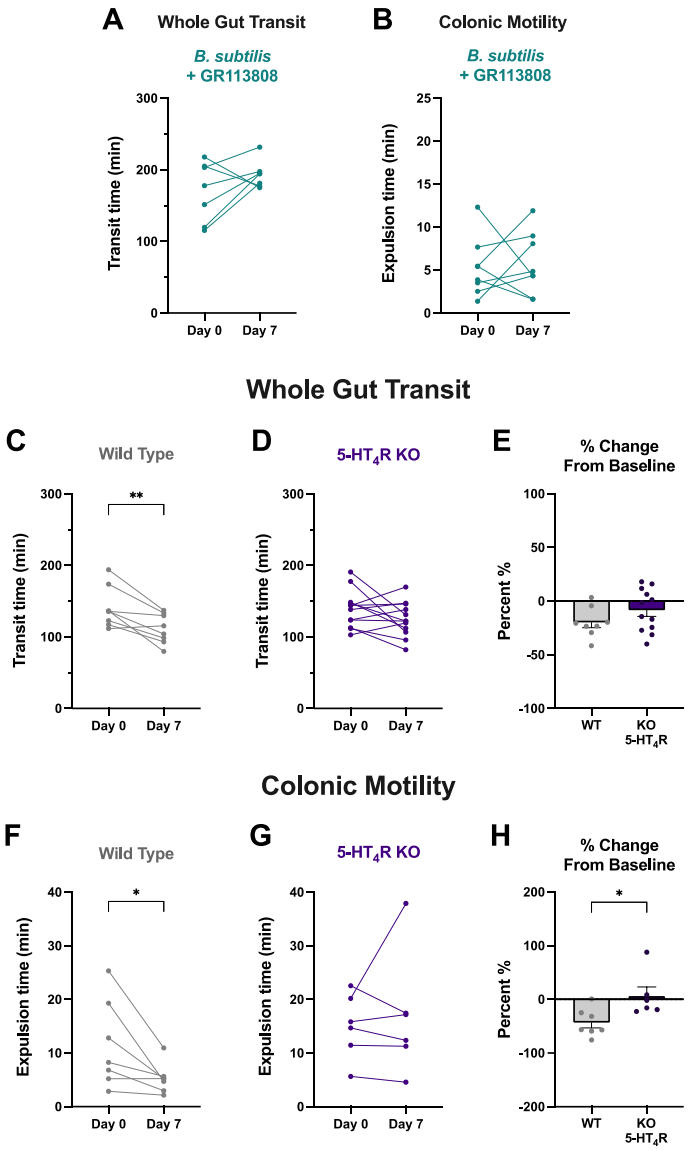

Figure 6. The prokinetic actions of *B. subtilis R0179* involve the 5-HT$_4$ receptor. The 5-HT$_4$ receptor (5-HT$_4$R) was targeted both pharmacologically and genetically to determine whether this receptor plays a role in *B. subtilis R0179*-induced prokinetic effects on colonic motility. (A-B) Mice that were co-treated with *B. subtilis R0179* and GR113808, a 5-HT$_4$R antagonist, exhibited no changes in whole gut transit time (n=7) or colonic motility (n=8) following a week of treatment. (C-E) Following a week of *B. subtilis R0179* treatment, wild type littermate control mice displayed significantly faster whole gut transit times (n=8), whereas there was no change in whole gut transit in 5-HT$_4$R KO mice (n=12). The two groups were not

significantly different in percent change from baseline. (F-H) Wild type littermate controls exhibited significantly faster colonic motility times after a week of *B. subtilis R0179* treatment (n=7), whereas there were no changes in colonic motility in 5-HT_4R KO mice (n=6). Furthermore, there was a significant difference in percent change from baseline between wild type and KO mice. Data are shown as individual animals (A,B,C,D,F,G), paired Student's *t*-test, or mean ± SEM (E,H), unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

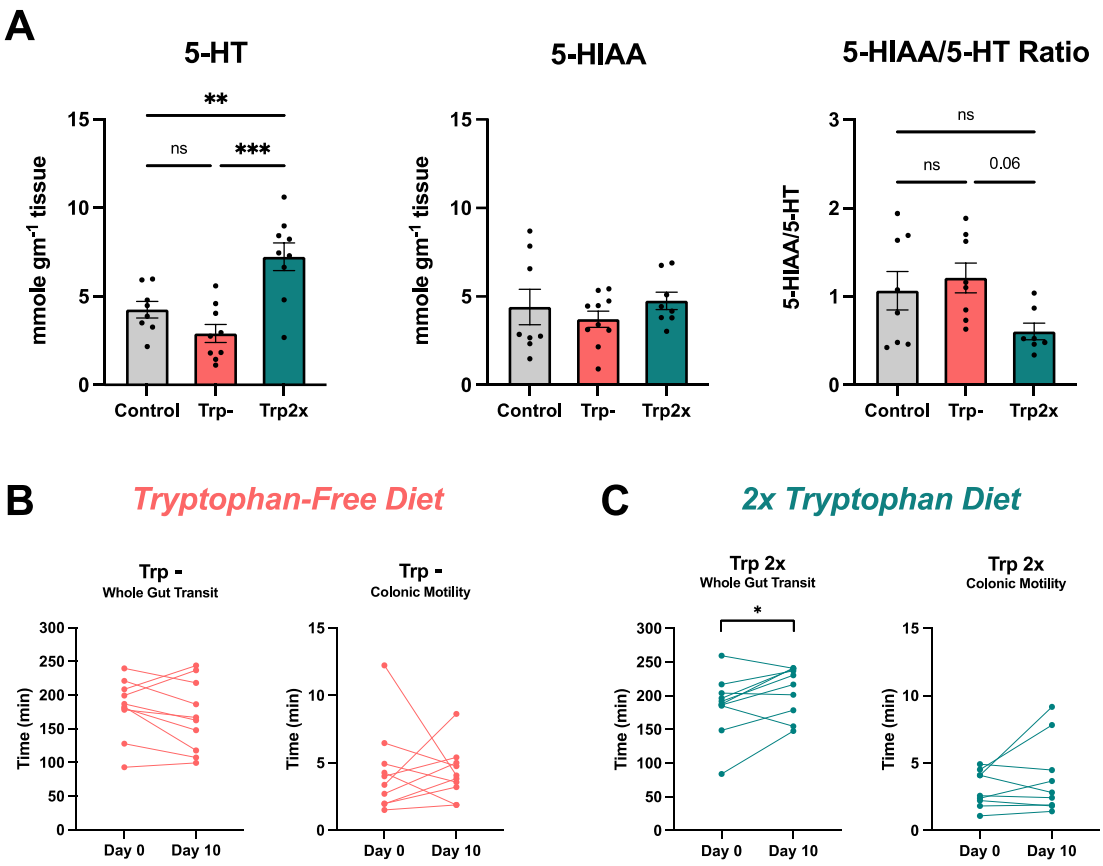

Figure 7. Modifications of Trp through the diet do not induce prokinetic effects on gut motility. To assess whether dietary-based alterations in Trp availability induce changes in colonic 5-HT signaling and intestinal motility, mice were fed for 10 days with either chow with no Trp (Trp-) or chow with twice as much Trp (Trp2x). (A) Mice that were fed the Trp2x diet (n=9) displayed significantly elevated levels of colonic 5-HT compared to mice that were fed the Trp- diet (n=9) and control diet (n=8). (B) There was no significant change in whole gut transit or colonic motility following 10 days on a Trp- diet (n=10). (C) Mice exhibited slower whole gut transit time after 10 days on the Trp2x diet (n=10). There was no significantly change in colonic motility. Data are shown as mean ± SEM (A), one-way ANOVA with Tukey's post-hoc multiple comparisons test, or as individual animals (B,C), paired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

The use of orally administered bacteria as therapeutic agents is an emerging strategy for the alleviation of GI symptoms, particularly constipation. As with standard pharmacology-based approaches, a deeper understanding of the mechanisms of actions of these agents is a necessary component of their development into predictable and personalized treatments for GI dysmotility. In this study, we explored a novel bacteria-mediated strategy to alter host 5-HT signaling and function in the gut. We tested the hypothesis that administration of the Trp-synthesizing strain *B. subtilis R0179* alters luminal Trp availability and promotes 5-HT biosynthesis in the gut, thereby inducing prokinetic actions on GI motility. We found that *B. subtilis R0179* administration enhances 5-HT signaling in the colon, as indicated by an elevated 5-HIAA/5-HT ratio, and accelerates colonic motility in both healthy and constipated mice. Notably, our findings indicate that these effects are dependent on spore viability as well as the ability to synthesize Trp, and that 5-HT_4Rs are involved in mediating the prokinetic response. Collectively, these data establish an underlying mechanism of action by which the orally administered bacteria *B. subtilis R0179* enhances intestinal 5-HT signaling and motility.

