THE ROLE OF SCFAS IN 5-HT MEDIATED COLONIC MOTILITY

THE ROLE OF SHORT-CHAIN FATTY ACIDS IN THE CONTROL OF SEROTONIN-MEDIATED COLONIC MOTILITY

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ABSTRACT

Introduction: The role of short-chain fatty acids (SCFAs) in colonic motility is controversial. Germ free (GF) mice are unable to produce SCFAs and serve as a model to study how their absence affects colonic motility. GF transit is slower than controls and colonization of these mice improves gastrointestinal (GI) transit and serotonin (5-HT) levels. Our aim was to determine the role SCFAs play in improving transit, and whether this is dependent on mucosal 5-HT signaling. Methods: Motility was assessed in GF mice via spatiotemporal mapping with intraluminal perfusion of either PBS or SCFA cocktail. Outflow from the colon was recorded to quantify propulsive contractions. Motility was then assessed in TPH1-KO mice with PBS, butyrate and then propionate. GPR43 and 5-HT staining was performed in control and GF colons. Mice were then given chow diet or high sugar diet (HSD) and motility was recorded. Fecal pellets were taken at baseline and just prior to motility experiments and SCFA levels were measured with mass spectrometry. Results: GF mice exhibit significantly lower proportion of propulsive contractions, lower volume of outflow per contraction and slower velocity of contractions compared to controls. SCFAs changed the motility patterns to that of the controls in all parameters. Butyrate administration significantly increased the proportion of propulsive contractions in controls, yet failed to in TPH1 KO mice. Propionate significantly inhibited propulsive contractions in both mice. HSD-fed mice were not different from chow-fed mice in any parameter. No SCFA was significantly reduced, but the change in butyrate concentration was significantly associated with LDC frequency. Conclusions: Our results reveal significant abnormalities in the propulsive nature of colon motor patterns in GF mice, explaining the decreased transit time in *in vivo* studies.

We show that butyrate, not propionate, activates propulsive motility and that this requires mucosal 5-HT, possibly released by ECs.

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5-HT	5-Hydroxytryptamine; serotonin
ASF	Altered Schaedler Flora
ATP	Adenosine Triphosphate
cAMP	cyclic Adenosine Monophosphate
CV	Conventionalized Mice
CMMC	Colonic Migrating Motor Complex
CNS	Central Nervous System
EC	Enterochromaffin Cell
ENS	Enteric Nervous System
FGID	Functional Gastrointestinal Disorders
FFA2/3	Free Fatty Acid Receptor 2/3; analogous to GPR43/42
GF	Germ free
GI	Gastrointestinal
GLP1	Glucagon-like Peptide 1
GPR43/42	G-protein coupled receptor 43/42; analogous to FFA2/3
HCl	Hydrochloric Acid
HDAC	Histone deacetylase
HM	Humanized Mice
HRCM	High-resolution Colonic Manometry
HSD	High Sugar Diet
IBS	Irritable Bowel Syndrome
ICC	Interstitial cells of Cajal
IPAN	Intrinsic primary afferent neuron
LDC	Long distance contraction; analogous to CMMC
LPS	Lipopolysaccharide
MMC	Migrating motor complex
MP	Myenteric plexus
MRCMS	McMaster Regional Centre for Mass Spectrometry
NO	Nitric Oxide

OCT	Optimal cutting temperature compound
PBS	Phosphate buffer solution
PCPA	p-chlorophenylalanine
PFA	Paraformaldahyde
PSD	Polysaccharide deficient diet
PYY	Peptide YY
RPMC	Rhythmic propulsive motor complex
SCFA	Short chain fatty acid
SERT	Serotonin transporter
SFB	Segmented filamentous bacteria
SMP	Sub-muscular plexus
SP	Spore-forming bacteria
TPH	Tryptophan hydroxylase (1 or 2)
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

DECLARATION OF ACADEMIC ACHIEVEMENT

This project was initially conceived by Dr. Jan Huizinga and myself, Alexander Vincent. Experiments were designed by Alexander Vincent and Dr. Jan Huizinga. All motility experiments were performed by Alexander Vincent. All histological experiments were performed my Dr. Xuanyu Wang. All data analysis was performed by Alexander Vincent under the guidance of Dr. Huizinga. Interpretation of results was conducted by both Alexander Vincent and Dr. Huizinga. Insight into further experiments was provided by Alexander Vincent's committee members: Dr. Premsyl Bercik, Dr. Waliul Khan and Dr. Michael Surette. This thesis was written by Alexander Vincent and edited by Dr. Huizinga.

1. INTRODUCTION

1.1 Bidirectional Relationship Between Motility and the Luminal Ecosystem

The large intestine is one of the most complex organs in the body. It is responsible for water absorption, the storage and clearance of feces, and maintaining an ecosystem of trillions of microbial cells and thousands of species (Eisenstein, 2016). In the colon, this ecosystem is comprised of the luminal contents including: the entirety of the microbiota, metabolites produced by microbiota and those that enter the colon through ingestion of food, and secretions from the endocrine system and immune cells. This ecosystem, like any other, is defined by its environment and is susceptible to evolutionary changes in relation to environmental alterations. The environment of the colon is not only defined as the contents of the lumen, but also the length of time these contents are present and thus how long they are able to interact with each other. This speed at which contents turnover is termed transit time, which is controlled by a variety of factors and is essentially decreased by the degree to which smooth muscle contractions propel contents aborally and increased by the amount they mix luminal contents for absorption.

Evolutionarily speaking, within this ecosystem, a given species' survivability (or ability to replicate) will depend on how well suited it is to compete within their environment. With respect to transit, a species that is able to reproduce rapidly will dominate in a diarrhea-prone individual whereas a species that grows slower yet is able to survive in a competitive environment will dominate in a more constipative state (Kashyap et al., 2013). With that being said, as much as transit can shape this ecosystem, the ecosystem can in turn affect transit which will be the focus of this thesis.

Just described is the bidirectional relationship between gut motility and the luminal ecosystem wherein a healthy gut remains in equilibrium. A better understanding of this relationship is vital to treat disorders related to when this equilibrium is perturbed.

1.2 Future for Functional Bowel Disorders

How colonic motility is controlled intrinsically by the enteric nervous system (ENS) and interstitial cells of Cajal (ICCs), and extrinsically by the central nervous system (CNS) is well (but not entirely) understood and has been studied extensively. Disorders directly affecting structural colonic physiology can be diagnosed with a specific cause and treated. For example, Hirshprung's disease results in complete colonic dysmotility caused by the congenital absence of the ENS from a segment of the colon. We can determine the etiology of the disease (ENS dysfunction) and treat it accordingly with removal of the affected tissue. On the other hand, functional gastrointestinal disorders (FGIDs) like irritable bowel syndrome (IBS), have often been described as disorders caused by an imbalance between gut and host physiology (Drossman, 2016). Where modern GI pathology falters is in trying to restore this evolutionarily symbiotic relationship. However, in order to do so, a better understanding of the etiology of these diseases is required.

The Rome foundation has been attempting to address this issue since the 1970s by developing a more integrated (systems) approach to dealing with FGIDs (Drossman, 2016). Rather than focusing on a specific biological etiology, clinicians are increasingly understanding these disorders as a product of multiple interacting biological, psychological and social systems. This is useful as it incorporates a broader range of

factors to explain the illness, and allows for a more general diagnosis to explain an illness when we may not understand the cause. This approach is especially useful when our understanding of the illness is lacking. However, a consequence of this is a tendency to focus on symptoms and improving quality of life rather than focusing on the underlying etiology. To combat this, treatment must be adaptive and evolve with basic and clinical scientific findings. As a result, treatment will move from general to targeted with the ultimate goal of individualized medicine. Thankfully the Rome Foundation recently moved a step in this direction by further categorizing general disorders into sub-groups in an attempt to delineate more detailed diagnoses (Drossman, 2016). Hopefully in the future with more mechanistic knowledge from basic research we can further develop these subcategories of functional diseases into precise etiological diagnoses. Only then will we be able to target the cause of the disequilibrium and subsequently restore it.

1.3 Physiological control of colonic motility

As mentioned above, extensive work has been done to understand how motor patterns are performed by the host. Colonic motility is a broad term that incorporates many types of contractile patterns of the colonic smooth muscle, be it propulsive or segmenting (Furness, 2008). The colon can be divided into multiple layers consisting of (from inside-out): the epithelial layer, the lamina propria, the muscularis mucosa, the submuscular plexus, the circular muscle layer, the myenteric plexus, the longitudinal muscle layer followed by the mesentery. ICC are dispersed throughout the muscle layers and within the plexuses (Komuro, 2012). Motor patterns are, ultimately, caused

by the contractions and relaxations of the upper two muscle layers. The 'dominant' pattern is caused by the release of excitatory and inhibitory neurotransmitters from the myenteric plexus onto the muscle layers via neurons of the ENS. These patterns, such as the long-distance contraction (LDC, synonymous with colonic migrating motor complex; CMMC), requires the ENS as it is blocked by TTX, a Na+ channel blocker, blocking neural action potentials. This begot the historic continued favor of the neurogenic control of motility. That being said, some motor patterns, such as myo*genic* ripples (a misnomer since they do not *originate* (-genic) from the muscle (-myo)), can occur without neuronal control and are thought to originate via ICC (Huizinga, 2016). Although they can function independently of the ENS, in vivo ICCs cooperate with the ENS to coordinate the dominant motor patterns (Huizinga, 2016). Thus, like most bodily systems, one wheel of control is no more important than the other as the entirety is functioning in a physiological setting; neurogenic and myogenic cooperate for the full effect.

The enteric nervous system is composed of a variety of different neurons classified based on their direction of projection, effector location, and neurotransmitter they release. Motor neurons, interneurons, and primary afferent neurons (both extrinsic and intrinsic) will be discussed here. The cell bodies of the motor neurons are located in the myenteric plexus, have endings on either circular or longitudinal muscle and are either inhibitory or excitatory. Inhibitory motor neurons release either nitric oxide (NO), vasoactive intestinal peptide (VIP) and/or adenosine triphosphate (ATP). Excitatory motor neurons on the other hand release acetylcholine and/or tachykinins (Furness, 2008). With respect to the LDC, it is historically thought that an excitatory motor neuron

fires above a bolus while an inhibitory motor neuron fires below a bolus, described as peristalsis by Starling & Baylis. However, this definition of peristalsis is more attune to the migrating motor complex (MMC) in the esophagus than the CMMC (or LDC) as they occur spontaneously in the absence of a fecal pellet and are thus not a reflex.

Interneurons are another class of neurons which are mostly located in the myenteric plexus of the ENS and project both orally and anally. These function similar to post-ganglionic neurons in the CNS whereby they convey information from a primary neuron to the effector neuron. NOS and 5-HT positive nerves are common interneurons (Furness, 2008). Interneurons also run between the submuscular plexus and the myenteric plexus, creating a form of communication between these two (Furness, 2008).

Primary afferent neurons are an interesting class of neurons who serve to sense stimuli and send signals to an effector. Different types of afferent neurons in the gut are depicted in Figure 1. They can be divided into extrinsic and intrinsic based on the location of their cell bodies. Intrinsic primary afferent neurons (IPANs) have cell bodies located in the gut wall whereas extrinsic primary afferent neurons have cell bodies in the nodose ganglion (vagal primary afferent neurons) of in dorsal root ganglia (spinal primary afferent neurons). Extrinsic neurons thus have the potential to mediate gut-brain communication; signals in the gut may reach the brainstem through vagal primary afferents (Fig. 1). IPANs on the other hand affect local responses; they synapse in the myenteric plexus onto ICC (Zhu et al., 2014), which in turn activate myenteric motor neurons in response to either chemical signals or distension. It is a misconception that IPANs sense the luminal environment themselves; neurons do not project to the lumen

(Fig. 1). In reality, luminal chemicals are sensed via specific receptors located on the luminal side of epithelial cells, which then secrete chemicals basally to receptors on the endings of IPANs. 5-HT (5-hydroxytryptamine, serotonin) is a strong stimulant of IPANs and has been found to be released by both mechanical distension and chemical sensing. The hormones CCK and motilin are also possibly involved in activating IPANs

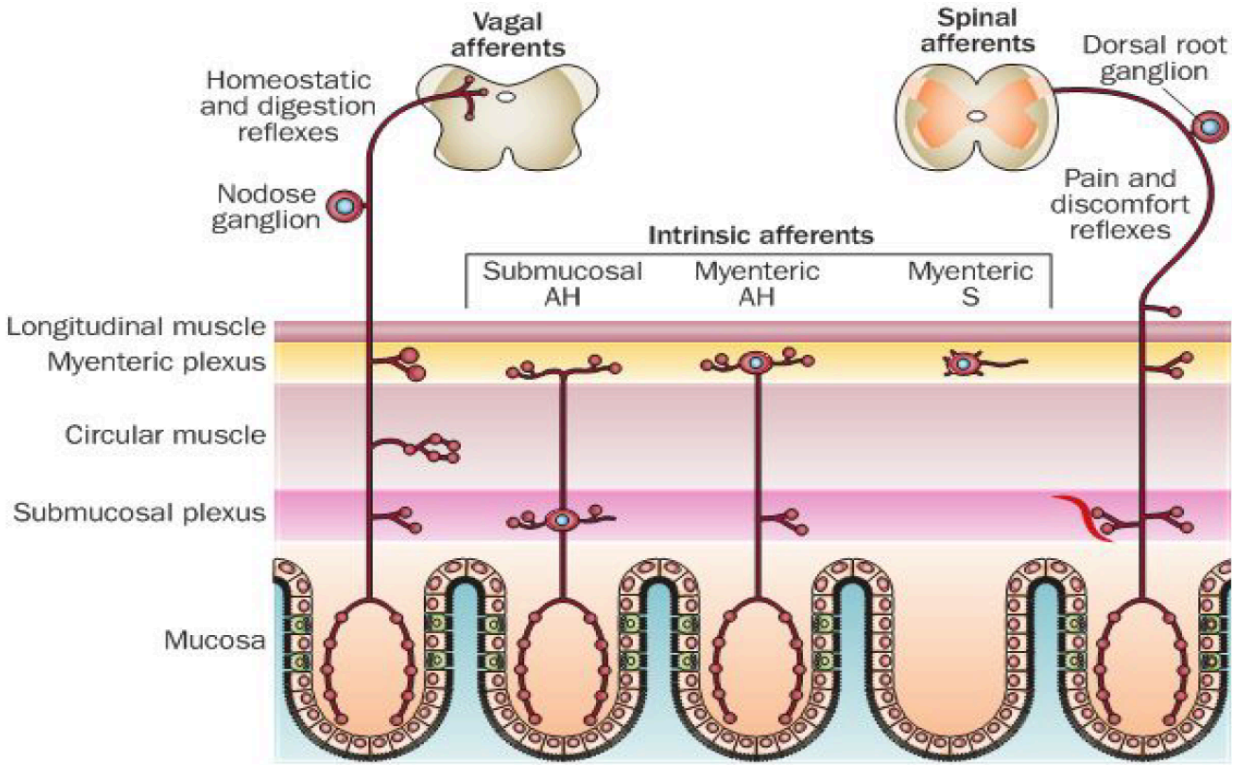

Fig. 1 Primary Afferent Neurons in the GI Tract

This schematic shows the different extrinsic and intrinsic primary afferent neurons in the gut. Most of these neurons have endings which project into the lamina propria where they are exposed to EC-cell (green epithelial cells) derived 5-HT. Depicted here are vagal afferents with cell bodies in the nodose ganglion, spinal afferents originating in the dorsal root ganglion. Intrinsic afferents depicted arise from the submucosal ganglion or myenteric ganglion. There are also mechanosensitive S-type neurons located in the myenteric plexus which respond to stretch. This figure was taken from Mawe & Hoffman (2013) without permission (yet).

as both have been shown to be released via nutrients (Furness, 2008). With respect to distension, it has long been known that muscle cells in the gut are directly sensitive to stretch and respond by contracting (Bulbring & Lin, 1958). It was only shown 40 years later, however, that stretch-sensitive IPANs are physically connected to the muscle layer and their activation is proportional to the degree of distension (Kunze et al., 2000). Thus IPANs are responsible for sensing both luminal signals indirectly via enteroendocrine system and for sensing the degree of distension. Interestingly, 60% of IPANs are responsive to mucosal chemicals and 80% respond when the intestine is stretched (Furness, 2008), meaning the majority of IPANs serve both functions. This is one of the examples of the robustness of colonic physiology; there are often multiple stimuli for the same phenomena. In this case, motility can be activated via both the composition of the luminal contents and the physical presence of stool, yet they most likely serve to amplify each other's effect.

