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THE IMPACT OF HOST-MICROBE INTERACTIONS ON MURINE COLONIC SECRETOMOTOR FUNCTION.

Ollscoil na hÉireann

THE NATIONAL UNIVERSITY OF IRELAND

Coláiste na hOllscoile, Corcaigh

UNIVERSITY COLLEGE CORK

Thesis presented by

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May 2014

Under the supervision of:
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Declaration

This thesis comprises original work carried out by the author and has not been submitted for any other degree at University College Cork, or elsewhere.

Author Contribution

All of the work described herein was performed independently by the author, with the following exceptions:

- **Chapter 1** Marcela Julio-Pieper provided artwork for figures (Figures 1.1 and 1.4) on intestinal ion transporters.

- **Chapter 2:** Patrick Fitzgerald and Collette Manley assisted with administration of the probiotics for *in-vivo* feeding studies.

- **Chapter 3:** Aileen Houston analysed the morphology of germ free and conventional mouse colonic tissues.

- **Chapter 4:** Lis London generated GABA-containing supernatants and associated controls and also analysed GABA content.

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"Be patient, hand," he said. "I do this for you."

Ernest Hemmingway - The Old Man and the Sea

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The Old Man and the Sea is a favourite story of mine and one which, by mere coincidence, I read shortly before beginning my post-graduate studies. It is a story which has stayed with me and not just for its lessons in perseverance. In his most difficult moments Santiago, the Old Man, would wish for his closest companion, Manolin. I am happy to say, that I have had many "Manolins" throughout my PhD. Cristina Torres-Fuentes (Bananita), Matteo Pusceddu (Winky Winky), Caroll Beltran (Gnam Gnam)...the Prickie Team....I also cannot forget Beatriz Lobo and Felicia De Palo. Just as Santiago persevered with thoughts of Manolin, I persisted with thoughts of you. You are my greatest friends and I am waiting for a time when we can all be together. KEEP GOING!

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I dedicate this work to you.

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Thesis Summary

There is increasing emphasis on microbiota-based strategies for treating disorders of the brain-gut axis. Probiotics are microorganisms which, when given in adequate amounts, can confer health benefits upon the host (Fontana et al., 2013). In this thesis I focus on the local effects of probiotics and germ free status on intestinal ion transport. I first examined the influence of two different probiotic strains *Bifidobacterium infantis* 35624 and *L. salivarius* UCC118 on active colonic ion transport in the mouse (Chapter 2). Importantly, I use the Ussing Chamber to examine and compare tissues, exposed chronically to these probiotics following *in-vivo* feeding for two weeks, and acutely following direct addition during *ex-vivo* experimentation. This permitted examination of the translational value of acute *ex-vivo* Ussing Chamber studies to *in-vivo studies*, thus determining the validity of the Ussing Chamber as a screening tool for potential probiotics. Furthermore, in addition to Ussing Chamber experiments, both *in-vivo* functional and molecular experiments were carried out following the two week feeding period, which provided information on the functional impact of the individual probiotic strains.

Next I examined the impact of the host microbiota itself on both baseline and stimulated colonic secretomotor function as well as probiotic induced changes in ion transport (Chapter 3). The Ussing Chamber was again used to examine segments of distal colon from both germ free and conventional mice, thus facilitating assessment of the impact of the host microbiota on secretomotor function in the mouse. Understanding the host-microbiota's role in the mouse is critical to truly appreciating the impact of host-microbe relationships on intestinal ion transport in this species. Moreover, taking into consideration the detailed characterisation of probiotic

induced changes in ion transport carried out in the previous chapter, the same probiotics were also tested in this study to determine whether the presence or absence of the host microbiota influenced responses to these microbes.

Finally, it has been well documented that many probiotics elicit their effects via secreted bioactives, however, identifying these bioactives has become a major limitation of many probiotic studies (Sánchez et al., 2010). Therefore, I studied the *ex-vivo* effects of an exogenous neuroactive compound, GABA, on murine colonic secretomotor and compared these with microbially produced GABA contained in supernatants from the probiotic *Lactobacillus brevis* DPC6108. This approach not only allowed for novel insight into GABAergic regulation of murine colonic secretomotor function, but more importantly, in the context of this thesis, allowed for the determination of probiotics as putative producers of bioactives with known ability to modulate intestinal secretomotor function. Furthermore, from a methodological point of view, this chapter allows for the validation of a new approach for screening potential probiotics and their bioactives.

Taken together I conclude that commensal microbes have an important and strain specific functional influence on colonic ion transport and secretomotor function and these effects can be mediated via extracellular bioactives. Moreover, I believe that functional *ex-vivo* studies such as those carried out in this thesis have a critical role to play in our future understanding of host-microbe interactions in the gut.

List of Publications

Published Manuscripts

Lomasney, K.W., Cryan, J.F., and Hyland, N.P. (2014). Converging effects of a Bifidobacterium and Lactobacillus probiotic strain on mouse intestinal physiology. **Am.J. Physiol. Gastrointest. Liver Physiol.** *In Press* doi:10.1152/ajpgi.00401.2013

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Papers To Be Submitted

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Lomasney K. W., Cryan J.F., Hyland N.P. (2012). γ-Amino-Butyric Acid Regulates Submucosal Cholinergic Signalling and Secretomotor Function in Mouse Colon. **Gastroenterology** *Vol. 142, Issue 5, Supplement 1, Page S-306*

Lomasney K.W., Cryan JF, Hyland NP (2011**).** Effects of the gut microbiota on colonic secretomotor function. **Proceedings of the British Pharmacological Society***PA2onlineVol.9,Issue3.athttp://www.pA2online.org/abstracts/Vol9Issue3abst034P.pdf*

Golubeva A.V., Crampton S.J., Desbonnet L., Edge D., **Lomasney K.W.**, Hyland N.P., O'Halloran K.D., Dinan T.G., O'Keeffe G.W., Cryan J.F. (2014). Differential negative impact of prenatal stress on physiological systems in adulthood**.** *European* **Neuropsychopharmacology**, *Volume 24, Supplement 1*, *March 2014*, *Pages S89-S90*

Lobo B., Tramullas M,. Finger B-C., **Lomasney K.W.**, Santos J., Hyland N.P., Dinan T.G., Cryan J.F. (2012). Psychosocial chronic stress induces intestinal dysmotility and changes in enteric nervous system in proximal colon**.** **Neurogastroenterology & Motility** *Vol. 24, Issue Supplement s2*

Chapter 1:
Introduction

1.1 Summary of Intestinal Ion Transport: Absorption and Secretion

The mammalian intestine contains a diverse array of electrolyte transporters upon which fluid transport depends. Ion channels and transporters allow for the establishment of electrical gradients which contribute to the movement of water across the intestinal wall (Kunzelmann and Mall, 2002). The intestinal epithelium is primarily absorptive, and the uptake of fluid is dependent on the absorption of solutes, in particular Na^+ and Cl^- (**Figure 1.1**). Na^+ absorption is mediated mainly through electroneutral ion exchangers. Three Na^+/H^+ exchangers (NHE) are expressed to various degrees along the intestine (Gawenis et al., 2002). NHE1 is located basolaterally and is considered a housekeeping transporter, regulating intracellular pH and cell volume. In keeping with such a role, NHE1 knockout mice display altered intracellular pH in pancreatic acinar cells; however they do not display any major changes in NaCl transport (Brown et al., 2003). In contrast to the basolateral expression pattern of NHE1, NHE2 and NHE3 are in general expressed on the apical side of epithelial cells, and are found in both the small and large intestine (Gawenis et al., 2010) (**Figure 1.1**). Absence of NHE3 in mutant mice results in severe and chronic diarrhea, thereby suggesting that this exchanger plays a significant absorptive role in mouse intestine (Schultheis et al., 1998).

Electrogenic Na^+ absorption on the other hand occurs primarily in the colon by amiloride-sensitive epithelial Na^+ channels (ENaC) located on the apical membrane of intestinal epithelia. ENaC is the major active ion channel for Na^+ reabsorption, and has been shown to compensate for electrolyte loss associated with diarrhoea in NHE3 knockout mice (Schultheis et al., 1998).

With respect to Cl^- ion transport, electroneutral Cl^- absorption is primarily facilitated by two well characterised anion exchangers, down regulated in adenoma (DRA) and putative anion transporter-1 (PAT-1), both demonstrated to be Cl^-/HCO_3^- exchangers (Jacob et al., 2002; Simpson et al., 2007). DRA and PAT-1 are expressed apically, and are present in both the small intestine and colon (Wang et al., 2002). However, DRA appears to have higher expression in the colonic epithelium compared to the small intestine, while PAT-1 displays an opposite pattern of expression (Wang et al., 2002). This would suggest that PAT-1 is more important for Cl^-/HCO_3^- exchange in the upper GI tract, while DRA is favoured in the colon (Wang et al., 2002). The clinical significance of DRA is highlighted by its absence, which is associated with a rare form of congenital Cl^- secretory diarrhoea (Höglund et al., 1996). Moreover, Cl^- absorption, at least via PAT-1, is dependent on NHE3 Na^+ absorption (Singh et al., 2010).

Absorption of ions also occurs by nutrient-coupled transporters, for example the sodium glucose transporter 1 (SGLT1), primarily responsible for electrogenic Na^+ absorption in the small intestine, and the short chain fatty acid (SCFA) transporter, SCFA/HCO_3^- (Bachmann and Seidler, 2011). SCFAs are the chief energy source for colonocytes and their production is dependent on host bacterial fermentation of carbohydrates or dietary fibre. SCFAs stimulate HCO_3^- secretion via SCFA/HCO_3^- anti-porters (Vidyasagar et al., 2005). However, they also inhibit Cl^-- and cyclic adenosine monophosphate (cAMP)-dependent HCO_3^- secretion (Vidyasagar et al.,

2004). Colonic Na^+ absorption and Cl^- secretion are also influenced by

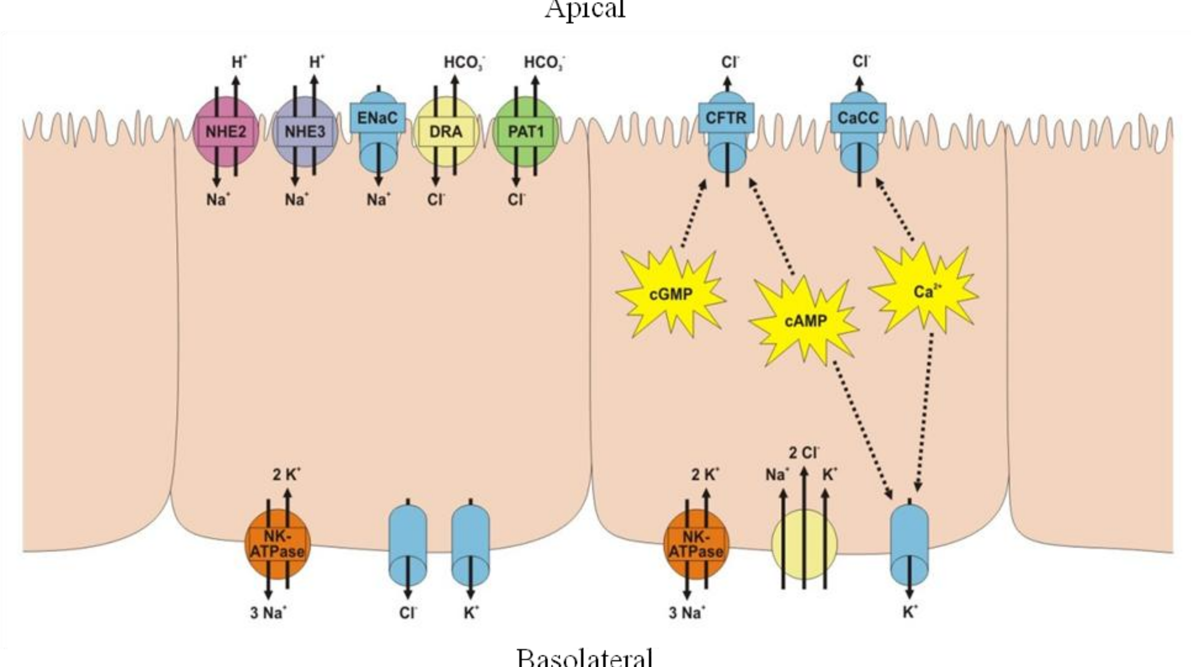

Figure 1.1 Na^+/H^+ cation exchangers (NHE) 2/3 in addition to down-regulated in adenoma (DRA) and putative anion transporter-1(PAT-1) anion exchangers import sodium (Na^+) and chloride (Cl^-) across the apical membrane into intestinal epithelial cells while exporting a proton (H^+) and bicarbonate (HCO_3^-) . Basolateral NK-ATPase and NHE-1 provides a gradient for the basolateral exit of ions and subsequent transport of water across the intestinal wall *(left side, absorption).* Chloride is pumped into the cell via the Na2ClK symporter the gradient for which is provided by the active basolateral NK-ATPase anti-porter which also recycles sodium out of the cell. Basolateral potassium channels recycle potassium (K^+) out of the cell causing a negative intracellular charge and thus a drive toward apical exit through cAMP/cGMP activated cystic fibrosis transmembrane conductance regulator (CFTR) or calcium activated chloride channes (CaCC) *(right side, secretion). (From* (Lomasney and Hyland, 2013)).

SCFAs which promote the expression of both NHE3 (Kiela et al., 2007) and ENaC (Zeissig et al., 2007) by intestinal epithelial cells, in addition to inhibiting cAMP- and cyclic guanosine monophosphate (cGMP)-mediated secretion (Vidyasagar et al., 2005). Collectively these studies suggest a primarily pro-absorptive role for SCFAs in the regulation of colonic fluid and electrolyte transport.

Though the intestine is considered primarily an absorptive organ, fluid secretion is pivotal to its proper physiological function, for example by facilitating passage of fecal content. Unlike fluid absorption however, secretion is primarily active, driven mainly by changes in intracellular Cl^- ion gradients and is regulated by apical ion channels and the activity of several basolateral transporters (Barrett and Keely, 2000). In brief, Cl^- is taken up by the basolateral sodium potassium chloride (NKCC1) symporter, considered a secondary active transporter, as the drive for Cl^- uptake is provided by the primary active transporter, NaKATPase located on the basolateral side of intestinal epithelia. The low concentration of intracellular Na^+ creates a net electronegative environment within the cell producing a driving force for Cl^- extrusion through the apical membrane (**Figure 1.1**) (Barrett and Keely, 2000).

In addition to Cl^- secretion, secretion of HCO_3^- occurs primarily by Cl^- and cAMP-dependent pathways, SCFA/ HCO_3^- electroneutral transporters as well as through DRA and PAT-1 (Binder et al., 2005). SCFAs also influence HCO_3^- transport by inhibiting activation of both cAMP- and Cl^--dependent HCO_3^- secretion (Vidyasagar et al., 2005), further supporting a pro-absorptive role for SCFAs.

1.2 Pharmacological secretagogues for the study of ion transport

The classic approach to investigating intestinal ion transport processes involves the use of specific pharmacological ligands with the ability to target and modulate ion channels, exchangers or effecter molecules of interest.

1.2.1 *Forskolin and cAMP induced ion transport*

As previously mentioned, the second messenger cAMP is the primary driver of electrogenic ion and fluid secretion in the intestinal epithelium, mainly through increasing the activity of chloride secretion through apical CFTR channels (Barrett and Keely, 2000). Indeed, as will be discussed later, severe diarrhoea following enteric infection with several pathogens including *Vibrio cholera* and Enteropathogenic *Escherichia coli* is vastly dependant on toxin induced increases in the levels of intracellular cAMP (Field, 2003) Therefore compounds that mimic such an effect are very useful in studying the ability of novel therapeutics to modulate active ion secretion in intestinal epithelia. Moreover, in the context of host-microbe interactions, such compounds would be useful for studying the therapeutic potential of probiotics for the treatment of pathogen-induced diarrhoea (Lomasney and Hyland, 2013) where there is an enormous unmet medical need especially in the developing and third world (Cheng et al., 2005).

Forskolin is one such compound and has been widely used, along with its derivatives for studying the role of cAMP in a variety of mammalian tissues and cell types including the intestine and intestinal epithelial cells (Alasbahi and Melzig, 2012). The compound itself is a labdane diterpenoid isolated from the roots of the Indian *Plectranthus barbatus* plant, also known as *Coleus forskolii* (Alasbahi and Melzig, 2012). It acts at an intracellular level to increase the activity of adenylyl cyclase (AC), the key enzyme responsible for catalyzing the conversion of adenosine triphosphate (ATP) to cAMP and pyrophosphate, thus increasing intracellular levels of cAMP (Sassone-corsi, 2012). There are at least 9 known isoforms of AC distributed throughout the body, of which forskolin is known to activate all but one

thus making it a relatively non-selective but useful tool for studying the role cAMP (Alasbahi and Melzig, 2012). cAMP has many downstream effecter molecules, however protein kinase A (PKA) is probably the most understood and relevant to intestinal ion transport. cAMP binds to the regulatory subunits of PKA causing its catalytic subunits to dissociate and mediate further effects down-stream (Sassone-corsi, 2012)

With regard to ion transport PKA phosphorylates apical CFTR and basolateral NKCC1 resulting in sustained trans-cellular efflux of chloride and water into the lumen (**Figure 1.2**). Moreover, to facilitate a sustained secretion, cAMP can also stimulate further recruitment of both CFTR and NKCC1 to the apical and basolateral membranes respectively resulting in increased channel density (Barrett and Keely, 2000). Basolateral potassium secretion via the voltage gated $K_V7.1$ channels is also activated which provides an essential driving force for apical chloride efflux (Matos et al., 2007). Finally, in addition to stimulating secretory activity in epithelial cells, cAMP also inhibits absorption, specifically via PKA induced phosphorylation of NHE2/3 regulatory proteins (Yun et al., 1997).

1.2.2 *Bethanechol as a cholinergic agonist and Ca2+ mediated ion transport.*

Bethanechol chloride also known as Carbamyl-β-methylcholine chloride is a synthetic ester which is structurally and pharmacologically related to acetylcholine but is resistant to degradation by acetylcholine esterase. It is a selective M_3 muscarinic acetylcholine (mACh) receptor agonist although it may also act on M_1 receptors (Hirota and McKay, 2006b). This compound, along with its less selective mACh receptor agonist carbachol, have been widely used to investigate cholinergic induced intestinal epithelial ion transport in a number of species including mouse

(Hirota and McKay, 2006). Both M_1 and M_3 mACh receptors are G_q protein coupled receptors which stimulate phospholipase C (PLC) leading to degradation of phosphatidylinositol 4-5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) which stimulate the release of intracellular Ca^{2+} stores from the endoplasmic reticulum (**Figure 1.2**) (van Koppen and Kaiser, 2003). It is this release of Ca^{2+} which mediates bethanechol-induced ion transport in intestinal epithelium in the form of Cl^- secretion. Similar to cAMP-induced Cl^- secretion, Ca^{2+} activates basolateral K^+ channels which provide a driving force for apical Cl^- efflux via Ca^{2+} activated Cl^- channels (CaCC). In the mouse colon in particular, Ca^{2+} induced basolateral K^+ efflux can occur via both a Ca^{2+} activated small conductance K^+ channel ($K_{Ca}3.1$), but also via the chromanol 293b sensitive voltage gated K^+ channels ($K_V7.1$) (Hirota *et al.,* 2006). Ca^{2+} activated K^+ channels also exist in the apical membrane of mouse colonic epithelial cells ($K_{Ca}1.1$), however these do not appear to be required for cholinergic induced Cl^- secretion but may allow for electroneutral secretion of KCl into the lumen (Hirota *et al.,* 2006).

Finally, bethanechol induced chloride secretion is relatively transient in comparison to cAMP induced ion transport. This is likely due to the increase in both PKC which is activated by DAG and the IP_3 derivative inositol 3,4,5,6-tetrakisphosphate (IP4) which downregulate Ca^{2+} activated basolateral K^+ channels and apical CaCC activity respectively (Hirota and McKay 2006).

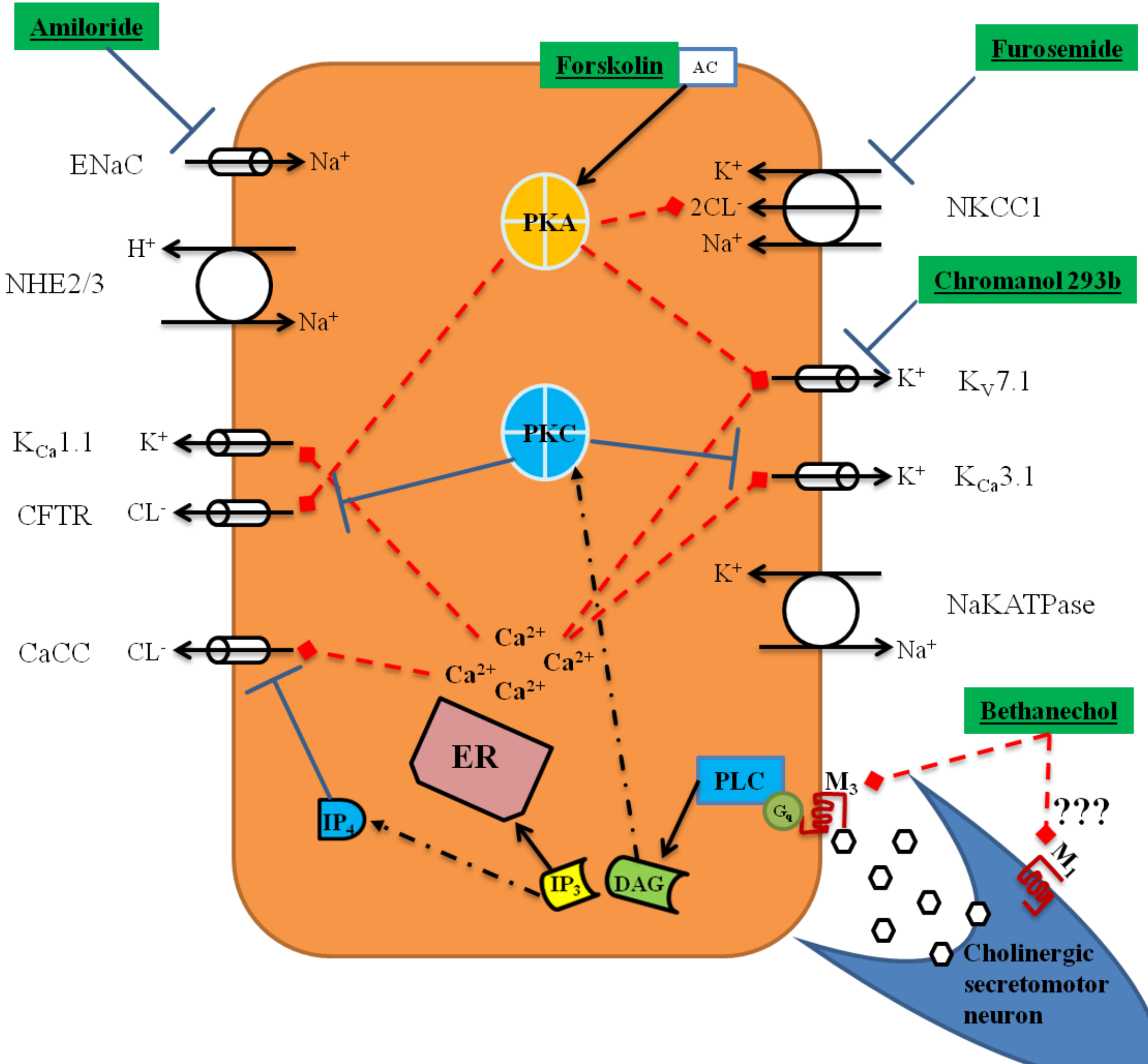

Figure 1.2 - Pharmacological Tools to Modify Ion Transport - *Clockwise* - Amiloride directly blocks ENaC on the apical side of the epithelium. Forskolin activates adenyl cyclase (AC), increasing intracellular levels of cAMP which binds to PKA which in turn phosphorylates apical CFTR and basolateral NKCC1 inducing a sustained apical efflux of Cl. Furosemide directly blocks NKCC1, thus indirectly inhibiting apical Cl^- secretion. Chromanol 293b acts directly on $K_v7.1$ to block basolateral K^+ exit. Bethanechol acts as an agonist at metabotropic muscarinic acetylcholine receptors (mAChR), in particular M_3 but possibly $M_{1.}$ Activation of epithelial mAChR stimulates phospholipase C (PLC) leading to degradation of phosphatidylinositol 4-5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) which stimulate the release of intracellular Ca^{2+} stores from the endoplasmic reticulum. The increased intracellular Ca^{2+} activates a transient apical Cl^- efflux via Ca^{2+} activated Cl^- channels (CaCC) which is further driven by basloteral exit of K^+ via Ca^{2+} activated potassium channels ($K_{Ca}3.1$). The transient nature of response to bethanechol is likely due to the inactivation of CaCCs by inositol 3,4,5,6-tetrakisphosphate (IP4), a derivative of IP_3, as well as the PKC (activated by DAG) induced inactivation of $K_{Ca}3.1$.