Gut bacteria influence the enteric serotonergic system

Our findings contribute to a growing body of research into how gut microbes can markedly impact host 5-HT signaling (Legan et al., 2022). Previous studies have shown that both germ-free and antibiotic-treated animals display deficits in colonic 5-HT levels and decreased Tph1 expression, demonstrating that normal enteric 5-HT signaling is microbiota-dependent (Ge et al., 2017; Reigstad et al., 2015; Yano et al., 2015).

Furthermore, microbially-secreted metabolites, including short-chain fatty acids (Fukumoto et al., 2003; Reigstad et al., 2015) and secondary bile acids (Yano et al., 2015), stimulate host 5-HT biosynthesis and release, which promotes prokinetic actions within the gut.

Administration of isolated bacterial strains that alter the host enteric serotonergic system is gaining appreciation as a potential therapeutic strategy to promote gut function. This work has involved studies of strain-specific effects of various bacteria acting through different mechanisms to ultimately promote 5-HT biosynthesis (Engevik et al., 2021; Nzakizwanayo et al., 2015; Yano et al., 2015). While it is widely accepted that the gut microbiota serve an active role in Trp metabolism, our study is the first to our knowledge to exploit the biochemical capacity of certain bacteria that synthesize Trp to promote intestinal 5-HT signaling.

B. subtilis R0179 as a prokinetic agent in the treatment of constipation

Clinical constipation is characterized by a variety of symptoms including infrequent bowel movements, hard stools, and distressful feelings of bloating and abdominal pain. A meta-analysis estimates that the global prevalence of constipation is ~14% in the adult population (Suares & Ford, 2011). Pharmacological interventions for patients who do not respond to first-line modifications to diet and lifestyle include osmotic and stimulant laxatives, prosecretory agents, and agents that target the serotonergic system. However, effective treatment options are limited.

Mounting evidence suggests that dysbiosis of the intestinal microbiota may contribute to chronic constipation (Mancabelli et al., 2017; Parthasarathy et al., 2016; Zhao & Yu,

2016). However, discrepancies exist in the methodologies of these studies, as well as in the inherent difficulty of drawing causal links between specific bacteria and physiological outcomes. It is clear that identification of mechanisms of action underlying bacterial alterations to gut physiology would be of great benefit in understanding how to develop novel microbiota-based therapeutic tools. Our finding that *B. subtilis R0179* restores colonic dysmotility in a model of constipation implicates the potential for this strain as a novel prokinetic agent for the treatment of constipation symptoms.

Enteric 5-HT$_4$Rs mediate the prokinetic actions of B. subtilis R0179

5-HT signals through a variety of receptors that are expressed by many cell types within the gut, including intestinal epithelial cells and enteric neurons (Mawe & Hoffman, 2013). Given that 5-HT is an important mediator of many regulated GI functions, 5-HT receptors are often targeted clinically to treat GI dysfunction. For example, clinical trial data demonstrate that 5-HT$_3$R antagonists and 5-HT$_4$R agonists are effective treatments for diarrhea (Andresen et al., 2008) and constipation (Degen et al., 2005; Evans et al., 2007), respectively, as well as the pain and discomfort symptoms of irritable bowel syndrome (IBS).

The 5-HT$_4$ receptor subtype has been targeted for its function as a prokinetic receptor in the GI tract. An emerging line of research identifies the mucosal 5-HT$_4$R as a salient therapeutic target for agents designed to treat constipation. Previous work in our lab has demonstrated that 5-HT$_4$R expression is differentially distributed in the intestinal mucosa along the GI tract, such that the expression levels are greatest in the colon in mice (Hoffman et al., 2012). Notably, 5-HT$_4$Rs are abundantly expressed by a variety of

colonic epithelial cells, including enterocytes, EC cells, and goblet cells. Activation of colonic mucosal 5-HT$_4$Rs evokes 5-HT release by EC cells and mucus release by goblet cells, both of which can lead to alleviation of constipation symptoms (Hoffman et al., 2012). Furthermore, luminally acting 5-HT$_4$R agonists have been shown to elicit prokinetic effects on GI function and promote colonic propulsive motility (Hoffman et al., 2012; Konen et al., 2021), lending support for the notion that mucosal 5-HT$_4$Rs act as important pharmacological targets in the treatment of constipation.

Our findings that the prokinetic actions of *B. subtilis R0179* were blocked by co-administration of a 5-HT$_4$R antagonist and were absent in 5-HT$_4$R KO mice demonstrate the involvement of the 5-HT$_4$R in enhancing intestinal motility with *B. subtilis R0179* treatment. It is worth noting that these experiments did not differentiate between epithelial and neuronal 5-HT$_4$Rs, as the KO mice are characterized by a full-body KO of the 5-HT$_4$R. Indeed, 5-HT$_4$Rs are known to also be located on enteric nerve terminals, and previous studies demonstrate that activation of neuronal 5-HT$_4$Rs facilitates synaptic transmission and increases neurotransmitter release from myenteric neurons (Liu et al., 2005; Pan & Galligan, 1994; Ren et al., 2008). However, a recent study by Keating and colleagues demonstrates that neuronal 5-HT is not a major contributor to the regulation of colonic motility in normal mice (Martin et al., 2022). Since we have found that the prokinetic actions of *B. subtilis R0179* are localized primarily to the colon, we would therefore hypothesize that mucosal 5-HT$_4$Rs are ultimately mediating these bacterially induced effects. Future investigations can test this hypothesis by administering *B. subtilis R0179* to conditional 5-HT$_4$R KO mice in which expression of the 5-HT$_4$R is down-regulated selectively within epithelial cells or enteric neurons.

In addition to 5-HT, other bioactive metabolites may also play a role in promoting GI function. One such molecule is tryptamine, a tryptophan-derived indoleamine. While bacterially-mediated metabolism of Trp into tryptamine is rare, two human commensal bacteria, *Clostridium sporogenes* and *Ruminococcus gnavus*, have recently been discovered by Williams et al to express tryptophan decarboxylase, the key enzyme that decarboxylates Trp to form tryptamine (Williams et al., 2014). In the gut, tryptamine has been shown to act as a ligand for the epithelial 5-HT_4R (Bhattarai et al., 2018). Indeed, tryptamine-induced 5-HT_4R activation increases colonic secretion which accelerates whole gut transit in mice (Bhattarai et al., 2018), and stimulates mucus release from goblet cells (Bhattarai et al., 2020). It is therefore possible that *B. subtilis R0179*-induced increases in luminal Trp availability could lead to the conversion of Trp into tryptamine to play a role in enhancing intestinal motility through activation of the 5-HT_4R.

While pharmacological treatments for constipation primarily target the 5-HT_4R, the 5-HT_3R also plays a role in activating propulsive motility and promoting secretory responses in the GI tract (Mawe & Hoffman, 2013). As such, partial agonists at the 5-HT_3R are being developed for the treatment of constipation predominant IBS (Evangelista, 2007). Recent work demonstrates that 5-HT_3Rs are expressed in the colonic mucosa and are thought to mediate host 5-HT secretory responses in a microbiota-dependent manner (Bhattarai et al., 2017). Therefore, it is possible that *B. subtilis R0179*-induced prokinetic effects may also involve the 5-HT_3R.