1.4 Short-Chain Fatty Acids (SCFAs)

1.4.1 Short-chain fatty acids in the colon

Speaking of luminal contents, short chain fatty acids (SCFAs) are the major anion in the colon. They are largely produced by the fermentation of undigested and unabsorbed carbohydrates by commensal bacteria and as such there are scarce levels present in the colons of germ free (GF) mice (Høverstad & Midtvedt, 1986). The major SCFAs are acetate (C2), propionate (C3), butyrate (C4), which together comprise >95% of all SCFAs in the colon (Cummings et al., 1987). In humans, the most commonly reported ratio of these SCFAs is 3:1:1 with a molar concentration of 60:20:20 mM,

respectively (den Besten et al., 2013). The highest concentration is found in the proximal colon at 70-140mM and decreases to 20-70mM in the distal colon (den Besten et al., 2013). 95% of all SCFAs are taken up by epithelial cells as a food source, with only 5% being excreted in the feces.

Metagenomics analysis led to the characterization of specific bacteria responsible for individual SCFA production. Interestingly, it was found that a small number of organisms, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, and *Ruminoccocus bromii* comprise the majority of butyrate production (Louis et al., 2010). Similarly, propionate production seems to be dominated by a few bacterial genera (Morrison & Preston, 2016). Acetate production however is more conserved among bacteria and thus would account for its relatively large composition.

SCFAs have been shown to act on the free fatty acid receptors 2 and 3 (FFA2 and FFA3, or GPR43 and GPR41, respectively) with differing potency (Brown et al., 2003; Le Poul et al., 2003). Propionate seems to be the most potent agonist for FFA3, based on cAMP dose response curves, followed by butyrate, then acetate (Le Poul et al., 2003), whereas FFA2 is equally sensitive to each (Tazoe et al., 2008). In the colon, FFA2 and FFA3 are dispersed within the mucosa and submucosa, and recently FFA3 has been shown to be co-localized to cholinergic neurons in the proximal but not distal colon (Kaji et al., 2016). Within the colonic mucosa, FFA3 was shown to be strongly expressed by peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) cells as well as neurotensin cells in the proximal colon (Nøhr et al., 2013). FFA2 is also strongly expressed by all PYY cells as well as 5-HT+ mast cells in the lamina propria, but not on neurons (Nøhr et al., 2013; Tazoe et al., 2008).

Recently, another receptor has been shown to use SCFAs as a ligand. The receptor olfr558 has been shown to be activated in response to SCFA signaling (Bellono et al., 2017). Unlike the FFA2/3 receptors, this receptor was selective to certain SCFAs; only isovalerate, butyrate and isobutyrate activate it (Bellono et al., 2017). Similar to the FFA2/3 receptors, it is also located on ECs. This study also went further and found that activation of this receptor causes intracellular depolarization followed by 5-HT release, which was then found to activate IPANs via 'synapse-like' connections between ECs and IPANs (Bellono et al., 2017). This provides a mechanism by which luminal contents can activate the enteric nervous system.

In addition to receptor activation, SCFAs have a number of other roles. SCFAs are the major fuel source in the colon, with butyrate being the most preferred by colonocytes (Clausen & Mortensen, 1995). Butyrate also has other regulatory roles on the colonic epithelia especially with respect to barrier integrity. Butyrate has been shown to improve tight junction permeability through increasing expression of tight junction proteins Zo-1 and claudin-1 (Liu et al., 2014; Wang et al., 2012), potentially reducing the amount of lipopolysaccharide (LPS) translocation and thus production of proinflammatory cytokines. Unlike butyrate, propionate and acetate have been shown to have roles in lipid metabolism, glucose homeostasis and appetite regulation (Chambers et al., 2015; Hong et al., 2005; Morrison & Preston, 2016). This effect seems to be mediated via FFA2 located on adipocytes (Kimura et al., 2013), pancreatic beta cells (Tang et al., 2015) and enteroendocrine L-cells (Chambers et al., 2015), respectively.

In addition to mediating actions through receptors, butyrate has also been shown to be a highly potent endogenous HDAC inhibitor, facilitating the transcription of certain

genes, namely the differentiation into regulatory T cells (Arpaia et al., 2013; Furusawa et al., 2013). Propionate was also shown to be a HDAC inhibitor, yet its potency was minimal compared to butyrate, and acetate did not exhibit this effect at all (Waldecker et al., 2008). At the epithelial layer and lamina propria, butyrate has been shown to enhance proliferation in colonocytes, induce apoptosis in cancerous colonocytes (Donohoe et al., 2012), down-regulate macrophage secretion of pro-inflammatory mediators (Chang et al., 2014) and inhibit SERT expression (Gill et al., 2013) via HDAC inhibition mechanisms. In addition to acting below the submucosal surface, rectal butyrate (but not propionate or acetate) application in vivo and in vitro was found to increase the density of cholinergic neurons, but not nitrergic neurons in the myenteric plexus (Soret et al., 2010). This would suggest that butyrate can directly influence ENS differentiation, and the authors attributed this to HDACi activity as trichostatin A, a potent HDACi was able to exert similar effects. It is clear, and interesting, that a difference of one hydrocarbon (between butyrate and propionate) can make a large difference in the function of the molecule.

Given the reported vast health benefits of SCFAs, they seem to be a panacea for all GI disorders. Simply give large doses orally and your patient's illness will be cured- albeit with some bloating. However, oral SCFA are rapidly oxidized and absorbed in the stomach and small intestine, respectively (Braden et al., 1995). Even if the SCFAs were to reach the colon, the organ exists in a perpetual state of homeostasis; both chemically and biologically. Drastically increasing the concentration of the major anions in your gut would cause serious imbalances in this homeostasis, most notably creating an acidic environment due to the pKa of these anions. It is thus no surprise that high circulating

concentrations of butyrate and propionate (>1mmol/L vs normal range of 5μmol/L) are associated with acidaemic disease due to their toxicity at high concentrations (Morrison & Preston, 2016). A more natural way to increase SCFAs in the colon is to supplement an animal or human diet with high amounts of fibre. Although there was not a linear correlation between fibre-intake an SCFA concentration (most likely due to an increase in excretion), fibre intake has been shown to have a profound effect on not only levels of SCFAs but also their composition (den Besten et al., 2013). For example, an increase in inulin intake was shown to increase relative production of butyrate and propionate over acetate (ibid.).

1.4.2 The effect of SCFAs on motility

As stated above, the relationship between microbiota and GI motility is bidirectional. The microbiota is an ecosystem that is dependent on transit time. It has been shown that accelerating and decelerating transit affects the microbiota as the former or latter evolutionarily select for species that are suited to excel in those environments (Kashyap et al., 2013). Vice versa, the microbiota is thought to affect motility via fermentation of resistant polysaccharides yielding SCFAs (Kashyap et al., 2013; Yano et al., 2015). Indeed, applying SCFAs to the colon in vivo has shown changes in motility, yet the exact role SCFAs play in modulating motility has been controversial. Nevertheless, the mechanism seems to involve hormone secretions.

Studies have reported results of SCFAs both accelerating transit and inhibit transit (Cherbut et al., 1998; Dass et al., 2007; Fukumoto et al., 2003; Grider & Piland, 2007; Hurst et al., 2014; Soret et al., 2010; Squires et al., 1992; Yajima, 1985). For example,

Yajima (1985) found propionate to induce contractile activity in segments of the rat colon whereas Hurst et al. (2014) found it to completely inhibit LDCs following administration to whole-tissue guinea-pig colons analyzed with spatiotemporal mapping. Interestingly, in the same study, Hurst et al. (2014) found butyrate, at the same concentrations of propionate, to increase the frequency of LDCs while at the same time decreasing the frequency of short-propagating contractions. This is an important distinction and calls into question the validity or interpretation of other motility studies which cannot distinguish between the propulsive nature of motor patterns in the colon. Cherbut et al. (1998) concluded that SCFAs (cocktail of acetate, propionate and butyrate) inhibit motility. They used electrical recordings along the colon to monitor myoelectrical activity. This was recorded as 'spike-bursts' and were synonymous with contractions (Cherbut et al., 1998). The study found SCFAs to significantly decrease the overall number of spike-bursts, especially the 'shortly propagated' bursts, while the totally propagated bursts, most likely the LDCs or propulsive contractions, actually increased (Cherbut et al., 1998, #49327). Thus, although there was a measured decrease in total contractions and motility was concluded to be decreased, the propulsive nature of the contractions was not looked at closely and it could be argued here that SCFAs increased propulsive motility, decreased segmenting motility and thus decrease transit time, or would decrease transit time in vivo.

Other studies report similar conflicting results, yet the consensus on the mechanism of action seems to involve the trigger of sensory neurons which either activate or inhibit myenteric contractions. Strong evidence for the role of 5-HT (Fukumoto *et al.*, 2003; Grider & Piland, 2007) and PYY (Cherbut et al., 1998) as

mediators between sensory neurons and luminal SCFAs show dose-dependent increases in these hormones following SCFA administration. Curiously, although Hurst et al. (2014) found opposite effects of propionate and butyrate ex vivo, their lab previously reported both metabolites to dose-dependently increase 5-HT release (Grider & Piland, 2007). Perhaps the most conclusive study for the role of SCFA in 5-HT release was done by Fukumoto et al. (2003). They performed elegant in vivo preparations of rat colons to show that SCFA stimulated fecal pellet output. They first found that treatment of SCFA produced a dose-response release of 5-HT and that endogenous 5-HT produced the same effect as SCFA. They then inhibited this effect using multiple antagonists to elucidate the mechanism. SCFA activity was blocked using a 5-HT_3 antagonist, alosetron. Pre-treatment of the colon with p-Chlorophenylalanine (PCPA; an inhibitor of tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-HT synthesis) and capsaicin, known to destroy primary afferent neurons, inhibited SCFA response. Hexamethonium, a nicotinic acetylcholine receptor blocker, also inhibited the response. Lastly, the response of SCFA was also inhibited following a vagotomy, which, given the reported effects of SCFA ex vivo, is interesting and begs the question of why the vagus nerve would be required in vivo but not ex vivo. In short, they found SCFA-activated motility produced 5-HT, and required TPH, 5-HT_3 receptors, primary afferent neurons, and acetylcholine receptors. The logical interpretation for a mechanism would be that SCFAs act to produce 5-HT, which acts on 5-HT_3 receptors on IPANs, which trigger acetylcholine release in the myenteric plexus, leading to a contraction.

Despite extensive work on the prokinetic hormones released by SCFAs, no study has measured inhibitory hormone release following *individual* SCFA administration.

Cherbut et al. (1998); however, measured PYY release following a SCFA cocktail. They were also able to inhibit the effects of SCFAs by pretreating tissue with a PYY antiserum. Given that PYY/GLP-1 (usually co-localized to the same endocrine cells) are known to be inhibitory hormones (Schirra & Göke, 2005), it is understandable that the study concluded that SCFAs inhibit motility via PYY. Nevertheless, there seems to be a dual response of SCFAs on motility. The fact that individual SCFAs have been associated with differential motility responses and both inhibitory and excitatory hormone release may explain the controversial results of SCFAs on motility. Future studies on the topic should use individual SCFAs and specific hormone release in order to elucidate this discrepancy.

1.5 Serotonin

1.5.1 Serotonin in the GI tract

Without even properly introducing serotonin, it should be clear by now that it is an integral neurotransmitter in the gut involved in the detection of stimuli, be it chemical or mechanical. This neurotransmitter is historically well known for its roles in the central nervous system, notably the role it plays in depression, anxiety, sleep, appetite, sex or temperature control (Gershon, 2013). However, (perhaps a cliché for modern gastroenterologists) many are surprised to discover that 95% of the body's 5-HT is found in the gut. It is rare in physiology for something produced in this quantity to be vestigial, and it is not the case with 5-HT. In fact, using the nematode *C. elegans*, 5-HT was recently found to have highly conserved ancestral regulatory mechanisms regarding food sensing and motor function (Lee et al., 2017). It seems new roles of 5-

HT in the gut are being discovered every year albeit not without controversy. The roles of 5-HT with respect to motility certainly highlight this controversy and will be discussed subsequently. For now, a basic introduction to 5-HT in the gut will be given.

The majority of 5-HT in the gut is produced by ECs and the rest (to a lesser but equally important extent) by serotonergic neurons located in the myenteric plexus (MP). In ECs, mucosal 5-HT is synthesized from 5-hydroxytryptophan (Zhu et al., 2011) by the rate-limiting enzyme TPH1. In neurons, 5-HT is produced by the rate-limiting enzyme TPH2 (Li et al., 2011). TPH1 knockouts (KO) have a depletion of mucosal, but not neuronal, 5-HT and show a decreased level of 5-HT, whereas a TPH2KO does not decrease gut 5-HT levels but removes neuronal 5-HT (Li et al., 2011). Following its release, serotonin re-uptake transporter (SERT) inactivates 5-HT and transports it back into epithelial cells (Gill et al., 2013).

In the gut, 5-HT has been shown to play several roles outside of motility. 5-HT has been shown to regulate mucosal growth (Gross et al., 2012), promote early development of myenteric neurons (Li et al., 2011), attenuate visceral hypersensitivity (Hoffman et al., 2012; Kanazawa et al., 2011), facilitate Cl^- and bicarbonate secretion (Hoffman et al., 2012; Mawe and Hoffman, 2013), neutralize luminal toxins (Sörensson et al., 2001), and exacerbate inflammation (Kim et al., 2015).

As previously mentioned, 5-HT research is riddled with controversial results. Given the numerous roles of 5-HT and the fact that there are over seven unique receptors, all with potentially different functions and a number of subtypes, definitive conclusions will be difficult to make. Exactly how 5-HT promotes motility is not currently known. The seven 5-HT receptors are dispersed throughout the mucosa, lamina

propria, and ENS (Dickson et al., 2010; Hoffman et al., 2012; Yaakob et al., 2015). In the human colon, 5-$HT_{2,3,4,7}$ were found to be the most common and subtypes are detected in virtually all layers of tissue (Chetty et al., 2009). It is understandable, knowing their ubiquity, how difficult it is to pinpoint a specific function for each receptor. With that being said, research into 5-HT receptor function has made much progress with respect to motility.

1.5.2 The role of 5-HT on colonic motility

The role of 5-HT in motility was championed by experiments done in the 1950s by Edith Bülbring (Bülbring et al., 1958; Bülbring & Crema, 1958; Bülbring & Lin, 1958). She showed that application of 5-HT mimics the peristaltic reflex and that it decreases the amount of pressure required to induce this reflex. She postulated that 5-HT is produced by pressure-sensing ECs to produce motility, yet never measured 5-HT release or was able to completely deplete the colon of 5-HT. Years later, with an understanding of serotonin's aforementioned role in stretch and/or sensory afferent neurons, this comes as no surprise. The ability of 5-HT to reduce the threshold of distension to activate LDCs was later reproduced (Gwynne et al., 2014).