1.2.3 *Capsaicin*

Capsaicin is an ingredient most often found in the genus of peppers known as *Capsicum* and responsible for the powerful sensation of heat associated with eating these vegetables. It is used in a vast range of culinary dishes, however, apart from its role in cuisine, capsaicin has been used for many years in numerous different countries as a therapeutic agent against a plethora of ailments (O'Neill et al., 2012). It has been used in particular for the treatment of pain, but pharmacological studies have also demonstrated physiological effects on cardiovascular, respiratory and gastrointestinal tissues (Holzer, 1991; Haanpää and Treede, 2012). The compound itself, *trans*-8-methyl-N-vanillyl-6-no-nenamide is a naturally occurring alkaloid derived from vanillyl amide, and similar to its precursor, is a potent antagonist of the transient receptor potential cation channel subfamily V member 1(TRPV1) (O'Neill et al., 2012). TRPV-1 is found all over the body and performs a variety of functions depending on location. In the periphery, including the GI tract, it is located mostly on primary sensory neurons and is believed to be responsible for the initial detection of noxious chemical and in particular heat stimuli, thus giving a "burning" sensation upon activation with capsaicin (Caterina et al., 1997). Indeed, TRPV-1 has been found in human colon and increased levels in patients with IBD is thought to be important in mediating visceral hypersensitivity (de Fontgalland et al., 2014). Studies in Ussing Chamber (See section 1.3; Figure 2) have also demonstrated an effect for capsaicin on intestinal ion transport (Vanner and Macnaughton, 2004a). Exposure to capsaicin activates sensory afferent neurons which in turn activate submucosal secretomotor neurons to release pro-secretory neuropeptides, such as substance P which can then cause an increase in luminal chloride efflux (See section 1.4).

1.2.4 *Tetrodotoxin*

Tetrodotoxin (TTX) is a powerful neurotoxin and is well known as the toxic substance found in the ovary and liver of the puffer fish; the fish itself is considered a supreme delicacy in Japan and indeed a licence is required to serve (Narahashi, 2001). However, this fish is not the only marine animal to contain this toxin and moreover, the toxin itself is not synthesised by these animals but rather specific species of bacteria to whom the animals play host (Cheng et al., 1995) Therefore, production of this toxin is an inherent property of these marine animals' host microbiota and indeed environmental factors, including the influence of symbiotic host-microbial relationships can affect the production of TTX (Daly et al., 1997). TTX acts by blocking voltage gated sodium channels VGSC) on nerves, and indeed skeletal muscle, thus preventing the influx of Na^{2+} which inhibits the propagation of action potentials thus eliminating any associated neural or muscle function (Nieto et al., 2012). Therefore, TTX is a particularly useful *ex-vivo* pharmacological tool for investigating the role of the neural component of tissue in regulating both homeostatic function as well as tissue responses to external stimuli (Miranda-Morales et al., 2010). Consequently it has been used extensively in organ bath studies of intestinal tissues to study the neural components involved in motility and ion transport (Vanner and Macnaughton, 2004a).

1.2.5 *Veratridine*

Veratridine (3-veratroyl-veracevine) (O’Neill (Ed.), 2013) is a natural plant alkaloid and neurotoxin most often derived from the genus of plants known as Veratrum (Catterall, 1975a, 1975b). It acts by reversibly binding to neural Na^{2+} channels and inducing a continual, TTX sensitive, activation of action potentials in the nerve cell,

increasing its excitability (Catterall, 1975a). The use of Veratridine as a pharmacological tool to examine enteric regulation of mucosal intestinal ion transport has provided invaluable functional insight into these process (Sheldon et al., 1990a). Due to its ability to act as a pan-neural stimulant, submucosal intestinal tissues exposed to veratridine have been shown to release a plethora of enteric neurotransmitters which combine to produce a powerful and sustained pro-secretory response resulting in the luminal efflux of NaCl (Sheldon et al., 1990a; Hyland and Cox, 2005). It should be noted however, that in the presence of extrinsic neural innervation, such as that found in whole intestinal tissues, stimulation with veratridine results in a net absorption of ions (Sheldon et al., 1990a).

1.3 Measurement of Intestinal Ion Transport: The Ussing chamber

The Ussing Chamber (**Figure 1.3**) was developed by the Danish physiologist and biochemist, Hans Henriksen Ussing (1911-2000), considered the "founder of epithelial transport" (Larsen, 2002; Palmer and Andersen, 2008). Using frog skin, Ussing discovered that the rate of active transport of ions across an epithelium could be measured as an electrical current provided that both sides of the epithelium were bathed in an identical electrolyte solution and that transepithelial electrical potential was eliminated (Ussing and Zehran, 1951).

The apparatus, to which Ussing gives his name, consisted of two chambers with epithelial tissue mounted in between. Each half chamber was filled with Krebs solution and both halves of the chamber were gassed, with carbogen (95% O_2, 5% CO_2) to oxygenate the tissue and to provide constant circulation. In order to eliminate transepithelial electrical potential, electrodes were used to apply an

external current across the tissue until the potential was brought to zero. The current required to achieve this was termed "short circuit current" or I_{sc}, and Ussing demonstrated that changes in this current matched the unidirectional flux of ions across the epithelium (Ussing and Zehran, 1951) (**Figure 1.3**). Since then, Ussing chambers have been used to measure ion transport across a multitude of epithelium including lung and intestine (Clarke, 2009a), with the first intestinal epithelial studies occurring shortly after Ussing's original experiments (Chaflin et al., 1958; Cooperstein and Hogben, 1959). To this day the Ussing chamber remains one of the most important tools to investigate electrolyte transport across epithelium (Larsen, 2002).

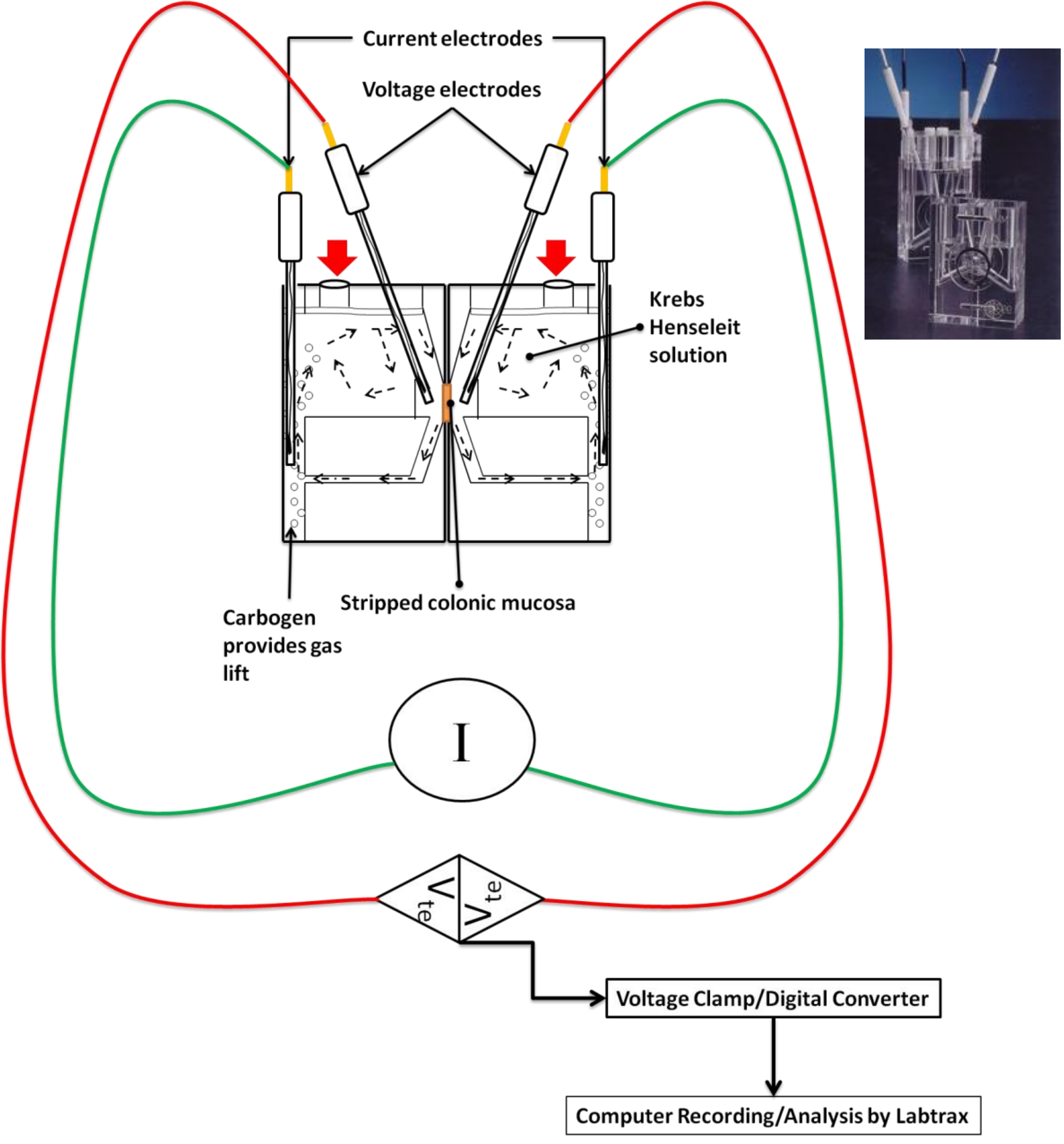

Figure 1.3. The Ussing Chamber is composed of two identical half chambers, representing the apical and basolateral reservoirs. Intestinal (colonic) tissue is mounted between both halves which are filled equally with chilled Krebs Henseleit (KH) buffer to eliminate osmotic and hydrostatic gradients. Carbogen (95%O_2, 5%CO_2) supplies the necessary oxygen and maintains pH as well as providing gas-lift to allow steady circulation of buffer and perfusion of the tissue. The chamber is kept at a constant temperature of 37°C. Tissue is voltage clamped to 0V by sending a set current directly across the tissue via voltage electrodes. The amount of current required to maintain the tissue at 0V (known as short circuit current) is measured by the current electrodes and recorded electronically. Test materials, compounds, drugs may be added to either side of the chamber (red arrows), and subsequent changes in short-circuit current recorded.

Short-circuit current represents net changes in electrogenic ion transport owing to the activity of several ion transporters, such as CFTR and ENaC (**Figure 1.1**). Nonetheless, epithelial electrolyte is also subject to significant influence by additional factors including the enteric nervous system (ENS) and local mucosal immune system (Cooke, 2000)

1.4 Influence of the Enteric Nervous System on in Ion Transport

The ENS is a complex neural network capable of regulating gastrointestinal function independent of the central nervous system (Furness 2000). Structurally it is composed of a network of ganglia, which form two major inter-connected and highly organised plexi within the wall of the intestinal tract. These plexi are known as the myenteric or Auerbach's plexus and the submucosal or Meisner's plexus (Furness, 2008).The myenteric plexus is located between the outer longitudinal and inner circular muscle layers of the external muscle coat of the intestine and is composed of numerous neurons which cluster and relay signals within neuronal ganglia (Furness, 2008). The submucosal plexus as its name suggests, is located within the tunica submucosa and similar to the myenteric complex, is composed of nerve strands which group and relay signals within enteric ganglia (Furness, 2008). Although the physical location of these plexi imply an obvious separation in function, with the myenteric plexus controlling intestinal muscle activity and the submucosal plexus regulating mucosal function, the plexi are not separate and communication between the two plexi is important for overall coordination of intestinal function (Furness, 2008). Moreover, the ganglia within both neural plexi is composed of a mix of afferent neurons, interneurons, and (secreto)motor neurons (Furness, 2008).

Moreover, these plexi are also innervated by extrinsic sensory afferents, originating from the vagal and spinal afferents of the nodose and dorsal root ganglia respectively, both of which form an important line of communication with the brain, thus providing coordination between GI reflexes and central behavioural responses (such as food intake) as well as allowing for pain perception (Berthoud, 2004).

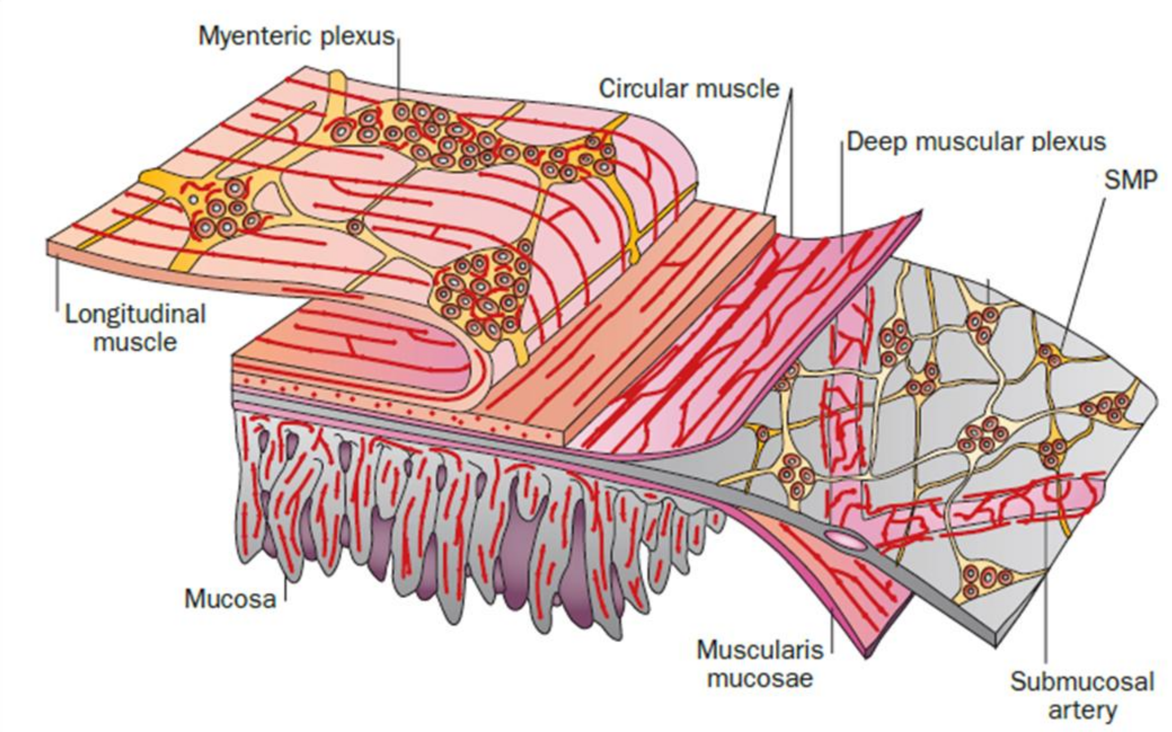

Figure 1.4 The enteric nervous system has two plexuses, the myenteric plexus between the longitudinal and circular muscle layers and the submucosal plexus (SMP) that has outer and inner components. Nerve fiber bundles connect the ganglia and also form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa (Adapted from Furness, 2008).

1.4.1 *Enteric nervous system and secretomotor function in the mouse*

While most of our understanding of the properties of enteric neurons and enteric neural reflexes come from studies in guinea pig and larger mammals such as the pig, the emergence in recent years of transgenic mouse models for the study of enteric disorders such as IBD presents a significant advantage for studying the

enteric nervous system in this species (MacDonald, 1997; Lomax and Furness, 2000; Brown and Timmermans 2004). The studies described in this thesis also employ the mouse throughout for studying the effects of microbes and their bioactives on neurally evoked intestinal ion transport in the Ussing Chamber. The following sections will describe what is currently known about murine enteric structure and function as well as the chemical coding and potential roles of specific neurons.

1.4.2 *Morphological and electrophysiological properties of mouse enteric neurons.*

The structure and electrophysiological properties of specific enteric neurons are important in determining their role within the ENS (Clerc and Furness, 2004; Nurgali *et al.,* 2004). The neurons within the mammalian enteric nervous system can be divided into two distinct morphological and electrophysiological classifications respectively (Wong *et al.,* 2008). Morphologically, the ENS is made up of Dogiel Type I and Dogiel Type II neurons (Furness, 2008). Dogiel Type I neurons are uni-axonal and contain cell bodies with lamellar dendrites, while Dogiel Type II, in general, have large round or oval cell bodies and a smooth surface with multiple process extending to various sites in and along the intestinal tract (Furness, 2003). Electrophysiological studies have divided enteric neurons into S-type and AH type (Brown and Timmermans, 2004). S-type neurons are characterised by their monophasic potentials, the presence of fast excitatory post synaptic potentials and the absence of slow afterhyperpolarising potential (AHP) while AH-type produce biphasic responses, containing a hump on the repolarising phase of the action potential and generally contain slow AHP's and do not produce fast excitatory post-synaptic potentials (EPSPs) (Nurgali *et al.,* 2004).

In the mouse the majority (~80-90%) of neurons in both neural plexi appear to be S-type neurons displaying Dogiel Type I morphology while the remaining (20%) are Dogiel Type II AH neurons (Nurgali *et al.*, 2004; Wong *et al.*, 2008). Dogiel Type I neurons in the myenteric plexus neurons innervate the circular muscle directly and appear to regulate longitudinal muscle reflexes through nerve projections in the tertiary plexus (layer of the myenteric plexus closest to the longitudinal muscle) (Sang *et al.,* 1997;1998). In the submucosal plexus these neurons project toward the mucosal layer of the intestine and act as secretomotor neurons regulating mucosal function including intestinal ion and fluid transport and mucous secretion (Furness et al, 2004). Dogiel Type II neurons are present in both neural plexi of the mouse and due to the similar pattern of innervation, morphology and neural chemistry in comparison to guinea pig, are believed to be intrinsic primary afferent neurons (IPANs), acting as the first step in enteric neural reflexes (Furness *et al.,* 2004). Moreover, myenteric Dogiel Type II neurons in the mouse colon project to other myenteric ganglia and also project into the submucosal plexus as far as the mucosa suggesting a key role in regulating coordination of enteric reflexes (Furness *et al.,* 2004).

1.4.3 *Chemical coding of murine enteric neurons*

1.4.3.(a) Myenteric Plexus

Although well defined in other small mammals (Furness, 2008) the ENS of the mouse has thus far been studied to lesser degree (Sang and Young, 1996, 1998; Sang et al., 1997; Furness et al., 2004; Nurgali et al., 2004; Wong et al., 2008). Immuno-histochemical staining of small intestine identified two major classes of

circular muscle motor neurons, those containing nitric oxide synthase, vasoactive intestinal peptide along with neuropeptide Y (NOS/VIP/NPY), and those containing calretinin along with substance P (CalR/SP) (Sang and Young, 1996). In addition myenteric ganglia in the small intestine were found to be innervated by seven classes of neuron, those containing NOS, VIP, NOS/VIP, NPY, CalR/calbindin (CalB), SP or 5-hydroxytriptamine (5-HT) (Sang and Young, 1996).(**Table 1.1**) Myenteric innervation of large intestinal circular muscle on the other hand proved to be considerably more diverse (at least within the range of neurotransmitters examined) with 5 major classes of neurons described and categorised as follows: NOS, NOS/VIP, GABA, SP, or CalR RSP containing neurons (Sang and Young, 1996). Ganglia within the myenteric plexus of the large intestine were found to be innervated by neurons containing NOS, VIP, CalB/CalR, CalR, SP, GABA or 5-HT (Sang and Young, 1996).

Circular motor neurons projecting anally are in general considered inhibitory neurons (as previously found in other species) and are largely immuno-reactive (IR) for NOS/VIP. In contrast to other species including rat and guinea pig, NPY-IR neurons are only located in a small subpopulation of small intestinal neurons (Sang et al., 1997). SP-IR neurons on the other hand projected orally and are therefore considered to be excitatory neurons, as previously observed in other species (Sang et al., 1997).

Table 1.1 Chemical coding of murine myenteric neurons in small (SI) and large (LI) intestine measured as neurotransmitter immunoreactivity (NT-IR) (Data taken from (Sang and Young, 1998)

		SP		**CalR**		**CalB**		**NOS**		**VIP**		**5-HT**		**GABA**	
		SI	**LI**	**SI**	**LI**	**SI**	**LI**	**SI**	**LI**	**SI**	**LI**	**SI**	**LI**	**SI**	**LI**
% Ach-IR neurons containing other NT-IR	Cell Body	~50%	~50%	75%	75%	60%	60%	~5%	<5%	15%	<15%	?	?	?	~5%
	Terminals in Circular muscle	90%	90%	<100%	<100%			0%	0%	0%	0%			?	25%
	Terminals in Myenteric Plexus	~66%	~75%	66%	>33%	15%	<25%	~5%	~2%	~5%	>5%	<5%	5%	?	<20%
	Terminals in Tertiary Plexus	90%		100%				0%		0%					
% of other NT-IR neurons containing ACh-IR	Cell Body	80%	80%	~99%	~100%	80%	80%	~10%	~10%	25%	25%	100%	100%	?	~33%
	Terminals in Circular muscle	100%	100%	100%	<100%			0%	0%	0%	0%			?	>75%
	Terminals in Myenteric Plexus	75%	>80%	<100%	>66%	50%	50%	>5%	~5%	>5%	15%	60%	>50%	60%	~30%
	Terminals in Tertiary Plexus	100%		100%				0%		0%					

GABA-IR neurons were only found in significant density in the large intestine and were found to project both orally and anally to the circular muscle suggesting both inhibitory and excitatory control over intestinal motility (Sang et al., 1997). Indeed, a small number of the anally projecting neurons were also NOS-IR. CalR-IR neurons in the mouse innervate both longitudinal and circular muscle and project anally. Moreover, some CalR-IR neurons were also found to project locally to the circular muscle (Sang and Young, 1996; Sang et al., 1997).