Colonization of B. subtilis R0179

Whether, and to what degree, orally administered bacteria can colonize the GI tract and interact with commensal bacteria remains an open question in the field. There are numerous factors that influence the survival and growth of orally administered bacteria in the gut (Han et al., 2021). For one, the region-specific diversity of microhabitats along the GI tract can be an important determinant of survival as the ingested bacteria are in transit through the tract. Notably, bacteria are exposed to high acidity in the stomach and to bile acids in the small intestine, both of which can markedly impact the survival of ingested bacteria (Bezkorovainy, 2001). Additionally, bacteria that successfully pass through the upper portions of the GI tract will be confronted with colonization resistance by competition from the commensal bacteria in the colon, the site of the largest bacterial density in the gut (Han et al., 2021; Zmora et al., 2018).

It has been proposed that in order to colonize the host's intestine, bacteria must be capable of attaching to intestinal mucosal cells (O'Sullivan et al., 1992). While *in vitro* studies suggest that certain bacteria do indeed demonstrate this ability to attach (Bernet et al., 1993), *in vivo* evidence is less conclusive. Indeed, emerging literature suggests that probiotic bacteria pass through the system without adhering or multiplying within the GI tract (Bezkorovainy, 2001; Kristensen et al., 2016; Zmora et al., 2018). For this reason, exogenous bacteria are typically required to be ingested continually for the desired duration of treatment effect.

In our experiments, we administered *B. subtilis R0179* daily for the entire treatment course. While the presence of the *B. subtilis R0179* strain was detected in the feces of bacteria-treated mice as validation of our experimental approach, we have not yet

explored the interactions between *B. subtilis R0179* and the host commensal microbiota, or whether its presence in the GI tract is transient. Further characterization of these interactions is essential to develop robust, predictable, and individualized treatment approaches for the use of *B. subtilis R0179* as a prokinetic agent.

Implications for the microbiota-gut-brain axis

Beyond the gut, recent studies highlight the potential for orally administered bacteria to alter CNS activity and behavior (Cryan & Dinan, 2012; Cryan et al., 2019). While the underlying mechanisms of action of different bacterial species and strains remain to be further elucidated, there is a growing appreciation for the notion that bacteria can regulate the production of microbial metabolites that possess neuroactive properties. Within the serotonergic system, gut 5-HT and CNS 5-HT are thought of as separate pools, since 5-HT does not cross the blood-brain barrier. However, 5-HT's metabolic precursor Trp represents a promising interface between microbially-mediated changes in the gut and CNS 5-HT synthesis (Legan et al., 2022). Indeed, preliminary data in our lab suggest that *B. subtilis R0179* administration elevates circulating levels of Trp in whole blood samples from bacteria-treated mice (data not shown). Future studies will explore the potential properties of the Trp-synthesizing strain *B. subtilis R0179* in altering CNS 5-HT synthesis and 5-HT-mediated behavior.

Dietary-mediated increases in Trp slow intestinal motility

Since Trp is an essential amino acid, the body's only source of Trp is through dietary consumption. It is known that Trp-varying diets can alter circulating Trp availability (Fernstrom, 2013; Richard et al., 2009). However, this is a dynamic process that involves

competition between Trp and other amino acids for absorption through transporters into enterocytes (Bröer, 2008).

The advantages of manipulating Trp through the diet are obvious. Consuming specific foods to benefit our health is common practice in our society and requires little extra work beyond acquiring the necessary dietary components. In contrast, altering Trp levels with bacteria is a more elaborate approach with greater hurdles to overcome, including preparation of the consistent dosage as well as the inherent unknowns regarding the consumption of live microorganisms that could interact with the host microbiome.

Our studies reveal that increasing Trp levels through dietary manipulations produces the opposite effects on colonic 5-HT signaling and gut motility compared to those seen following *B. subtilis R0179* treatment. Since dietary Trp is absorbed primarily in the small intestine, it may be the case that too much Trp absorption leads to a detrimental effect on the 5-HT system, such that it induces a slowing of gut function in the upper GI tract. Our approach for altering Trp availability through a bacterially-mediated mechanism, rather than through the diet, is unique in that it localizes the effects of increased Trp to the colon, thereby acting primarily to stimulate colonic motility.

Concluding remarks

Taken together, our findings highlight the therapeutic potential of the Trp-synthesizing strain *B. subtilis R0179* to enhance intestinal 5-HT signaling and induce prokinetic actions in the gut. Bacteria that are capable of altering intestinal Trp availability represent a novel approach to altering the enteric serotonergic system. Further elucidation of the mechanisms of action of specific bacteria such as this one will drive the

field forward towards the targeted use of isolated bacteria to promote intestinal function in a transient and predictive manner.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The gut microbiome plays a pivotal role in maintaining our health and well-being. It is now widely recognized that these microbes, residing within our GI tracts on the scale of tens of trillions, contribute significantly to host physiological processes. One such process is GI function, which is regulated by a series of neural reflexes and signaling molecules, including 5-HT. Notably, some gut microbiota are known to produce microbial metabolites that act to promote 5-HT biosynthesis and release, thereby altering gut function of the host. Despite the exciting promise of orally delivered bacteria to beneficially alter GI function, there remains a need for the elucidation of underlying mechanisms by which specific bacterial species and strains act to exert effects on host physiology. The studies described in my dissertation demonstrate that an orally administered, Trp-producing bacterial strain, *B. subtilis R0179*, can increase mucosal 5-HT signaling and enhance intestinal motility. This work contributes to a growing body of research that aims to identify novel, predictive strategies for the targeted use of bacteria as interventions in the treatment of gut dysfunction and dysbiosis.

I. PROKINETIC EFFECTS OF *B. SUBTILIS R0179*

A. Summary and conclusions

The use of orally administered bacteria as therapeutic agents is an emerging strategy for the alleviation of GI symptoms, particularly constipation. As with standard pharmacology-based approaches, a deep understanding of the mechanisms of actions of bacterial agents is a necessary component of their development into predictable and personalized treatments for GI dysmotility. Various bacteria are known to express key enzymes that alter the synthesis of bioactive metabolites, which can then exert downstream effects on host physiology. The strain used in this dissertation, *B. subtilis R0179*, expresses tryptophan synthase, a key enzyme that catalyzes the final steps of biosynthesis of Trp from indole. *In vitro* analysis conducted by Lallemand Health Solutions has demonstrated that *B. subtilis R0179* produces Trp during a 48 hour incubation period, confirming that tryptophan synthase is active in this strain.

This dissertation describes a series of experiments that establish how this Trp-synthesizing property of *B. subtilis R0179* can be exploited to alter intestinal 5-HT signaling and induce prokinetic effects on GI function. We have found that one week of treatment with *B. subtilis R0179* increases colonic Trp levels and 5-HT turnover. Moreover, this elevated 5-HT signaling produces prokinetic actions in the colon, both in healthy mice as well as in a mouse model of constipation. We further demonstrate that the administration of live bacteria is necessary for this increased 5-HT signaling and function, and that treatment with a tryptophan synthase knockout strain of *B. subtilis* does not induce changes in colonic Trp or 5-HT levels nor produce prokinetic effects. Taken

together, the findings of these studies implicate the Trp-synthesizing bacteria *B. subtilis R0179* as a novel therapeutic agent to target the 5-HT system in the colon and produce prokinetic effects on GI motility in the treatment of constipation.