The controversy that has followed 5-HT in motility has to do with whether or not 5-HT is *required* to produce LDCs (Smith et al., 2010; Smith & Gershon, 2015; Spencer et al., 2015; Spencer et al., 2015). The requirement of mucosal 5-HT was first tested in multiple ways via dissection of the mucosal layer (Keating and Spencer, 2010), breeding TPH1 KO mice (Heredia et al., 2013) and using various 5-HT antagonists. It was found that LDCs could still occur in the absence of a mucosal layer and that TPH1

KO mice have seemingly normal transit times, concluding *mucosal* 5-HT was not required for peristalsis. This does not mean that 5-HT is not important for motility, simply that *mucosal* 5-HT does not initiate LDCs. Furthermore, in TPH1 KO mice, administration of a 5-HT_3 antagonist inhibited spontaneous LDCs (Keating and Spencer, 2010), suggesting another source of 5-HT initiates motility. The focus was then shifted to neuronal 5-HT, as a TPH1 KO mouse retains its 5-HT produced by TPH2. Motility was then assessed in TPH2 KO mice (Li et al., 2011). TPH2 KO mice have significantly slower transit times than controls and a double TPH1 and TPH2 KO had this same effect on transit (Gershon, 2013; Li et al., 2011). However, whether this is solely due to a global lack of gut serotonin is not certain, as these mice had significantly lower densities of ENS neurons than WT mice, attesting to the importance of TPH2 as an essential growth factor for ENS development (Li et al., 2011, #42191). As such, it is not clear whether the difference in motility in TPH2 KO mice is due to the loss of neuronal 5-HT transmission specifically, a loss of neurons in general, or both. It was concluded that neuronal 5-HT was more important for initiating motility than TPH1, yet this does not belittle the physiological role of TPH1, which was elucidated in elegant experiments with TPH1 KO mice (Heredia et al., 2013).

Despite the seemingly normal transit times of TPH1 KO mice, at closer inspection, TPH1 KO mice have largely perturbed fecal pellet production and propulsion due to a dissonance between stretch and mucosal reflexes. Again, with an understanding of 5-HTs role in activating IPANs, this comes as no surprise. In their study, Heredia *et al.*, (2013) essentially dispel the notion that 5-HT is not required for normal peristalsis. They found that TPH1 KO mice were not able to propel an artificial fecal pellet smaller than

2.5mm in diameter, but could propel a fecal pellet larger than 2.5mm whereas control mice could expel both (Heredia et al., 2013, #47553). KO mice also exhibited significantly more retrograde propulsive activity. These findings would suggest mucosal 5-HT is required to amplify distension-induced reflexes, which function to propagate content aborally and whose absence can be compensated for by intact stretch reflexes from neurons in the myenteric plexus. This would explain the larger fecal pellets and lower pellet output in these mice; the fecal pellets can only be propagated once they reach a large enough size to trigger 5-HT-independent stretch neurons in the myenteric plexus. As such, the absence of EC-derived 5-HT is not detrimental to murine health, but is certainly involved in normal motility and these KO mice could be used as a model for constipation. It is thus undeniable the requirement for both neuronal and mucosal serotonin stores for the integrity of *physiological* peristalsis.

Clinically, 5-HT concentrations have been found to be increased in IBS-D and celiac disease and decreased in constipation compared to controls, however the exact role this deviation plays in the aetiology of the disease is unknown as 5-HT levels do not coincide with symptom severity (Camilleri, 2009). Despite our lack of mechanistic understanding, 5-$HT_{3/4}$ receptor antagonists/agonists are used to treat diarrhea and constipation, respectively (Mawe and Hoffman, 2013). As such, many studies have been done in attempts to elucidate the precise role 5-HT and its receptors play in governing the function of the large bowel.

1.5.3 5-HT receptors involved in motility

The most studied 5-HT receptors with respect to motility are 5-HT_3 and 5-HT_4. The 5-HT_3 receptor is expressed on sensory nerve endings in the MP and submucosal plexus where they are thought to contribute to sensations of bloating and pain (Chetty et al., 2009). 5-HT_3 antagonists showed decreased frequency of bowel movements, nausea and sensitivity in clinical trials (Crowell, 2004). 5-HT_4 receptors on the other hand are expressed throughout intestinal tissues (Hoffman et al., 2012). In the mucosa, 5-HT acts on 5-HT_4 receptors on colonocytes, EC cells and goblet cells all with the proposed function to decrease transit time; colonocytes increase luminal Cl^- secretion and thereby H_2O eflux, EC cells stimulate motility through releasing 5-HT and goblet cells increase mucous secretion (Hoffman et al., 2012). 5-HT_4 receptors are also located on smooth muscle cells where agonist action induces relaxation and on cholinergic neurons (Chetty et al., 2009). Because of their diverse expression and potentially different function at each location, one cannot understand the complexity of 5-HT on motility simply by measuring transit times. A more worthwhile and comprehensive approach to studying this relationship is done by observing motility patterns ex vivo. For example, it was found that 5-HT_3 and 5-HT_4 receptors are individually involved in two distinct motor patters: the LDC and the rhythmic propulsive motor complex (RPMC) (Yu et al., 2015). If a drug is given in vivo and transit is measured to be longer, one would conclude that the drug is inhibiting motility whereas it could be exciting segmenting motility patters so as to increase absorption. This discrepancy would only be recognized through a visualization of motor patterns.

1.6 Measuring Colonic Motility

Until a reliable way to measure colonic motor patterns in vivo is discovered, our understanding of colonic motility will be lacking. The easiest and most common way to currently measure motility in animal models in vivo is through the carmine red dye method. A dye is gavaged orally and the time taken to expel a red fecal pellet is recorded as total transit time. As alluded to earlier, this method has its limitations. Not only can one not visualize motor patterns, but also one cannot determine which organ is contributing most, if at all, if one observed an abnormal time. In vivo motility studies focused on a specific organ, as performed by Fukumoto et al. (2003), use the geometric center method where a radiolabeled substance is introduced via injection to the proximal colon and the amount left over is measured in the colonic tissue; the more distal a substance is measured, the faster the motility. Although this is a reliable method, it lacks in its ability to monitor motor patterns.

One study was able to measure motor patterns in an isolated organ in vivo using a delicate dissection of the small intestine (Bogeski et al., 2005, #83338). They were able to anesthetize the animal, remove the jejunum from the abdominal cavity with the mesentery connected, cannulate two ends, video-record the tissue and create spatiotemporal maps of the motor patterns. They also measured the amount of outflow from the tissue, giving them an indication of the propulsive nature of the motor patterns. This would be an optimal method of measuring motility in vivo in the colon, however their method is restricted to the small intestine due to the difficulty one would face with cannulating the distal colon without severing an artery in the mesentery.

Spatiotemporal mapping is a technique designed in 1994 by Bercik et al. to visualize motor patterns of the gut. Although it can be adapted with difficulty to be used

in vivo, it is typically performed ex vivo. The technique involves video-recording a dissected intestinal organ and using software to plot the diameter changes over time along the length of the organ. The product is a map of contractions where one can visualize unique motor patterns, quantify them based on parameters like frequency, amplitude and velocity and, with the use of outflow recordings as done by Bokeski et al. (2005), determine the propulsive nature of the contraction. The obvious drawback from this technique is its ex vivo nature. Any results cannot be assumed to be reproducible in vivo as the tissue is devoid of any connection to the CNS, PNS or mesenteric blood supply, yet results are still valuable in their ability to determine physiological responses of organ in question independent of the rest of the body.

In humans, a relatively new technique, championed by Phil Dinning called high resolution colonic manometry (HRCM; an improvement upon low-resolution), gives us the ability to monitor motor patterns in the colon in vivo (Dinning et al., 2015). This technique has also been performed in animals as well (Chen et al., 2013), yet is limited in that it measures pressure changes, and not contractions, which is an important distinction when one attempts to differentiate an artifact from a true motor pattern (Corsetti et al., 2017). As such, the most reliable method of monitoring colonic contractile patterns in an animal model is spatiotemporal mapping with outflow analysis. It is a relatively simple technique whose insights outweigh its limitations and can be applied to delicate tissues (like those in germ free mice).

1.7 Coming Full Circle; Microbiota, SCFA, 5-HT and Motility

1.7.1 Germ free motility

It is difficult to eliminate completely SCFAs from the colon. One can treat the animal with antibiotics to reduce microbial fermentation, yet bacterial niches still exist in mice treated as such (Stefka et al., 2014). The germ free (GF) mouse is a great model to use in order to evaluate what the absence of microbiota and by extension, microbial-byproducts, have on motility. Although many other metabolic processes are disturbed in these mice, including those related to bile acids (Sayin et al., 2013), SCFAs are present at negligible levels, nearly 100-fold lower than control mice (Høverstad & Midtvedt, 1986).

In vivo motility studies on GF mice have shown that they have significantly longer transit times (Kashyap et al., 2013; Yano et al., 2015). By humanizing (HM; administering human microbiota to GF mice) or conventionalizing (CV; adding murine microbiota) the GF mouse (Yano et al., 2015) the effect on transit time was reversed three days after colonization. This would indicate that the difference in motility between mice with and without microbiota is more due to a lack of stimuli than a detrimental effect on morphology in GF mice; if transit time can be reversed in three days, it is not likely these mice are undergoing development of new cells in this time-period. Giving HM mice a polysaccharide rich diet (increasing fermentation of SCFAs) accelerates transit time, giving them a polysaccharide-deficient diet (PSD) slows transit and giving GF mice PSD had no change on transit (Kashyap et al., 2013). This would indicate that polysaccharide fermentation by microbiota increases transit through production of SCFAs.

In vitro and ex vivo experiments on the GF colon are lacking. One study looked at myoelectical activity of GF rat small intestine and found the interval between LDCs

larger in these mice than controls (Husebye et al., 2001). Other studies have concluded similar lower frequency of major propulsive contractions in the small intestine (Collins et al., 2014; Samuel et al., 2008; Yajima et al., 2016), yet no study has looked at either basal motor patterns in the GF colon or those induced with short-term SCFAs.

1.7.2 Colonic serotonin in GF mice

GF mice have lower levels of 5-HT in their gut tissues, and the number of EC cells in GF mice appears to be controversial. Some studies show strongly diminished numbers (Yano et al., 2015) whereas others have reported that they have the same number of colonic EC cells (Reigstad et al., 2015) and react to 5-HT stimuli to the same degree that HM mice do (Kashyap et al., 2013). With that being said, the expression of *TPH1* mRNA in the EC cells in these mice is lower compared to controls (Reigstad et al., 2015; Yano et al., 2015). Taken together it would seem that 5-HT levels are indeed lower in GF mice, yet the subjectivity of measuring EC cells calls into question whether this is simply due to lower TPH1 levels, or both lower EC cell density and TPH1 levels.

In an attempt to elucidate which bacteria in CV or HM cultures are mediating this change in transit time, Yano et al. colonized GF mice with different strains or cocktails of bacteria. They found that only spore forming bacteria (Sp), and not altered Schaedler flora (ASF), Segmented filamentous bacteria (SFB), or *Bacteroides spp*. from SPF microbiota significantly ameliorated the GF-associated abnormalities in both transit time, *TPH1* expression and tissue 5-HT levels; increasing the values to CV/HM levels (Yano et al., 2015). These Sp. bacteria are known to produce SCFAs (Atarashi *et al.*, 2013) and metabolomic analysis of these Sp-colonized mice found that levels of SCFAs

(including propionate and butyrate) were higher in these mice compared to GF (Yano et al., 2015). Additionally, administration of butyrate and acetate to an isolated line of human EC cells (BON cells) increased *TPH1* expression (Reigstad et al., 2015). Interestingly, this increase was not observed with LPS administration, indicating that it is the product of microbial fermentation that is triggering the response rather than the microbiota themselves (Reigstad et al., 2015).

Exactly how SCFAs increase TPH1 expression and thereby 5-HT levels in EC cells is unknown as EC cells are not thought to express the SCFA receptors FFA2 and FFA3 in the colon (Tazoe et al., 2008), however a recent study in the duodenum showed co-localization between FFA2-IR cells and 5-HT-IR cells (Akiba et al., 2015), and their method has yet to be replicated in the colon. Another possibility is that SCFAs, particularly butyrate, directly modulates TPH1 activation via HDAC inhibitor pathways, yet it is unclear if the timeframe for this mechanism can explain immediate effects of acute administration of SCFAs to the colon (Hurst et al., 2014). A third, more plausible, mechanism suggests that only certain SCFAs act on the receptor Olfr558 on ECs to elicit 5-HT release (Bellono et al., 2017). Despite the mechanism by which TPH1 is increased, the literature seems to show that 5-HT signaling mediated by SCFAs is the key difference between motility changes in GF and HM mice.

1.8 Impact of the Western Diet on Human Health

It is undeniable how much progress humans have made in the industrial age. Globalization and mass production of foods have made it easier than ever to feed oneself, yet this has come at a cost which we are only beginning to understand. The

increased availability of processed, tasty, cheap and convenient foods has changed the diet in industrial societies from one rich in fibre to one of low fibre, but high in fat, sugar and calories. A recent study estimates that our ancestors used to eat around 135g of resistant starch per day (Leach & Sobolik, 2010), whereas a study of 15,000 Americans from 1999 reported an intake of only 2.6g per day (Moshfegh et al., 1999). With an understanding of the role fibre plays in maintaining and enriching our microbiota, and in turn the effects this symbiosis has on several aspects of human health from blood glucose levels to mental health, it comes as no surprise that cultures who have retained our ancestral high-fibre diet have a significantly lower risk of developing IBD, IBS, heart disease, diabetes and colon cancer (Galisteo et al., 2008). Furthurmore, a Western-diet was shown to decrease colonic 5-HT levels in rats (Bertrand et al., 2012) and increase it in the small intestine (Bertand et al. 2011). Moving away from the gut, given the significance of 5-HT in the brain, it is worthwhile to discuss whether gut 5-HT release can impact brain 5-HT neurotransmission. Research seems to indicate a link; a recent epidemiological study found an inverse relationship between the prevalence of depressive symptoms and fibre intake (Gopinath et al., 2016), yet whether this is *caused* by perpetually low SCFAs and thereby low gut 5-HT levels is not clear.

1.9 Objectives and Hypothesis

The overall aim of this project is to understand how SCFAs alter motility patterns in the mouse colon. It is hypothesized that SCFAs are required to produce normal motility patterns, that this effect can be activated with acute SCFA administration, that mucosal

5-HT is required to elicit this effect and that motility can be altered by changing one's diet. These hypotheses will be tested with the following objectives:

1. Evaluate the phenotypic changes, if any, in motor patterns of GF mice and whether they are altered following intraluminal administration of SCFAs;
2. Determine if mucosal 5-HT is required to produce the effects of SCFAs on colonic motor patterns using TPH1 KO mice, and;
3. Determine if lowering fibre content in the diet will adversely affect SCFA production and colonic motor pattern phenotype.

2. METHODS

2.1 Animals

For GF experiments, 10 8-10 week-old, female, wild-type, c57Bl/6 mice were used as controls (Charles River Laboratories, Wilmington, MA, USA). 10 age and sex matched C57Bl/6 GF mice were received from McMaster University's Gnotobiotic Facility.

For TPH1 KO experiments, 5 8-10 week-old, female, C57/Bl/6, TPH1 KO mice, bred in-house, were received by Dr. Waliul Khan. 5 age and sex matched C57Bl/6 wild-type mice were used as controls for comparison.

For diet experiments, 10 C57/Bl6 female mice were used and caged individually. 5 mice were given a high-sugar diet (50% sucrose; prepared in-house) and 5 were given a standard chow diet for one week. Mice were weighed before and after feeding and four fresh fecal pellets from each mouse on a high sugar diet were collected: two were taken before being placed on the diet and two afterwards. Samples were weighed and frozen in liquid nitrogen and placed in a -80°C freezer before being processed for analysis via mass spectrometry.

For all experiments, female mice were used exclusively as reports of gender-specific alterations in microbiota and serotonin levels have been reported (Clarke et al. 2013). Due to the small sample size in experiments, all sources of variation were minimized wherever possible.

All animals were fed *ad libitum* and kept on a 12-hour light cycle. Mice were housed in cages of 3-5 animals (with the exception of diet experiments) and transported individually to the lab two hours prior to experimentation. Two hours were given to allow

the animals to acclimatize to their new environment and to mitigate any stress following transport. All experimental procedures were done in accordance with McMaster University Animal Research Ethics Board (AUP: 14-12-49).