This group also examined the orientation of inter-neurons in the mouse myenteric plexus and found that both in the small and large intestine, the NOS-, VIP-, CalB and 5-HT-IR neurons projected predominantly or exclusively anally. In the large intestine, most of the GABA-IR neurons also projected anally. Thus the majority of identified interneurons projected anally. In both regions, the SP-IR neurons projected orally and locally. In addition, the calretinin-IR neurons in both regions projected locally and the NPY-IR neurons in the small intestine also projected locally (Sang et al., 1997). This is a pattern previously demonstrated in guinea pig and may suggest that a larger diversity of neural transmission is projected anally to facilitate the major GI functional actions such as peristalsis. Moreover, the projection patterns for 5-HT throughout the mouse intestine were similar to those of both guinea pig and porcine ENS, while the polarity of VIP/NOS maintained a pattern which appears to be conserved across guinea pig, rat and human suggesting conservation of these neural patterns is important for mammalian ENS function in general (Sang et al., 1997).

Acetylcholine (ACh), considered an excitatory neurotransmitter in the ENS (Furness, 2008), was consistently co-localised with SP-IR neurons (all nerve terminals innervating muscle) in the small intestine, while as expected NOS/VIP-IR

neurons contained little or no ACh (none in the nerve terminals innervating muscle) supporting the view of these motor neurons as excitatory and inhibitory respectively within the small intestine of the mouse (Sang and Young, 1998). The large intestine on the other hand, due to the presence of GABAergic and CalR-IR non-cholinergic motor neurons, appears to be more complex (Sang and Young, 1998)(Table 1). Although most GABAergic neurons within the myenteric plexus appeared to be non-cholinergic, the majority of GABAergic nerve terminals in the circular muscle were indeed cholinergic, indicating an excitatory influence over migration complexes in that area (Sang and Young, 1998) (**Table 1.1**). The remaining population of GABAergic nerve terminals innervating the circular muscle may be non-cholinergic inhibitory neurons as a subpopulation of neurons was previously detected which also contained NOS (Sang and Young, 1996). Therefore the specific role of GABAergic neurons in the ENS remains unclear and due to their apparently more diverse nature relative to SP/VIP/NOS neurons, they likely play a more varied role in intestinal homeostatic regulation (Sang and Young, 1998).

While it was previously demonstrated that the majority of interneurons projected anally to other myenteric ganglia, this study clearly demonstrated a more wide-ranging population in comparison to motor neurons (Sang and Young, 1998). Although, no cholinergic VIP/NOS motor neuron terminals were detected in either small or large intestine, small subpopulations of cholinergic VIP/NOS nerve terminals were detected in the myenteric ganglia of both intestinal sections, indeed 15% of large intestinal VIP neurons were shown to be cholinergic (Sang and Young, 1998). Calbindin neurons also appeared to be relatively evenly divided between cholinergic and non-cholinergic. Although all 5-HT neuron cell bodies appeared to be cholinergic, this did not translate to nerve terminals as only around 50% and 60%

of 5-HT-IR nerve terminals in large and small intestine respectively, appeared to be cholinergic (Sang and Young, 1998). This observation was attributed to three possible reasons: nerve terminals arising from cell bodies not located in the myenteric plexus (e.g. submucosal plexus), potential problems with detection, and differences in the sub-cellular location of 5-HT and the cholinergic marker used (VChAT) (Sang and Young, 1998).

1.4.3.(b) Submucosal Plexus

Although not described in detail Sang and Young noted immuno-reactivity for NPY, VIP, SP and CalR nerves in the submucosal plexus of mouse small and large intestine (Sang and Young, 1996). Furthermore, immuno-reactivity for NOS and 5-HT was restricted to large and small intestine respectively. Interestingly, following separation from the myectomy, there was a loss of NOS-IR neurons from the submucosal plexus of the large intestine indicating that these may innervate the submucosal plexus from the myenteric plexus, a further indication of coordination between enteric plexi in this species (Sang and Young, 1996). In addition, the volume of cholinergic innervation of the submucosal plexus was demonstrated to be 40% and 20% in the small and large intestine respectively, and due to the presence of IR nerve fibres close to the mucosa, these were suggested to be secretomotor neurons (Sang et al., 1997).

Table 1.2. Distribution of neurotransmitter immuno-reactivity in the submucosal plexus of mouse intestine (Data adapted from Sang and Young, 1996)

Location	**Location in Neuron**	**Neurotransmitter**							
		SP	**CalR**	**CalB**	**NOS**	**VIP**	**NPY**	**GABA**	**5-HT**
Small Intestine	Cell Body	✓	✓✓	✓	×	✓✓	✓✓	×	-
	Fibres	✓✓	✓	✓	×	✓	✓✓	✓✓	-
	Fibers in perivascular plexus	×	✓✓	×	×	✓✓	✓✓	×	-
	Fibres in Mucosa	×	✓	×	×	✓✓	✓✓	-	-
Large Intestine	Cell Body	×	✓✓	✓✓	✓✓	✓	✓✓	×	-
	Fibres	✓✓	✓✓	✓✓	✓	✓✓	✓✓	✓✓	-
	Fibers in perivascular plexus	✓✓	✓✓	×	×	✓✓	✓✓	×	-
	Fibres in Mucosa	×	✓✓	×	×	✓✓	✓✓	-	-

The presence of Dogiel Type II AH neurons in the mouse submucosal plexus is uncertain, although the evidence thusfar appears to point against their existence.(Furness et al., 2004; Qu et al., 2008; Wong et al., 2008; Mongardi Fantaguzzi et al., 2009; Foong and Bornstein, 2013). With regard to the chemical coding of submucosal neurons, the most comprehensive study thusfar has been carried out in mouse ileum (Mongardi Fantaguzzi et al., 2009). In this study five groups of nerve cells in submucosal ganglia were described: cell bodies of VIP/NPY/CalR neurons (30%), VIP/NPY/CalR/TH neurons (20%), ChAT/CGRP/SOM neurons (30%; including a small sub-group of ChAT/CGRP/SOM/NPY neurons), neurons with ChAT and no other marker (about 10%) and neurons with neither ChAT nor VIP (~ 8%). VIP/NPY/CalR/TH neurons have been shown to innervate the mucosa while VIP/NPY/CalR innervated submucosal blood vessels suggesting their roles as secretomotor and vasodilator neurons respectively (Vanner and Macnaughton, 2004b; Furness, 2008). This would suggest that VIP is a primary neurotransmitter of secretomotor neurons, as has been demonstrated in other species, including human, rat and guinea-pig (Furness, 2008, 2012). CGRP/SOM/ChAT chemically coded neurons, which also innervate the mucosa are also likely to be the primary cholinergic secretomotor neurons as they share similar chemical coding to cholinergic secretomotor neurons in guinea pig (Furness et al., 2003). The functions of the remaining subgroups of neurons is not clear and will require further study.

Hence, three neuron types that account for most neurons in the submucosal ganglia of the mouse small intestine are: VIP-immunoreactive non- cholinergic secretomotor neurons (~30% of neurons), VIP- immunoreactive non-cholinergic

vasomotor neurons (~20% of neurons), and cholinergic secretomotor neurons, many of which also contain CGRP and somatostatin (~45% of neurons).

1.4.4 *The role of prominent submucosal secretomotor neurotransmitters in the regulation of mouse intestinal ion transport.*

1.4.4.(a) Summary

It is interesting to note that the same neurotransmitter systems (ACh/SOM and VIP/NPY) are expressed in secretomotor neurons across several mammalian species indicating their importance as primary neurotransmitters of mammalian secretomotor function (Furness, 2008). With regard to intestinal fluid and electrolyte transport both ACh and VIP stimulate ion and fluid secretion while SOM and NPY inhibit fluid and electrolyte secretion (Furness, 2008; Tough et al., 2011). In addition, it has been previously shown, at least for VIP/NPY, that these neuropeptides also co-localise within the same neurosecretory vesicles, suggesting they are released together (Cox et al., 1994). This likely represents the requirement for the enteric nervous system to tightly regulate fluid secretion and prevent excess fluid loss. Therefore, given the likely importance of these neurotransmitters in controlling intestinal ion transport, it is important to understand how they may impact on epithelial ion transport in the mouse. The following sections will briefly describe what is known about these major neurotransmitters in mouse.

1.4.4.(b) Acetylcholine(ACh)

The neurotransmitter, acetylcholine, is synthesised by the enzyme, actetyltransferase from the precursors acetyl-CoA and choline. Cholinergic receptors are divided into two major categories, the G-protein coupled mACh receptors, of which there are five

subtypes (M_1-M_5), and the ionotropic nicotinic, ACh receptors of which there are 5 subunits which can be made up of five subunits that may be in homomeric or heteromeric combinations (Hirota and McKay, 2006a)

Atropine, a mACh receptor antagonist, has been used extensively in Ussing Chamber to confirm cholinergic regulation of ion transport in several species, including mouse, which display a decrease in baseline I_{sc} (Diener et al., 1989; Chandan et al., 1991b; Sayer et al.,2002). nACh receptor also causes very small decreases in baseline I_{sc} and is consistent between species including mouse (Sun et al., 2000; Sayer et al., 2002). However, the majority of studies examining cholinergic regulation of ion transport have focused on cholinergic stimulation of ion transport (Hirota and McKay, 2006a). In particular, carbachol and bethanechol have been used as cholinergic agonist, which can stimulate Cl^- and fluid secretion resulting in a transient increase in I_{sc} (Hirota and McKay, 2006a). The accepted dogma in most species states that cholinergically driven ion transport is due to activation of M_1 mACh receptor on submucosal secretomotor neurons followed by activation of M_3 mACh receptors on intestinal epithelial cells (Hirota and McKay, 2006a). However, in mouse intestine, cholinergic regulation of ion transport is not well understood (Hirota and McKay, 2006b). In mouse small intestine cholinergic response were insensitive to neural blockade with TTx in the small intestine (Sheldon et al., 1989) while in the mouse large intestine, carbachol induced changes in I_{sc} were sensitive and insensitive to neural blockade in the mid and distal colon respectively (Carew and Thorn, 2000; Sayer et al., 2002). Bethanechol however, displays sensitivity to TTX in the distal colon of mice (Sagmanligil and Levin, 1993). However, a recent study has revealed that unlike most other species such as rat and guinea pig (Hirota and McKay, 2006a), bethanechol induced changes in I_{sc} are independent of epithelial

M_3 mACh receptors and may rely on M_1 mACh receptors in M_3 deficient mice (Haberberger et al., 2006; Hirota and McKay, 2006a).

1.4.4.(c) Vasoactive intestinal Peptide

VIP is a neuropeptide with wide distribution in the enteric nervous system of the mouse (Sang and Young, 1996, 1998; Sang et al., 1997; Mongardi Fantaguzzi et al., 2009), where it plays important regulatory role in many physiological processes (Furness, 2008). VIP may mediate its effects at two receptor subtypes (VPAC1 and VPAC2), which are members of class B receptors belonging to the super-family of GPCR (Couvineau and Laburthe, 2012). Activation of these receptors increases AC levels and causes increases in I_{sc} in intestinal mucosa due to cAMP driven ion Cl^- ion secretion (Schwartz et al., 1974). Furthermore, this response is sensitive to inhibition by NPY (Holliday et al., 2000; Hyland et al., 2003), supporting the idea that NPY plays an important regulatory role over VIP stimulated ion transport. Moreover, both neuropeptides co-localise within submucosal secretomotor neurons (Mongardi Fantaguzzi et al., 2009). In the mouse, I_{sc} responses to this neuropeptide in descending colon, VIP responses were insensitive to neural blockade, suggesting that VIP stimulation of electrolyte transport occurs at the level of the epithelium (Hyland and Cox, 2005).

1.4.4.(d) Neuropeptide Y

Recent studies in mouse colon have established a clear anti-secretory role for NPY (Y) receptors with regard to regulation of epithelial fluid and ion transport (Hyland et al., 2003; Cox, 2007). In the murine submucosa there are two receptors are involved in NPY regulation of intestinal transport, Y_1 receptors located primarily on

intestinal epithelial cells and Y_2 located pre-junctionally on enteric secretomotor neurons (Hyland et al., 2003). Both Y receptors are involved in intestinal anti-secretory tone, although NPY only mediates Y_2 receptor anti-secretory tone while peptide YY, another Y receptor ligand, is responsible for Y_1 mediated tone (Tough et al., 2011). However, with regard to regulation of stimulated epithelial ion transport, epithelial Y_1 receptors appear to play a more substantial role in limiting VIP induced increases in I_{sc} (Hyland et al., 2003). The anti-secretory effect is likely due to the fact that all Y receptors belong to a family of $G_{i/o}$ coupled receptors and thus inhibit intracellular AC production, which would in turn reduce cAMP induced chloride secretion by VIP (Michel et al., 1998). Although Y_2 receptors are considered to be mostly located on inhibitory secretomotor neurons, a residual Y_2 receptor mediated inhibition of ion transport is present following neural blockade with TTx suggesting the existence of non-neural Y_2 receptors in the murine colonic mucosa (Hyland et al., 2003).

1.4.4.(e) *Somatostatin*

Currently 5 somatostatin receptors (SSTRs) have been described SSTR 1-5 and similar to NPY receptors, SSTRs are also functionally coupled to the inhibition of AC via *pertussis* toxin sensitive G-proteins (Patel et al., 1994; Pepe et al., 2012). This appears to be the case in mouse colon as somatostatin was shown to inhibit cAMP dependant increases in I_{sc} in the Ussing Chamber following stimulation with either prostaglandin E2 or dibutyryl-cAMP. However, the same effect was not seen in duodenum suggesting that SOM may be more important in the regulation of stimulated ion secretion in the large intestine (Samson et al., 2000). Furthermore, in addition to inhibiting cAMP induced ion transport, SOM also inhibited carbachol

induced increases in I_{sc} in both mouse duodenum and the large intestine, further supporting the importance of this peptide in the regulation of cholinergic ion transport in the mouse (Samson et al., 2000). However, pre-treatment with TTX demonstrated that the effects of SOM were neurally independent with the exception of SOM inhibition of cholinergic induced ion transport in mouse colon (Samson et al., 2000).

Although the presence of SSTR 1-3 and 5 have has been detected in the murine intestinal mucosa (Wang et al., 2011), there are a lack of studies localising SSTRs in the mouse intestine. A recent study has demonstrated that SOM can increase electroneutral NHE8 ion exchanger expression, in the mouse small intestinal mucosa (Wang et al., 2011). Moreover, following addition to Caco2 cells, changes in NHE8 expression could be observed after one hour (Wang et al., 2011).

1.4.4.(f) *Gamma-aminobutyric acid*

GABA, long recognised as one of the major inhibitory neurotransmitters in the central nervous system, is now being acknowledged as a key regulator of ENS and neuroendocrine function (Krantis, 2000). GABAergic neurons in the mouse appear to be classified predominantly as inter-neurons (Sang et al., 1997), an observation which has been noted across several mammalian species examined (Krantis, 2000). In the ENS glutamate through decarboxylation of glutamate by the enzyme glutamic acid decarboxylase (GAD), is converted to GABA which can then generate physiological signals through interaction with GABA receptors (Krantis, 2000). GABA receptors are divided into two main types, ionotropic ($GABA_A$ and $GABA_C$) and metabatropic ($GABA_B$). $GABA_A$ and $GABA_C$ receptors are members of the superfamily of ligand gated ion channels (Chebib and Johnston, 1999).

$GABA_A$ receptors are hetero-oligomeric composed of a combination of α1-6, β1-4 and γ1-4 subunits, while $GABA_C$ receptors are homo-oligomeric consisting of ρ1 or ρ2 subunits (Chebib and Johnston, 1999). $GABA_B$ on the other hand are hetero-oligomeric seven transmembrane G-protein coupled receptors, composed of $GABA_{B1}$ and $GABA_{B2}$ subunits, which can activate Ca^{2+} and K^+ channels (Chebib and Johnston, 1999).

Recent studies have confirmed the presence of both $GABA_A$ and $GABA_B$ receptors in mouse mucosa and submucosal plexus respectively (Casanova et al., 2009b; Li et al., 2012a). When tagged with green fluorescent protein, the $GABA_{B1}$ receptor subunit was found to be highly expressed in submucosal neurons of both ileum and colon, however, no evidence of epithelial or enteroendocrine $GABA_{B1}$ expression was found in the mouse (Casanova et al., 2009b). Although other subunits have not been identified thus far, given that $GABA_B$ receptors form via heterodimerization, this would suggest $GABA_B$ receptors in the mouse mucosa and submucosa are primarily neuronal in nature (Takahashi et al., 2000a; Casanova et al., 2009b). This is in line with previous studies in myenteric plexus of mouse and other species indicating that they are pre-junctional in nature and are involved in inhibitory regulation of intestinal motor complexes (Chebib and Johnston, 1999; Sanger et al., 2002). However, with regard to intestinal ion transport, a role thusfar in any species, has yet to be fully elucidated (Hyland and Cryan, 2010).

Conversely, $GABA_A$ receptors, in the small intestine at least, have been demonstrated to influence mucosal ion transport in guinea pig, rat and mouse, however the mechanisms appear to differ between each species (Hardcastle et al.,

1991; MacNaughton et al., 1996; Li et al., 2012a). In the rat, $GABA_A$ antagonism with bicuculline caused a Cl^- and neurally dependant decrease in basal ion transport as measured by $I_{sc,}$ while GABA itself had no effect (Hardcastle et al., 1991). However, this effect was lost upon removal of the myenteric plexus indicating that myenteric rather than submucosal neurons are responsible for the $GABA_A$ mediated effects and may be important for coordination of enteric reflexes in this species (Hardcastle et al., 1991).

In guinea pig small intestine, both GABA and a $GABA_A$ receptor agonist caused a Cl^- dependant and neurally-dependant biphasic increase in I_{sc}, suggesting a role for $GABA_A$ receptor signalling in the regulation of mucosal fluid and electrolyte transport compared to rat (Hardcastle et al., 1991; MacNaughton et al., 1996). Indeed, the biphasic response was believed to be due to activation of cholinergic induced chloride transport during the first phase while an indirect stimulation of mucosal mast cell histamine release was proposed to account for a large proportion of the second phase. (MacNaughton et al., 1996). In the mouse, at least in the ileum, $GABA_A$ are expressed in the mucosa and apically on the intestinal epithelium where it was demonstrated they can directly mediate Cl^- efflux and fluid secretion due to their ionotropic nature (Li et al., 2012a). Moreover, this effect was demonstrated to be neurally independent, as pre-treatment with TTx did not prevent GABA stimulated intestinal fluid secretion (Li et al., 2012a)

1.5 The Impact of the host microbiota on ENS

The gut microbiota is a complex and dynamic ecosystem which contains ten times the the number of cells in the human body and consists of up to five hundred

different bacterial species. This "superorganism" which consists primarily of seven different phyla, most notably Bacteroidetes and Firmicutes, forms its own functioning genome, known as the gut microbiome, containing up to 3.3 million genes and is almost 150 times larger than its human counterpart (Fraher et al., 2012). These organisms live in symbiosis with the host and indeed are believed to play a critical role in host development and health (Lutgendorff et al., 2008; Sommer and Bäckhed, 2013). More recently it is becoming more and more evident that an abnormal gut flora contributes to alterations in the mucosa and local immune system leading to gastrointestinal disease (DuPont and DuPont, 2011). For example, disturbances in host-microbiota populations of Bacteroidetes and Firmicutes species due to anti-biotic treatment, can open the way for opportunistic enteric pathogens such as *Clostridium difficile* to cause infection and diarrhoea (Khoruts, 2014; Shankar et al., 2014). *Salmonella* and *Campylobacter infection* have also been associated with prior anti-biotic treatment(Effler et al., 2001; Varma et al., 2006). Moreover, an important aspect of susceptibility to these infections is old-age, which has also been associated with marked perturbations in normal enteric flora (Rampelli et al., 2013) further emphasising the link between microbiota and host. Interestingly *Clostridium difficile* associated diarrhea is highly amenable to a radical form of therapy known as fecal microbiota transplantation, which is believed to restore "healthy" populations of the host-microbiota (Petrof and Khoruts, 2014). Chronic inflammatory disorders of the gut such as IBD and IBS are also highly associated with disturbances in the host microbiota (Shanahan and Quigley, 2014). Indeed, IBS, a disorder which displays remarkable symptom heterogeneity, particularly disturbances in motility and ion transport, can be divided in to three subtypes

(Diarrheal, Constipation predominant and alternating IBS)- all associated with a characteristic host-microbe population (Parkes et al., 2012).

Given that functional GI disorders such as IBS are heavily influenced by the host-microbiota, it is reasonable to assume that alterations in host microflora alter ENS activity and function and studies on motility in germ-free (GF) animals have given strong support to this proposal (Caenpeel, 1989; Husebye, 1992, 1994, 2001). GF or gnotobiotic animals, usually rodents, are animals which have been bred in specific isolated and bacteria free conditions and thus contain no host-microbiota (Smith et al., 2007). Some of the earliest studies carried out in GF rats demonstrated a slower intestinal transit associated with a reduction in intestinal migrating motor complexes (MMC) responsible for propelling food along the gut, after meals and following fasting. (Dupont et al., 1965; Abrams and Bishop, 1967; Gustafsson and Norman, 1969; Van Eldere et al., 1988; Caenepeel et al., 1989). As MMC are influenced by the ENS innervating the intestinal muscle, these studies were some of the first to indicate a critical role for the host microbiota in the development and normal function of the enteric nervous system.

Moreover, when GF rats were exposed to conventional intestinal microflora the occurrence and spatial distribution of MMC were increased to the level observed in conventional rats after a period of 7-10 days (Husebye et al., 1992;1994). Furthermore, changes in neuropeptide Y expression were also observed indicating an effect on neurochemical coding (Husebye et al., 1994). Interestingly however, certain migratory patterns such as slow wave frequency were not reversed upon conventionalisation and this was suggested to be due to developmental maturity, indicating that timing of colonisation is important for complete and proper development of the motility function (Husebye et al., 1994). Another study carried

out by the same group examined the influence of specific bacterial species on motility in germ free rats (Husebye et al., 2001). It was found that primary metabolisers and anaerobic microbes, including *Bifidobacterium* and *Lactobacillus* have the most effect, whereas aerobic *E. coli* X7 has little or no effect (Husebye et al., 2001). Moreover, different microbes had differential effects on motility, while *Clostridium tabicum* n sp. VP 04 was most effective, a combination of *L. acidophilus* A10 and *B. bifidum* B11 was more effective than *L. acidophilus* A10 alone, which had little effect, demonstrating the strain dependant effects on enteric neural function (Husebye et al., 2001).

More recently the influence of the host microbiome in mice has been investigated (McVey Neufeld et al., 2013; Collins et al., 2014). McVey-Neufeld and colleagues examined the impact of the host microbiome on the electrophysiological parameters of jejunal myenteric neurons (McVey Neufeld et al., 2013). Although, no difference was observed for Dogiel Type I (S neurons), Dogiel Type II (IPANs) demonstrated significantly less excitability in comparison to specific pathogen free (SPF) and conventionalised mice (McVey Neufeld et al., 2013). This was interesting as previous studies in probiotics had demonstrated the strain specific ability of *L. rhamnosus* JB-1 to increase the excitability of IPANs in rats by acting on specific calcium dependent K^+ channel (Wang et al., 2010b, 2010c). In the latter study, the influence of the host microbiome on ENS development was examined at an early postnatal time period (P3) in mouse small intestine and major differences in myenteric plexus structure and chemical coding were observed (McVey Neufeld et al., 2013). The GF jejunum and ileum, but not duodenum, displayed abnormal morphology with unevenly spaced ganglia and thinner, less abundant connecting nerve fibers, and a significantly decreased neuron density in comparison to SPF mice

(McVey Neufeld et al., 2013). The number of cells per myenteric ganglia was decreased in both jejunum and ileum of germ free mice, the number of NOS-IR cells was increased in both segments and this corresponded with a decrease in circular muscle contraction frequency and amplitude in both segments (McVey Neufeld et al., 2013). Moreover, circular muscle contraction frequency and amplitude could be increased in GF jejunum, but not SPF, following nitrergic blockade, giving a further indication that over prominence of this signalling network may be responsible for decreased motility in GF animals (McVey Neufeld et al., 2013).