B. Alternative approaches and future directions

Bacterial administration method

Determining the optimal route of delivery for the bacteria was an important step when initiating and optimizing these experiments. For all of the studies described in Chapter Three, we utilized oral gavage as the mode of delivery of *B. subtilis R0179*. Initially, we administered *B. subtilis R0179* in the drinking water because it seemed to be an easier, less stress-inducing approach for the animals. However, this strategy presented several logistical challenges, including the difficulty of suspending the bacteria in the water, as well as frequent cage water bottle clogs due to the viscosity of the mixture. Furthermore, while this strategy allows for minimal stress to the animals, it was not possible to control the bacterial dosage per mouse by measuring water consumption since all mice are co-housed in groups of 4-5 as per our protocol. Another complicating factor of adding the bacteria to the drinking water was that the bacterial spores were stored in a maltose powder, so animals in the vehicle group exhibited change due to consumption of the sweetened water.

The primary advantage of gavage, on the other hand, is that it ensures that each mouse receives the same dose of bacteria and in a consistent manner at the same time of day. Some studies have reported that repeated oral gavage treatment provokes stress-induced physiological changes (Balcombe et al., 2004; Brown et al., 2000). However,

thorough technique training by Animal Care staff was done to ensure that the gavage technique was performed correctly to minimize stress to the animals. To confirm that this procedure was not a confounding variable in these experiments, we performed an ELISA to assess plasma corticosterone concentrations in animals that had been gavaged for one week compared to mice that were handled but not gavaged, and found that daily oral gavage did not produce elevated stress (Fig. 1). Furthermore, the gavage administration approach avoids the confounding effects of maltose. Therefore, we concluded that oral gavage is the ideal delivery method for bacteria treatment because we were able to administer the bacteria in a consistent and controlled manner, and without inducing stress in the animals.

Strain incongruence between Trp+ and Trp- strains

Our study utilized an additional bacterial control treatment to determine whether the prokinetic actions of *B. subtilis R0179* were due to the presence of a functional tryptophan synthase gene. Initially, we attempted to genetically manipulate the *B. subtilis R0179* strain to knockout the tryptophan synthase gene cassette as a strain-congruent control. However, we were not successful in producing this knockout in *B. subtilis R0179*. Therefore, we turned to an alternative approach using a different *B. subtilis* strain, *B. subtilis 1A2*, which is a non-revertible loss of function mutant in the tryptophan synthase gene. As a result, it is important to note this strain incongruence between the Trp+ and Trp- strains used in this study. Future experiments should compare the effects of Trp+ and Trp- strains from the same strain background.

Compensatory changes in 5-HT-related genes

It is known that microbes can directly and indirectly alter the expression of important 5-HT-related genes (Legan et al., 2022). Certain isolated strains, when administered to mice, can promote 5-HT bioavailability by increasing intestinal SERT expression (Cao et al., 2018; Engevik et al., 2021; Wang et al., 2015), elevating host colonic Tph1 and MAO-A expression (Yano et al., 2015), or upregulating the 5-HT_4R (Engevik et al., 2021). Furthermore, alterations in 5-HT-related gene expression levels are associated with pathophysiological conditions. For example, decreased colonic expression of SERT is detected in patients with ulcerative colitis as well as irritable bowel syndrome (IBS) (Coates et al., 2004).

In our study, we found that *B. subtilis R0179* administration increases levels of Trp, the precursor for 5-HT synthesis, in colonic tissue and also leads to increased 5-HT signaling, as demonstrated by an increase in the 5-HIAA/5-HT ratio. We therefore sought to determine whether *B. subtilis R0179* induces compensatory changes in gene expression levels of key 5-HT-related proteins as a result of this altered 5-HT signaling.

RT-qPCR was used to compare the relative amounts of mRNA present in proximal and distal colon tissue for various genes of interest between bacteria-treated mice and vehicle control-treated mice. In the proximal colon, *B. subtilis R0179*-treated mice exhibited significantly lower relative mRNA expressions of SERT and MAO-A compared to vehicle-treated mice (Fig. 2a). There were no significant differences between groups in Tph1 or 5-HT_4R expression. In the distal colon, on the other hand, *B. subtilis R0179*-treated mice displayed significantly lower relative mRNA expression

levels of Tph1 compared to vehicle-treated mice, but no significant differences in SERT or MAO-A (Fig. 2b).

These data suggest that *B. subtilis R0179* does indeed induce compensatory changes in key 5-HT-related gene expression levels. However, the direction of these changes was puzzling and warrants future investigation. In our study presented in Chapter Three, we observed a significantly elevated 5-HIAA/5-HT ratio in the distal colons of mice that received *B. subtilis R0179* compared to vehicle. Since this indicates that *B. subtilis R0179* treatment promotes an increase in 5-HT release and breakdown, we hypothesized that SERT and MAO-A, which are responsible for 5-HT clearance and breakdown, respectively, would be upregulated in the colonic tissue of these animals. Along similar lines, we observed an increase in colonic Trp levels in *B. subtilis R0179*-treated mice, yet RT-qPCR analysis revealed a decreased relative mRNA expression level of Tph1, the rate-limiting enzyme in 5-HT synthesis.

These findings are also inconsistent with the literature on bacteria-mediated changes in 5-HT-related gene expression levels. While different species act on different aspects of the 5-HT signaling pathway, bacteria-induced changes all appear to involve the upregulation of the expression levels of these genes. For example, Hsiao and colleagues found that spore-forming bacteria act to increase 5-HT availability by upregulating colonic Tph1 expression (Yano et al., 2015). Moreover, upregulation of colonic mRNA expressions of SERT (Cao et al., 2018; Wang et al., 2015) and MAO-A (Mandić et al., 2019) are associated with increases in 5-HT bioavailability in other bacterial treatments. As such, the observed decreased expression levels of SERT, MAO-A, and Tph1 in bacteria-treated mice should be further explored.

Trp absorption along the GI tract

As with other amino acids, the body's nutritional needs for Trp are provided by dietary intake. Once consumed, amino acids are absorbed along the small intestine by several interdependent transport systems located in the apical and basolateral membranes of enterocytes (Bröer, 2008). Intestinal absorption of Trp occurs primarily through the cotransporter B^0AT1 (SLC6A19) (Böhmer et al., 2005; Keszthelyi et al., 2009). Relative measurements of mRNA expression of the *SLC6A19* gene in rats reveal that B^0AT1 is predominantly located in the membranes of jejunal and ileal enterocytes (Chen et al., 2019). Additional support for the notion that Trp absorption occurs mainly in the small intestine is that an oral gavage of an amino acid mixture produces the highest levels of Trp content in the later portions of the small intestine, primarily the jejunum and ileum, whereas Trp is present at very low levels in the cecum, and at indetectable levels in the colon (Singer et al., 2012).

Our finding that *B. subtilis R0179* administration increases Trp levels in the distal colon raises the question of whether and to what degree Trp absorption is possible in the large intestine. Few studies have investigated the expression of B^0AT1 in distal portions of the GI tract. Notably, one study determined that the mRNA expression levels of B^0AT1 were significantly lower in colonic biopsies taken from patients with IBD compared to controls (Nikolaus et al., 2017), suggesting that alterations in colonic Trp absorption may be implicated in pathophysiological conditions.

Preliminary data from our lab indicate that relative mRNA expression of B^0AT1 is present, albeit at low levels, in the colon (Fig. 3a). When we examined the relative

mRNA expressions of B^0AT1 in the proximal colon following a week of *B. subtilis R0179* administration, we did not detect a significant difference between bacteria- and control-treated mice (Fig. 3b). Since the colon harbors the highest numbers and biodiversity of bacteria (Hillman et al., 2017), the presence of B^0AT1, or some other transporter located on colonocytes capable of absorbing Trp, warrants future exploration in order to better understand possible bacterially-induced changes to colonic Trp levels and metabolism.

Colonization of B. subtilis R0179

The gut microbiome represents an intricate and finely tuned ecosystem. An open question in the field is how orally consumed bacteria interact with host commensals in the GI tract. The complexity of these interactions is further highlighted by strain- and region-specific effects of different bacteria. Thus, it will be important to characterize these interactions to determine the viability and potential therapeutic applications of various bacterial species and strains.