2.2 Tissue Preparation

The mice were anesthetized with isoflurane and euthanized via cervical dislocation. The entire colon was dissected from the animal and placed in 4^{o}C oxygenated (5% CO_2, 95% O_2) Krebs' solution containing (in mM) 120 NaCl, 6 KCl, 15.5 $NaHCO_3$, 1.2 NaH_2PO_4, 0.1 Citric acid, 0.1 Aspartic acid, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 6 glucose for dissection. The tissue was pinned inside a Sylgard-bottomed dish where the mesentery was carefully cut and the cecum removed. Fecal pellets were initially difficult to remove. Preliminary techniques involved removal by sliding a set of tweezers across the length of the tissue, yet this was found to be too damaging as no LDCs were observed following this preparation. Fecal pellets were successfully removed by gently flushing the colon with PBS using a gavage needle. Care had to be taken while gavaging as too much distension may damage the mucosa and subsequently its response to mucosal stimulation. In order to aid the clearance of fecal pellets, the tissue preparation was done in Krebs' solution devoid of $CaCl_2$ to avoid smooth muscle contractions, thereby relaxing the tissue. Once the technique of gavaging was practiced, normal Krebs' solution was used. Another technique practiced in the literature is to place the tissue inside the bath and allow the fecal pellets to be evacuated naturally. This can take up to one hour, however, and the organ bath must be replaced in order to remove the contaminated solution. This would drastically increase the amount of time

that the tissue is in the hot solution, decreasing the amount of time the tissue is viable for actual recording.

After removing the fecal pellets, the oral and anal ends of the tissue were cannulated with a steel tube (made from an 18-gauge needle) with a plastic collar and fastened with nylon string. The size of the plastic collar was adjusted such that it was as thin as possible. This was done to minimize both distension and damage to the tissue while being inserted.

2.3 Experimental Setup

The prepared tissue was placed into an organ bath lined with Sylgard gel, containing 500 mL of Krebs' solution continuously bubbled with carbogen (5% CO_2, 95% O_2). Carbogen was used to prevent the Sodium Bicarbonate from precipitating out of solution. The degree of the bubbling had to be compromised as too little would cause precipitation, and thus a change in the pH of the Krebs' solution, and too much would cause ripples at the top of the water, which would interfere with the creation of Dmaps. Preliminary baths with room for only one bubbling tube along the length of the bath had to be replaced for a larger a one to accommodate two bubbling tubes to avoid any precipitation of Sodium Bicarbonate. A heating tube, connected to a hot water bath, was placed into the organ bath and used to adjust the temperature to 35 – 37 ^{o}C. 35 ^{o}C was found to be the optimal temperature, especially for longer experiments, with the assumption that the tissue was warm enough to produce peristalsis and as cool as possible to limit deterioration of the mucosa. The oral cannula was attached to polyethylene (PE) tubing, which was wrapped around the heating tube before being

connected to a Peristaltic Pump P-1 (Pharmacia, Sweden). It was important to wrap the inflow tube around the heating tube such that by the time the solution reached the colon, it was at the same temperature as the bath. The anal cannula was attached to a PE outflow tube and placed on top of an adjustable crank that was used to set the level of intraluminal pressure to 2 cmH_2O above the oral end. The level of the crank was set to maintain a consistent flow of fluid. If the crank was set too low, there would be an excess in outflow pressure, causing fluid to flow out of the tissue faster than it entered, resulting in a flaccid colon. Conversely, if the crank was set too high, there would be a backwards pressure gradient and the fluid would build up inside the tissue, resulting in a damaging level of distension. The fluid from the outflow was collected inside a small glass cylinder. PE tubing was placed inside the cylinder and connected to a pressure transducer (Argon, TX, USA), which measured the height of the outflow fluid collected inside the cylinder (once calibrated). The transducer was connected to a Grass amplifier (Astro-Med, Inc., RI, USA), which was connected to a computer used to record and analyze the data with AxoScope (Molecular Devices, CA, USA) software. The volume of outflow was calculated by multiplying the cross-sectional area of the cylinder by the measured change in height in the following equation.

$$V(cm^3) = A(cm^2) * h(cmH_2O)$$

After 30 minutes of equilibration in the organ bath, motility was recorded with a miniature CDD board camera (SONY, Hong Kong), which was used to create spatiotemporal diameter maps (Dmaps) as described in detail by Parsons and Huizinga (2015). These maps are shown as images, with time running horizontally and distance

along the colon running vertically from proximal (top) to distal (bottom). The intensity of the Dmap is the width ("diameter") of the colon with black signifying a contraction and white a relaxation.

2.4 Spatiotemporal Map Analysis

All analysis was done on ImageJ with custom made plugins developed by SP. An LDC was defined as a contraction running from the oral to anal end propagating uninterrupted over at least 50% of the length of the colon with a propagation velocity of control proportions (~1 mm/s) (West et al., 2017). The velocity was calculated by measuring the slope of a contraction. The frequency of contractions was calculated by dividing the total number of contractions with an amplitude greater than 0.04 cm per map by the length of that map in minutes. A cut-off of 0.04 cm was used to eliminate myogenic ripple contractions, yet still include the proximal pacemaker contractions. Initial observations indicated that 0.04 cm was the lower threshold for a proximal pacemaker contraction to produce outflow.

2.5 Outflow Analysis

Outflow recording was initiated at the same time as motility video recordings. As the outflow dripped into the collection cylinder, 'steps' of pressure increase were plotted by the software (Fig. 1). It was crucial that the outflow tube was placed such that each drip was not impeded in any way, as this would create an anomaly in the recording. The time and change of pressure at a 'step' was measured and plotted onto the spatiotemporal map. Preliminary experiments showed that the amount of outflow for a

given contraction was correlated with the amplitude, propagation distance and the origin of the contraction. Outflow that was associated temporally with a contraction was assigned to that contraction and, conversely, any contraction that was not associated with outflow was termed non-propulsive. The efficiency of the colon for a given period of time was calculated by dividing the change in outflow per contraction by the number of contractions greater than 0.04 mm in amplitude for that time period.

2.6 Intraluminal Solutions

PBS was pumped through the lumen as a control instead of Krebs' solution as the glucose contained in Krebs' may interfere with mucosal 5-HT release (Zelkas *et al.*, 2015). As we hypothesized that 5-HT release would be triggered by SCFAs, we wanted to eliminate any 5-HT stimulus from the baseline condition to better observe this effect following treatment.

In GF experiments, a solution of SCFAs was dissolved in PBS (5 mM acetate, 1 mM propionate and 1 mM butyrate) (Sigma, Oakville, CA). The pH was corrected to 7.4 with Tris base. Tris base was used instead of NaOH to correct pH as the added sodium would interfere with the positive ion concentration in the Krebs' solution. Slow perfusion to the colon was used to optimize epithelial cell survival and to be able to induce drugs to the lumen without any change in luminal distention. The SCFA concentrations used have been shown to maximally increase TPH1 activity in a cultured cell line of ECs (Reigstad *et al.*, 2015). This study showed a parabolic relationship between TPH1 activity and SCFA concentrations. It was thus important to use a concentration that was large enough to produce a reaction, yet not too large so as to inhibit mucosal 5-HT.

Solutions were perfused at speeds of 30 µL/min or 140 µL/min. A lower speed was chosen to limit distension-induced contractions (Heredia *et al.*, 2013), to be able to better assess stimulus-induced contractions, while still being fast enough to perfuse solutions through the lumen. A higher speed was used to examine motility at a greater degree of distension without damaging the tissue.

In TPH1 KO experiments, 10 mM of butyrate and 10 mM of propionate were perfused individually as these metabolites were shown to have differential effects and produce a maximal effect at these concentrations in control mouse colons (Hurst *et al.*, 2014). These solutions were perfused at only 30 µL/min, again to limit the amount of baseline activity and better observe a potential increase in activity following treatment with SCFAs. After each experiment, much care was taken in order to completely remove traces of SCFAs inside the perfusion tubes as this would affect subsequent experiments. Tubes were flushed with water for approximately 10 minutes, sprayed with ethanol and left to dry overnight.

2.7 Experimental Design

After a 30 minute equilibration period, motility recordings were initiated. Each recording session was 20 minutes long. For GF experiments, two baseline records were made with PBS perfusion, one at 140 µL/min, and another at 30 µL/min. The PBS solution was then changed to SCFAs, without causing distension in the colon. This was done by attaching the inflow tube to a three-way valve. This way, because the flow is so slow and would take ~10 minutes to reach the tissue at the current speed, the speed could be increased, brought near the tissue and flow into a waste basin without

distending the colon. After this, the flow would be turned back down and the valve switched such that the new solution would be allowed to enter the tissue. SCFAs were then perfused continuously for 20 min at 30 µL/min before initiating recording. After this, one recording was made at 30 µL/min and another at 140 µL/min. In total, four 20 min maps from one colon were used for analysis, and results from the two speeds were combined to produce one baseline value and one treatment value. For all TPH1 KO experiments, only the lower speed was used to reduce distension-induced motility to better observe a change following treatment. Two baseline recordings were made with PBS infusion. Another two recordings were made with perfusion of 10mM butyrate dissolved in PBS. And lastly, two more recordings were made with perfusion of 10mM propionate. Results from both maps were averaged. For all diet experiments, the lower speed was again used in all recordings. First, one 30-minute baseline recording was made with PBS infusion. The solution was then switched to 10mM butyrate until the butyrate reached the tissue. The solution was then switched back to PBS and a 15-minute recording was made. This was done to determine if butyrate activates a mechanism that occurs in the absence of the stimulus, or if butyrate is required continuously to produce its effects. This period was termed “PBS post-Butyrate”. After this, the PBS was switched back to 10mM butyrate and continuously perfused for a final 30-minute recording. LDC integrity in this set up has been shown to be consistent for several hours (Gwynne *et al.*, 2004) and in both of our experiments the tissue was in the bath for a maximum of 2.5 hours.

2.8 Immunohistochemistry for 5-HT and GPR43

Immunohistochemistry and quantification was done by Xuan-Yu Wang as follows. The whole colon from three CD1 and three germ free mice were removed, thoroughly washed in Kreb's. All colons were divided into proximal and distal ends by cutting at the center. Half of the proximal and distal colon were fixed in 4% paraformaldahyde (PFA) for 6-8 hours at 4^0C and then soaked in 20% sucrose in PBS overnight before embedding in optimal cutting temperature compound (OCT) in liquid nitrogen. The other half of the fresh colons were directly embedding in OCT in liquid nitrogen. Frozen sections of 10 μm were cut from both fixed and fresh tissues, mounted on positive charged slides.

For 5-HT staining, fixed tissue sections were used. Non-specific binding was blocked by 2% bovine serum albumin (BSA, Sigma, St. Louis, MO) incubation for one hour at room temperature. Tissues were then incubated overnight at room temperature with polyclonal rabbit anti 5-HT (1:1500, ImmunoStar, Hudson, WI). After rinsing several times, tissues were incubated with Alexa 488 conjugated donkey anti-rabbit IgG (1:200, Jackson Immuno Research, West Grove, PA) for 1 hour.

For GPR43 staining and GPR43/5-HT double staining, fresh tissue frozen sections were used and fixed in ice-cold acetone for 15 min before incubated with Alexa Fluor 594 conjugated rabbit anti-GPR43 (1:250, Bioss Antibodies, Woburn, MA) overnight at 4^0C. After several rinses in PBS, sections were fixed again with 4% PFA for 3 hrs at 4^0C and then used for 5-HT staining with the same procedure as mentioned above.

All antibodies were diluted in 0.05M PBS (pH 7.4) with 0.03% triton-X-100. Negative controls included the omission of primary antibodies from the incubation

solution. Immunostaining was examined using a Leica DMRXA2 microscope with 20x objective lens. Each cross section was divided into four equal quadrants for analysis. All pictures were taken with a Retiga Imaging digital camera attached to the microscope and analyzed using an Apple computer with Volocity software (Improvision, Montreal, QC).

2.8.1 Immunohistochemistry Quantification

EC quantification was performed by counting the number of positive ECs per power field (20x objective lens). Other positive cells were distinguished from ECs by their location (lamina propria vs epithelia). Only positive cells within the epithelium of both luminal surface and crypts were counted. (n=24 power-fields for both proximal and distal colon from CD1 and germ free mice).

2.10 Mass Spectrometry Fecal Sample Preparation

As mentioned briefly above, mice placed on a high-sugar diet were weighed before and after starting the diet. At the same time, two fecal pellets were collected, weighed accurately to 20-50 mg with an analytical balance and placed in a 1.5mL Eppendorf tube where they were frozen in liquid nitrogen and stored in a -80°C freezer prior to processing. Once ready, weight equivalent amount of 3.7% (10x dilute) HCl was added to the fecal sample. The samples in HCl were then delivered to McMaster regional center for Mass Spectrometry (MRCMS) in an ice box where samples were analyzed for the presence of butyrate, propionate and acetate.

2.11 Statistics

Averaged values are presented as mean ± SEM. Differences between motility results were tested with a two-way ANOVA and Tukey's multiple comparison test. For immunohistochemistry, parametric t-tests were performed to compare the number of positive ECs per picture between control and GF mice assuming unequal variance. For comparisons between two unpaired groups with a sample size of less than 20, the non-parametric Mann-Whitney test was used. For comparisons between two paired groups with a sample size of less than 20, the Wilcoxon test was used. Differences showing $p < 0.05$ were deemed significant.

3.0 RESULTS

3.1 Motor Patterns in Control and GF Mice

LDCs: Typical baseline maps for control and GF mice are seen in Fig. 2A,C with PBS perfusion. The motor activity of the colon in the control mice was dominated by typical LDC activity, characterized by highly rhythmic rapidly propagating contractions that propagated the entire length of the colon while keeping the entire colon contracted for the duration of the LDC. (Fig. 2A,B & 2D-F). In contrast, the colon of GF mice was dominated by slowly propagating contractions that were often interrupted by short transient relaxations in the mid-colon, a motor pattern we call “slow LDCs” (Fig. 2C & 2A-C). The rhythmicity of slow LDCs was variable (Fig. 2C). The GF mouse colon was capable of generating normal LDC activity, however, the slow LDC was far more dominant (Fig. 2C).

Proximal Activity: In control mice, the motor pattern in the proximal colon was highly rhythmic; short lasting contractions with a relatively constant frequency of ~1.00 cpm. LDC’s emerged from this activity such that every 2-3 proximal contractions developed into an LDC. The proximal activity can be clearly seen in the control mouse recordings in Fig. 2A,B dispersed between LDCs, and Fig. 3D-F after LDCs. This motor pattern was occasionally observed in the GF colon (Fig. 3A,B), but was much more variable compared to control mice. The average frequency of proximal activity in the GF mouse was significantly lower than in the control mouse (control: 1.00 +/- 0.05 cpm vs GF: 0.81± 0.05 cpm, $p = 0.02$). The proximal activity in the GF mouse was not rhythmic like that of the control mouse and occurred irregularly.

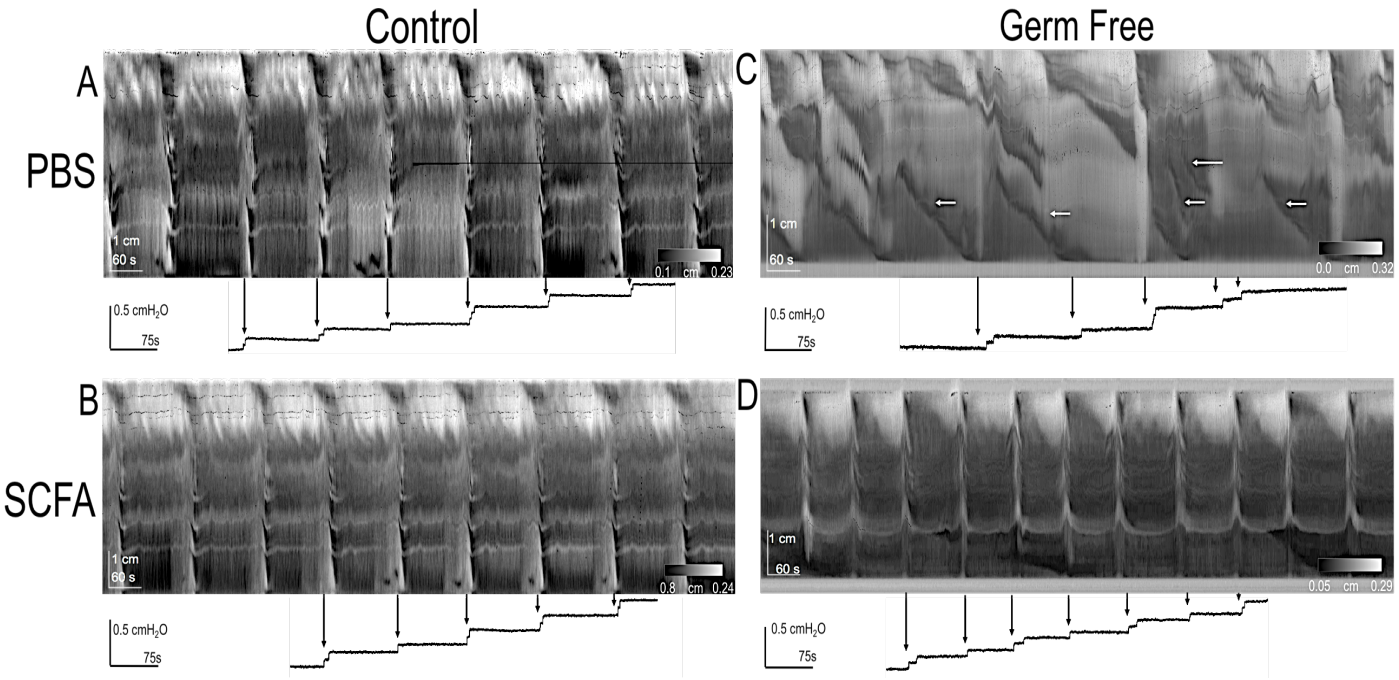

Fig. 2 Spatiotemporal Maps of the Control and GF Mouse Colon.