However, while our understanding of host microbial influences on myenteric ENS development is increasing, very little is known with regard to the influence of the host microbiota on intestinal secretomotor function. No studies thus far have directly examined the influence of the host microbiota on submucosal plexus structure and function, however, one study in pig has demonstrated the ability of a probiotic *Pediococcus acidilactici* to increase the number of CGRP-IR neurons in the submucosal plexus of the ileum (di Giancamillo et al., 2010). With regard to ion transport, however, in a study carried out in GF rat large intestine. This study focused on Na^+ transport and found significant increases in mucosal to serosal flux J_{ms} of Na^+ in the caecum and proximal colon corresponding with an increase in I_{sc} and decrease in tissue conductance were noted (Rösel and von Engelhardt, 1996). No differences in J_{ms} of Na^+ were observed in the distal colon, however, an increase in I_{sc} was observed only in the distal part of the distal colon. In the caecum and proximal colon, the changes in Na^+ transport were likely due to increased activity of apical electroneutral Na^+/H^+ activity (**Figure 1.1**) as amiloride treatment failed to alter the increased in I_{sc} while blocking of NaKATPase (a driving force for Na^+/H^+ activity) abolished net Na^+ fluxes (Rösel and von Engelhardt, 1996). In the distal

part of the distal colon, the increased I_{sc} was associated with a change from electroneutral Na^+ transport to primarily electrogenic transport as amiloride had significant effects on the increased I_{sc}. This study demonstrates the significant segmental differences in Na^+ transport, believed to be due to differences in aldosterone levels, in the large intestine and thus demonstrates how these differences can impact on host microbe interactions (Rösel and von Engelhardt, 1996). Moreover, it provides some of the first evidence that the enteric microflora can impact on intestinal secretomotor function and thus warrants further study into possible mechanisms.

1.6 Probiotic Secreted Bioactives

In the gut, bacteria, including the commensal host microbiome are separated from the intestinal epithelial wall by anti-bacterial peptides in the small intestine and by two layers of mucous in the large intestine (Johansson et al., 2011a, 2011b). Indeed the thick layer of nutrient rich mucous is essential for colonisation of commensal microbes in the large intestine (Liu et al., 2013). Therefore, given the lack of direct contact between commensal or probiotic microbes and the host intestinal wall, much attention has recently been focused on extracellular secreted bioactives which may mediate many of the beneficial host-microbe interactions with probiotics (Sánchez et al., 2010). Studies using cell free probiotic supernatants or conditioned media have discovered that peptides and other bioactive molecules secreted by the bacteria are responsible for many of the positive effects of probiotics in both pathogen induced and inflammatory bowel disorders (Sánchez et al., 2010)

L. rhamnosus GG ATCC 53103 is an extremely well characterised probiotic (Lebeer et al., 2012) which has demonstrated effects on intestinal barrier function

and health. As previously mentioned extracellular proteins produced by this probiotic have been shown to upregulate barrier function and TJ protein function in epithelial cells under normal conditions (Escamilla et al., 2012; Patel et al., 2012; Sultana et al., 2013). Moreover, some of these effects could be attributed to a secreted (Escamilla et al., 2012) and heat stabile protein (Patel et al., 2012). However the precise identity of these proteins remains elusive. Other studies have demonstrated protective effects of identified soluble factors from *L. rhamnosus* GG ATCC 53103 on barrier function and survival during inflammatory challenge (Tao et al., 2006; Seth et al., 2008; Yan et al., 2011). Identified proteins p40 and p75 can stimulate cell survival mechanism in the intestinal epithelial cells via epidermal growth factor receptor, activating Akt and via a PIK3 dependant and p38 ERK1/2 independent mechanism protecting against intestinal injury in inflammatory bowel conditions (Li et al., 2012a).

The production of self-produced antibiotics or bacteriocins is a relatively common trait among probiotics, in particular *Lactobacilli* (Dobson et al., 2012). Several lactobacillus species are known to produce bacteriocins including *L. rhamnosus* GG ATCC 53103 (Lu et al., 2009), which produce NPSRQERR and PDENK and *Lactobacillus salivarius* UCC 118 which produces the bacteriocin Abp 118 (Corr et al., 2007). Interestingly, although it is not believed that bacteriocin interact directly with the intestinal mucosa, a recent study has demonstrated that these factors may interfere with the ability of the probiotic to protect against H_2O_2 induced barrier dysfunction and change in TJ protein expression (Miyauchi et al., 2012b).

With regard to intestinal ion transport, thus far no bioactives have been identified, which impact directly on intestinal ion transport although several

probiotics appear to function through secreted bioactives (Lomasney and Hyland, 2013). CM from *B. breve* BBC50, could inhibit both carbachol and forskolin induced changes in I_{sc} via downregulation of PKC, while CM from both *L. acidophilus* ATCC 4357 and *L. rhamnosus* ATCC 53103 where able to increase Cl^-/OH^- exchange in Caco-2 IEC (Borthakur et al., 2008) (**Table 1.4**) could upregulate DRA expression acutely via a PI3K dependant mechanism (Borthakur et al., 2006) and chronically via upregulation of promotor genes (Raheja et al., 2010) *L. acidophilus* ATCC 4357 secreted factors, which are heat labile, can also increase Na^+/H^+ exchange via direct upregulation of NHE3 expression and function suggesting that the probiotic bioactives act on NaCl absorption (Singh et al., 2012). Furthermore, although a direct effect on intestinal ion transport was not demonstrated, CM from *Bifidobacterium infantis* Y1 was able to restore baseline and secretagogue induced changes in I_{sc} in mice with colitis, demonstrating the ability of secreted factors of this probiotic to protect against inflammation induced changes in ion transport (Ewaschuk et al., 2008). Again however, although the effects of these bioactives was associated with signalling via PI3K, possibly indicating effects on cell survival (Seth et al., 2008) and epithelial ion transporter expression (Borthakur et al., 2008), their identity and thus mechanism of action remains elusive.

1.7 Cytokines as prominent targets for probiotics modulation

1.7.1 *Indirect effects of commensal microbes on intestinal ion transport*

Microbes with the ability to modulate both pro- and anti-inflammatory inflammatory cytokines may be useful in the context of ulcerative colitis associated with hyper-secretion, such as those which target IFN-γ, TNF-α (Resta-Lennert and Barrett 2006) or Il-10 (Ewaschuk et al., 2008). However, a decrease in secretory responses has in general been observed in inflamed tissues (Sandle et al., 1990). Such observations are supported by studies demonstrating a reduction in the expression of CFTR in human colonic cell lines *in vitro* following exposure to IFN-γ and TNF-α, previously demonstrated amongst other cytokines to be increased in patients with inflammatory bowel disease (Hering et al., 2012). However, the data with respect to changes in CFTR expression are not consistent in this regard (Lohi et al., 2002;Sanchez et al, 2002; Bertelsen et al., 2004). Nonetheless, CFTR is not the only ion channel sensitive to inflammatory mediators, and both NKCC1 and NaKATPase also appear to be targets for the inflammatory cytokine, IFN-γ in human intestine, where it results in a down regulation in their activity (Bertelsen et al., 2004). As both these basolateral transporters are critical in influencing electrogenic Cl^- secretion, the influence of IFN-γ on their activity likely accounts for CFTR-independent effects (Bertelsen et al., 2004). Similarly, a study in T84 epithelial cells, in which IFN- γ reduces NaKATPase and NKCC1 expression support this observation (Sugi et al., 2001). It is perhaps not surprising therefore that probiotics such as *Lactobacillus acidophilus*, known to have protective effects in inflammatory bowel disease (Greenhill, 2011), have also been shown to prevent IFN-γ induced -

changes in ion transport (Resta-Lennert and Barrett, 2006). The apical channel, DRA has also been shown to be downregulated in ulcerative colitis patients and animal models of the disease (Yang et al., 1998), while expression of ENaC displays an insensitivity to aldosterone in human ulcerative colitis colonic biopsies (Amasheh et al., 2004). Here again, this may be due to the presence of the pro-inflammatory cytokines, TNF-α and Il-1β, both of which have been shown to downregulate ENaC in rat distal colon (Barmeyer et al., 2004). IFN-γ has also been shown to downregulate both NHE2 and NHE3 expression in rat intestine and human intestinal epithelial cells (Rocha et al., 2001). As such, microbes with the ability to upregulate specific ion channels or transporters, known to be decreased during inflammation, may be of benefit in restoring fluid and electrolyte homeostasis in inflammatory bowel diseases. For example, *Lactobacillus acidophilus* has been demonstrated to increase the expression of DRA *in vitro* as well as in mice (Raheja et al., 2010). However, and as has already been alluded to, the anti-inflammatory effects of microbes and probiotics are likely to indirectly influence intestinal and colonic ion transport.

With regard to specific diseases, animal models of colitis are associated with alterations in colonic fluid and electrolyte transport, I_{sc} and ion transporter expression and these alterations appear to be amenable to modulation by probiotics (Madsen et al., 2001; Ewaschuk et al., 2008) or indeed elements of the host microbiota (Ghosh et al., 2011). The probiotic mix, VSL-3(containing 9×10^{10} colony forming units (CFU) of bifidobacteria (*Bifidobacterium longum, Bifidobacterium infantis, and Bifidobacterium breve*), 8×10^{10} lactobacilli (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii* subsp. *Lactobacillus bulgaricus, and Lactobacillus plantarium*), and 20×10^{10} of

Streptococcus salivarius subsp. *Thermophilus*) partially recovered baseline I_{sc} and forskolin-induced Cl^- secretion in the IL-10 knockout mouse model of colitis (Madsen et al., 2001). Despite the ability of VSL-3 to reduce TNF-α and IFN-γ, recovery of baseline I_{sc}, and the diminished response to forskolin, were only observed in IL-10 knockout mice, suggesting that the effects of VSL-3 on I_{sc} may occur via an immune-independent mechanism (Madsen et al., 2001). *In vitro* exposure of T84 colonic epithelial cell monolayers to VSL-3 increased I_{sc}, and this effect appeared to be specific to VSL-3, as two unrelated bacterial strains (*Lactobacillus reuteri* and *Streptococcus bovis*) were unable to produce the same result in the T84 monolayers (Madsen et al., 2001). Though the precise mechanisms through which VSL-3 exerts its effects have yet to be fully characterised, there is evidence that one of the bacterial components of VSL-3, *Bifidobacterium infantis*, could potentially be responsible for the change in Isc induced by VSL-3, as *Bifidobacterium infantis* produced a recovery of baseline I_{sc} in IL-10 knockout mice (Ewaschuk et al., 2008). Indeed, bacterial-induced modulation of IL-10 has also been associated with changes in I_{sc}, as *Bifidobacterium breve*-induced reductions in I_{sc}, in a mouse model of hypersensitivity, were sensitive to neutralisation of IL-10, suggesting this cytokine either directly or indirectly reduces I_{sc} (Zhang et al., 2009). However not all bacteria exert similar effects in the same context, and *Lactobacillus salivarius* UCC118, in two models of experimental colitis, one of which was the IL-10 knockout mouse, had no effect on I_{sc}, but prevented an inflammation-induced increase in permeability (Feighry et al., 2008).

An elegant study by (Ghosh et al., 2011) demonstrated that inflammation induced changes in expression of the colonic ion transporter, DRA are sensitive to modulation by the microbiota. *Citrobacter rodentium*-induced colitis in susceptible

C3H/HeOuJ mice, resulted in a marked reduction in DRA gene expression relative to non-susceptible C57BL/6 mice (Ghosh et al., 2011). However, susceptible C3H/HeOuJ mice with the micribiota of a non-susceptible C57BL/6 donor displayed a significantly smaller change in expression of DRA following infection with *Citrobacter rodentium* (Ghosh et al., 2011). Given that mice infected with *Citrobacter rodentium* display severe dehydration, one could speculate that changes in key colonic ion transporters like DRA may account such an observation. These data not only suggest that the host microbiota may influence susceptibility to infection, but that it may also account for symptom severity through modulation of colonic ion transporters.

1.7.2 *Cytokine regulation of short circuit current*

Given the prominent role of cytokine modulation in mediating the effects of many probiotics, understanding the direct role of cytokines in regulating intestinal ion transport can give insight into the possible pathways through which probiotics take effect. Therefore, some of the prominent cytokines which have gained attention with regard to their ability to regulate both baseline and stimulated changes in I_{sc} will be discussed, thus giving insight into the possible role of cytokines in mediating probiotic induced responses observed in the Ussing Chamber (**Table 1.3**)

Interleukin-10 (IL-10) is one such cytokine which has received much attention due to its impact in inflammatory bowel disorders and which can also influence in electrolyte transport in the gut (Leach et al., 1999). Some of the first work demonstrating the impact of IL -10 on ion transport were carried out in the Ussing Chamber on rat small intestine (Madsen et al., 1996). Serosal but not

mucosal addition of exogenous IL -10 dose dependently decreased baseline I_{sc} in a neurally independent manner believed to be due an increase in net NaCl absorption (Madsen et al., 1996). IL -10 also increased cAMP, but not calcium induced changes in I_{sc} which correlated with a decrease in intracellular cAMP levels. However, although I_{sc} was increased, net Cl^- fluxes decreased following stimulation with either forskolin or carbachol and a residual secretory flux, believed to be due to bicarbonate secretion, remained. Therefore IL -10 induces its anti-secretory effects by decreasing cAMP-mediated Cl- efflux while increasing electroneutral NaCl absorption. In the absence of this cytokine IL $-10^{-/-}$ mice display a decrease in baseline I_{sc} and responses to forskolin in the colon (Ewaschuk et al., 2008).

Like IL -10, initial studies carried out in T84 intestinal epithelial cell cultures identified an anti-secretory function for IL -4 (Colgan et al., 1994). When exposed for 48h, Il-4 attenuated both carbachol and forskolin mediated increases in I_{sc} and this was believed to be independent to changes in barrier function suggesting a direct effect on trans-cellular ion transport (Colgan et al., 1994). Interestingly, a later study comparing the effects of IL-4 and IL-10 on T84 cells co-cultured with *Staphylococcus aureus* enterotoxin B (SEB) activated immune cells, demonstrated that IL-10 but not IL-4, when added to the co-culture, or to immune cells before co-culture and SEB activation, restored losses in forskolin stimulated ion transport as well as barrier permeability (Lu et al., 1998). This strongly suggests, when taken together with studies from IL-$10^{-/-}$ (Ewaschuk et al., 2008), that IL-10, during immunological compromise, plays a powerful immunologically mediated role in the restoration of ion transport, while IL-4 is ineffective and may play more direct role in homeostatic regulation of epithelial chloride ion transport (Colgan et al., 1994). Importantly, this study not only points to differential regulation of intestinal ion

transport by cytokines, but demonstrates that cytokine induced effects are situation dependent, relying greatly on environment, in particular the presence or absence, as well as the type of physiological stress (Madsen et al., 1996, 1999; Lu et al., 1998).

Indeed, IL-11 causes a decrease in basal I_{sc} in both rat small and large intestine (Greenwood-Van Meerveld et al., 2000). However, segmental differences were observed. In the colon, IL-11 induced changes in baseline I_{sc} were TTx sensitive and took 8 times longer than TTx insensitive responses in small intestine indicating a significant difference in cytokine regulation of ion transport between small and large intestine (Greenwood-Van Meerveld et al., 2000). The exact mechanistic effects of IL-11 are unknown, although studies in mouse stomach demonstrate a change in mRNA expression of genes corresponding to several major mucosal ion transporters also present in intestine, including an increase in CFTR mRNA levels (Howlett et al., 2012). This suggests IL-11 may act to modulate I_{sc} by regulating the activity and expression of key transporters involved in trans- epithelial intestinal fluid and electrolyte movement.

Indeed this appears to be the case for interferon gamma (IFN-γ), a pro-inflammatory cytokine highly implicated in the pathogenesis of IBDs, such as ulcerative colitis, which are associated with severe diarrhea (Binder, 2009). With regard to ion transport IFN-γ has been shown to down regulate both baseline and stimulated ion transport in several studies (Yoo et al., 2000; Rocha et al., 2001; Bertelsen et al., 2004). In these studies cAMP induced increases in I_{sc} are decreased following exposure to IFN-γ and this has been associated with a decrease in expression of CFTR (Resta-Lenert and Barrett, 2006), NKCC1 and NaKTPase (Sugi et al., 2001; Bertelsen et al., 2004). However, this cytokine has also been shown to

down-regulate regulate Na^+ coupled glucose transport (Yoo et al., 2000) as well as the expression of key transporters involved in fluid and electrolyte absorption, including NHE2/3 (Rocha et al., 2001). Moreover, the effects of IFN-γ on I_{sc} in mice, are also present in immuno-deficient mice suggesting that it's effects are not immunologically dependent (Yoo et al., 2000). Indeed it is believed that one of the main mechanisms for the effects of IFN-γ on ion transport is via rearrangement of F-actin filaments in epithelial cells thus disruption. This suggests that IFN-γ acts by decreasing ion transport function rather than targeting specific secretory or absorption processes. Indeed, mouse models of IBD, including the IL-$10^{-/-}$ mouse are associated with an increase in the levels of IFN-γ, and treatments to decrease this, including probiotics are associated with decreased levels of IFN-γ secretion (Ewaschuk et al., 2008).

Table 1.3 **Influence of cytokines on I_{sc} current**

Cytokine	Pro or Anti-inflammatory	Overall Effect	Isc responses	Molecular Changes	Model	Reference
IFN-γ	Pro	Anti-secretory	↓ baseline Isc ↓cAMP stimulated ΔIsc	↓CFTR expression ↓NKCC1expression ↓NaKATPase expression	CD-1 Mouse small intestinal primary culture Human fetal small intestine xenograft Rat small intestine Caco-2 IEC	Yoo et al., 2000; Rocha et al., 2001; Bertelsen et al., 2004
IL-1β	Pro	Pro-secretory	↑↑↑ baseline Isc	Increase in prostaglandin (PGE2) stimulation of Cl- and K+ secretion ↓ENaC expression and activity ↑NO release via neurkinin receptors???	Mouse colon Rabbit distal colon	Homaidan et al., 1995 Schumann et al., 2012 Eutamene et al., 1995
TNF-α	Pro	Pro-secretory	↑↑↑ baseline Isc	Increase in prostaglandin (PGE2) stimulation of Cl- and K+ secretion ↓ENaC expression and activity	HT-29 IEC Human Sigmoid Colon Rat Distal Colon	Kandill et al., 1994 Amasheh et al., 2004 Zeissig et al., 2008

IL -6	Pro	Pro-secretory	↑ baseline Isc ↑veratridine stimulated ΔIsc ↑bethancehol stimulated ΔIsc	Stimulation of cholinergic secretomotor neurons ↑cholinergic secretomotor activity	Rat	O'Malley et al., 2011
IL -4	NA	Anti-secretory	↑cAMP stimulated ΔIsc ↑carbachol stimulated ΔIsc	???	T-84 IEC	Colgan et al., 1994 Lu et al., 1998
IL -13	NA	Pro secretory	↑ baseline Isc ↑cAMP stimulated ΔIsc ↑carbachol stimulated ΔIsc	↑ CFTR activity and expression	Mouse jejunum	Wu et al., 2011
IL -10	Anti	Anti-secretory	↓baseline in conventional tissue ↑cAMP stimulated ΔIsc	Increase in NaCl absorption ↑HCO3- secretion ↑Na+ absorption ↓Cl- secretion ↓intracellular cAMP	Rat small intestine	Madsen et al., 1996 Ewaschuk et al., 2010
IL -11	Anti	Anti-secretory	↓ baseline Isc	Increase in CFTR	Rat Small and Large intestine	Greenwood-Van Meerveld et al., 2000 Howlett et al., 2012

In contrast to those discussed thus far, several cytokines have been known to cause an increase in $I_{sc.}$ Tumour necrosis factor alpha (TNF-α) regulates a variety of cell types involved in inflammatory responses and is also thought to be a critical cytokine involved in the pathogenesis of IBD (Binder, 2009). One of the first studies examining the effects of TNF-α on epithelial ion transport was carried out in porcine ileum mounted in Ussing Chamber (Kandil et al., 1994). Serosal application of TNF-α caused a chloride dependant increase in $I_{sc,}$ comparable in magnitude to prostaglandin E2 (PGE2) stimulated changes in I_{sc} and which was sensitive to indomethacin, an inhibitor of prostaglandin production (Kandil et al., 1994).

This effect was not be observed in T84 cell mono-layers in the absence of fibroblasts, which produce high levels of PGE_2, amongst other prostaglandins (Kandil et al., 1994). This strongly suggests that TNF-α acts indirectly via PGE_2, to increase baseline epithelial Cl^-efflux. Moreover, studies in HT-29 cells demonstrated no direct effect of TNF-α on basal I_{sc}, supporting an indirect regulation of epithelial ion transport by TNF-α (Schmitz et al., 1999; Gitter et al., 2000). In addition to stimulating Cl- ion transport, TNF-α can also decrease intestinal sodium absorption by downregulating apical ENaC activity and subunit expression (Amasheh et al., 2004; Zeissig et al., 2008). TNF-α mediated decreases in aldosterone stimulated sodium absorption in human sigmoid colon were accompanied by downregulation of γ and β ENaC subunits (Amasheh et al., 2004). Furthermore, similar results were observed in rat distal colon, where ENaC regulation is thought to be similar to that of human sigmoid colon, and the effect could be reversed following inhibition of ERK1/2, but not p38, MAPK (Zeissig et al., 2008). This suggests that TNF-α inhibits electrogenic sodium absorption by downregulating apical ENaC expression via an ERK signalling mechanism.

Finally, IL-6, has been shown to mediate colonic hypersecretion in rats via a cholinergic, neurally dependent mechanism (O'Malley et al., 2011). This effect is also dependent on downstream activation of ERK, JAK-STAT, and NF-κB signaling cascades. Moreover, IL-6 potentiated bethanechol and veratridine, induced changes in I_{sc} and depending on strain, capsaicin responses (O'Malley et al., 2011, 2012).

1.8 Direct effect of microbes on intestinal epithelial ion transport and ion channel, transporter or exchanger expression

1.8.1 Pathogen-induced effects on intestinal ion transport and ion channel, transporter or exchanger expression

Ussing chambers have proved to be a useful tool in understanding the way in which pathogens such as *Vibrio cholera* and *Escherichia coli* influence intestinal ion transport and fluid secretion (De et al., 1959). These pathogens secrete a variety of virulence factors such as *E. coli* heat stable/labile toxin, cholera toxin and *Shigella* toxin which have the capacity to disrupt intestinal ion transport, and aggressively activate ion secretion pathways resulting in excess fluid secretion.