In our study, we administered *B. subtilis R0179* every day for the treatment course. This is a standard administration approach based on the notion that exogenous bacteria do not adhere or multiply within the GI tract and therefore must be ingested continually. After a week of treatment, we then utilized fecal PCR to confirm the presence of the *B. subtilis R0179* strain in the feces of bacteria-treated mice to validate our experimental approach.

The existence of the *R0179* strain in the feces suggests that at least some of the bacteria likely survived the passage through the GI tract. However, it does not provide

any indication of colonization within the host. A follow-up study is currently underway in the Mawe lab to determine how long the presence of *R0179* is detected in fecal samples of bacteria-treated mice. To do this, fecal samples from bacteria-treated mice will be assessed for the presence of *B. subtilis R0179* at varying time points following cessation of treatment, including acute (1 day, 2 days, 3 days, 7 days) and chronic (14 days) time points. If *B. subtilis R0179* does not colonize, we might expect to detect only trace amounts, if any, of the bacteria at the 1-3 day time points and not at any later time points.

Furthermore, future investigations will employ 16S rDNA analysis to examine the microbiome composition in mice that receive *B. subtilis R0179* treatment. This sequencing technique can be used to measure the relative abundance and diversity of microbes that are present in fecal samples (Cryan et al., 2019). It is important to note the caveat that assessment of stool microbiome is not a complete proxy for determining the effects of certain ingested bacteria on host mucosal GI composition and function (Zmora et al., 2018). Nevertheless, these future directions will provide a more comprehensive understanding of the interactions between *B. subtilis R0179* and the host microbiome. Ultimately, these data would be informative to help guide a more robust treatment plan and course for the use of *B. subtilis R0179* as a prokinetic agent.

Implications for the microbiota-gut-brain axis

The high co-morbidity between chronic brain diseases, neuropsychiatric symptoms, and GI dysfunction highlights the importance of the gut-brain connection in pathophysiological conditions (Mayer, 2011). Indeed, it is thought that at least 60% of

individuals that are diagnosed with anxiety and/or depression also report symptoms of GI dysmotility (Liu & Zhu, 2018).

The microbiome is now recognized for its role in the gut-brain axis, and for its importance in the regulation of the brain and behavior (Cryan & Dinan, 2012). A landmark study by Sudo and collaborators first demonstrated that absence of a normal microbiome in early life results in HPA axis-mediated hypersensitive stress responses in adulthood (Sudo et al., 2004). Subsequent studies demonstrate that germ-free mice exhibit reduced anxiety-like behavior (Clarke et al., 2012; Diaz Heijtz et al., 2011; Neufeld et al., 2011) and prominent neurochemical changes, including elevated monoamine turnover (Diaz Heijtz et al., 2011) and altered hippocampal brain-derived neurotrophic factor levels (Neufeld et al., 2011; Sudo et al., 2004), compared to their conventionally colonized counterparts. Furthermore, fecal microbiota transplant studies highlight the remarkable transfers of microbiota-driven disease symptomologies in both rodent models and humans, including transfers of anxiety-like (De Palma et al., 2017; Sudo et al., 2004) and depressive-like behavior (Kelly et al., 2016).

While it is clear that some bacteria can induce changes in CNS activity and behavior, the underlying mechanisms of action of different bacterial species and strains remain to be further elucidated. One emerging mechanism is through the production and secretion of microbial metabolites that possess neuroactive properties (Engevik et al., 2021). Additionally, some bacteria exhibit the capacity to generate neurotransmitters themselves, including GABA (Barrett et al., 2012), dopamine (Tsavkelova et al., 2000), and 5-HT (Özogul, 2011; Shishov et al., 2009), though further *in vivo* evidence is

necessary to understand how these bacteria and their products interact within a host microbiome.

We have begun to investigate the potential CNS-related effects of *B. subtilis R0179*. Using HPLC, we have measured 5-HT and Trp levels in whole blood samples from mice that received *B. subtilis R0179* administration. Following one week of treatment, there was no significant difference in whole blood 5-HT levels between bacteria-treated mice and vehicle-treated mice (Fig. 4). There was, however, a significantly greater level of Trp in the blood of mice that received *B. subtilis R0179* compared to vehicle control. This indicates that there are elevated levels of circulating Trp due to treatment with *B. subtilis R0179*. Given that an intraperitoneal injection of Trp can lead to elevated concentrations of brain Trp and 5-HT synthesis in rats (Carlsson & Lindqvist, 2004), it is possible that increased circulating Trp as a result of *B. subtilis R0179* administration could induce changes within the CNS 5-HT system. Future studies will examine whether there are changes in 5-HT levels in the brainstems of *B. subtilis R0179*-treated mice, and whether Trp-synthesizing bacteria can affect 5-HT-mediated behaviors such as anxiety and depression.

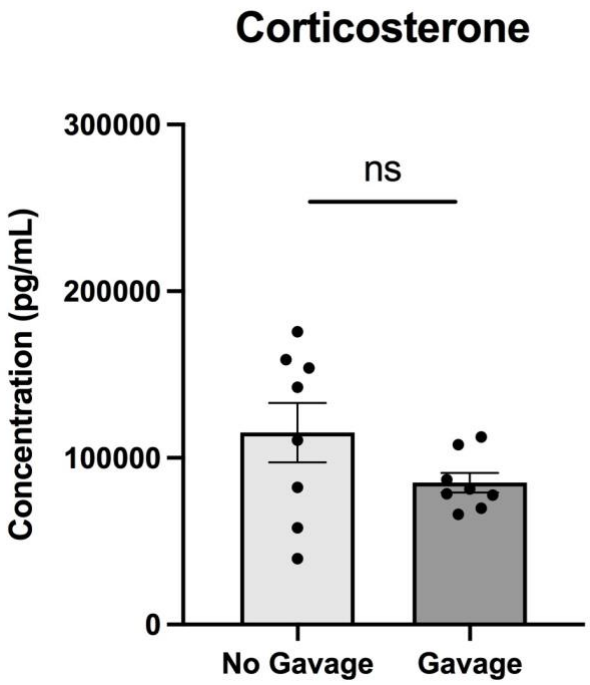

Figure 1. One week of daily gavage does not induce stress in mice. To determine whether daily gavage induces stress in mice, plasma corticosterone levels were assessed by an ELISA after a week of either daily handling without gavage or daily gavage treatment of 100µl PBS. There was no significant difference in plasma corticosterone levels between mice that received daily handling only (n=8) and mice that received daily gavage (n=8). Data are shown as mean ± SEM. Unpaired Student's *t*-test.