Beneath each spatiotemporal map is an actual concomitant recording of the outflow for that experiment. ***A****. Regular LDC activity from a control mouse with intraluminal PBS infusion.* ***B****. LDC activity during intraluminal SCFA infusion in a control mouse.* ***C****. Predominant slow LDC activity in a GF mouse with PBS infusion where white arrows highlight significant contractions that did not produce outflow.* ***D****. Regular*

Myogenic ripples: High frequency myogenic contractions paced by the ICC-SMP (Chen et al., 2013; Plujà et al., 2001) occurred during short periods of quiescence in between the dominant motor patterns in control mice (Fig. 3D-F). In GF mice, these contractions were rarely observed given the lack of quiescence, however they were present (Fig. 3B).

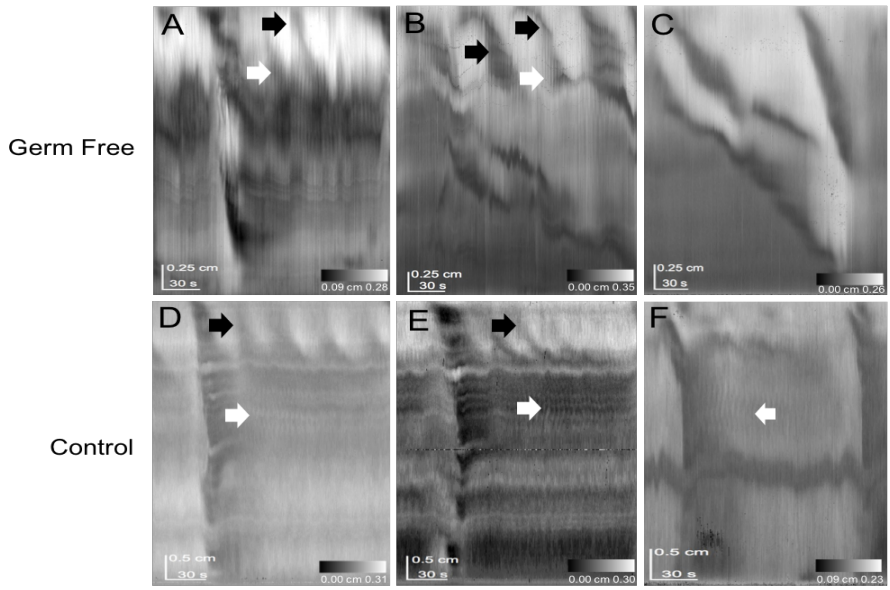

Fig. 3 Different Motor Patterns in GF and Control Mice.

Black arrows indicate proximal rhythmic contractile activity; white arrows indicate myogenic ripple contractions. ***A:*** *A regular LDC observed in a GF mouse colon; LDCs can show interruption, i.e. a transient period of relaxation (termed interrupted LDCs (Chen et al., 2013, #90335)). The LDCs in GF mice most often showed this interruption.* ***B,C:*** *Slow LDCs in GF colons.* ***D-F****: LDCs in control mice. Note the faster velocity and uninterrupted propagation.*

3.2 Outflow Analysis in GF Mice

The GF mice had a significantly decreased volume of outflow per contraction compared to controls (Fig. 3A). Although the volume of outflow per LDC in control mice was not different from outflow per LDC (both slow and normal LDCs) in GF mice (controls: 0.118 ± 0.010 mL/contraction vs GF: 0.106 ± 0.009 mL/contraction p=0.86), the percent of all contractions that were LDCs was lower in GF mice than control during baseline (p=0.004, Fig. 3B). The GF mice also displayed an increased frequency of contractions overall, (p<0.001, Fig. 3D), yet this was mostly due to an increased frequency of non-propulsive contractions (p<0.001, Fig. 3C), indicating a decreased

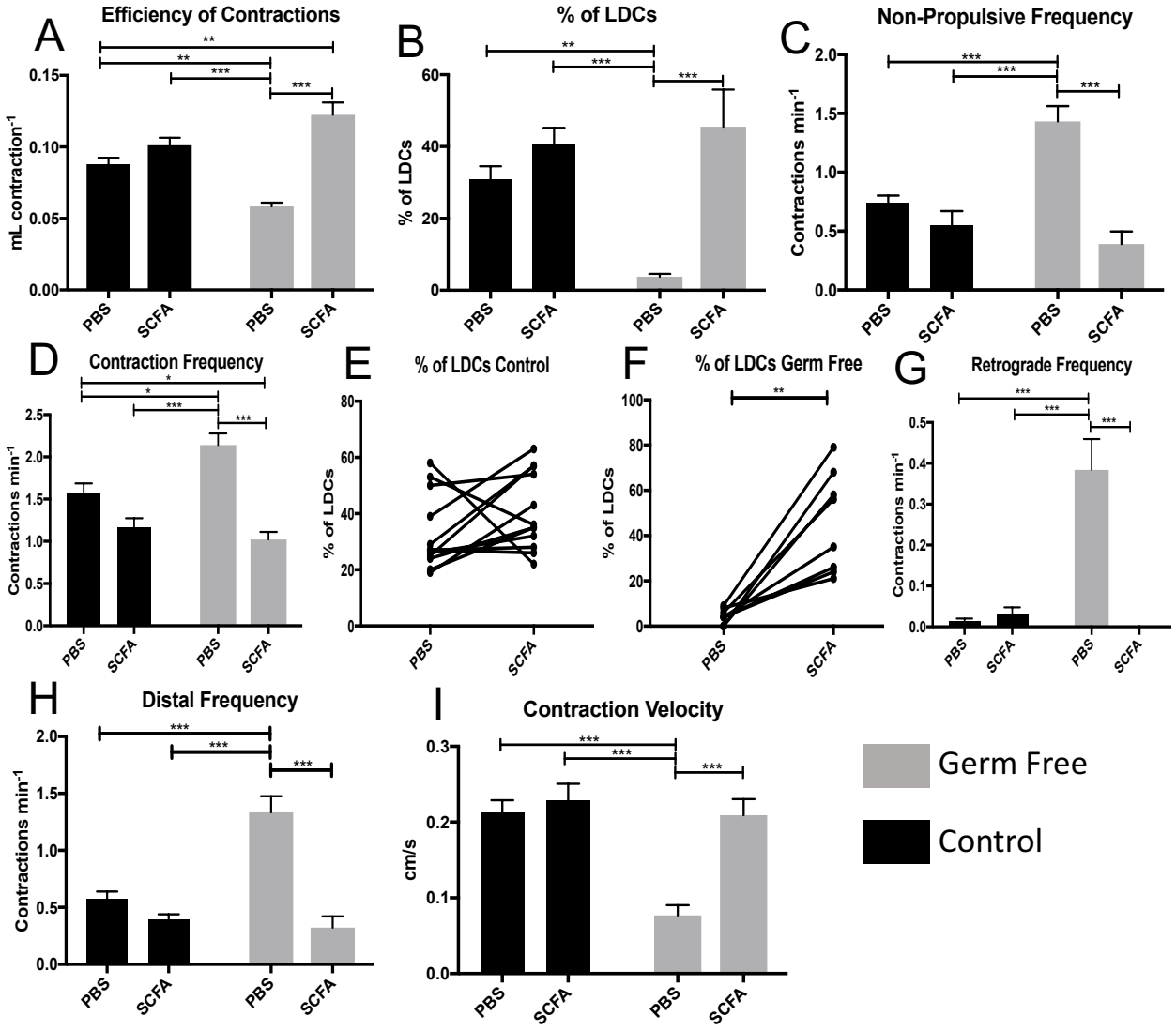

Fig. 4 Quantification of SCFA-induced activities in GF and Control Mice.

Grey bars represent GF experiments and black bars represent control experiments. ***A****: Efficiency of contractions in both mouse models measured in mL/contraction.* ***B****: The percentage of all contractions that were LDCs.* ***C****. The frequency of non-propulsive contractions (contractions that did not produce outflow).* ***D****. The frequency of total contractions.* ***E,F****. Line-plots of Fig. B showing no change in controls (****E****) and an increase in % of LDCs in GF mice (****F****).* ***G****: The frequency of retrograde contractions.* ***H:*** *The frequency of contractions originating in the distal 2/3 of the colon.* ***I****: The velocity of contractions in both mouse models. Data were analyzed with a two-way ANOVA followed by Tukey's post-hoc test, or a Student's t-test where appropriate (n=4-8 for each group). * indicates p < 0.05, ** indicates p <0.01 and *** indicates p<0.001.*

ratio of propulsive activity to non-propulsive activity. These non-propulsive contractions contributed to an increased distal contraction frequency ($p<0.001$, Fig. 3H) as well as increased retrograde frequency ($p<0.001$, Fig. 3G).

3.3 Effect of SCFAs on Motility

3.3.1 SCFAs: Germ free mice

7mM SCFA administration (5mM acetate, 1mM butyrate, 1mM propionate) to the lumen of GF colon changed the motor pattern to a pattern that was similar to that of the control colon (Fig. 2D). The most obvious change was the change from slow LDCs to rhythmic LDC activity and a lower number of contractions overall. SCFAs increased the percentage of LDCs relative to all observed contractions (Fig. 3B,F). Furthermore, the frequency of non-propulsive contractions in the GF mouse decreased, signified by a decrease of distal and retrograde contractions following SCFAs ($p<0.001$, Fig. 3C,G,H). In agreement with this, SCFAs significantly increased the efficiency of contractions in the GF mouse (Fig. 3A).

3.3.2 SCFAs: Control mice

SCFAs did not significantly affect motility in control mice, under our standard control conditions, in any of the measured parameters at a total concentration of 7mM (5mM acetate, 1mM butyrate, 1mM propionate; Fig. 4). In order to determine if SCFAs have potential effects on the control mouse colon, we reduced the amount of baseline activity by: 1) decreasing distension (intraluminal pressure was decreased to 1 cmH_2O, and the speed of inflow to 30 µL/min), 2) increasing the concentration of SCFAs to 10

mM each and 3) administering SCFAs individually as they were found to differentially effect LDCs (Hurst et al., 2014). When butyrate was administered alone at a higher concentration of 10 mM, and a low inflow speed, the percentage of contractions that were LDCs and the frequency of LDCs significantly increased ($p<0.05$) (Fig. 4A,C,G,H). At the same concentration and inflow speed, propionate significantly inhibited LDC activity ($P<0.001$) (Fig. 4A,E,G,H).

3.3.3 SCFAs: TPH1 KO mice

The baseline activity in TPH1 KO mice consisted of rhythmic LDCs, similar to control with normal proximal myogenic activity (Fig. 5B). The frequency of LDCs during the baseline activity was not different from controls (controls: 0.25 +/- 0.03 cpm vs 0.17 +/- 0.03 cpm, $p = 0.14$, Fig. 5). 10mM butyrate was not able to increase the frequency of LDCs in these mice despite their similar, if not lower, level of baseline activity (Fig. 5B,D). There was no difference in the frequency of LDCs or the percentage of LDCs following butyrate perfusion (Fig. 5B,D,G,H, $p=0.84$). However, 10 mM propionate had the same effect in TPH1 KO mice as it did in control mice, significantly diminishing the occurrence of LDCs (Fig. 5B,F,G,H, $p=0.001$).

3.4 Immunohistochemistry

3.4.1 Immunoreactivities of GPR43 and 5-HT

Staining of both SCFA receptor GPR43 and 5-HT was done in order to determine structural support for the hypothesis that SCFAs evoke 5-HT release directly through this receptor. Immunohistochemistry revealed co-localization between GPR43+ and 5-HT+ cells in both the GF mouse and control mice (Fig. 6). The observed co-localization

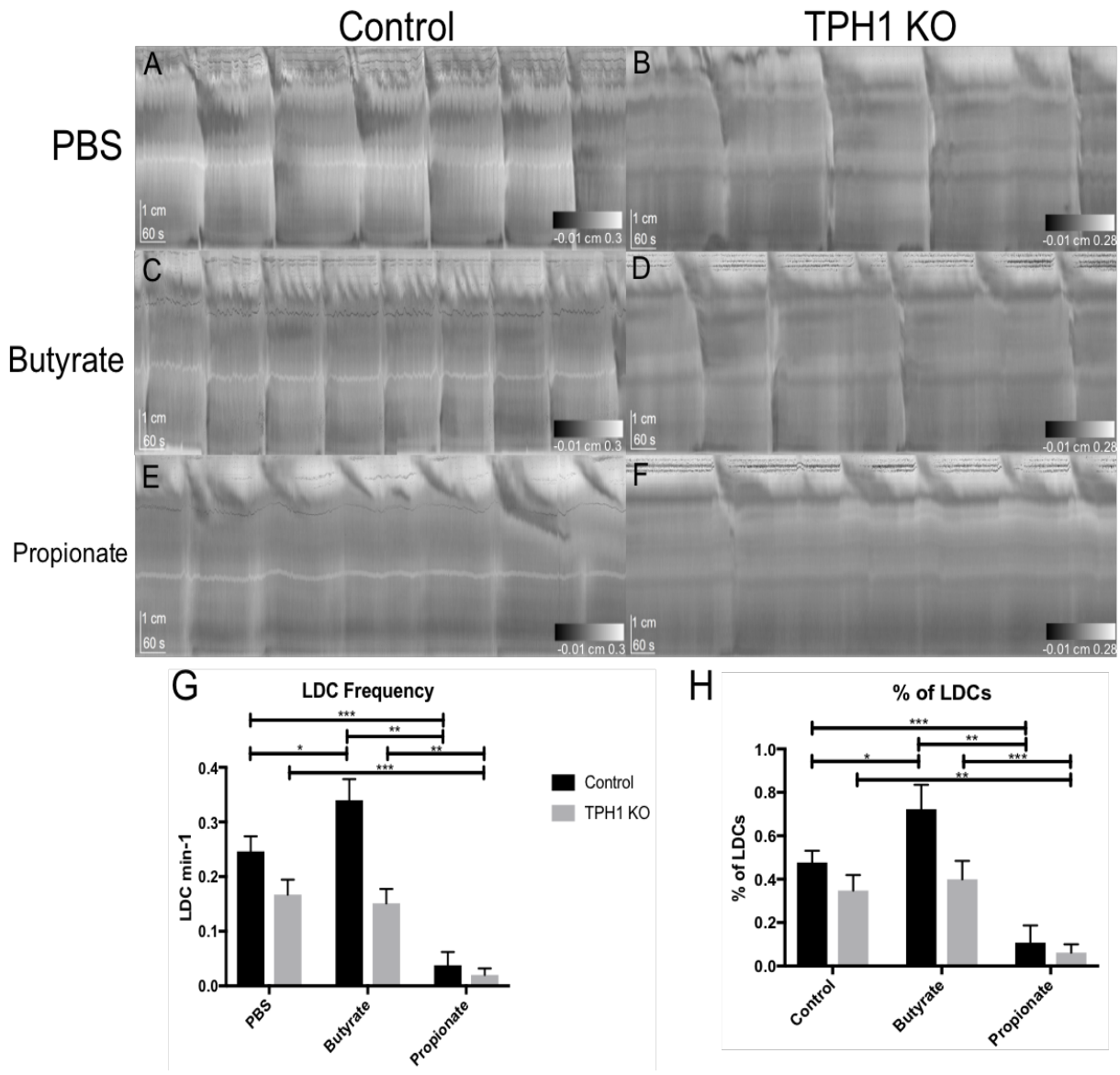

Fig. 5 Motility Analysis in TPH1 KO Mice and Controls.