Mechanistically, increased epithelial Cl^- transport is the most common target of bacterial toxins. Cl^- secretion typically occurs as a result of increased CFTR or calcium activated chloride channel (CaCC) activity. In the case of cholera toxin, CFTR activation is as a result of ADP-ribosylation of AC leading to increased intracellular cAMP, activation of PKA which, in turn, phosphorylates the CFTR regulatory domain resulting in increased Cl^- secretion (Kimberg et al., 1971; Sharp and Hynie, 1971). Similarly, *E. coli* heat stabile toxin increases cGMP via ribosylation of guanylate cyclase (Field M. 2003). CaCC on the other hand is

targeted by *Vibrio parahaemolyticus*, via a thermostabile direct hemolysin, though the precise mechanism are unclear (Takahashi et al., 2000a). Ion absorption is also disrupted by pathogens. For example, intestinal cells exposed to *E. coli* display increased expression of DRA through direct injection of a virulence factor into the cell the cytosol by a type 3 secretion system (T3SS) (Gill et al., 2007). These proteins disrupt intra-cellular microtubule networks thereby causing significant internalisation, and thus decreased cell surface expression of DRA (Gill et al., 2007).

In addition to its effects on Cl^- secretion, cholera toxin can also inhibit Na^+/H^+ exchange by both NHE2 and NHE3 via post-translational and post-transcriptional mechanisms respectively (Subramanya et al., 2007). In contrast, *E. coli* increases the activity of NHE2 (Hodges K. 2006), however in common with cholera toxin decreases NHE3 activity *in-vitro* in intestinal epithelial cells (Hodges et al., 2008). While the precise mechanisms involved are still under investigation, it is known that alterations in NHE3 exchange has greater implications with regard to diarrhoeal diseases (Gawenis et al., 2007) Finally, *E. coli* can also decrease intestinal SGLT-1 activity within 30 minutes by up to 85%, in human intestinal epithelial cells *in-vitro* though it is not currently understood how this occurs (Dean et al., 2006).

1.8.2 *Effects of probiotics and commensal organisms on intestinal ion transport and ion channel, transporter or exchanger expression*

While the mechanisms underlying pathogen-induced effects on intestinal ion transport have been relatively well characterised, the effects of commensal organisms or probiotics on the absorptive and secretory processes of the mammalian intestine are less well studied (Table 1). *Lactobacillus acidophilus* (LA), for example, has been shown to induce the expression of apical DRA exchangers both *in*

vitro, in Caco-2 intestinal epithelial cells, and *in vivo* in murine colon (Borthakur et al., 2008). In Caco-2 cells Cl^-/OH^- exchange was increased following a three hour treatment with LA, and this corresponded with increased expression of DRA. This effect was not specific to LA, and a similar response was also observed for *Lactobacillus reuteri*. Further characterisation of the LA-induced effect on Cl^-/OH^- exchange demonstrated that viable bacteria were required to alter Cl^-/OH^- exchange. Moreover, the LA effect was mediated via a secreted factor and via a phosphatidyl-inositol 3 kinase (PI3K)-dependent pathway (Borthakur et al., 2008). Following their initial findings, the same group subsequently analysed the long-term effects of LA on Cl^-/OH^- exchange in Caco-2 cells and in mouse colon. Following an eight hour treatment with LA, Cl^-/OH^- exchange was significantly increased as predicted from previous studies. This effect functionally correlated with increased, 4,4'-diisothiocyano-2,2'-stilbene disulphonic acid (DIDS)-sensitive, Cl^- uptake, and occurred in a concentration- and time-dependent manner (Raheja et al., 2010). At a genetic level, the LA-induced effect appeared to occur through transcriptional regulation, resulting in increased DRA promotor activity, and a resultant increase in both DRA gene and protein expression in mouse colon (Raheja et al., 2010) (**Figure 1.4**).

A combination of LA and *Streptococcus thermophilus* (ST) have also been demonstrated to influence the expression of key ion transporters, namely CFTR and NKCC1 (**Figure 1.4**) by preventing their down-regulation by IFNγ (Resta-Lennert and Barrett, 2006). As inhibition of PI3K functionally mimicked the effects of ST/LA on IFN-γ-induced reductions in forskolin-stimulated Isc in HT-29 cells, the authors speculated that PI3K may mediate the effects of ST/LA (Resta-Lennert and

Barrett, 2006). However, the biochemical pathways involved in the combined effects of LA and ST on the expression of CFTR and

NKCC1 were insensitive not only to inhibition of PI3K, but also to inhibitors of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK)1/2 (Resta-Lennert and Barrett, 2006). However, other bacteria such as *Bifidobacterium breve* Bb C50 (Bb C50) have more clearly defined biochemical pathways.

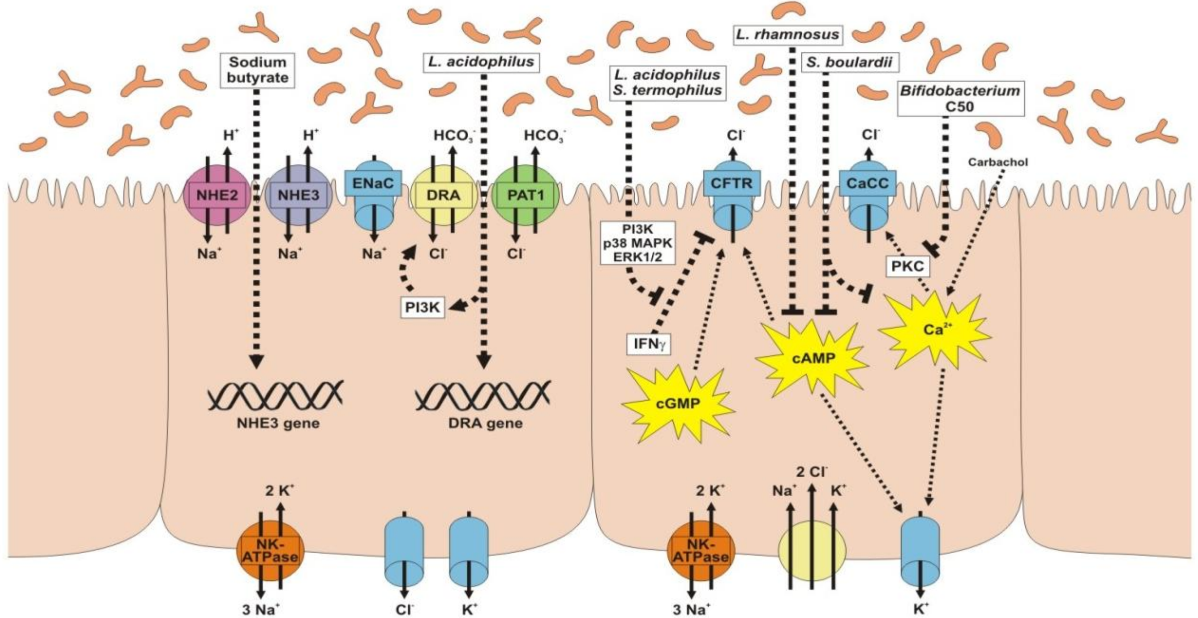

Figure 1.4. From left to right, sodium butyrate increases sodium hydrogen cation exchanger 3 (NHE3) promotor activity. Secreted factors from Lactobacillus acidophilus (LA) increase down-regulation in adenoma (DRA) promotor activity associated with an increase in mRNA and protein expression of DRA, and increases channel activity via a phosphatidyl-inositol 3 kinase (PI3K)-dependent pathway. Lactobacillus acidophilus/Streptococcus thermophilus restore interferon-γ (IFN-γ) induced inhibition of chloride (Cl–) secretion possibly through restoration of cystic fibrosis transmembrane conductance regulator (CFTR) involving PI3K/p38MAPK and ERK1/2 signalling. Lactobacillus rhamnosus attenuates cAMP induced Cl– secretion, while S. boulardi reduces cAMP and calcium-induced Cl– secretion. Bifidobacterium breve C50 reduces carbachol (CCh) induced Cl– secretion, possibly involving protein kinase C (PKC).

Decreased serine/threonine phosphorylation of important transcriptional regulators, namely inhibitors of NF-kappa-B alpha and p38 MAPK, have been identified as being responsible for the effect of Bb C50 on Cl^- secretion (Heuvelin et al., 2009). However, Bb C50 does not appear to alter CFTR expression (Heuvelin et al., 2010).

Nutrient transporters involved in the transport of electrolytes are also sensitive to modulation by microbes or probiotics, and this has been demonstrated to be the case for *Enterococcus faecum* with respect to Na^+/glucose transport and Na^+-coupled L-glutamine transport (Lodemann et al., 2006), and to a lesser extent for *Bacillus cereus* var toyoi (Lodemann et al., 2008). Fourteen days feeding with *Enterococcus faecum* increased glucose-stimulated Isc following mucosal addition

and appeared to enhance L-glutamine-stimulated Isc, though not significantly (Lodemann et al., 2006). In contrast, tissues from animals fed *Bacillus cereus* var toyoi displayed no significant difference in response to glucose or L-glutamine, however tissues from *Bacillus cereus* var toyoi -fed animals displayed a significantly enhanced I_{sc} response to stimulation with PGE_2 (Lodemann et al., 2008). Such studies highlight the ability of bacteria to selectively target specific nutrient transporters or PGE_2-signalling pathways.

Supporting the *in vivo* effects of *Enterococcus faecum* on glucose-stimulated Isc, *in vitro* studies with culture supernatants obtained from another bacterium, *Lactobacillus acidophilus*, dramatically increased glucose absorption by Caco-2 colonic epithelial cells following acute exposure (Rooj et al., 2010). This effect was sensitive to blockade of SGLT-1 with phloretin, while blocking GLUT glucose transporters had no effect, suggesting that SGLT-1 activity is selectively targeted by a secreted component of *Lactobacillus acidophilus*. However, other studies with *Enterococcus faecum* and two *Lactobacilli* (*Lactobacilus reuteri* and *Lactobacilus salivarus*), suggest that more gross structural changes in intestinal tissues, such as increased villus length, increased surface area or alterations in barrier function may account for increased glucose transport (Awad et al., 2009,2010; Scharek et al., 2005)

Table 1.4 Summary of microbial/probiotic effects on identified molecular targets and fluid and electrolyte transport

Microbe	Direct/ Indirect effect	Possible molecular target	Effect on Target	Effect on ion transport	References
Lactobacillus acidophilus	Direct	**1.** DRA **2.** CFTR **3.** NKCC1	**1.** Increased apical expression and activity **2/3** Protection from inflammatory mediated down-regulation	**1.** Increased $Cl^-/3.^-$ apical exchange. **2.** Restoration of secretagogue induced I_{sc}	Raheja et al., 2010. Borthakur et al., 2008. Resta-Lennert and Barret, 2006
Lactobacillus rhamnosus	Direct	DRA	Possible increased expression and activity	Possible increased apical Cl^-/OH^- exchange	Raheja G. et al., 2010
Streptococcus thermophilus	Direct	**1.** CFTR **2.** NKCC1	**1/2.**Protection from inflammatory mediated down-regulation	**1/2.**Restoration of secretagogue induced I_{sc}	Resta-Lennert and Barret, 2006
Bifidobacterium infantis	Indirect	Il-10	Possible upregulation of pathway	Restoration of baseline and secretagogue induced I_{sc}	Ewaschuk J.B. et al., 2008
Bifidobacterium breve	Indirect	Il-10	Possible activation or upregulation	Reduced allergy induced increases in I_{sc}	Zhang L.L. et al., 2009
Bifidobacterium breve C50	Direct	PKC	Decreased activity	Reduced carbachol induced I_{SC}	Heuvelin E. et al., 2010
Saccharomyces boulardii	Direct	**1.** PKC **2.** Nitric oxide pathway	**1.** Increased activity **2.** Pathway induction	**1/2.**Reduced calcium and cAMP induced increases in I_{sc}	Czerucka D. et al., 1994 Girard P. et al., 2003
Enterococcus faecum	Indirect	Na^+/Glucose transporters	unknown	Increase in glucose stimulated I_{sc}	Awad A. et al., 2009
Bacillus cereus var toyoi	Indirect	Na^+/Glucose transporters	unknown	Increase in glucose stimulated I_{sc}	Lodemann U et al., 2010
Echerechia coli F18	Direct	PKC/cAMP	Decreased activity	Decrease in basal and cAMP induced changes in I_{sc}	Ohland C.L. et al., 2012.

1.9 *The Influence of Microbes and Putative Probiotics on Short Circuit Current (Isc) and Epithelial Cell Signalling*

1.9.1 *The influence of probiotics on secretagogue induced Isc*

Microbes and probiotics can also influence secretagogue-evoked responses. For example, both Bb C50 and its culture medium inhibited carbachol-induced $I_{sc,}$ and similarly, though to a lesser extent, forskolin-induced Isc in human intestinal epithelial cells (HT29-19A) (Heuvelin et al., 2010). In the same study *Lactobacilus rhamnosus* was also shown to reduce forskolin- but not carbachol-induced increases in I_{sc}, suggesting a species-specific effect of Bb C50 on calcium induced ion transport (Heuvelin et al., 2010). Moreover, the effect of BB C50 on carbachol-induced changes in I_{sc} correlated with a decrease in protein kinase C (PKC) activity, which may in turn decrease CFTR channel activity and subsequently I_{sc} (Akabas, 2000). In contrast forskolin-induced protein kinase A (PKA) activity was unaffected by Bb C50, suggesting a PKA-independent pathway may be responsible for the decrease in Isc in response to forskolin in the presence of Bb C50.

Like Bb C50 and *Lactobacillus rhamnosus*, the yeast, Saccharomyces *boulardii* (*Sb*), also affects epithelial cAMP signalling. In human colonic epithelial cells (T84), *Sb* attenuates both receptor- and non-receptor mediated increases in intracellular cAMP, and subsequent serosal to mucosal flux of ^{125}I, representative of Cl^- ion transport (Czerucka & Rampal, 1999). The reduction in calcium-mediated responses in the presence of conditioned media from *Sb*, however, occurred independent of a decrease in $Ins(1,4,5)P_3$, and therefore the authors speculate that *Sb* may induce the expression of negative regulators of calcium-induced secretion (Czerucka & Rampal, 1999). Consistent with such effects been partially receptor-

mediated, previous studies, by the same group, demonstrated that the *Sb*-induced inhibition of stimulated cAMP displayed sensitivity to pertussis toxin, implicating the involvement of G protein coupled receptors in mediating the effect of *Sb* (Czerucka et al., 1994). *In vivo* studies with *Sb* support the hypothesis that the yeast may have anti-secretory properties. Following administration of *Sb* for eight days to pigs, intestinal baseline Isc was lower, as were theophylline-induced increases in Isc, and these effects were accompanied by decreased Na^+ absorption and Cl^- secretion respectively (Schroeder et al., 2004.). However, 16 days following administration such effects were no longer apparent, suggesting that further adaptation may occur following longer term administration of the yeast.

1.9.2 *Probiotics can prevent adverse pathogen induced changes in intestinal ion transport.*

Given that a number of beneficial microbes have been functionally characterised with respect to their effect on intestinal ion transport, their application to counter pathogen-induced changes in intestinal fluid and electrolyte secretion have also been examined. In particular, a *Sb*-derived 120kDa heat labile protein inhibits cholera toxin (CT)-induced Cl^- secretion in IEC-6 intestinal epithelial cells (Czerucka et al., 1994). Subsequent studies using S*b* further supported the functional significance of this finding *in vivo*, and demonstrated an anti-diarrhoeal effect of *Sb* in a castor oil-induced rat model of diarrhoea (Girard et al., 2003). Moreover, both a pig model of *Escherichia coli* Abbotstown-induced diarrhoea and Caco-2 intestinal epithelial cells exposed to pathogenic *Escherichia coli*, displayed sensitivity to the probiotics *Escherichia coli* nissle (Schroeder et al., 2006) and *Lactobacillus plantarum* 299V respectively (Michail et al., 2002). However, neither of these studies provided a mechanistic explanation for the observed effects on diarrhoea or

I_{sc}, though the effects of *Lactobacillus plantarum* 299V are thought to occur via competitive inhibition of *Escherichia coli* rather than via direct effects on intestinal ion transport (Michail et al., 2002). Similar studies have also demonstrated the ability of *Steptococcus thermophilus* and *Lactobacillus acidophilus* to prevent enteroinvasive *Escherichia coli* (EIEC)-induced increases in basal Cl^- secretion, however neither probiotic had a significant effect on baseline I_{sc} *per se* (Resta Lennert and Barrett 2003). Whether these probiotics act via competitive inhibition, directly through stabalising certain ion transporters, and/or indirectly through associated associated signalling pathways is unknown and likely depends on the specific strain (**Figure 1.4**)

A timely study has however provided an interesting and comprehensive bacterial strain comparison using several strains of both pathogenic (EPEC), non-pathogenic (*Escherichia coli* F18), mutant (*Escherichia coli* Δescn (type III secretion virulence factor deleted), commensal (*Escherichia coli* C1 and C28/2) and probiotic bacteria (*Escherichia coli* Nissle 1917) to shed some light on the mechanisms through which pathogenic and non-pathogenic bacteria may differentially regulate intestinal ion transport (Ohland et al., 2012). Hyporesponsiveness to forskolin *in vitro* in intestinal epithelial cells (T84) was a characteristic feature for all strains, including enterohaemorragic *Escherichia coli* (EHEC; Ohland et al., 2012). Functionally, this initial inhibition of forskolin-induced I_{sc} may be important for facilitating bacterial adhesion to the intestinal mucosa, however, conversely, some EPEC caused an initial increase in baseline I_{sc} *in vitro*, and this may represent a protective physiological response to prevent EPEC adhesion (Ohland et al., 2012). Despite EPEC initially inducing an increase in I_{sc}, both EPEC and EHEC ultimately resulted in a hyposecretory state, the mechanisms

underlying which varied between pathogenic and non-pathogenic strains (Ohland et al., 2012). Both EPEC and commensal (F18) bacteria appeared to cause this hyposecretion downstream of cAMP production; whereas EPEC caused mislocalisation and thus reduced activity of apical CFTR (Ohland et al., 2012). It is suggested that intra-cellular signals known to regulate CFTR activity may also be involved in mediating the hypo-secretion induced by pathogenic and non-pathogenic bacterial strains used by (Ohland et al., 2012), such as PKC/PKA.

1.10 Probiotics and the Intestinal Epithelial Barrier

1.10.1 *Summary*

The regulation of the gut barrier is critical to successful maintenance of a healthy relationship between the host and its enteric inhabitants, the host microbiota . Under certain conditions such as infections or stress, the integrity of the epithelial barrier is compromised and para-cellular permeability to non desirable luminal content , such as pathogenic bacteria is increased, allowing access to sites where direct interaction with immune cells can occur (Gareau et al., 2006, 2008; Catalioto et al., 2011). Moreover enhanced ENS cholinergic signalling is believed to play a major role in intestinal permeability implying a link between increased barrier permeability and ENS dysfunction (Cameron and Perdue, 2007; Gareau et al., 2007) Indeed, increased para-cellular permeability is believed to be major underlying factor in the pathogenesis of functional GI disorders such as IBS where a leaky gut causes over exposure of sensory neurons to luminal antigens (Piche et al., 2009; Piche, 2014).

The intestinal barrier itself is a complex defensive system which consists of a tight epithelial cell wall, a mucous layer, which varies in thickness along the

intestine, active immunoglobulins, in particular immunoglobulin A, and epithelial produced anti-microbials (McGuckin et al., 2009). In addition to protecting against invading pathogens, the intestinal barrier, in particular the epithelial cell layers is critical for maintaining efficient trans-cellular ion transport. Cell-cell adhesion proteins are critical in this regard, as they maintain the structural integrity of the epithelium, and thus the osmotic electrical gradients vital for ion transport regulation. The most well characterised of these are the tight junction (TJ) protein complexes which are composed of several transmembrane proteins and include, occludins, several members of the claudin family as well as scaffolding proteins which include the zonula occludens (ZO) family,ZO1-3 (Tsukita et al., 2001) The occludins and claudins are major components of the para-cellular junctional complex, forming a barrier which is selectively permeable to several small molecules and ions including Cl^- (**Figure 1.5**, Hartstock et al., 2008). This selective permeability is made possible by several members of the claudin family which have can directly regulate passive transport of ions and nutrients via an extracellular loop domain, with selectivity for cations (Hartstock et al., 2008). The scaffolding proteins (ZO-1-3), as suggested by their name, provide a structural link for the main transmembrane proteins of the junctional complex to the actin cytoskeleton of the intestinal enterocyte (**Figure 1.5**; Hartstock et al., 2008).

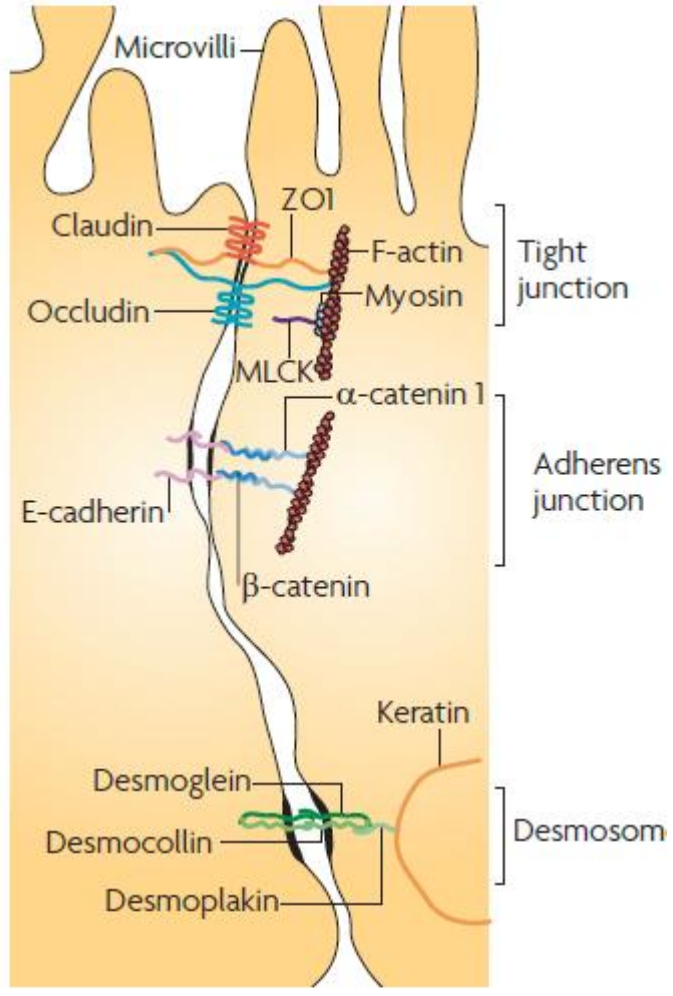

Figure 1.5. Transmembrane proteins occludin and claudin seal the paracellular space between adjacent epithelial cells and claudins in particular allow for selective transport of small molecules such nutrients and cations through the paracellular space. The ZO family proteins, act as adaptors that connect transmembrane proteins to the actin cytoskeleton of the enterocyte. (Image adapted from; (Turner, 2009)

1.10.2 *Interactions between the microbiota and Tight Junction Proteins*

Various pathogens, including *E. coli*, have been shown to affect TJ proteins indirectly via phosphorylation of the myosin light chain, a key component of the contractile actomyosin ring which interacts with TJ complexes (Scott 2002). However, pathogens such as *Shigella flexneri, Salmonella enterica serovar Typhimuriu* can also directly interrupt TJ proteins by altering phosphorylation states and overall distribution of TJ proteins, such as occuldin and ZO1(Kohler 2007,

Boyle,. 2006).In contrast, however, probiotics have been demonstrated to have a positive influence with regard to maintenance and protection of the epithelial barrier, and do so by a number of mechanisms including increasing secretion of mucous, anti-microbials such as beta defensins and altering tight junction proteins (Ohland and Macnaughton, 2010a).