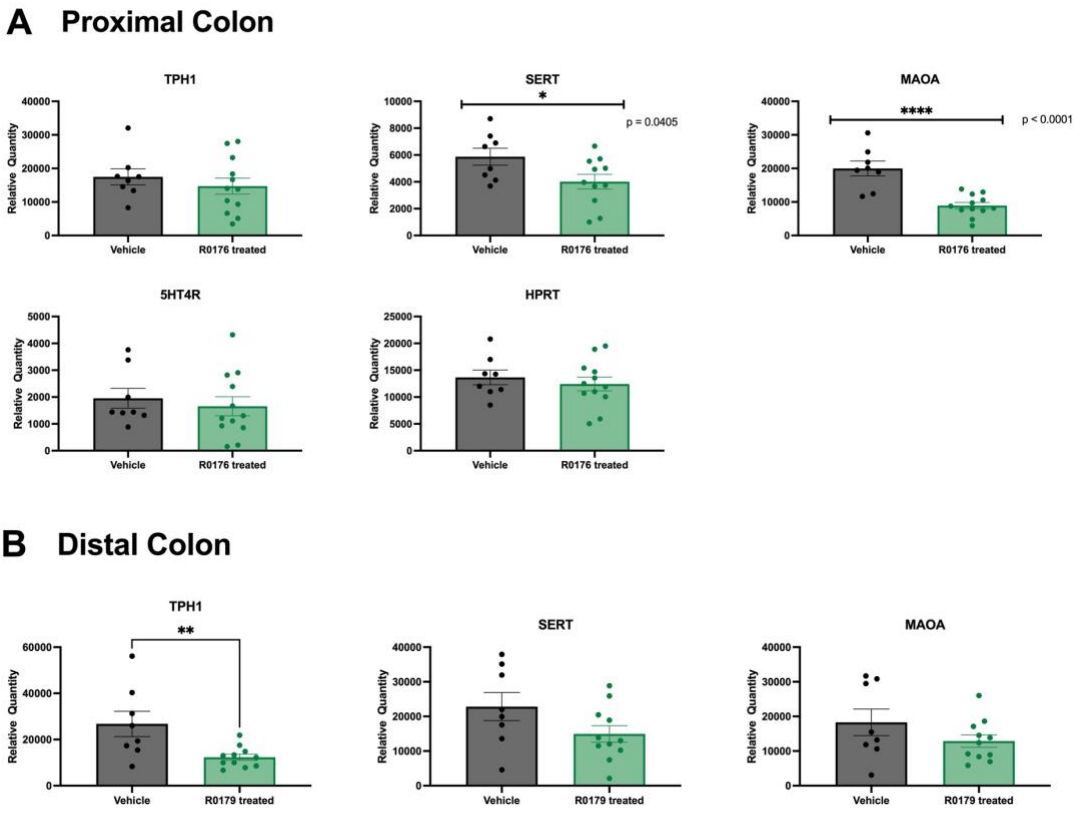

Figure 2. *B. subtilis R0179* induces compensatory changes in gene expression levels of key 5-HT-related proteins. To examine whether *B. subtilis R0179* treatment alters the expression levels of proteins that are involved in 5-HT synthesis, reuptake, and breakdown, RT-qPCR was employed to compare the relative quantities of mRNA present in colonic tissue for each gene of interest between vehicle-treated and *B. subtilis R0179*-treated mice. (A) *B. subtilis R0179*-treated mice (n=12) displayed significantly lower relative mRNA expression levels in proximal colon tissue of the serotonin transporter (SERT) and monoamine oxidase A (MAO-A) compared to vehicle-treated mice (n=8). There were no significant differences between groups in tryptophan hydroxylase 1 (TPH1), the 5-HT4 receptor (5HT4R), or the housekeeping gene (HPRT). (B) *B. subtilis R0179*-treated mice (n=11) exhibited significantly lower relative mRNA expression levels in distal colon tissue of TPH1 compared to vehicle-treated mice (n=8), whereas there were no significant differences between groups in relative mRNA expression levels of SERT or MAO-A in distal colon tissue. Data are shown as mean ± SEM. Unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

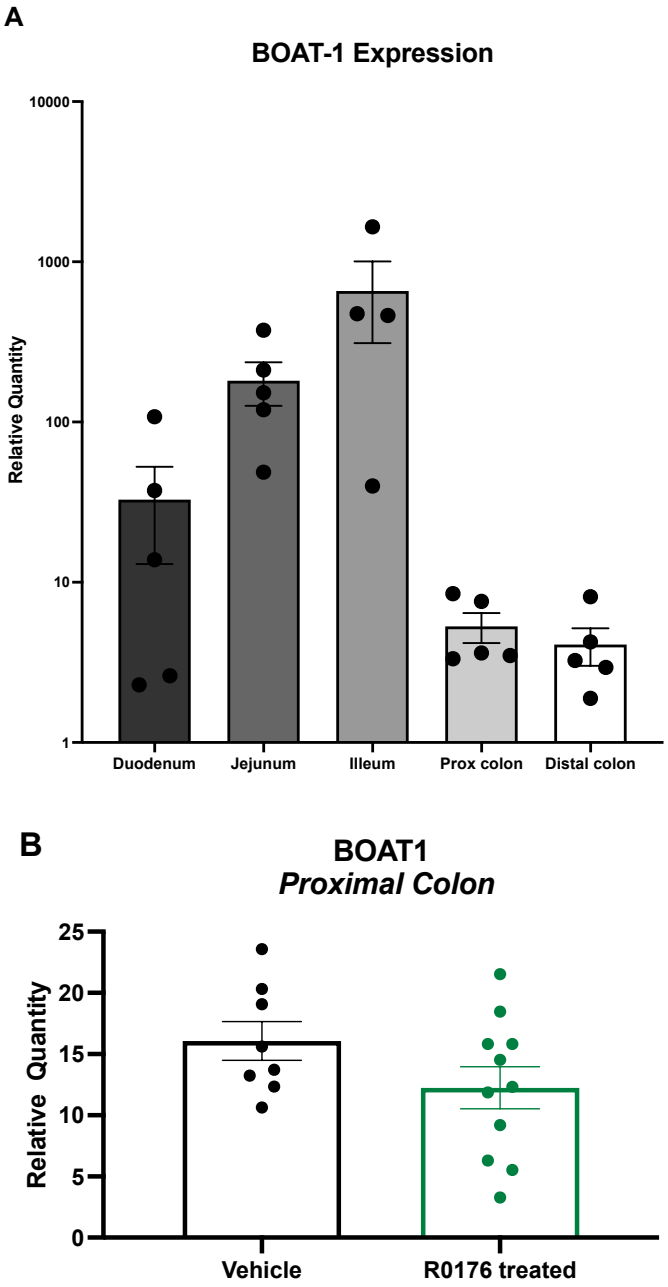

Figure 3. Preliminary investigation of intestinal B^0AT1 expression. Trp is absorbed predominantly in the small intestine through the cotransporter B^0AT1. *B. subtilis R0179* treatment alters colonic levels of Trp, raising the question of whether and how Trp is absorbed in distal portions of the GI tract through the B^0AT1 transporter. (A) To investigate the relative distribution of B^0AT1 throughout different regions of the GI tract, RT-qPCR was used to compare the relative quantities of mRNA present in five distinct regions

(duodenum, jejunum, ileum, proximal colon, and distal colon) of mouse gut tissue (n=5). While these data are preliminary, they suggest that relative mRNA expression of B^0AT1 in colonic tissue is present, though at low levels. Data are shown as mean ± SEM. (B) To assess whether *B. subtilis R0179* treatment induces a compensatory change in colonic B^0AT1 expression, RT-qPCR was employed to compare the relative quantities of mRNA present in proximal colon tissue for B^0AT1 between vehicle-treated (n=8) and *B. subtilis R0179*-treated mice (n=11). There was no significant difference between groups. Data are shown as mean ± SEM. Unpaired Student's *t*-test.

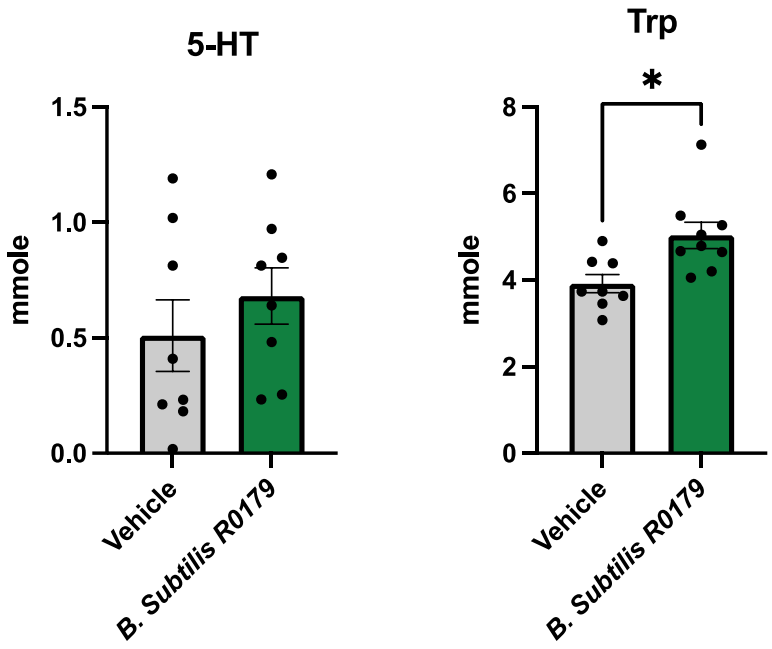

Figure 4. *B. subtilis R0179* treatment produces elevated levels of circulating Trp. Following one week of treatment with *B. subtilis R0179*, whole blood levels of 5-HT and Trp were measured using HPLC to determine whether the Trp-synthesizing bacteria alters circulating levels of these metabolites. There was no significant difference in whole blood 5-HT levels between vehicle-treated mice (n=8) and *B. subtilis R0179*-treated mice (n=9). However, there was a significantly greater level of whole blood Trp in bacteria-treated mice compared to vehicle-treated mice. Data are shown as mean ± SEM. Unpaired Student's *t*-test. * $p < 0.05$.