A,B: *Baseline activity with intraluminal PBS perfusion in control and TPH1 KO mice, respectively.* ***C,D****: The effect of intraluminal perfusion of 10mM butyrate on LDC activity.* ***E,F:*** *The effect of 10mM propionate perfusion.* ***G:*** *The LDC frequency in cpm.* ***H:*** *The percent of contractions that are LDCs. Black bars indicate control mice (n=4) and grey bars indicate TPH1 KO mice (n=5). Data were analyzed with a two-way ANOVA followed by Tukey's post-hoc test. * indicates p < 0.05, ** indicates p <0.01 and *** indicates p<0.001.*

would suggest the possibility of direct involvement of 5-HT in inducing SCFA-induced motor patterns in the GF mouse. 5-HT+ epithelial cell quantification revealed no difference between control and GF mice (Fig. 5). There was a decrease in GPR43+ cells in the distal colon of the GF mouse compared to control, but no difference in total numbers compared to control (Fig. 5).

3.4.2 c-Kit immunoreactivity

c-Kit immunohistochemistry revealed the presence of all subtypes of ICC in control mouse colon; ICC associated with the myenteric plexus (ICC-MP), submuscular plexus

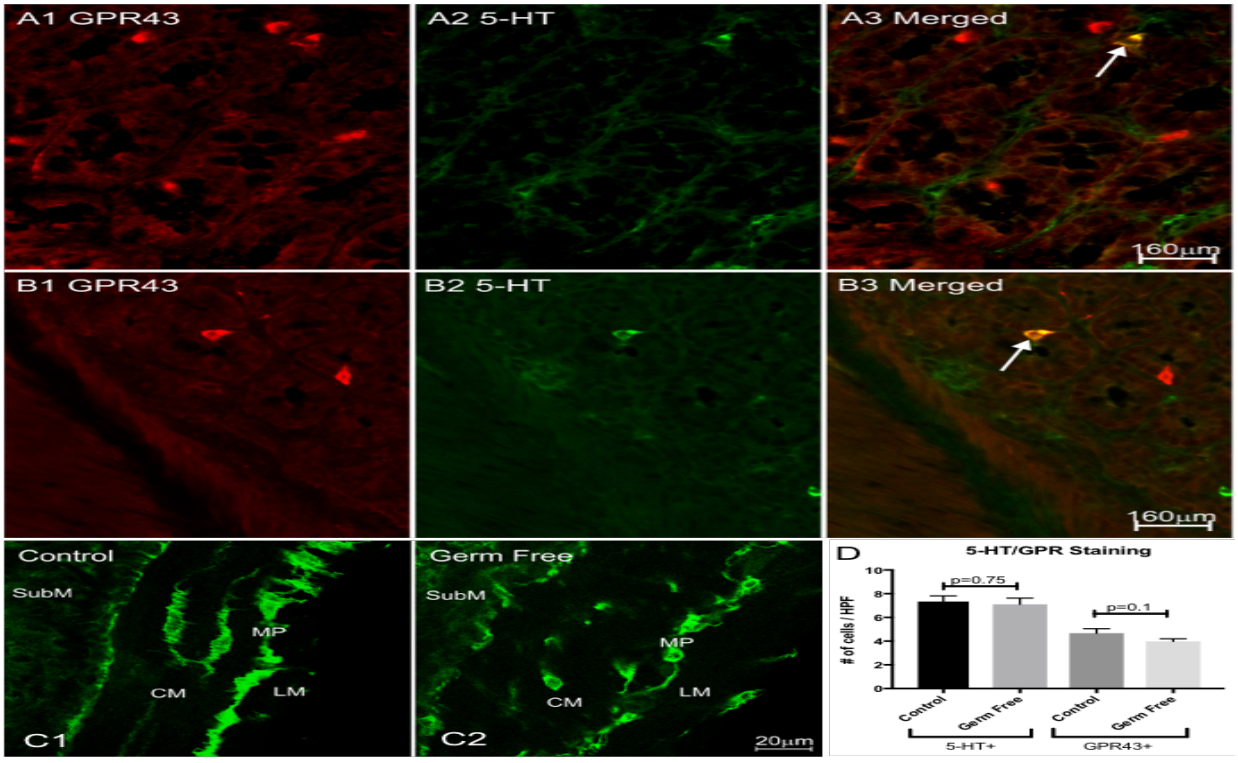

Fig. 6 GPR43, 5-HT and c-Kit Immunoreactivity Control and GF Mouse Colons.

*Co-localization between GPR43+ and 5-HT+ in a control (**A1-3**) and GF mouse (**B1-3**), indicated with a white arrow. **C1,2:** c-Kit+ staining revealing the three subtypes of ICC in control and GF mouse colon, respectively. SubM: submucosal; CM: circular muscle; LM: longitudinal muscle; MP: myenteric plexus. **D:** Quantification of 5-HT+ and GPR43+ epithelial cells. There was no difference between control and GF.*

(ICC-SMP) and intramuscular ICC (ICC-IM) (Fig. 6C1). Both the distribution and quantity of the three ICC subtypes in GF was similar to controls (Fig. 6C2).

3.5 High-Sugar Diet

3.5.1 Consumption and metabolomics

Mice were placed on a high sugar diet for one week as this technique was presented to specifically eliminate butyrate and acetate, but not propionate from the gut (CDDW Abstract). HSD mice ate significantly less food in grams than mice on a chow diet (Fig. 7A). Given the HSD had a higher energy density (3.46 kcal/g vs 3.00 kcal/g for chow diet), there was no difference in weight gain or energy intake between the two groups after one week (Fig. 7B, C). The HSD had a composition of 8.7% fibre whereas the chow diet consisted of 16.7% fibre, significantly decreasing the amount of fibre intake over the one week period for HSD-fed mice (Fig. 7D). Mass spectrometry analysis revealed no significant decrease in any SCFA measured (acetic acid, butyric acid, propionic acid, isobutyric acid, isovaleric acid, pentanoic acid or lactic acid) in HSD-fed mouse stool samples after one week on the diet (Fig 7 E-G). With that being said, both butyric and propionic acid levels decreased in every mouse, yet the variability between samples restricted a significant result (Fig. 7F, G $p=0.06$).

3.5.2 Motility changes after HSD

Spatiotemporal maps from HSD-fed mice and controls are shown in Figure 7. There was no difference in either LDC frequency or LDC velocity between HSD mice and controls (Fig. 8I, J). Some baseline results from certain experiments in both HSD-

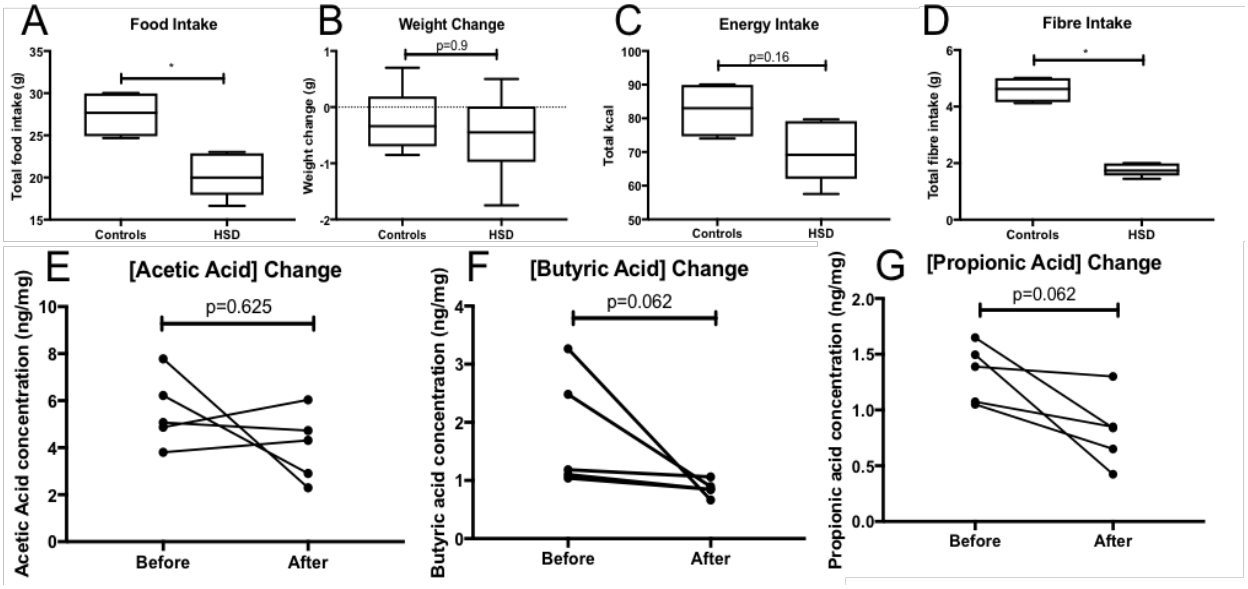

Fig. 7 Consumption analysis and associated metabolomics changes in HSD mice.

A: *Difference in food intake (in g) between controls and HSD mice after 1 week.* ***B****: Weight change after 1 week on each diet.* ***C:*** *Total energy intake (in kcal) after 1 week in HSD and chow-fed mice.* ***D:*** *Difference in fibre intake (in g) after 1 week.* ***E-F:*** *Changes in levels of acetic acid, butyric acid, and propionic acid (in ng/mg of stool) in HSD-fed mice after 1 week on the diet.*

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fed mice and controls showed motor patterns that closely resembled that of the GF mouse (Fig. 8A, B). This is not typically seen in control experiments in any other aforementioned protocol. The protocol for this experiment differed from previous ones in that mice had to be housed individually in order to monitor food consumption.

After baseline recordings, a 10mM concentration of butyrate was introduced into the colon and quickly washed away before starting another recording. This phase was termed ‘PBS post-Butyrate’ and was done in order to determine if SCFAs activates a prokinetic mechanism which remains active in their absence, or if continuous SCFA perfusion is required to induce motility. In HSD-fed mice, the former was the case; pre-

treatment with butyrate was able to significantly increase the frequency and velocity of LDCs (Fig. 7E, F, I, J). Curiously, this was not observed in control mice. After pre-treatment, a continuous supply of 10mM butyrate was perfused through the colon. This significantly increased the frequency of LDCs (Fig. 7G, H, I, J) in both groups, and also

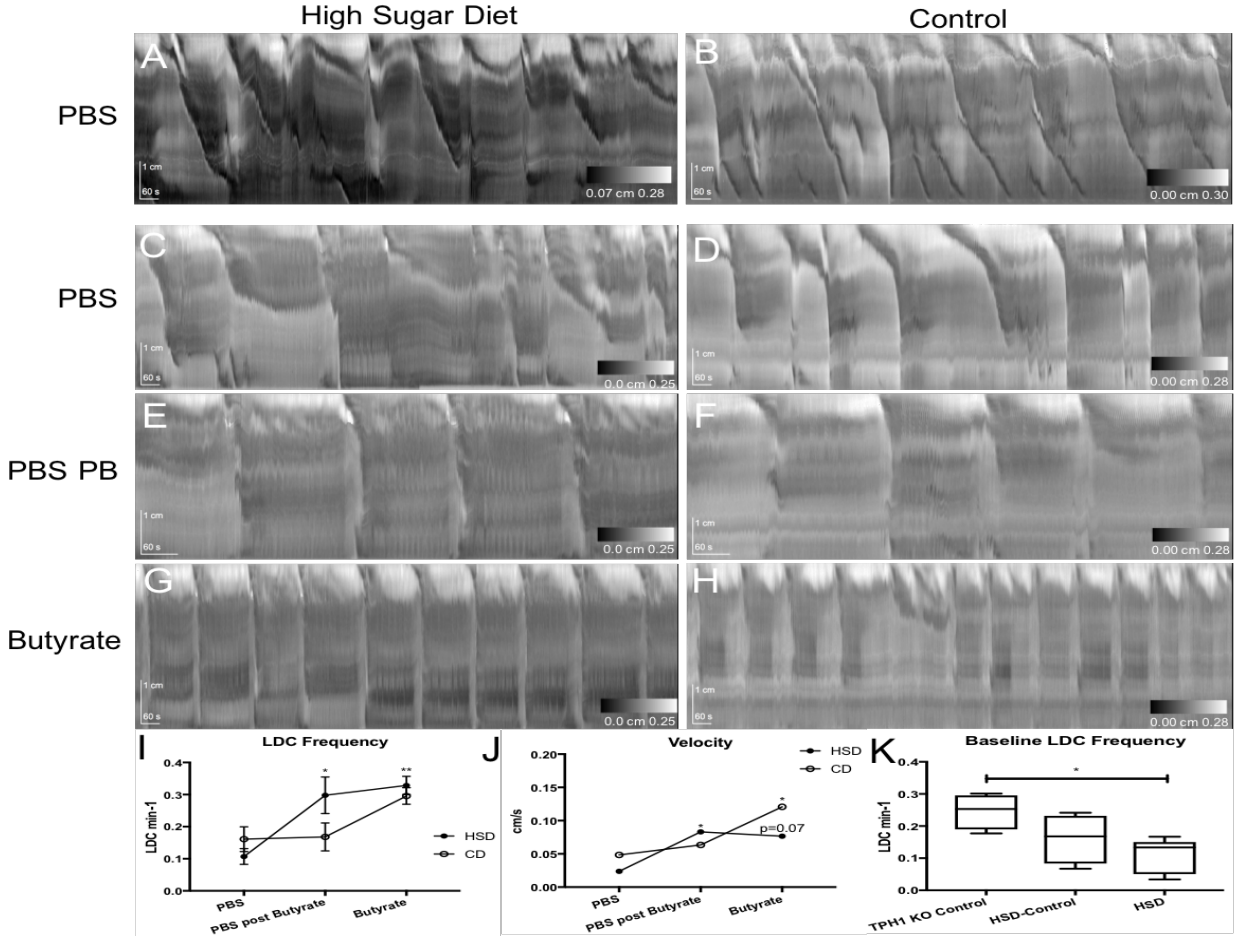

Fig. 8 Motility analysis in control and HSD-fed mice.

A-H *show spatiotemporal maps from HSD-fed mice (n=5; left panel) and chow-fed mice (n=4, right panel).* ***A,B:*** *A selected baseline map from each group showing the presence of 'slow-LDC' motor pattern seen in GF mice.* ***C,D:*** *Common baseline maps for each group.* ***E-F:*** *Typical response of each group to pre-treatment with butyrate.* ***G-H:*** *Typical response of each group to continuous butyrate treatment.* ***I,J:*** *Quantified LDC frequency and velocity changes, respectively.* ***K:*** *Comparison of LDC frequency between TPH1 KO controls, HSD-controls and HSD-mice.*

significantly increased the velocity of LDCs in control, but not HSD-fed mice (Fig. 8G, H, I, J). As results from control mice in this experiment did not seem typical, their baseline results were compared to the control mice used in TPH1 KO experiments as the tissue preparation and baseline protocol were exactly the same. Although the higher frequency of LDCs in TPH1 KO controls over HSD controls was not significant (p=0.2; Fig. 8K), TPH1 KO controls did have a greater LDC frequency when compared to HSD-fed mice themselves (Fig. 8K). This would suggest that some intervention done to the HSD control mice had altered their baseline motility response.