Most studies to date have implied a protective role for probiotics against pathogen or inflammatory induced changes in TJ protein expression, activity and organisation. For example, several species of *Lactobacillus* including *L. salivarius* UCC 118 are capable of protecting against hydrogen peroxide induced disruption in barrier function and TJ membrane localisation, and this ability is inversely related to bacteriocin production (Miyauchi et al., 2012a). Furthermore a recent study has demonstrated powerful potential probiotic treatment of barrier dysfunction. This study demonstrated the ability of a probiotic, *B. fragilis* NCTC 9343, previously effective in correcting model colitis (Mazmanian et al., 2008), to reverse barrier permeability in the maternal immune activation (MIA) model, a model of autism spectrum disorders (Hsiao et al., 2013). Moreover reversal was also associated with amelioration of MIA induced changes in TJ proteins, claudins 8 and 15. Such studies highlights the broad impact of barrier modulating probiotics and emphasise the need for further investigation of therapeutic potential. However, although much is known about the potential for probiotics to protect against disease induced changes in TJ function and expression, the effects of probiotic on normal TJ function is relatively underappreciated and more recent studies have begun to show the ability of probiotics to directly interact with and modulate TJ proteins in the absence of physiological insults (**Table 1.5**).

One of the first studies to examine direct interactions of probiotics with TJ proteins examined the effects of *Lactobacillus acidophilus* ATCC4356 and *Streptococcus thermophilus* ATCC1925 on barrier function in Caco-2 intestinal epithelial cells demonstrating differential effects when using the probiotics in combination rather than separately (Resta-Lenert and Barrett, 2003). When used separately, both probiotics induced an increase in TER after 12 hours and up to 24 hours, however, when used in combination, a larger increase in TER was observed after only 6 hours and the effect also persisted after 24 hours indicating a lasting change in para-cellular permeability and barrier integrity (Resta-Lenert and Barrett, 2003). The increase in TER following the probiotic combination was associated with an increase in phosphorylated ZO-1 and Occludin. Interestingly, although pre-treatment with *L. acidophilus* ATCC4356/*S. thermophilus* ATCC1925 combination prevented a pathogen induced down regulation of actinin phosphorylation, the probiotics had no effects on actinin in non-infected cells (Resta-Lenert and Barrett, 2003). Furthermore, as pre-treatment was required for the protective effects of the probiotics this suggests that, the protection of actinin phosphorylation was likely due to direct microbe-microbe interaction (possibly competitive antagonism), while the effects on ZO-1 and Occludin-1 were due to direct host-microbe interactions between the probiotics and the intestinal cells. Therefore, the importance of studying baseline effects of probiotics is evident to distinguish direct effects of probiotic host-microbe interactions and thus establish the mechanisms involved. Furthermore, as will be highlighted by the following studies, the importance of examining individual strains is crucial.

A recent study examined the influence of several *Lactobacillus* strains on barrier function and TJ proteins in T84 IECs, demonstrating strain specific

influences (Hummel et al., 2012). All probiotic strains used , *L. acidophilus* PZ 1041, *L. gasseri* PZ 1160, *L. fermentum* PZ 1162, and *L. rhamnosus* PZ 1121 increased TER. The increase in TER correlated with an increased expression of E-cadherin following incubation with *L. acidophilus* PZ 1041, *L. gasseri* PZ 1160, *L. fermentum* PZ 1162, but not *L. rhamnosus* PZ 1121 while an increase in activated β-catenin, was induced in particular by *L. acidophilus* PZ 1041 and *L. rhamnosus* PZ 1121 (Hummel et al., 2012). Moreover, membrane expression of activated PKC-δ, which can bind to E-cadherin and disturb the AJ complex, was decreased by all four Lactobacillus strains suggesting this is a possible probiotic target. Interestingly, in contrast to the gram negative strain *E coli* Nissle 1917 which augmented ZO-2 protein expression likely through a decreased activation and expression of PKC-ζ (Zyrek et al., 2007), little or no upregulation of ZO-2 was observed following *Lactobacillus* strains in this study, while *L. acidophilus* PZ 1041 even caused a decreased in ZO-2 protein expression (Hummel et al., 2012). Together, these results highlight the impact of specific extracellular envelopes on host-microbe interactions and though a clear difference in molecular targeting between gram-postive and gram-negative probiotics is apparent, strain differences are also seen between strains of the same species.

L. rhamnosus GG ATCC 53103 is an extremely well characterised probiotic and has been noted for its positive and protective immuno-modulator effects through secretion of effector proteins (Yan et al., 2011). A recent study in neonatal C57/BL6 mice demonstrated that *L. rhamnosus* GG ATCC 53103 could accelerate small intestinal barrier maturation and this was associated with an increase in Claudin-3 protein expression (Patel et al., 2012). Interestingly, while the bioactive involved was heat resistant up to 70°C, heat killing the bacteria at this temperature prevented

cytotoxic effects associated with a high dose of probiotic while maintaining the positive effects on barrier maturation and TJ protein expression (Patel et al., 2012). This suggests that bioactive molecules produced by probiotics may be isolated and used in the absence of live bacterial cultures thus avoiding dangers associated with administering live bacterial cultures to hosts with compromised barrier and immune function (Liong, 2008). *L. rhamnosus* GG ATCC 53103 has also been shown to upregulate tight junction proteins in human colonic epithelial cells via an unidentified secreted molecule and increased TER and TJ protein expression in human epidermal skin cells in a strain specific manner independent of Toll Like Receptor (TLR) 2 signalling (Escamilla et al., 2012; Sultana et al., 2013)

In contrast to *L. rhamnosus* GG ATCC 53103, *Lactobacillus plantarum* WCFS1 has been shown to increase apical but not cytoplasmic expression of Occludin and ZO-1 in both human duodenal biopsies and Caco-2 cell mono-layers, and the increase in Occludin could be recapitulated using a TLR-2 agonist suggesting this as a possible molecular target of this probiotic strain (Karczewski et al., 2010).

Lactobacillus plantarum MB452 is probiotic used in the probiotic mix VSL#3 which has previously shown efficacy for the treatment of pain, TJ proteins disruption and barrier dysfunction in inflamed intestine (Madsen, 2001; Schlee et al., 2008; Dai et al., 2012). When isolated and exposed to T84 cell mono-layers, *Lactobacillus plantarum* MB452 induced an increase in TER and increased protein expression of ZO-1, ZO-2, Occludin and cingulin (Anderson et al., 2010). Furthermore, gene expression analysis demonstrated an up-regulation and downregulation respectively of genes associated with tight junction stability and disassembly (Anderson et al., 2010). Moreover, genes altered in this study differed

from those altered in previous studies using probiotic mix, VSL#3, such as decreasing inflammatory cytokines (Jijon et al., 2004; Schlee et al., 2008) indicating the specific effects of each probiotic.

Conditioned medium (CM) from *Bifidobacterium infantis* Y1, used in the probiotic mix VSL#3 (previously demonstrated to enhance epithelial barrier function (Madsen et al., 2001)) demonstrated a superior ability to increase TER in T-84 cells following 4 hours of incubation (Ewaschuk et al., 2008). This was associated with decreased paracellular permeability to mannitol and correlated with an increase in Occludin, ZO-1, Claudin 1,3 and 4 expression (Ewaschuk et al., 2008). Moreover, when tested in Il-10$^{-/-}$ mouse model of colitis, CM from this probiotic restored barrier function (TER) and this was associated with a restoration of baseline and forskolin induced changes in I_{sc} (Ewaschuk et al., 2008). This suggests that restoration of barrier function through modulation of tight junction proteins also plays an important role in the ability of this probiotic to protect against loss in epithelial ion transport function, although effects on trans-cellular ion transporters cannot be discounted (Resta-Lenert and Barrett, 2006).

Two other strains have been demonstrated to have direct effects on TJ proteins. Caco-2 cell mono-layers exposed to cell-free supernatants of *Bifidobacterium lactis* 420, demonstrated an increase in TER as well as a modification of TJ proteins associated with a change in cyclo-oxygenase activity (Putaala et al., 2008). Finally, although carried out in human epidermal keratinocytes, *Bifidobacterium longum* ATCC 51870 was able to strain dependently increase TER after 24 hours incubation and this correlated with an increase in the expression of ZO-1, Occludin, Claudins 1 and 4 (Sultana et al., 2013). Moreover, this effect appeared to be TLR-2 mediated as, pre-treatment of the cells using a TLR-

2 neutralising anti-body abolished the effect of the probiotic on TER (Sultana et al., 2013). Peptidoglycan, a known TLR-2 agonist and known to be expressed on the cell surface of gram positive bacteria, a may have been partially responsible for effect of *Bifidobacterium longum* ATCC 51870, as this peptide also induced an increase in TER but did not appear to alter TJ protein expression (Sultana et al., 2013).

Finally, *E. coli Nissle 1917,* one of the earliest identified probiotic strains and also one of the most studied (Nissle, 1916; Jacobi and Malfertheiner, 2011), has been demonstrated to have positive effects in both pathogen induced and inflammatory bowel disorders (Jacobi and Malfertheiner, 2011). However, recently the effects of this species on baseline TJ protein fuction have been tested in human and mouse intestinal cell monolayers (Ukena et al., 2007; Zyrek et al., 2007; Veltman et al., 2012; Hering et al., 2013). In comparison to gram positive Lactobacillus strains which target PKC-δ and upregulate AJ protein complexes in T-84 IEC's (Hummel et al., 2012), *E. coli Nissle 1917,* a gram negative bacteria, exposed to the same cell line, appears to target PKC-ζ causing, increasing its membrane expression but decreasing phosphorylation and thus activity resulting in increased TER correlated with increased ZO-2 expression and TJ localisation (Zyrek et al., 2007). In contrast, although increases in ZO-1 mRNA and protein expression and decreased permeability were observed in murine IEC isolated from germ free mice, no changes were observed in ZO-2 or TER, (Ukena et al., 2007). Although this may indicate a role for the host-microbiota in regulating host responses to *E. coli Nissle 1917*, given that previous effects were observed in T-84 IEC's, devoid of functioning microbiome, the differential effect is likely due to methodology as cells were isolated from whole tissue following *in-vivo* treatment with the probiotic (Ukena et al., 2007). Interestingly, although no effects were observed on baseline $I_{sc,}$ *E. coli*

Nissle 1917 restored sodium absorption in germ free mice with DSS induced colitis demonstrating that the positive impact on barrier function can also impact ion transport (Ukena et al., 2007).

In slight contrast to these studies, a more recent study has managed to identify a secreted bioactive factor produced by *E. coli Nissle 1917* which positively modulates barrier integrity and TJ protein expression (Hering et al., 2013). HT-29/B6 IEC's were treated with supernatant from *E. coli Nissle 1917,* which resulted in an increase in TER, and Claudin-14 with no changes in ZO-1, Occludin, Claudin 1-5, 7,8,10 or baseline I_{sc} (Hering et al., 2013). In divergence from previous the previous studies, this effect was mediated through increased phosphorylation and activity of PKC-ζ as well as ERK1/2, and this effect was lost in *E. coli Nissle 1917* mutants lacking TcpC protein (Hering et al., 2013). As previous studies used live bacterial cells, this may suggests that isolation of secreted bioactives from *E. coli Nissle 1917* can produce different effects in the absence of the cell, similar to *Lactobacillus rhamnosus GG ATCC* 53103 (Patel et al., 2012), demonstrating the importance of fully elucidating the bioactive content and activity of individual probiotic strains.

However, as this experiment was carried out in a different cell line, further studies are required to verify the differential effects of *E. coli Nissle* 1917 and its extracellular bioactives on intestinal function. Overall, these studies demonstrate the ability of probiotics to directly modulate TJ protein function and barrier function, including ion permeability and transport function, in healthy tissue, warranting further investigation of probiotic effects on baseline intestinal barrier function.

Table 1.5. Direct Effects of probiotics on TJ function in normal conditions

Probiotic	Physical Effect	Signalling Molecule	Signalling Mechanism	Model	Reference
Bifidobacterium infantis Y1 (VSL#3)	↑ TER ↓ paracellular permeability (to mannitol) ↑ Occludin, ZO-1, ↑ Claudin 1,3 and 4 ↓ Claudin 2	Unidentified (Present in cultured supernatant)	↑ ERK1/2 phosphorylation ↓p38 phosphorylation	T84 (Human IEC)	Ewaschuk *et al.*, 2010
Bifidobacterium lactis 420	↑ TER	Unidentified secreted metabolite	Unknown	Caco-2 (Human IEC)	Putaala *et al.*, 2008

Bifidobacterium longum ATCC 51870	↑ TER ↑ZO-1, Occludin, Claudins 1 and 4	May be partially mediated by peptidoglycan	TLR-2 mediated	Normal human epidermal keratinocytes (NHEK)	Sultana *et al.,* 2013
Escherichia coli Nissle 1917	↑ TER, ↑ZO-2 expression and TJ localisation	Unidentified	↑PKC-ζ expression ↓PKC-ζ phosphorylation (↓ activity)	T84 (Human IEC)	Zyrek *et al*., 2006
	↑ ZO-1 expression (non-DSS treated) No changes in TER ↓ permeability (to Evans blue) ↓	Unidentified	Unknown	Isolated IEC from DSS treated Germ free Balb/c	Ukena *et al.,* 2007

	inhibition of sodium absorption			mice	
	↑ TER, ↑ Claudin-14 expresson. ↓ permeability to Na+ and Cl- (No influence on ZO-1/2)	TcPC **(Present in cultured supernatant)**	↑PKC-ζ phosphorylation (↑ activity) ↑ERK 1/2 phosphorylation (↑activity)	HT-29/B6 (Human IEC)	Hering *et al.,* 2013
Escherichia coli Nissle 1917	↑ TER (TJ proteins??)	Unidentified	Differential regulation of miR-203, miR-483-3p, miR-595.	T84 (Human IEC)	Veltman *et al.,* 2012
Lactobacillus acidophilus *ATCC4356 /Streptococcus*	↑ TER, ↓ permeability;	Unidentified heat inactivated bacterial	↑ occludins, ZO-1, ERK 1/2	Caco-2 (Human IEC)	Resta-Lennert

thermophilus ATCC1925		cell factor	phosphorylation (↑activity)		and Barret, 2003
Lactobacillus acidophilus PZ 1041	↑TER, ↑E-cadherin expression	Unidentified (Possibly Gram positive dependant)	↓PKC-δ membrane expression ↑β-catenin phosphorylation (↑activity)	T84 (Human IEC)	Hummel *et al.*,2012
Lactobacillus casei ATCC 334	↑ZO-1	Secreted molecule ≥ 100kDa	Unknown	Human colon carcinoma cell line HCT-116	Escamila *et al.,* 2012
Lactobacillus fermentum PZ 1162	↑TER, ↑E-cadherin expression	Unidentified (Possibly Gram positive dependant)	↓PKC-δ membrane expression	T84 (Human IEC)	Hummel *et al.*,2012

Lactobacillus gasseri PZ 1160	↑TER, ↑E-cadherin expression	Unidentified (Possibly Gram positive dependant)	↓PKC-δ membrane expression	T84 (Human IEC)	Hummel *et al.,*2012
Lactobacillus plantarum MB452 (VSL#3)	↑TER, ↑ZO-1 and 2, Occludin and cingulin expression among others (See ref. for full list) ↓ expression of genes associated with barrier degredation (See ref. for full list)	Unidentified	Unknown	Caco-2 (Human IEC)	Anderson *et al.,* 2010
Lactobacillus plantarum WCFS1	↑Apical Occludin and	Unidentified but not present in conditioned	Possibly TLR-2	Healthy Human	Karczweski

	ZO-1	medium, possibly cell envelope proteins e.g. lipoteichoic acids and lipoproteins (Buwitt-Beckmann et al., 2006)	mediated	duodenal biopsy	et al., 2010
	↑Apical ZO-1			Caco-2 (Human IEC)	
Lactobacillus rhamnosus GG ATCC 53103	↑ TER ↑ZO-1, Occludin, Claudin-1 expression	Unidentified	Unknown	Normal human epidermal keratinocytes (NHEK)	Sultana *et al.,* 2013
	↑ZO-1	Secreted molecule ≥	Unknown	Human colon carcinoma cell	Escamila *et*

		100kDa		line HCT-116	*al.,* 2012
	↑Claudin-3, ↓ permeability, ↑maturation of intestinal barrier	Heat stabile to 70°C	Unknown	C57BL/6J mouse ileum (distal 3rd of small intestine)	Patel *et al.,* 2012
Lactobacillus rhamnosus PZ 1121	↑ TER	Unidentified (Possibly Gram positive dependant)	↓PKC-δ membrane expression ↑β-catenin phosphorylation (↑activity)	T84 (Human IEC)	Hummel *et al.,*2012

1.11 Microbial Endocrinology

The relationship between enteric microbiota and the enteric nervous system has resulted in the evolution of new field termed microbial endocrinology (Lyte, 2004). Microbial endocrinology is a relatively new inter-disciplinary field bringing together microbiology and neuroscience with the aim of gaining greater insight into host-microbial interactions and their role in health and disease (Lyte, 2011). It has been well established that the neuroendocrine system can influence the growth and pathogenesis of certain pathogenic bacteria, including *Clostridium perfringens* (Lyte, 2004). However, for a long time this was considered to be an indirect effect due to ENS depression of the host immune system (Evans et al., 1948). It wasn't until the early 1990's that several well characterised neurotransmitters, in particular, norepinephrine were shown to directly enhance the growth of numerous gram negative species of bacteria (Lyte and Ernst, 1992) .This implied the existence of neurotransmitter receptors on bacterial cells and together with the known ability of bacteria to produce the corresponding ligands, led to the proposal of a bi-directional axis of communication between the nervous system and host microbes (Lyte , 2010).

Therefore, given the vast role of the ENS in intestinal function and regulation as well as the relatively in depth understanding achieved to date, an approach examining the influence of microbially produced neuropeptides may indeed go long way in the quest to fully understand host-microbe interactions and the bioactive peptides involved.

In conclusion it is becoming clear that understanding host-microbe relationships is now critical to further advancing our knowledge of the gastrointestinal tract and developing microbial based therapies. Moreover, given the

vast milieu and variety of known microbial strains, their secreted bioactives and indeed those yet to be identified, it is essential that appropriate experimental methodologies and techniques are developed and validated to screen for strains with specific therapeutic potential.

Aims and Objectives

Intestinal ion transport is an important and highly regulated process, disturbances of which have been implicated in numerous intestinal disorders, and in particular in diarrheal disease, and are a common underlying cause of symptoms associated with inflammatory bowel diseases and irritable bowel syndrome (IBS) (Seidler et al., 2006)(Binder, 2009)

Emerging evidence now suggests a relationship between the occurrence of major GI disorders and dysbiosis of the host microbiota. Recently, several studies have begun to uncover evidence of a microbial influence on intestinal ion transport, including direct effects on $Na^+$and Cl^- transport processes (Raheja et al., 2010), active Cl^- secretion (Heuvelin et al., 2010) as well as indirectly through immune and barrier function modulation (Bron et al., 2012). Indeed, the promise of therapies including probiotic therapy and fecal microbiota transplant, targeted at altering the host-microbiome, in treating gastrointestinal(GI) disorders associated with adverse disturbances in ion transport, such as IBS and colitis, emphasise the importance of understanding host-microbe interactions (Shanahan and Quigley, 2014).

The overall objective of this thesis was to gain further insight into the mechanisms underlying commensal microbial influences on intestinal ion transport. In this regard, I examined the impact of commensal host-microbe interactions on colonic secretomotor function in mouse. I set out to achieve this by investigating:

- The impact of two *specific* probiotic microbe strains *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 on murine colonic secretomotor function, both *in-vivo* and *ex-vivo*. **(Chapter 2)**.

- The impact of the host-microbial environment on colonic secretomotor function and host-microbe-probiotic (*Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118) interactions using germ free mice. **(Chapter 3)** .
- An alternative approach to identifying the bioactives involved in mediating the physiological influences of probiotics. The influence of supernatants from a probiotic (*Lactobacillus brevis* DPC6108) known to produce a high, but specific concentration of a known probiotic metabolite, in this case, the neuroendocrine molecule γ-amino butryic acid (GABA). Secretomotor responses measured in Ussing Chamber were compared to those produced by the same concentrations of pure exogenous GABA, thus allowing us to determine the role of GABA as a probiotic bioactive with the ability to influence intestinal ion transport. **(Chapter 4)**
- The Ussing Chamber, an important physiological instrument both historically and currently for the study of intestinal ion transport and barrier function, as a tool to study the impact of commensal microbes and their bioactives on ion transport **(Chapters 2-4)**.

Chapter 2:

Converging effects of a *Bifidobacterium* and *Lactobacillus* probiotic strain on mouse intestinal physiology

2.1 Abstract

Evidence has grown to support the efficacy of probiotics in the management of gastrointestinal disorders, many of which are associated with dysregulated fluid and electrolyte transport. A growing body of evidence now suggests that the host microbiota and probiotics can influence intestinal ion transport, and that these effects often occur in a strain-dependent manner. In this study, I sought to investigate the effects of two therapeutically relevant organisms, *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118, on small intestinal transit, fecal output and water content, transepithelial resistance (TER) and colonic secretomotor function. Mice fed either strain for two weeks displayed significantly reduced small intestinal transit *in vivo* compared to vehicle, though neither bacteria influenced fecal pellet output or water content. Colon from mice fed both strains displayed increased colonic TER, without a concomitant change in the gene expression of the tight junction proteins, claudin 1 and occludin. However, *L. salivarius* UCC118 selectively inhibited neurally-evoked ion secretion in tissues from animals fed this particular strain. Consistent with this finding, the neurotoxin, tetrodotoxin (TTx), significantly inhibited the short-circuit current response induced by *L. salivarius* UCC118 following addition to colonic preparations in Ussing chambers. Responses to *Bifidobacterium infantis* 35624 also displayed sensitivity to TTx, though to a significantly lesser degree than *L. salivarius* UCC118. Both strains similarly inhibited cholinergic-induced ion transport after addition to Ussing chambers. Further studies, therefore, are now warranted with respect to investigating the effects of *B. infantis* 35624 and *L. salivarius* UCC118 in disease contexts associated with

increased small intestinal transit and neurally-mediated infectious diarrhea in particular.

Keywords: Commensal, enteric nervous system, short-circuit current, tight junction protein, Ussing Chamber

2.2 Background

Probiotics are defined as live microorganisms, that, when ingested in adequate amounts, provide health benefits to the host (Fontana et al., 2013). *Lactobacilli* and *Bifidobacteria* represent two extensively studied probiotic species with demonstrated strain-dependant efficacy in a number of gastrointestinal diseases (Ritchie and Romanuk, 2012). In particular, *Bifidobacterium longum* subsp. *infantis* 35624 (*B. infantis* 35624) specifically relieves many of the symptoms associated with the functional bowel disorder, irritable bowel syndrome (IBS) (O'Mahony et al., 2005; Whorwell et al., 2006). Moreover, preclinical studies demonstrate that *B. infantis* 35624 selectively inhibits colorectal distension-induced abdominal pain in contrast to *Lactobacillus salivarius* UCC118 (*L.salivarius* UCC118) and *Bifidobacterium breve* UCC2003 (McKernan et al., 2010). These findings recapitulate those observed clinically, in which *B. infantis* 35624 was more effective at improving IBS symptoms when compared to a lactobacillus strain or placebo (O'Mahony et al., 2005). However, in the context of experimental colitis both *B. infantis* 35624 and *L. salivarius* UCC118 had similar effects (McCarthy et al., 2003). Therefore, these two commensal organisms appear to exert different effects on the host depending on the context in which they are studied. However, the effects of *B. infantis* 35624 or *L.salivarius* UCC118 on host gastrointestinal physiology remain largely unknown.