II. INVOLVEMENT OF THE 5-HT$_4$ RECEPTOR

A. Summary and conclusions

Activation of the 5-HT$_4$R elicits prokinetic actions in the GI tract including the release of 5-HT by EC cells and of mucus by goblet cells. As such, the 5-HT$_4$R is a principal target for pharmacological interventions in the treatment of GI dysmotility. Previous work in our lab has demonstrated that 5-HT$_4$R expression is greatest in the mouse colon and that luminally acting 5-HT$_4$R agonists promote colonic propulsive motility (Hoffman et al., 2012; Konen et al., 2021).

In our study, we found that the prokinetic effects of *B. subtilis R0179* administration are localized primarily to the colon. To test our hypothesis that the 5-HT$_4$R is involved in mediating these bacteria-induced effects, we targeted the 5-HT$_4$R pharmacologically with a 5-HT$_4$R selective antagonist, and genetically with 5-HT$_4$R knockout mice. We found that co-administration of *B. subtilis R0179* and the 5-HT$_4$R antagonist GR113808 blocked the prokinetic effects of *B. subtilis R0179* treatment alone. Furthermore, *B. subtilis R0179* administration in 5-HT$_4$R KO mice elicited no changes in motility following a week of treatment. Taken together, these findings highlight that the 5-HT$_4$R plays a key role in mediating the *B. subtilis R0179*-induced enhancement of intestinal motility.

B. Alternative approaches and future directions

Tissue specificity of 5-HT$_4$Rs

The 5-HT$_4$R is expressed not only by epithelial cells of the mucosa, but also by enteric neurons within the GI tract. Indeed, activation of 5-HT$_4$Rs located on enteric neurons increases acetylcholine release from myenteric neurons and facilities synaptic transmission (Liu et al., 2005; Pan & Galligan, 1994; Ren et al., 2008), demonstrating that neuronal 5-HT$_4$Rs play a role in regulating intestinal motility. However, the precise mechanisms by which neuronal 5-HT$_4$Rs contribute to prokinetic effects need to be further elucidated. For example, Keating and colleagues recently proposed that neuronal 5-HT is not a primary contributor to propulsive colonic motility in normal mice (Martin et al., 2022).

Although our study demonstrates the overall involvement of 5-HT$_4$Rs in the prokinetic actions of *B. subtilis R0179*, our approach did not differentiate between epithelial 5-HT$_4$Rs and neuronal 5-HT$_4$Rs. GR113808, while highly selective for the 5-HT$_4$R in general, does not target specific tissues that express 5-HT$_4$R. Additionally, the 5-HT$_4$R KO mice we used are characterized by a full-body KO of the 5-HT$_4$R.

Future experiments can explore the relative contributions of epithelial and neuronal 5-HT$_4$Rs by using conditional 5-HT$_4$R KO mice in which 5-HT$_4$R expression is down-regulated selectively within epithelial cells (villin1-CRE) or enteric neurons (ChAT-CRE). Our lab has established breeding colonies for both of these conditional KO mice and we have begun characterizing their baseline motility patterns.

Possible role of tryptamine

In addition to 5-HT, other bioactive metabolites can act as signaling molecules to promote GI functions. One particular metabolite of interest is tryptamine, an indoleamine formed by the decarboxylation of L-tryptophan. Tryptamine's physiological significance in the ENS was first brought about by Takaki and colleagues who demonstrated that tryptamine promotes the release of endogenous 5-HT in the GI tracts of guinea pigs (Takaki et al., 1985). Subsequent work has revealed that tryptamine is a ligand for the mucosal 5-HT_4R (Bhattarai et al., 2018), and that tryptamine-induced activation of 5-HT_4Rs exerts prokinetic effects on intestinal motility (Bhattarai et al., 2020; Bhattarai et al., 2018).

As tryptamine is a Trp-derived metabolite, it is possible that the *B. subtilis R0179*-mediated increases in Trp availability promote the conversion of supplemental Trp into tryptamine. This conversion is carried out by the enzyme tryptophan decarboxylase. Notably, the recent discovery of two human commensal bacteria that express tryptophan decarboxylase, *Clostridium sporogenes* and *Ruminococcus gnavus* (Williams et al., 2014), opens the door to future investigations into the potential contributions of tryptamine in the prokinetic effects of *B. subtilis R0179*. We have begun optimizing the HPLC system for metabolite analysis to detect and measure levels of tryptamine in gut tissue of *B. subtilis R0179*-treated mice.

Furthermore, our lab plans to explore the possibility of synergistic effects of co-administration of *B. subtilis R0179* and other bacterial strains with known biochemical properties. Dr. Purna Kashyap and colleagues at the Mayo Clinic have genetically

engineered a strain of *Bacteroides thetaiotaomicron* to express tryptophan decarboxylase (Bhattarai et al., 2018). In a recent study, they demonstrate that this engineered strain increases luminal tryptamine and accelerates whole gut transit times, highlighting its potential as a therapeutic agent in the treatment of constipation symptoms. The co-administration of *B. subtilis R0179*, which synthesizes Trp, and engineered *Bacteroides thetaiotaomicron*, which can convert this Trp to tryptamine, could yield a novel approach to treating GI dysmotility in a mechanistic manner. This research underscores the exciting potential of bacterial treatment strategies once the underlying mechanisms of bacterial actions are well elucidated.

Potential involvement of the 5-HT$_3$ receptor

5-HT acts through a variety of receptors to regulate intestinal functions. As described, our study has focused primarily on the 5-HT$_4$R due to its known involvement in the prokinetic actions of luminally acting agents, and because our lab is well equipped to target this receptor pharmacologically and genetically. However, it is also possible that the prokinetic actions of *B. subtilis R0179* involve the 5-HT$_3$R. Indeed, the 5-HT$_3$R plays important roles in regulating gut functions, including activating propulsive motility and promoting secretory responses (Mawe & Hoffman, 2013). Furthermore, the 5-HT$_3$R is targeted in clinical treatments of GI symptoms. For example, partial 5-HT$_3$R agonists are used to promote GI motility in the treatment of constipation predominant IBS (Evangelista, 2007), and 5-HT$_3$R antagonists induce slowing of GI motility in the treatment of diarrhea predominant IBS (Talley et al., 1990). While we do not have a breeding colony to test the involve 5-HT$_3$R using a genetic approach, we could

pharmacologically target the 5-HT_3R using an antagonist such as ondansetron to determine whether the 5-HT_3R contributes to the prokinetic actions of *B. subtilis R0179*.

III. THERAPEUTIC APPLICATIONS AND CONSIDERATIONS

A. Summary and conclusions

Clinical constipation is characterized by several manifestations of GI dysfunction including hard stools and infrequent bowel movements. Despite the high global prevalence of constipation symptoms, treatment options remain limited (Ramkumar & Rao, 2005). Emerging literature suggests that intestinal dysbiosis contributes to chronic constipation (Mancabelli et al., 2017). For example, it is thought that constipation is associated with a relative decrease in beneficial bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* spp., as well as a relative increase in pathogenic bacteria, such as *Campylobacter jejuni* (Zhao & Yu, 2016). However, more research is necessary to determine causal links between alterations in microbiota composition and function and GI dysmotility.