3.5.3 Linear regression analysis: [Butyrate] predicts LDC frequency

In order to determine if the magnitude of change in a metabolite for a given mouse was associated with any baseline motility parameter, linear regression analysis was performed by plotting the percentage change of a metabolite for each mouse with that mouse's baseline motility

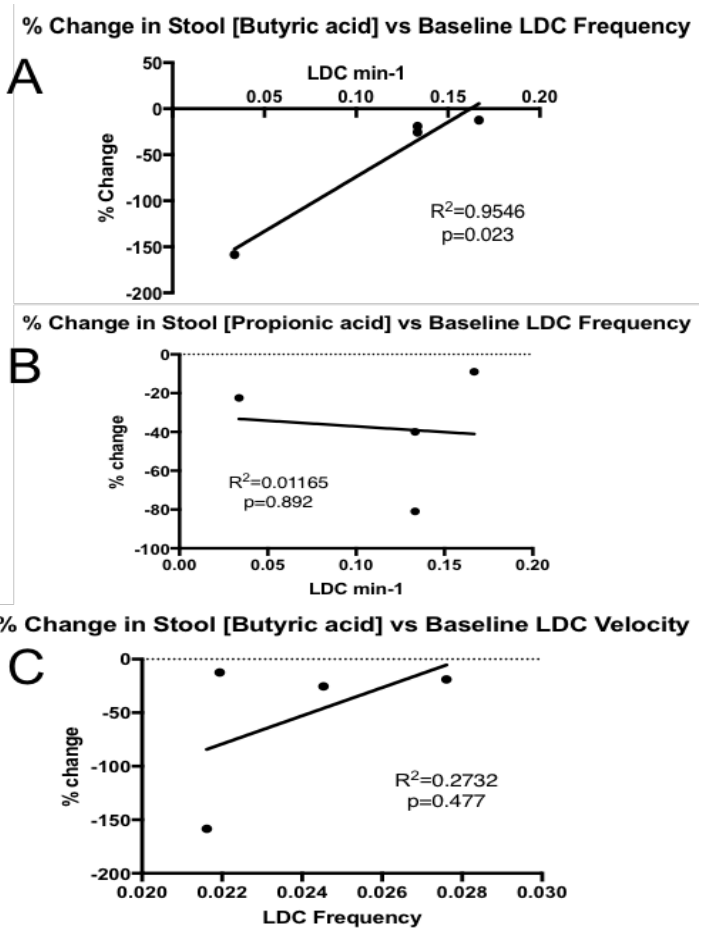

Fig. 9 Linear Regression Analysis of Metabolite Content and Motility.

A: *% change of [Butyric acid] with baseline LDC frequency.* ***B:*** *% change of [Propionic acid] with baseline LDC frequency.* ***C:*** *% change of [Butyric acid] with baseline LDC velocity.*

measurements (Fig. 9A-C). It was found that the degree of change in butyrate, but not propionate (Fig. 9B), significantly predicted LDC frequency (Fig. 9A; R^2=0.9546; p=0.02) but not velocity (Fig. 9C; R^2=0.27; p=0.477).

4. DISCUSSION

4.1. LDC Activity

Gastrointestinal motility has been studied in detail in the small intestine of GF mice (Collins et al., 2014; Husebye et al., 2001; Wichmann et al., 2013; Yajima et al., 2016), yet few studies have reported on motility in the colon of these mice. The dominant propulsive motor pattern in the colon is the LDC. The present study suggests that the efficiency of distention-induced LDC activity is greatly diminished in the absence of SCFA in GF mice, emphasizing the important role of luminal metabolites in the control of luminally-induced motor activity.

Although GF mice have abnormal colonic motor patterns under our experimental conditions compared to control mice, they have the potential to elicit all normal activity under physiological stimuli, including LDCs. Given that LDCs are inhibited by TTX (Fida et al., 1997), their presence in GF mice indicates that the neural control mechanism that generates LDCs is present. Furthermore, the fact that a stimulant of IPANs (i.e. SCFAs) (Fukumoto et al, 2003), was able to exert its effects at such a low concentration would suggest that either IPAN excitability is intact in GF mice, (contrary to findings that they are hampered in these mice compared to controls (McVey Neufeld et al., 2015)), or that they are indeed hampered and there is a compensation to account for it.

LDC activity was also present at baseline in TPH1 KO mice (Fig. 5). This indicates that LDC activity does not necessarily require EC-derived 5-HT, giving evidence for this side of the long debate. However, this does not mean that 5-HT does not play an important role in modulating LDCs physiologically.

4.2 Propulsive Nature of Contractions

There are several motor patterns in the colon, some serve to propel contents, like the LDC, and others serve to segment contents so as to increase absorption, like retrograde contractions or possibly the low-velocity LDCs described here (similar to segmenting contractions described in Li et al. (2016)). In the absence of SCFAs, the ratio of propulsive to non-propulsive activity is much lower in GF mice compared to controls, seen in the decreased percentage of contractions that are LDCs (Fig. 4). This is consistent with in vivo studies that show a decreased transit time in these mice (Kashyap et al., 2013; Yano et al., 2015). Whereas the colon of control mice at optimal levels of distension show predominantly LDC activity, GF mice, at the same levels of distention and perfusion speeds, show predominantly a combination of slowly propagating LDC-like activity, retrograde activity and isolated contractions (Fig. 2).

A careful outflow analysis associated with each contraction shows that the majority of the contractions in the GF mice are non-propulsive and inefficient in expelling fluid (Fig. 2, 3). This type of conclusion highlights the value of outflow analysis in motility studies; although the total number of contractions was increased in these mice, the efficiency of the contractions decreased, indicating that it is the propulsive or absorptive nature of the contractions that is important in determining an effect on functional motility. We infer that the observed decrease in efficiency of contractions contributed to the slower total transit measured in previous studies (Kashyap et al., 2013; Yano et al., 2015). As we did not observe a difference in the efficiency of slow LDCs in GF mice compared to LDCs in controls ($p>0.05$), the decreased efficiency is most likely due to a decreased ratio of propulsive contractions to non-propulsive

contractions (Fig. 2 & 3B, C). Another variable that may have contributed to slower transit in these mice is the velocity of contractions, which was significantly slower when compared to controls (Fig. 4I). Although the velocity of contractions in our study did not seem to correlate with less propulsion of fluid, we expect that it would correlate with a slower propagation of solid fecal pellets.

4.3 SCFA-induced Motility in GF Mice

Although SCFAs are not the only microbial metabolite missing from GF mice, their established pro-kinetic effect made them a potential candidate for restoring motility in GF mice and we found positive evidence for this in the present study. Nevertheless, we cannot conclude that it was solely the absence of SCFA that caused abnormal motility as GF mice have substantial differences compared to controls in all facets of their physiology. With that being said, SCFA administration did restore the abnormal motility in GF mice to the control phenotype (Fig. 2 & 3), suggesting that their absence does play a large role in the observed differences in GF mouse motility. Interestingly, SCFAs decreased the frequency of contractions overall, which might indicate a decrease in motility, which has been reported previously (Cherbut et al., 1998; Squires et al., 1992). However, we have shown that transit promoting motility is more predicated by the propulsive nature of the contraction rather than the overall frequency. In GF mice, SCFAs restored the efficiency of contractions through increasing the ratio of propulsive to non-propulsive contractions (Fig. 4B&C) and the velocity of the contractions to control levels (Fig. 4I).

The differences between control and GF mice were observed under predetermined conditions of optimal propagating LDC activity, that is, an average inflow rate of 85 μL/min and an intraluminal pressure of 2 cmH_2O and tested under relatively low concentrations of SCFA. The concentration chosen for these experiments was 7mM in total (5mM of acetate, and 1mM of butyrate and propionate) as these concentrations were shown to maximally induce TPH1 activity in vitro in an isolated cell line of ECs (Reigstad et al., 2015). With that being said, this effect on TPH1 was seen over chronic (>24hrs) exposure and the concentration they used is approximately $1/10^{th}$ the physiological concentration seen in control mice (Soret et al., 2010). Thus, we believe that the acute administration of low concentrations we used failed to elicit a further response in controls under the already optimal baseline conditions. SCFAs may have also been present at physiological levels in control mice as a gavage of fecal pellets may not remove the strongly-adhesive, metabolite-rich inner mucous layer of the colon (Li et al., 2015). This begs the question of whether 'normal' baseline conditions in control mice are predicated by the degree to which SCFAs are removed prior to the experiment. Contrary to control mice, a response was observed in GF mice at these concentrations as they had not been prior exposed to these metabolites and we were thus observing a nascent reaction.

Recent studies have attributed the slower transit in GF mice to increased levels of either GLP-1R or GLP-1 (Wichmann et al., 2013; Yang et al., 2017). Curiously, colonization with SCFA producing bacteria decreased GLP-1 levels to normal (Wichmann et al., 2013), despite various reports of SCFAs directly activating the release of this hormone in vitro (Tolhurst et al., 2012). Although we hypothesize that the

slower transit is from a lack of 5-HT signaling rather than GLP-1, we do agree with the authors' theory that a decrease in motility would be beneficial so as to optimize any absorption of the scarce amount of nutrient-rich SCFAs. It seems plausible that GF mice compensate for a lack of colonic nutrients by slowing transit. We may be observing an evolutionary motility response to when the colon lacks nutrients and this would also give insight to the role of diet in constipation.

Absorptive motor patterns have been discussed in detail and have been evoked by low doses of the gut neurotransmitter neurotensin (Li et al., 2016). Interestingly, the motor patterns in the GF mice closely resemble that of neurotensin-induced motor patterns (Li et al., 2016, neurotensin has been coupled with SCFA receptors (Nøhr et al., 2013), and neurotensin-positive cells in the colon have been shown to be enlarged in GF mice, indicating hypertrophy (Nogueira & Barbosa, 1994). However, whether slow transit in GF mice is caused by a lack of SCFA signaling, an increase in neurotensin signaling, or both is unclear and remains an interesting avenue to pursue.

4.4 SCFA-induced Motility in TPH1 KO mice

After optimizing our protocol to observe altered motility following individual SCFAs, we found that butyrate significantly increased the LDC frequency and the percentage of contractions that were LDCs in control mice (Fig. 5C, G, H), indicating an induction of propulsive activity. Furthermore, in agreement with Hurst et al. (2014), we found that 10 mM propionate completely inhibited LDC activity in the control mouse colon (Fig.4 E, G, H). The effect of butyrate alone is similar to the effect seen in GF mice and would suggest a dominant pro-kinetic effect of this metabolite over the

inhibitory effect of propionate. Acetate, although the most ubiquitous SCFA, was not tested in this study as Hurst et al. (2014) found it to be weakly inhibitory similar to propionate, but had no effect on LDC activity, the main dependent variable in our study.

We then tested the role of mucosal 5-HT in this differential response of SCFAs using the same protocol on TPH1 KO mice. TPH1 KO mice are mice devoid of mucosal 5-HT as they lack the rate-limiting enzyme to produce 5-HT, TPH1. Although this mutant mouse was found to have normal transit times (Li et al., 2011), they have abnormal propulsive mucosal reflexes (Heredia et al., 2013); control mice could sense and propel small fecal pellets whereas TPH1 KO mice could not. It was thus interpreted that mucosal 5-HT is used to amplify distension-induced motor patterns but that other, stretch-activated neurons in the myenteric plexus can work in their absence only when distension reaches a higher threshold. Despite using the lowest possible baseline amount of distension, TPH1 KO mice were still able to elicit LDCs in our experiment (Fig. 5B). This would indicate that our level of distension approached this threshold whenever an LDC occurred. In their study, Heredia et al. (2013) found that TPH1 KO mice could only propel a fecal pellet over 2.5mm in diameter. Interestingly, whenever the diameter of the proximal colon of TPH1 KO mice reached 2.5mm in diameter, an LDC occurred and there was never a diameter of over 2.5mm that did not produce a contraction.

In addition to distension, ECs also respond to various chemical signals- microbial metabolites, catecholamines and toxins- via 5-HT release (Bellono et al., 2017; Furness et al., 2013). In particular, ECs have been shown to increase 5-HT release in response to SCFAs (Fukumoto et al., 2003; Grider & Piland, 2007) and promote peristalsis in this

way. We tested whether SCFA-induced 5-HT release is causing motor activity or if the 5-HT release is an effect of the increase in contractions. In TPH1 KO mice, 10 mM butyrate failed to increase LDC frequency or the percentage of LDCs, suggesting that this response requires mucosal 5-HT (Fig. 5D, G, H). However, 10 mM propionate had the same inhibitory effect in TPH1 KO mice as it did in control mice, suggesting that this action is independent of mucosal 5-HT (Fig. 5E, F, G, H). This provides evidence for mucosal 5-HT as a sensor of luminal contents and an inducer of motility. It was surprising to find that although propionate was previously shown to produce 5-HT release (Grider & Piland, 2007), its affect was not abolished with removing mucosal 5-HT. This would indicate that even if propionate releases some 5-HT, any pro-kinetic effect this may have is dominated by strong induction of an inhibitory pathway.

4.5 Possible Mechanisms of Action

4.5.1 GPR43

We describe, for the first time, co-localization between 5-HT+ ECs and the SCFA receptor GPR43 in the colon (Fig. 6). This would suggest that SCFAs have the potential to release 5-HT via direct action on this receptor. However, propionate has been described as a more selective agonist to this receptor than butyrate in vitro by comparing dose-response curves of cAMP accumulation (Le Poul et al., 2003), and propionate has been shown to release luminal 5-HT to a similar extent as butyrate when applied luminally to the rat colon (Grider & Piland, 2007). Although we do suggest the possibility that butyrate increases peristalsis by acting directly on ECs, this does not explain the observed opposite effect of propionate on motility, as it has a similar

potential to act on ECs. We hypothesize that this difference is due to an effect of propionate on other enteroendocrine cells and the fact that butyrate may also increase 5-HT signaling through other means.

SCFA receptors GPR43 and GPR42 have been well characterized with respect to L-cells and immune cells in the colon (Le Poul et al., 2003; Tazoe et al., 2008). They are mostly located within the lamina propria on mast cells, yet are also located on epithelial L-cells coupled with hormones GLP-1 and PYY. Of the three major SCFAs, propionate seems to be the most potent inducer of in vitro GLP-1 and PYY release from enteroendocrine L-cells (Chambers et al., 2015), whereas butyrate did not significantly increase GLP-1 release compared to vehicle (Tolhurst et al., 2012). Although no studies have looked at the responses of butyrate on PYY release, PYY seems to be more closely regulated via GPR43 than GLP-1 (Brooks et al., 2017), suggesting that propionate would have a greater effect on PYY release than butyrate given their respective affinities for this receptor. With respect to motility, PYY was shown to be increased following SCFA administration to the rat colon, where a decrease in overall spike bursts was measured (Cherbut et al., 1998). This decrease was mostly seen in short-propagations and stationary spike bursts, whereas total-propagations over the entire length of the colon were actually increased, suggesting SCFAs increased propulsive activity and decreased non-propulsive contractions, as we have shown in this paper. With that being said, this study used a combination of SCFAs and although propionate has been shown to release inhibitory hormones to a greater degree than butyrate, one cannot attribute the inhibitory effect of PYY seen by Cherbut et al. (1998) to propionate over butyrate. It would be worthwhile to measure individual hormone

release from the colon following individual SCFA administration to elucidate if one metabolite solely activates inhibitory hormones and another, excitatory neurotransmitters.

4.5.2 Histone deacetylase inhibition

In addition to being a ligand for GPR42/43, butyrate is also uniquely a histone-deacetylase (HDAC) inhibitor, being able to epigenetically modify gene expression. Interestingly, butyrate, and trichostatin A, a potent HDAC inhibitor, were shown to decrease the serotonin transporter SERT activity (Gill et al., 2013), which facilitates serotonin clearance. Inhibiting SERT would cause an increase in local 5-HT activity, possibly affecting motility. Butyrate was also shown to influence TPH1 activity in isolated ECs (Reigstad et al., 2015), suggesting it may directly alter the TPH1 gene expression independent of SERT activity. Either of these mechanisms may explain the selective requirement of 5-HT for butyrate-induced activity. However, butyrate was able to affect motility in our preparations within 30 minutes of administration and although HDAC activity may explain long term changes in serotonin transcription, it is unlikely to affect motility in this timeframe.