In this regard, I sought to investigate the effects of *B. infantis* 35624 and *L. salivarius* UCC118 on intestinal transit and colonic ion transport. Intestinal fluid and electrolyte transport is a tightly regulated process which maintains a balance between electrolyte secretion and absorption (Kunzelmann and Mall, 2002). Electrogenic chloride ion secretion is driven by the apically expressed cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channels, while epithelial sodium channels are responsible for active sodium absorption (Barrett and Keely, 2000).

Several studies now suggest that probiotics can directly or indirectly influence intestinal epithelial ion transport, and subsequently short-circuit current (I_{sc}) (Lomasney and Hyland, 2013). Specifically, *Lactobacillus acidophilus* can influence the activity of key regulators of intestinal ion transport including CFTR, the downregulated in adnoma (DRA) anion exchanger and the basolaterally-expressed sodium-potassium-chloride cotransporter (NKCC1) (Borthakur et al., 2008; Resta-Lenert and Barrett, 2009; Raheja et al., 2010). These effects, however, are not unique to *Lactobacillus acidophilus*, and other strains can also influence these targets, for example *Lactobacillus rhamnosus* (DRA)(Raheja et al., 2010) and *Streptococcus thermophiles* (CFTR and NKCC1)(Resta-Lenert and Barrett, 2009). Moreover, the host microbiota (Lomasney et al., 2014), as well as specific probiotic and commensal organisms (Czerucka et al., 1994; Ewaschuk et al., 2008; Resta-Lenert and Barrett, 2009; Heuvelin et al., 2010; Ohland et al., 2012), have the capacity alter secretagogue-evoked ion transport.

Studies examining the effects of *B. infantis* 35624 and *L.salivarius* UCC118 on intestinal ion transport, in the context of intestinal inflammation at least(Sommerfield et al., 2003; Feighery et al., 2008), suggest that these two strains may differentially influence colonic secretomotor function. Therefore, in this study I sought to determine the effects of *B. infantis* 35624 and *L. salivarus* UCC118 in healthy animals on small intestinal transit, fecal output and water content. I also characterized the effects of both strains on colonic ion transport and secretomotor function after either two weeks feeding or following acute addition to Ussing chambers.

2.3 *Materials and Methods*

All drugs were obtained from Sigma-Aldrich Ltd. (Ireland) unless otherwise stated. The following compounds were used, with the final concentration and diluent in parenthesis, amiloride (100µM dissolved in dH_2O) (Matos et al., 2007), bethanechol (100µM; dissolved in dH_2O)(Hirota and McKay, 2006b), forskolin (10µM dissolved in DMSO) (Hyland and Cox, 2005), furosemide (100µM dissolved in DMSO)(Cuthbert et al., 1999), tetrodotoxin (300nM dissolved in sodium citrate buffer)(Ikehara et al., 2010) and veratridine (30µM; dissolved in 70% ethanol) (Hyland and Cox, 2005). Krebs buffer was prepared as follows, 1.2mM NaH_2PO_4 , 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 11mM $CaCl_2$ and 10mM glucose. Chloride-free Krebs buffer was prepared to an equivalent osmolarity by replacing Cl^- with appropriate amounts of nitrate (Johns, 1980). of 116mM $NaNO_3$, 5.4mM KNO_3, 1.2mM NaH_2PO_4, 2.5mM $Ca(NO_3)_2$, 1.2mM $Mg(NO_3)_2$, 22mM $NaHCO_3$ and 11.2mM glucose . *B. infantis* 35624 and *L. salivarius* UCC 118 were from Alimentary Health Ltd. (Cork, Ireland).

2.3.1 *Animals*

Male Swiss Webster mice (22-35g) were obtained from Harlan, UK. All animals were kept on a 12-12h dark-light cycle (lights on at 7am) with food and water *ad libitum*, and were fasted overnight prior to measurement of small intestinal transit. All mouse experiments were conducted following institutional ethics guidelines and were in full accordance with the European Community Directive (86/609/EEC).

2.3.2 *Preparation of probiotics*

Freeze-dried preparations of *B. infantis* 35624 and *L. salivarus* UCC118 were obtained from Alimentary Health Ltd. Briefly, both *B. infantis* 35624 and *L. salivarus* UCC118 were produced in large-scale fermenters, the bacterial pellet harvested, washed and the resultant supernatant discarded. The biomass was subsequently freeze-dried with cryoprotectant (Alimentary Health Ltd) was used as vehicle for comparison throughout. CFU was determined by the spread plate technique.

2.3.2.(a) Probiotic feeding

Freeze-dried probiotics and vehicle were reconstituted daily in dH_2O, and a volume of 300µl containing 1 x 10^9 CFU/ml was administered to the mice daily by oral gavage for two weeks. Mice in the same treatment groups were housed in pairs.

2.3.2.(b) Preparation of probiotics for Ussing chamber studies

For Ussing chamber studies bacteria were resuspended in Krebs buffer and added to the mucosal reservoir of the Ussing chamber to yield 1 x 10^9 CFU/ml. The pH of the final suspensions was assessed; Krebs buffer (pH 7.0), vehicle + Krebs buffer (pH 7.01), *B.infantis* 35624 + Krebs buffer (pH 6.94) and *L. salivarius* UCC118 + Krebs buffer (pH 6.97). To heat kill the probiotics, suspensions were maintained at 100°C for 30 minutes and were subsequently allowed to cool for 15 minutes prior to addition to the Ussing chamber. Probiotics were determined to be viable after resuspension in Krebs buffer, and failed to grow following heat treatment (data not shown).

2.3.3 *Measurement of small intestinal transit*

To examine small intestinal transit, mice fed *B. infantis* 35624*, L. salivarus* UCC118 or vehicle for two weeks were administered activated charcoal (0.5g in 10ml + 0.5% methylcellulose) by oral gavage (0.1ml/10g). 20 mins later mice were euthanized by cervical dislocation, the small intestine immediately removed and the distance travelled by the charcoal measured.

2.3.4 *Measurement of stress-induced fecal output and fecal water content*

To measure fecal output, mice fed *B. infantis* 35624, *L. salivarus* UCC118 or vehicle for two weeks, were placed individually in open single house rat cages lined with white photographic paper under direct light (lux reading of 1000). Fecal pellet output was measured every 15 mins for a period of 90 mins. Feces were collected in glass vials and sealed upon collection. To measure fecal water content, faecal samples were weighed before and after desiccation at 60°C for 16 hours. The difference in weight was calculated as wet weight.

2.3.5 *Ussing Chamber Studies*

Mice fed *B. infantis* 35624 or *L. salivarus* UCC118 for two weeks were euthanized by cervical dislocation, the distal colon removed and placed in chilled Krebs solution. Seromuscular stripping was carried out by blunt dissection under a stereomicroscope, and both the longitudinal and circular muscle layers removed. The resulting mucosal-submucosal segments were mounted in Ussing chambers (exposed tissue area of 0.12cm^2), maintained in Krebs solution at 37°C and oxygenated with carbogen gas (95% O_2, 5% CO_2). Tissues were voltage clamped to zero using an

automatic voltage clamp (DVC-1000/EVC-4000, World Precision Instruments, Sarasota, USA). Once a stable baseline was achieved, basal short-circuit current (Isc) and TER were recorded. Tissue responses to the neural activator, veratridine, as well as to bethanechol and forskolin, to assess calcium- and cAMP-mediated ion secretion respectively, were then measured. All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using DataTrax software (World Precision Instruments).

Using a separate group of experimentally naïve mice, I assessed the acute effects of *B. infantis* 35624 or *L. salivarus* UCC118 on colonic ion transport. Mice were euthanized and colonic tissue prepared as above. Once a stable baseline was achieved, basal Isc and TER were recorded, and either *B. infantis* 35624*, L. salivarus* UCC118 or vehicle added to the mucosal reservoir. The resultant peak change in Isc was recorded, after which bethanechol (15 mins) and forskolin (15 mins) were routinely added to the serosal reservoir. To investigate the contribution of chloride ions to the change in Isc induced by *B. infantis* 35624 or *L. salivarus* UCC118, Krebs was replaced with chloride-free Krebs buffer. To examine the contribution of ENaC, NKCC1 and the enteric nervous system on probiotic-stimulated changes in Isc, tissues were pre-treated with amiloride (mucosal), furosemide (serosal) or TTx (serosal) respectively.

2.3.6 *Analysis of tight junction protein gene expression*

Total RNA was extracted using a commercially available kit (Qiagen,Valencia, CA, USA). mRNA was reverse transcribed using a high-capacity cDNA reverse transcription kit

(Applied Biosystems, Foster City, CA, USA) in a G-Storm thermocycler (G-Storm, Ringmer, East Sussex, UK). Gene expression was analysed by qualitative real-time polymerase chain reaction using TaqMan Gene expression assays and the AB7300 system (Applied Biosystems). Occludin (Ocln) was detected using the probe # Mm00500912_m1and claudin (Cldn 1) using probe #Mm00516701_ml. The expression of each gene was normalised to β actin. All samples were analysed in duplicate.

2.3.7 *Statistics*

Data were analysed and graphed using Graphpad Prism 5 by either one- or two-way ANOVA followed by post-hoc analysis using Bonferroni's Multiple Comparison Test. Data are presented as mean ± SEM and a $P < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 Effects of B. infantis 35624 or L. salivarus UCC118 on small intestinal transit, fecal output and fecal water content after two weeks feeding

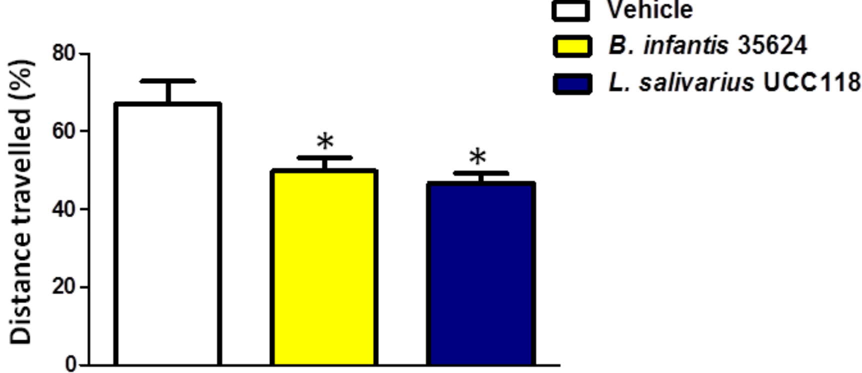

Figure 2.1: Small intestinal transit was significantly decreased in animals fed *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 relative to vehicle *in vivo*. *P<0.05. *n*=7-8.

B. infantis 35624 and *L. salivarus* UCC4331 differentially influenced bowel movement difficulty in IBS patients without a concomitant change in stool consistency (O'Mahony et al., 2005). Therefore, I assessed whether B. *infantis* 35624 or *L. salivarus* UCC118 differentially influence small intestinal transit as well as fecal output and water content. Both *B. infantis* 35624 and *L. salivarus* UCC118 significantly decreased small intestinal transit relative to vehicle after two weeks of feeding (**Fig. 2.1**; P < 0.05). However neither strain significantly altered stress-induced fecal output or water content (**Fig. 2.2** A & B). Therefore, both strains exert similar inhibitory effects on small intestinal transit, but do not appear to influence fecal output or water content.

Table 2.1: Gene expression of the tight junction proteins Occludin 1 and Claudin 1 in the mouse colon following two weeks of feeding with *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118.

Gene	Gene expression following probiotics (delta Ct)		
	Vehicle (n=8)	*B.infantis* (n=11)	*L.salivarius* (n=9)
Occludin	0.88±0.15	0.85±0.13	0.99±0.15
Claudin 1	0.48±0.11	0.43±0.07	0.47±0.08

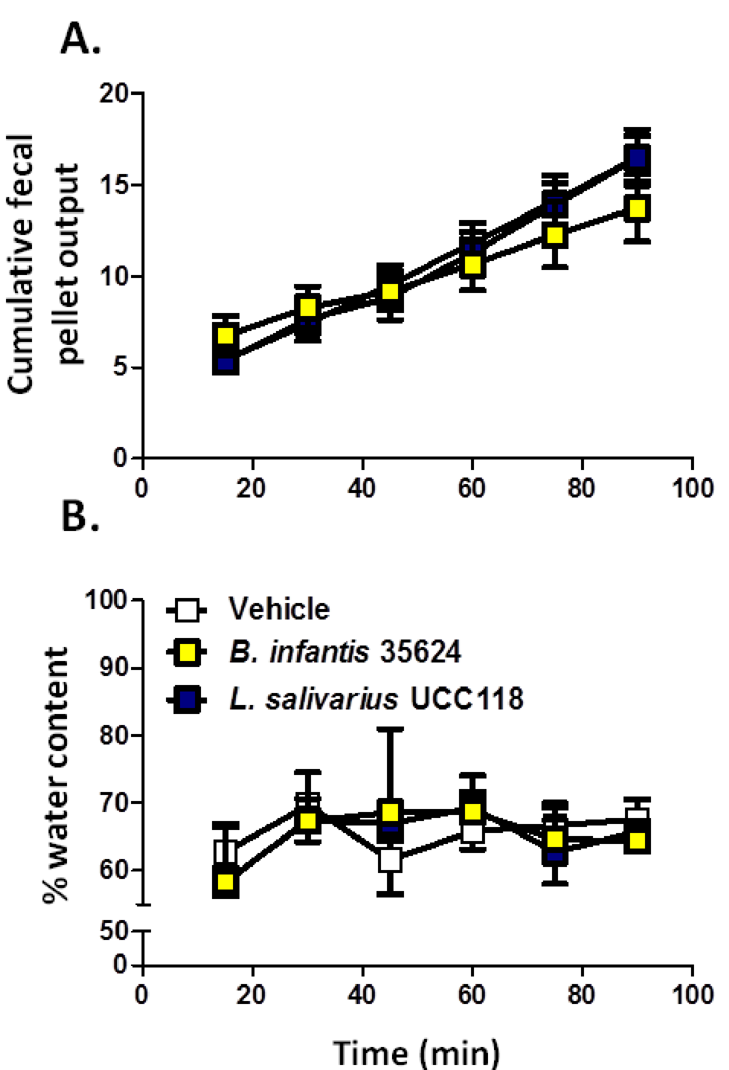

Figure 2.2: Neither *Bifidobacterium infantis* 35624 nor *Lactobacillus salivarius* UCC118 significantly influenced stress-induced fecal pellet output (A) or water content (B) in response to a mild stress. *n*=10-11.

2.4.2 *Effects of B. infantis 35624 and L. salivarus UCC118 on transepithelial resistance and tight junction protein gene expression after two weeks feeding*

A number of probiotics have the capacity to influence intestinal epithelial barrier function (Ohland and Macnaughton, 2010b). In particular, *L. salivarus* UCC118 has demonstrated beneficial effects on colonic permeability during colitis (Feighery et al., 2008), and can protect against hydrogen peroxide-induced reductions in TER *in*

vitro (Miyauchi et al., 2012). Therefore, after feeding mice *B. infantis* 35624 and *L. salivarus* UCC118 for two weeks I assessed colonic TER in Ussing chambers, and analyzed tissues for the expression of two tight junction protein genes known to be influenced by *L. salivarus* UCC118 (24). Both *B. infantis* 35624 and *L. salivarus* UCC118 increased TER, the later significantly so (**Fig. 2.3**). However, neither probiotic altered the gene expression of either claudin 1 or occludin after two weeks feeding (**Table 2.1**). The observed increase in TER may be indicative of potential barrier-enhancing properties of the strains.

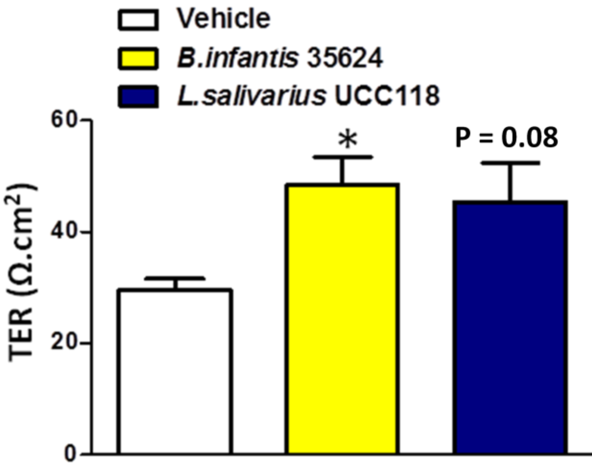

Figure 2.3: Colonic transepithelial resistance (TER) was assessed in mucosa-submucosa preparations mounted in Ussing chambers. Tissues from animals fed *Bifidobacterium infantis* 35624 displayed a significantly increased TER relative to those from the vehicle-fed group. *P<0.05. *n*=8-9.

2.4.3 *Ex vivo assessment of baseline and secretagogue-evoked colonic ion transport in animals fed B. infantis 35624 and L. salivarus UCC118*

To further investigate the effects of *B. infantis* 35624 and *L. salivarus* UCC118 on colonic physiology I examined tissue responses to a number of secretory stimuli in animals fed both bacterial strains for two weeks. Specifically, I examined calcium-stimulated ion transport using the cholinomimetic, bethanechol, cAMP-mediated ion

transport using forskolin and neurally-mediated ion transport using the sodium channel activator, veratridine. Both strains reduced baseline I_{sc} relative to vehicle (P < 0.05, **Fig. 2.4**A), however neither strain significantly influenced the tissue response to either bethanechol (**Fig. 2.4**B) or forskolin (**Fig. 2.4**C). Veratridine-induced ion transport was, however, significantly decreased in tissues collected from animals fed *L. salivarus* UCC118 only (**Fig. 2.4**D; P<0.05). Thus, both *B. infantis* 35624 and *L. salivarus* UCC118 similarly influence basal colonic ion transport. However, only *L. salivarus* UCC118 inhibits neurally-mediated responses.

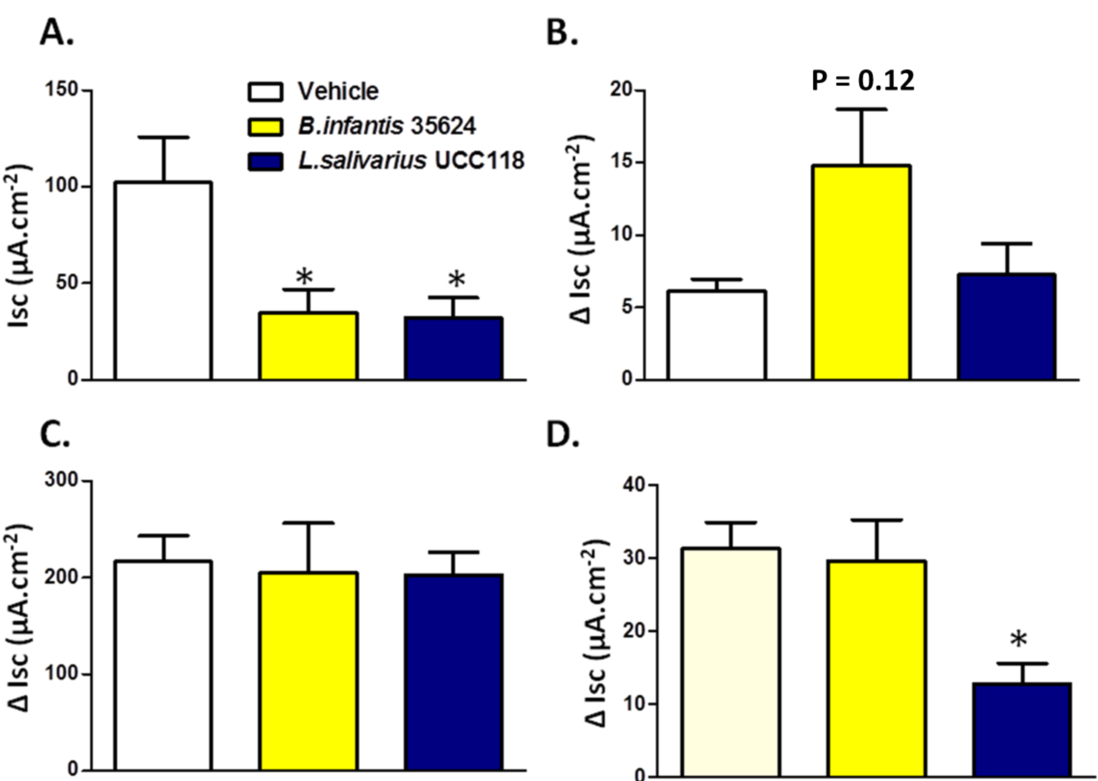

Figure 2.4: Baseline (A) and stimulated (B-D) short-circuit current (I_{sc}) responses were assessed in colonic tissues from animals fed *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118. Both bacteria significantly decreased basal Isc (A), and neither had a significant effect on either bethanechol- or forskolin-induced ion transport (B & C). *Lactobacillus salivarius* UCC118 significantly inhibited veratridine-induced ion transport relative to vehicle and *Bifidobacterium infantis* 35624(D). *Vehicle *versus* probiotic, *P<0.05; # *Bifidobacterium infantis* 35624 *versus Lactobacillus salivarius* UCC118, # P<0.05. *n*=5-8.

2.4.4 *Characterization of the acute effects of B. infantis 35624 or L. salivarus UCC118 on colonic ion transport in Ussing chambers*

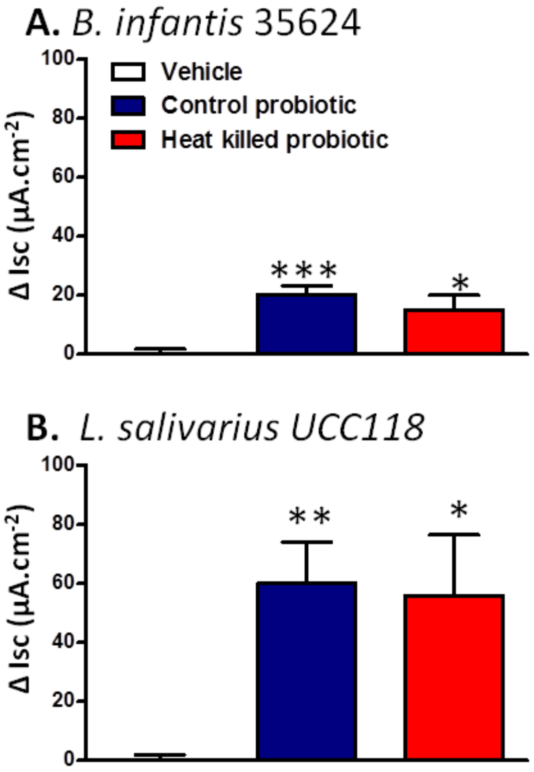

Figure 2.5: The acute effects of *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 on colonic short-circuit current (Isc) responses were measured following addition of either microbe to the mucosal compartment of the Ussing chamber. Both viable and heat-killed *Bifidobacterium infantis* 35624 (A) and *Lactobacillus salivarius* UCC118 (B) significantly increased baseline Isc relative to vehicle. *P<0.05, **P<0.01, ***P<0.01. *n*=5-9.