In our study, we found that administration of the Trp-synthesizing strain *B. subtilis R0179* ameliorated intestinal dysmotility in a mouse model of constipation. Constipated mice that received *B. subtilis R0179* displayed significantly faster colonic motility times following one week of treatment. As we saw in healthy mice, the prokinetic actions induced by bacteria treatment were most evident in the colon. These data emphasize the potential of *B. subtilis R0179* as a novel prokinetic agent, and underscore the promise of targeted uses of isolated bacteria to promote GI function in a predictive treatment strategy.

B. Alternative approaches and future directions

Other *in vivo* models of constipation

The EAE mouse model of multiple sclerosis (MS) was used for our evaluation of prokinetic actions of *B. subtilis R0179* treatment in the context of constipation. Previous work in our lab has demonstrated constipation symptoms in mice in which EAE has been induced with several different immunization strategies (Spear et al., 2018), and that colonic motility is improved in these constipated mice by treatment with a luminally acting 5-HT_4R agonist (Konen et al., 2021). Furthermore, we have extensive experience using this model to test prokinetic pharmacological agents. Very few experimental models are without limitations, however. In this case, EAE is used primarily as a model of MS, which is a multifaceted autoimmune disease characterized by neurological symptoms as well as inflammation and a compromised intestinal barrier.

An alternative *in vivo* model of constipation that we have previously used in our lab is aged animals (Konen et al., 2021). It is well established that aging is associated with altered GI function, and there is an increased prevalence of constipation in the human aging population. Aged animals exhibit symptoms that are consistent with chronic constipation, such as slowed intestinal transit, reduced fecal output, and reduced fecal water content (Patel et al., 2014). This model of constipation is characterized by fewer confounding factors than EAE, since it is not grounded in a neurological basis of disease. However, the primary limitation of using aged mice as a model is the time scale necessary for the development of constipation. Studies using aged-induced constipation typically perform experimental manipulations only once the mice reach ~72 weeks of

age, which makes this model quite logistically challenging, particularly for graduate studies.

Drug-based induction of constipation is another alternative approach. Pharmacological strategies to induce constipation in animal models utilize the administration of a variety of different agents that ultimately act to slow intestinal transit, decrease fecal output, and reduce the weight and water content of feces. The most commonly used drug to induce constipation is loperamide, an opioid receptor agonist that induces an antidiarrheal effect to inhibit fluid secretion through direct stimulation of enteric μ-opioid receptors (De Luca & Coupar, 1996). Another constipation-inducing opioid receptor agonist used is diphenoxylate, which has been shown to decrease GI motility and reduce intestinal fluid (Kongdang et al., 2022). Additionally, an α_2 adrenergic receptor agonist known as clonidine acts within the sympathetic nervous system to relax intestinal smooth muscle. Mice treated with clonidine display decreased number and weight of feces as well as delayed intestinal transit times (Kojima et al., 2009; Zhou et al., 2013). While these drugs are often employed to test the efficacy of various prokinetic pharmacological treatments, effective agents typically act at the neuromuscular end of the reflex circuit because synaptic transmission is inhibited throughout the circuitry. Nonetheless, future experiments will be conducted with *B. subtilis R0179* in the opiate model because opiate-induced constipation is a major problem for individuals receiving chronic opiate therapy to inhibit pain (Camilleri et al., 2014).

Trp-catabolizing strains of bacteria

GI dysfunction is, of course, not limited to slower transit times as seen in constipation, but rather exists on both ends of the spectrum. Diarrhea predominant IBS is a prevalent subtype of the functional bowel disorder IBS that is characterized by loose stool and increased sudden urgencies of bowel movements (Longstreth et al., 2006). Consequently, some treatment strategies for the management of diarrhea symptoms seek to slow intestinal transit time, thereby allowing more fluid absorption during stool transit.

Since Trp-synthesizing bacteria increase Trp level and therefore promote 5-HT signaling, it is conceivable that Trp-catabolizing bacteria which break down Trp could reduce 5-HT signaling through the diminished precursor availability for 5-HT synthesis. With this in mind, we conducted a preliminary investigation into the functional impacts of bacteria that catabolize Trp. The strain *Bifidobacterium infantis R0033*, provided by Lallemand Health Solutions and utilized in their probiotic formulations, has been demonstrated to break down Trp *in vitro*.

Using the same experimental design as the studies described in Chapter Three, we administered *Bifidobacterium infantis R0033* via daily oral gavage and assessed changes in motility as a result of bacteria treatment. Mice that received the Trp-catabolizing strain *Bifidobacterium infantis R0033* exhibited significantly slower colonic motility following a week of treatment (Fig. 5). Furthermore, there was no significant change in whole gut transit time, which is consistent with *R0179*-induced changes that localize to the colon and are not reflected in the net measure of whole gut transit. These preliminary data support our overarching hypothesis that bacterially-induced alterations of Trp can impact

5-HT-mediated gut functions. Notably, these findings provide an exciting future direction to investigate the use of Trp-catabolizing bacteria to slow GI motility in cases of diarrhea-presenting GI symptoms.

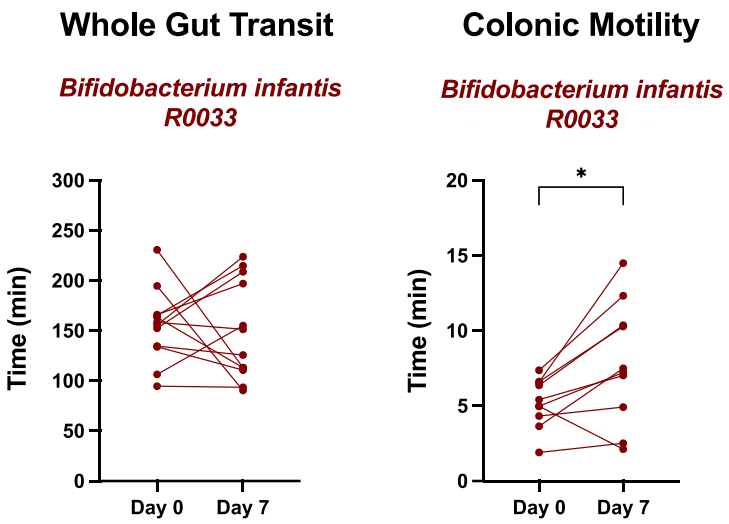

Figure 5. Treatment with the Trp-catabolizing strain *Bifidobacterium infantis R0033* slows colonic motility. To investigate the functional impacts of treatment with a bacterial strain that breaks down Trp, motility was assessed in mice that received *Bifidobacterium infantis R0033* (n=12) via daily oral gavage for one week. There was no significant difference in whole gut transit time following a week of treatment. However, mice exhibited significantly slower colonic motility times as a result of treatment with the Trp-catabolizing bacteria.

IV. CONCLUDING REMARKS: THE FUTURE OF "PRECISION PROBIOTICS"

The gut microbiome is a highly dynamic system that plays a key role in promoting our overall health and well-being. Ranging from acute strategies to temporarily alleviate GI dysmotility to more long-term approaches to treat chronic diseases, microbial interventions offer exciting promise as avenues of future targeted therapeutic outcomes. This dissertation contributes to a growing field of research seeking a predictive strategy for the use of bacterial interventions to promote human health.

Ultimately, this field must address the wide gaps in our knowledge regarding how exactly orally administered bacteria interact within a given host system, and to what degree these effects are sustained from individual to individual. Not only is it imperative that we gain a deeper understanding of bacterial species- and strain-specific effects, but we must also acknowledge the lack of a globally recognized consensus of what exactly defines a "normal" or "healthy" microbiome in which these bacteria are acting. Innovative strategies for the development of personalized probiotic interventions will be critical steps for the future of precision therapies.

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