4.5.3 Activation of 5-HT through Olfr558

It is possible that GPR42/43 or HDACi activity is not involved in butyrate's prokinetic mechanism of action and that there is simply a different EC receptor that butyrate acts on where propionate does not. A study just published in *Cell* has suggested just this (Bellono et al., 2017). A taste receptor, Olfr558, was found to be

colocalized to 5-HT+ ECs and its activation electrically excited the cell, causing cell depolarization and subsequent 5-HT release. Interestingly they found that only certain metabolites depolarized cultured EC-cells. Isovalerate, another SCFA metabolite, produced the strongest Ca^{2+} response, followed by isobutyrate and butyrate (Bellono et al., 2017). Acetate and propionate did not produce a Ca^{2+} response at all. This would be the simplest and most likely explanation for the induction of motility by butyrate but does not address how propionate can inhibit motility. It would be interesting to see if isovalerate and isobutyrate were able to exert similar effects on LDC activity as butyrate.

4.6 ICC involvement

To evaluate the possibility of developmental changes to ICC that might influence motor patterns in GF mice, we first evaluated the presence of the three subtypes of ICC within the musculature. Qualitative assessment showed the presence of all ICC subtypes in cross-sectional stains in both controls and GF mice, suggesting there were not any structural changes to ICCs. With that being said, it cannot be determined from histological observation that the synaptic connections of ICCs in GF mice are normal, yet motor pattern analysis may provide clues; the rhythmicity and propagation characteristics of the motor patterns in GF mice may reflect functional changes of ICC. GF mice have normal ripple activity (Fig. 3), the high frequency motor activity that is governed by the dominant pacemaker in the mouse colon, derived from ICC-SMP (Yoneda et al., 2004).

Low frequency contractions with much higher amplitudes compared to the ripples have been shown to be mediated by rhythmic electrical activity from ICC-MP (Plujà et al., 2001; Yoneda et al., 2004), suggesting it likely mediates the LDC. Like the LDC, the ICC-MP pacemaker activity is stimulus-dependent and its frequency may be variable (Chen et al., 2013). Slow waves at the ICC-MP frequency travel along the mouse colon at a similar frequency, at the same propagation velocity as LDCs (0.5-1 cpm, 1-2 mm/s) (Bywater et al., 1989) and are dependent on neural activity (Bywater et al., 1989; Plujà et al., 2001). Furthermore, IPANs directly communicate with the ICC-MP to affect pacemaker frequency (Zhu et al., 2014). Given that IPANs are directly or indirectly activated via EC cells through distention and chemicals (Bellono et al., 2017; Gwynne et al., 2014), it seems likely that the ENS and ICC-MP work in conjunction to respond to a combination of stimuli to produce the LDC. Thus, butyrate may alter the frequency of LDCs by differentially activating the ICC-MP through signaling IPANs.

4.7 High-Sugar (Low-Fibre) Diet

Mice were placed on a 50% sucrose diet based on preliminary findings from the Madsen lab at the University of Alberta which showed that this diet, after 2 days, eliminated the detection of butyrate from the stool, whereas propionate concentrations were unaffected (Fedorak et al. 2017). Given the results from our TPH1 KO and GF experiments, this was a perfect model to test what effect targeted removal of butyrate would have on motility phenotype. One can remove total levels of SCFAs from the gut with antibiotics or by placing mice on a low-fibre diet as one is killing the producers of SCFAs and removing the substrate for SCFA fermentation, respectively. However, there

is no model which exclusively removes butyrate but not propionate from the colon. The fact that butyrate was depleted after only 2 days suggests that sugar is actively reducing the production (or increasing the absorption) of butyrate. This has substantial clinical implications with respect to patients on high fibre-diets but needs more evidence to substantiate these claims. The only other study to look at SCFA levels after a HSD looked at their levels in the plasma and found that a high fat, high sugar diet depleted both propionate and butyrate from the plasma, and after supplementation with inulin, only propionate levels increased (Sugatani et al., 2008, #14639). This would suggest that sugar inhibits butyrate concentration independent of fibre composition. As it pertains to patients of a certain diet, this would imply that any high-sugar desert would have detrimental effects on gut flora outside of its direct caloric burden.

4.7.1 Food composition

Using the protocol from the aforementioned lab, we were unable to create a high-sugar diet with the same composition of fibre as the chow diet (Fig. 7D). We decided to use the diet anyway in the hopes of replicating the described effect on butyrate regardless of the composition of diet; as long as the diet acted as a model for low butyrate, we would accept interpretational limitations. The chow diet had twice the amount of fibre as our HSD, and we thus suggest data be interpreted as mice going on a low-fibre diet with sucrose added to increase energy intake. Although, surprisingly, mice ate significantly more of the chow diet than HSD (Fig. 7A), they did not gain any more weight as their energy intake was not different (Fig 6B, C).

4.7.2 Metabolomics

We were unable to replicate the aforementioned results with respect to the concentration of butyrate and propionate. Both butyrate *and* propionate decreased in all samples after placing mice on a HSD, although the overall effect was not significant ($p=0.06$; Fig. 7F, G). Acetate concentration was also, unlike previous results (Fedorak et al. 2017), unaffected after a week on a HSD (Fig. 7E). Isobutyric acid, isovaleric acid, pentanoic acid and lactic acid concentrations were also measured and shown to not change (data not shown).

4.7.3 Baseline Motility after HSD

Motility was assessed in control mice and mice on a HSD with spatiotemporal mapping. The baseline protocol was identical to TPH1 KO experiments previously described. Although HSD mice did show abnormal motor patterns similar to the GF mouse, this was also curiously seen in mice on a control diet (Fig 7. A, B). Thus, overall the baseline LDC frequency and velocity was not different between HSD-mice and HSD-control mice (Fig. 8I, J). The only difference between the controls used for the HSD experiments and TPH1 KO controls was that all HSD-controls had to be individually housed in order to monitor food intake. There is some evidence to suggest that housing mice individually influences their behavior and stress response (Arndt et al., 2009). Whether individual housing causes enough stress to directly alter motility is unclear, yet is indeed a legitimate concern given the evidence suggesting the impact of acute stress on colonic motility (West et al., 2017).

In order to determine if baseline motility in HSD-controls was different than what we previously considered normal control motility, LDC frequency in these mice was compared to controls used in TPH1 KO experiments. Although this was not significant ($p=0.2$; Fig. 8K), there seems to be a difference and whether a larger sample size would reach significance is something worth investigating. TPH1 KO controls were also compared to HSD-mice and there was a significant decrease in the LDC frequency of HSD-mice (Fig. 8K). There thus appears to be more of an affect from the HSD on the mice than individual housing, indicating that an acute diet intervention had an affect on motility phenotype. With that being said, this conclusion can only tentatively be made as one cannot ascertain whether the difference between HSD-mice and TPH1 KO controls was due to the diet change, individual housing or any other change that may have gone overlooked. It would be necessary to test whether mice fed a HSD and housed socially display a different baseline motility phenotype than our 'normal' TPH1 KO controls.

4.7.4 Effect of butyrate on HSD-fed mice

Pre-treatment with butyrate was done in attempt to understand whether constant butyrate signaling is required to exert its effects or if butyrate signaling activates a mechanism that persists in its absence. Surprisingly, although baseline results from HSD-mice were not different than HSD-controls, their response to pre-treatment with butyrate was. Pre-treatment with butyrate significantly increased the LDC frequency and velocity in HSD-mice compared to baseline whereas it did not in controls. It might be the case that these mice were more sensitive to this intervention due to a potential decrease in butyrate. A more logical reasoning follows the nutrient-availability theory

outlined by Wichman et al. (2013). Mice fed a high-sugar-low-fibre diet obtained most of their energy from simple carbohydrates, which are absorbed in the small intestine. The colon however is presented with increasingly less fibre to produce nutrient-rich metabolites. Following this logic, the colons of HSD-mice are relatively nutrient deprived and a homeostatic response to this would be to increase the absorption of nutrient rich SCFAs, possibly by upregulating receptors for its transport. If this was the case, for the pro-kinetic reactivity to butyrate to have increased, receptors for nutrient sensing would also have had to be increased. Although we have no evidence for an upregulation of Olfr558, leading to an increased reactivity to butyrate, it is a possible explanation for the observed findings from Figure 7I. Furthermore, the fact that pretreatment with butyrate had any affect at all indicates that a pro-kinetic mechanism was activated and persisted, at least, for the 15 minutes of PBS infusion without butyrate. Lastly, as expected, both HSD-controls and HSD-mice responded to constant butyrate perfusion through an increase in LDC frequency.

4.7.5 Associations Between Butyrate and Motility

Although there was no significant decrease in any metabolite after 1 week on a HSD, there was a large range in the magnitude of change for both butyrate and propionate as well as for motility (Fig. 8A,C, Fig. 7F,G). Thus, we wanted to determine if there was a relationship between the amount of decrease in a specific SCFA with baseline motility results. This was done by plotting the percent decrease in a metabolite with the baseline motility parameter (LDC velocity or frequency) (Fig. 9). As expected, the magnitude of butyrate (but not propionate) decrease accurately predicted LDC

frequency (but not velocity) (Fig. 9A). This would suggest first and foremost that not all mice responded to the HSD in the same way, but that when butyrate decreased substantially, baseline LDC frequency was also lower. Although we did not measure SCFAs in HSD-control mice, as we did not foresee an effect of individual housing, we hypothesize that these mice would also display the same relationship between butyrate concentration and LDC frequency as HSD-fed mice.

4.8 Limitations

This study is limited in its ability to make comparisons to physiological or in vivo results. We adapted the spatiotemporal mapping technique to provide some indication of transit in vivo. However, these comparisons should be taken with a grain of salt and be limited to ones made between similar ex vivo experiments until the methodology is validated. Furthermore, liquid-perfusion through the lumen was done instead of the more physiological use of a solid fecal pellet. Because of this, we were not able to show a direct effect of LDC velocity on transit time. With that being said, the goal of the study was to evaluate motor patterns to gain an understanding of the nature of LDC activity in select mouse models. Any suggested comparisons apart from motor pattern analysis is simply a guide for subsequent experiments.

GF mice are an incredibly useful tool when studying the effect microbiota play on physiology. However, without a microbiota, GF mice have profoundly different physiologies. For example, GF mice have developmental retardation and cannot be weaned until one week later than control counterparts. They are noticeably smaller, their colons are shorter, and their gut walls are thinner and weaker. Not only did this

result in tearing the gut wall and forfeiting one experiment, but may have resulted in different relative levels of intraluminal pressure in these mice. For consistency, 2 cmH_2O was used as a level of intraluminal pressure for all experiments, but if a GF mouse had a thinner colonic diameter, they would have a relatively higher level of intraluminal pressure which may have increased motility in our experiments. The same limitations follow for TPH1 KO mice who have many other biological abnormalities including lower fat mass (Crane et al., 2015).

With respect to HSD experiments, limitations are vast as diets were not isocaloric, nor did they have similar levels of fibre. One cannot reason whether lower fibre or higher sugar directly impacted SCFA composition. Furthermore, metabolomics was performed on fecal samples. Given that only 5% of SCFAs are excreted into the feces, we are only observing a fraction of total SCFAs in the gut (den Besten et al., 2013). One may observe a decrease in stool SCFAs if absorption of SCFAs was increased but production stayed the same. Thus, stool SCFA levels should be compared to caecum SCFAs levels to account for any changes in absorption. With that being said, we were unable to collect *baseline* samples from the caecum of HSD-mice without invasive surgery, as this may have damaged the colonic tissue prior to motility experiments.

4.9 Future Directions

4.9.1 Short term projects

There are many avenues to pursue following this project. Two projects that immediately continue from aforementioned results will be addressed here and then one

long term study will be discussed afterwards. Motility will be assessed in GF mice by using fecal pellets instead of intraluminal perfusion in order to validate our conclusions from outflow analysis. Fecal pellet propulsion will give us a more tangible comparison to a physiological setting as having two independent experiments showing the same effect is much stronger than only one. We could also continue this project by repeating experiments with other microbial metabolites such as isovalerate, shown to be a strong agonist of EC (Bellono et al., 2017).

Another continuing project would involve investigating the mechanism by which propionate deactivates LDCs. This may be done by using spatiotemporal mapping with propionate before and after antagonists to PYY or GLP-1. It might be useful primarily to administer agonists to receptors of these hormones (their short half-life limits direct pharmacological administration (Brooks et al., 2017), to see if they mimic the effect of propionate. Subsequent experiments may involve the use of GPR43 KO mice, in the same way TPH1 KO were used, to see if propionate inhibits motility in these mice.

4.9.2 Long-Term Project

Lastly, the fact that SCFA-released-EC-cell-derived-5-HT acts directly on IPANs has incredible implications for the gut-brain axis when one considers Figure 1. It depicts results that have shown vagal primary afferent neurons with synapses exactly where EC-cell-derived-5HT acts on IPANs, as described by (Bellono et al., 2017). 5-HT does not distinguish between different classes of afferent neurons; it is likely that when SCFAs trigger 5-HT release, it acts on both 5-HT_3 receptors on IPANs as well as on vagal afferent neurons travelling to the brainstem. Demonstrating this dual effect of

afferent neurons, luminal 5-HT application was shown to attenuate vagal-dependent vesicomotor reflex in response to colorectal distension in rats (Zhang et al., 2011). However, more interesting is the relationship that gut 5-HT and the vagus play in behavior and IBS.

It is not a coincidence that symptoms of depression and anxiety are common comorbidities with IBS (Lee et al., 2017). This has been linked to changes in responses to stress, which has in turn been hypothesized to be due to low-vagal tone (Weber et al., 2010). If the vagus can be activated by fibre through 5-HT, supplementing diets with fibre may prove to be effective in treating constipated IBS patients with comorbid depression. In agreement with this, there is a large body of evidence which suggests fibre intake is inversely associated with mood disorders like depression. A recent retrospective study showed that there is an association between fibre intake and depressive symptoms (Gopinath et al., 2016). A multi-centre randomized controlled trial that gave participants either instruction on following a vegan diet or no instruction showed significantly lower depressive and anxiety symptoms in the vegan-trained group (Agarwal et al., 2015). Lastly, vagal nerve stimulation is currently an effective treatment for patients with otherwise treatment-resistant depression (Carreno & Frazer, 2017), yet the mechanism is not clear.

To test whether increased fibre ultimately leads to vagal nerve stimulation via the SCFA-5HT pathway described herein, a basic and clinical study will be designed. In mice, a high-fibre diet supplemented with inulin will be given chronically and half of the mice will receive a vagotomy. Transcription levels of genes related to depression and

anxiety, subjective tests measuring depression-like or anxiety-like symptoms, gut 5-HT, and stool SCFAs will be measured and compared.

In humans, a randomized control trial will be designed to administer either an inulin-butyrate ester, inulin alone, or a placebo. The ester, like that created in Chambers et al. (2016), will ensure increased production of butyrate in the colon. Similar to mouse studies, stool SCFA and 5-HT will be measured. To assess subjective depressive/anxiety symptoms, standard depression/anxiety questionnaires will be given. To objectively measure any changes in CNS activity, especially those connected to vagal activity, an fMRI will be given prior to and after the diet intervention. A recent study found that anterior insula fMRI activity was able to detect changes in vagal tone in addition to being able to predict success of vagal-nerve stimulation (Fang et al., 2017). To substantiate fMRI findings, we can use heart rate variability (HRV), which has been widely used as a measurement of vagal tone (Hayano et al., 1991).

4.10 Conclusions

In conclusion, the present study has shown that GF colonic motility is abnormal and characterized by a predominance of non-propulsive motility. The results can be explained by abnormal luminal-mucosal signaling and is not dependent on a postulate of abnormal ENS development. Low-dose of a mixture of SCFAs activates propulsive activity, signified by an increase in the frequency and predominance of LDCs in these mice. By administering individual SCFAs to TPH1 KO mice we found that butyrate is mediating this propulsive switch and that this activity requires mucosal 5-HT signaling. We then showed for the first time in the colon, the presence of SCFA receptor GPR43

on 5-HT producing ECs, providing a potential direct mechanism by which butyrate may exert its effects. Lastly, after placing mice on a HSD, we found there to be no appreciable differences in average baseline motility, however we did find evidence to suggest that baseline motility is correlated with the concentration of butyric acid in the stool.

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