To characterize the potential mechanisms underlying the effects of *B. infantis* 35624 and *L. salivarus* UCC118 on colonic ion transport , I took a number of experimental approaches; (i) ion exclusion studies, using chloride-free Krebs, to investigate the contribution of chloride ions to the Isc response evoked by *B. infantis* 35624 and *L. salivarus* UCC118 (ii) pharmacological studies using furosemide to inhibit basolateral NKCC1 and amiloride to inhibit epithelial sodium channels and (iii) the neurotoxin, TTx, to the examine the effects of *B. infantis* 35624 and *L. salivarus*

UCC118 on enteric neurons. In additional experiments, the bacterial strains were heat-killed to investigate whether viable bacteria were required to induce their effects on colonic ion transport. The acute effects of *B. infantis* 35624 and *L. salivarus* UCC118 on bethanechol- and forskolin-induced changes in Isc were also examined. Both viable and heat-killed *B. infantis* 35624 and *L. salivarus* UCC118 significantly increased baseline Isc relative to vehicle following mucosal addition to the Ussing chamber (**Fig. 2.5**).

Table 2.2: The effects of pharmacological manipulation and ion exclusion on the baseline response of mouse colon to *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 in Ussing chambers.

Manipulation	***B.infantis* 35624**	***L.salivarius* UCC118**
Krebs	18.91±4.21 μA.cm^{-2} (n=6)	22.53±7.45 μA.cm^{-2} (n=6)
Chloride-free Krebs	17.30±3.24 μA.cm^{-2} (n=6)	52.70±12.00 μA.cm^{-2} *(n=6)
Vehicle (dH_2O)	20.74±5.18 μA.cm^{-2} (n=6)	25.52±3.32 μA.cm-2 (n=5)
Amiloride	26.07±1.31 μA.cm^{-2} (n=10)	27.47±4.47 μA.cm^{-2} (n=5)
Vehicle (DMSO)	25.24±3.71 μA.cm^{-2} (n=6)	28.81±0.71 μA.cm^{-2} (n=6)
Furosemide	21.77±7.94 μA.cm^{-2} (n=7)	23.12±3.04 μA.cm^{-2} (n=6)

* P <0.05, Krebs *versus* Chloride-free Krebs.

In experiments conducted with chloride-free Krebs, the response to *L. salivarus* UCC118 was significantly increased relative to vehicle (**Table 2.2**). Neither basolateral addition of furosemide nor apical addition of amiloride significantly altered the I_{sc} response elicited by either bacterial strain (**Table 2.2**). Pre-treatment of tissues with TTx significantly inhibited the response to both *B. infantis* 35624 (P < 0.01; **Fig. 2.6**A) and to *L. salivarus* UCC118 (P < 0.001; **Fig. 2.6**B), with the extent of inhibition greater for *L. salivarus* UCC118 (approx. 60% reduction in I_{sc} response; **Fig. 2.6**A & B).

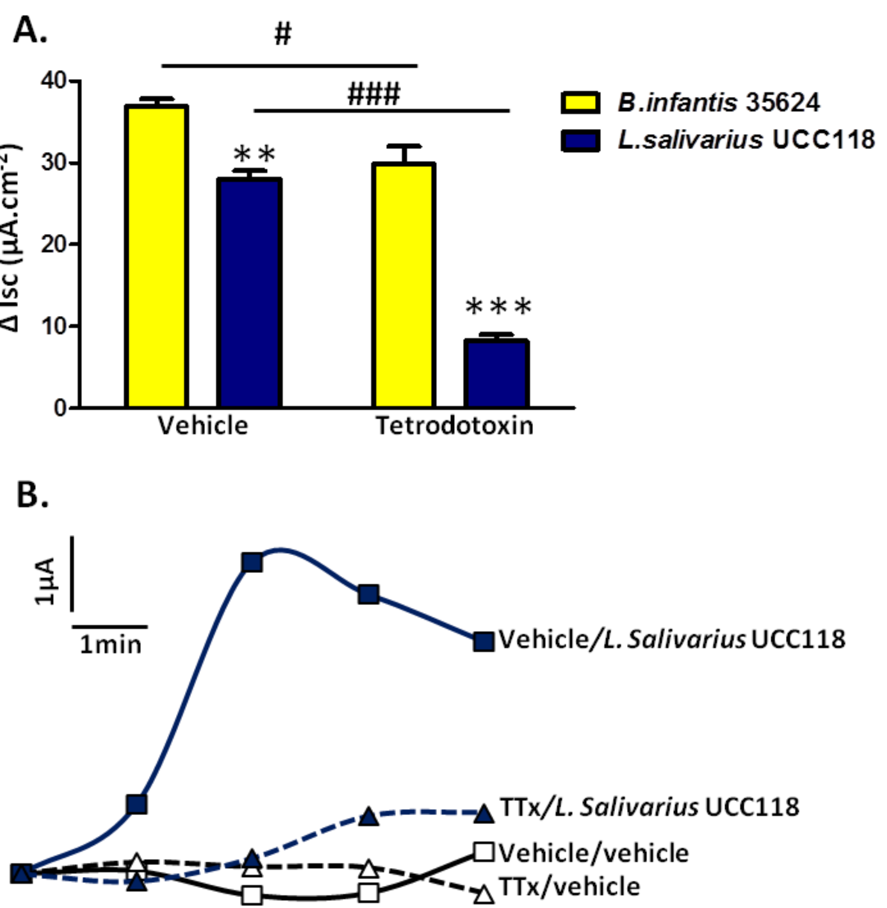

Figure 2.6: To investigate the sensitivity of the *Bifidobacterium infantis* 35624- and *Lactobacillus salivarius* UCC118-induced effects on short-circuit current (I_{sc}) to neural blockade, tissues were pre-treated with tetrodotoxin (TTx) prior to luminal addition of the microbes. The magnitude of the Isc response to *Lactobacillus salivarius* UCC118 was significantly greater than that of *Bifidobacterium infantis* 35624, and the response to both bacteria displayed sensitivity to TTx (A). Representative trace demonstrating the effect of TTx on the *Lactobacillus salivarius* UCC118-induced response in mouse colon (B). * *Bifidobacterium infantis* 35624 *versus Lactobacillus salivarius* UCC118, **P<0.01,***P<0.001; $^{\#}$ vehicle *versus* TTx, $^{\#}$ P<0.05, $^{\#\#}$ P<0.01. *n*=5-6.

Both *B. infantis* 35624 (P < 0.05) and *L. salivarus* UCC118 (P < 0.01) significantly inhibited bethanechol-induced I_{sc} (Fig. 2.7A), and neither strain influenced cAMP-mediated ion transport (Fig. 2.7B). Thus, chloride ions do not appear to significantly contribute to the increase in I_{sc} observed in response to acute exposure of tissues to *B. infantis* 35624 or *L. salivarus* UCC118, though their removal influences the

response to *L. salivarus* UCC118. Both strains interact with the enteric nervous system, with *L. salivarus* UCC118 displaying greater sensitivity to the neurotoxin and both bacteria similarly inhibit cholinergic-induced ion transport.

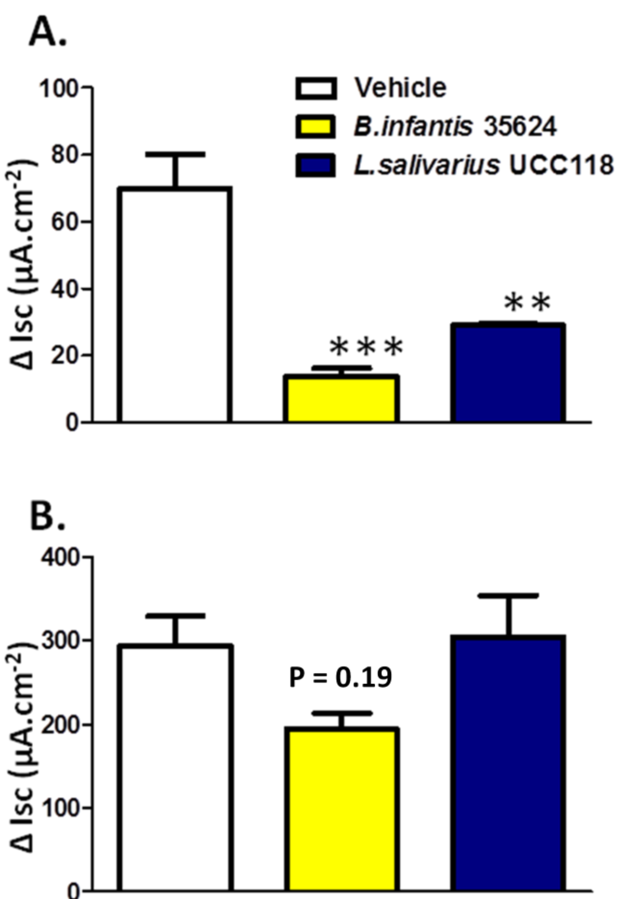

Figure 2.7: The acute effects of *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 on bethanechol- and forskolin-induced ion transport were assessed following mucosal addition of the microbes to the Ussing chamber. Both *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 significantly decreased bethanechol-induced ion transport (A), while neither bacteria had an effect on forskolin responses (B). *P<0.05. *n*=4-6.

2.5 Discussion

I have demonstrated that both *B. infantis* 35624 and *L. salivarius* UCC118 similarly inhibit small intestinal transit, increase TER and decrease baseline short-circuit current in tissues obtained from mice fed each strain for two weeks. However, in animals fed *L. salivarius* UCC118, neurally-evoked colonic ion transport was selectively inhibited. The TTx sensitivity of the *L. salivarius* UCC118 response observed in Ussing chamber studies further supports a predominantly neural effect of this organism on secretomotor function. Also notable, following acute exposure of tissues to either *B. infantis* 35624 or *L. salivarius* UCC118, was the similar inhibition of cholinergic-induced ion transport by both microbes. Additional experiments revealed that removal of chloride ions significantly increased the magnitude of the baseline response to *L. salivarius* UCC118 only, and that both strains induced similar effects on baseline short-circuit current irrespective of heat-killing in acute tissue experiments.

Recent evidence suggests that probiotics exert both strain- and region-specific effects on gut physiology(Wu et al., 2013). For example, *Lactobacillus rhamnosus* and *Lactobacillus reuteri* differentially influence migrating myoelectric complex parameters in mouse jejunal and colonic preparations (Wu et al., 2013). Moreover, the probiotic mix, VSL#3, also exerts region-specific effects on gastrointestinal motility in isolated tissue preparations (Massi et al., 2006a). Our data suggest that the inhibitory effects of *B. infantis* 35624 and *L. salivarius* UCC118 on small intestinal transit are not strain-dependent, and do not influence fecal output or water content when examined under mildly stressful conditions. I did not investigate the mechanism by which *B. infantis* 35624 or *L. salivarius* UCC118 inhibit small

intestinal transit further. Nevertheless, evidence suggests that probiotics may influence motility at the level of the enteric nervous system (Kunze et al., 2009a; Wang et al., 2010a, 2010b) or through direct effects on smooth muscle cells (Bär et al., 2009). Clinically, increased small bowel transit has been associated with diarrhoea-predominant IBS, stress and anxiety (Husebye, 1999). Therefore, probiotic interventions, using bacterial strains with the capacity to slow small intestinal transit, may be efficacious in these contexts. However, consideration must be given to the possibility that changes in small intestinal transit observed in this study could also be due to a change in the rate of gastric emptying (GE). For example *Lactobacillus reuteri* DSM 17938 has been shown to accelerate GE in human infants (Indrio et al., 2009, 2011) while a combination of *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 delayed GE in a mouse model of post-infectious gut dysfunction (Verdu et al., 2008). Moreover, the delay in GE correlated with an improvement in para-cellular permeability(Verdu et al., 2008).

It has been suggested that an altered microbiota may contribute to intestinal inflammation, and therefore disrupted epithelial barrier function (McKernan et al., 2010). Several probiotics exert beneficial effects on epithelial barrier function by regulating, for example, mucin gene expression, production of β-defensins, bacteriocins and secretory IgA as well as modulation of tight junction proteins (Ohland and Macnaughton, 2010a). I observed an increase in colonic TER in tissues from animals fed *B. infantis* 35624 or *L. salivarius* UCC118 for two weeks, which suggests that both strains may have the capacity to increase epithelial barrier function. However, I did not find an associated change in the gene expression of two tight junction genes, claudin 1 and occludin. *In vivo* studies, in the context of

experimental colitis, support a beneficial effect of *L. salivarius* UCC118 on intestinal permeability and suggest that this effect is bacteriocin-dependent, as bacteriocin-negatve *L. salivarius* UCC118 did not significantly improve colitis-associated deficits in permeability *in vivo* (Miyauchi et al., 2012b). Moreover, *in vitro* studies further confirmed the beneficial effect of *L. salivarius* UCC118 on hydrogen-peroxide induced changes in both TER and permeability, and this beneficial effect was accompanied by a redistribution of tight junction proteins, including claudin 1 and occludin (Miyauchi et al., 2012b). The later findings may account for the absence of any change in gene expression observed in our study. However, changes in TER may not necessarily accompany changes in permeability. For instance, the beneficial effect of *L. salivarius* UCC118 on colitis-associated deficits in permeability were not accompanied by a change in TER or short-circuit current (Feighery et al., 2008). Our study, however, is the first to indicate a role for *B. infantis* 35624 in the modulation of TER. Potential mechanisms underlying this effect may include changes in tight junction gene expression, localisation or protein kinase activity previously described for other strains of *B. infantis* (Ewaschuk et al., 2008; Bergmann et al., 2013).

There is now a growing appreciation that one of the mechanisms by which microbes interact with the host is through bacterial signalling to the enteric nervous system (Sharkey and Savidge, 2014). With respect to secretomotor function, our data indicate that *L. salivarius* UCC118 preferentially inhibits neurally-driven responses following chronic administration. Acute exposure of colonic preparations to *L. salivarius* UCC118 in the presence TTx further suggests a role for the enteric nervous system in mediating the effects this particular microbe on colonic ion

transport. The enteric nervous system also appears, although to a lesser degree, to contribute to the effects of *B. infantis* 35624 on the short-circuit current response elicited after short-term exposure *in vitro*. It is known, however, that different microbes have the capacity to differentially influence enteric nerve activity (Kunze et al., 2009a; Bercik et al., 2011; Khoshdel et al., 2013; Mao et al., 2013b). For example, *Lactobacillus reuteri* (Kunze et al., 2009a) and *Bacteroides fragilis* (Mao et al., 2013b) enhance, or activate, enteric nerve activity while *Bifidobacterium longum* exerts an inhibitory effect (Bercik et al., 2011; Khoshdel et al., 2013). The inhibitory effect of *L. salivarius* UCC118, in particular, on enteric nerve activity may be of therapeutic importance in pathogen-mediated diarrheal diseases, in which neural pathways have been implicated, such as those caused by *Salmonella* spp., *Clostridium difficile* (Jones and Blikslager, 2002) and rotavirus (Jodal et al., 1993).

The inhibition of cholinergic-induced ion transport by *L. salivarius* UCC118, in acute Ussing chamber studies, provides further evidence for an anti-secretory effect of this microbe. I also observed a similar effect of *B. infantis* 35624 on cholinergic responses. However, others have observed strain-dependent effects for different microbes on this response (Heuvelin et al., 2010). Notably, *Bifidobacterium breve* C50 selectively inhibited carbachol-induced ion transport through an inhibition of protein kinase C activity (Heuvelin et al., 2010). In contrast, *Bifidobacterium breve* 15698, *Lactobacillus rhamnosus* 10893 and *Escherichia rectale* L15 had no such effect (Heuvelin et al., 2010). In the same study, strain-dependency was also noted with respect to the effects of the different bacterial strains on forskolin-induced ion transport (Heuvelin et al., 2010). However, I observed no effect of *B. infantis* 35624 or *L. salivarius* UCC118 on forskolin-induced responses. Therefore,

both *B. infantis* 35624 and *L. salivarius* UCC118 appear to selectively inhibit cholinergic-induced ion transport relative to cAMP-mediated responses, and do not display strain-selectivity in this regard.

Our pharmacological studies did not discriminate whether the increase in short-circuit current observed in response to *B. infantis* 35624 or *L. salivarius* UCC118 after mucosal addition to the Ussing chamber were secretory or absorptive in nature. However, removal of chloride ions significantly increased the tissue response to *L. salivarius* UCC118. In the absence of chloride ions, increased secretion of bicarbonate could account for an increase in short-circuit current, similar to that which I observed in response to *L. salivarius* UCC118. However, Cl^-/HCO_3^- exchange, facilitated by apically expressed DRA for example, is dependent on the presence of chloride ions, the activity of which would likely be reduced in the absence of chloride. Nonetheless, bicarbonate secretion may also occur via CFTR (Grubb, 1997). Moreover, in the absence of external chloride, the permeability of CFTR to bicarbonate may increase (Shcheynikov et al., 2004). Further characterisation of the ionic nature of the response elicited by both *B. infantis* 35624 and *L. salivarius* UCC118 is now warranted in order to better understand the ion transport processes underlying their effects on short-circuit current. Likewise, I can only speculate on the divergence observed between studies in which animals were fed both strains and those in which tissues were acutely exposed to *B. infantis* 35624 or *L. salivarius* UCC118. Based on the TTx sensitivity of the response elicited by *B. infantis* 35624 and *L. salivarius* UCC118 observed in tissues acutely exposed to both strains, I speculate a neural reflex underlies the relatively immediate change in short-circuit current. However, following longer periods of exposure, several

microbes have been shown to alter the expression of key ion channels or exchangers in addition to indirectly influence ion transport through immune modulation (Lomasney and Hyland, 2013).

Collectively, our data suggest that both *B. infantis* 35624 and *L. salivarius* UCC118 significantly affect gastrointestinal physiology; slowing small intestinal transit and significantly influencing colonic secretomotor function. Both display anti-secretory effects following addition to Ussing chambers, inhibiting cholinergic-induced responses. Moreover, *L. salivarius* UCC118 preferentially inhibits neurally-evoked ion transport in animals fed the microbe for two weeks, suggesting that this strain, in particular, may be of benefit in countering diarrhea associated with neural activation. Further studies are now justified with respect to investigating the effects of *B. infantis* 35624 and *L. salivarius* UCC118 in disease contexts associated with increased small intestinal transit and neurally-mediated diarrhea in particular.

Chapter 3:

Selective Influence of Host Microbiota on Colonic Secretomotor Function

3.1 Abstract

More microbes are resident in the distal colon than any other part of the body, and this microbiota has the capacity to influence enteric nerve development, excitability and gastrointestinal function. Germ free (GF) mice are a valuable tool in interrogating the communication between microbiota and host. Despite the intimate relationship which exists between the microbiota and the colonic mucosa-submucosa, there is a paucity of studies examining the influence of the microbiota on secretogogue-evoked responses. To this end, I investigated both epithelial and neural-evoked ion transport, and the response elicited by two commensal organisms, in colonic mucosa-submucosa preparations from GF mice in Ussing chambers. Baseline electrical parameters, short-circuit current and transepithelial resistance, were comparable between tissues from GF and conventional animals. Noteworthy, however, was a hyper-responsiveness of GF colon to forskolin stimulation. In contrast, the absence of the microbiota did not influence the tissue response to bethanechol. Moreover, responses to the sodium-channel activator, veratridine and the TRPV1 receptor agonist, capsaicin were preserved in GF mice relative to conventional tissues. Similarly, the short-circuit current response to two well-characterised commensal organisms occurred independent of an interaction with the host microbiota. This is the first comprehensive characterisation of secretomotor responses in GF colon.

Key words: Germ free; mucosa; probiotics; secretomotor; submucosal

3.2 Background

The impact of the microbiota on host physiology and behaviour has become increasingly appreciated particularly in the context of brain-gut interactions (Cryan *et al.*, 2012). Moreover, its influence on the development of the enteric nervous system (Collins *et al.*, 2013) and on the excitability of enteric neurons has recently been shown (McVey Neufeld *et al.*, 2013). Additionally, the expression of Toll Like Receptors by enteric neurons (Barajon *et al.*, 2009), in particular in the submucosal plexus, suggests that the microbiota can directly activate intestinal neural responses and may, therefore, influence local secretomotor reflexes in the gut. Despite some overt secretomotor deficits (e.g. semi-solid feces) being a characteristic feature of GF animals (Gordon *et al.*, 1971), to date, there is limited evidence describing a direct role for the host microbiota in the regulation of intestinal fluid and electrolyte transport in GF tissues (Rösel *et al.*, 1996). As the activity of the microbiota is likely to influence host secretory or absorptive function, and given the role the microbiota plays in physiology and pathophysiology, particularly in the etiology of inflammatory conditions of the gut, I sought to characterise the response of GF mucosa-submucosa preparations to neural and epithelial stimulants, and to the commensal organisms, *Bifidobacterium longum* subsp. *infantis* 35624 and *Lactobacillus salivarius* subsp. *salivarius* UCC118, in Ussing chambers.

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3.3 Materials and Methods

3.3.1 *Animals*

GF and conventional (CONV) Swiss Webster breeding pairs were supplied by Taconic (Germantown, New York, USA) and first-generation offspring were used in all experiments. GF Swiss Webster mice were housed 4–5/cage in flexible film gnotobiotic isolators under a strict 12-h light/dark cycle. CONV mice were similarly housed 4–5/cage in the standard animal facility under the same controlled conditions (temperature 20–21 °C, 55–60% humidity) on the same 12 h light/dark cycle. In all cases, both GF and CONV mice received the same pelleted diet after it was autoclaved (sodium dodecyl sulphate diets, product code 801010). Experiments were conducted in accordance with the European Directive 86/609/EEC and the Recommendation 2007/526/65/EC, and were approved by the Animal Experimentation Ethics Committee of University College Cork.

3.3.2 *Materials*

All drugs were obtained from Sigma-Aldrich Ltd. (Ireland). The following compounds were used, with the final concentration and diluent in parenthesis, bethanechol (100µM; dH_2O) (Cameron and Perdue, 2007), capsaicin (3µM; 70% ethanol)(Matsumoto et al., 2011), forskolin (10µM; DMSO) (Hyland and Cox, 2005) and veratridine (30µM; 70% ethanol) (Hyland and Cox, 2005). *B. infantis* 35624 (1 x 10^9 CFU/ml; Krebs) and *L. salivarius* UCC 118 (1 x 10^9 CFU/ml; Krebs) were obtained from Alimentary Health Ltd. (Cork, Ireland). Krebs buffer consisted of the

following, 1.2mM NaH_2PO_4 , 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 11mM $CaCl_2$ and 10mM glucose.

3.3.3 *Ussing Chamber Studies*

Mice were euthanized by cervical dislocation and distal colon was removed and placed in chilled Krebs solution. Seromuscular stripping was carried out by blunt dissection under a dissecting microscope and both the longitudinal and circular muscle layers, as well as the myenteric plexus, were removed. The resulting mucosal-submucosal segments were mounted in Ussing chambers and bathed with Krebs solution (maintained at 37°C, exposed tissue area of 0.12cm^2). Carbogen (95% O_2, 5% CO_2) provided gas lift to the chambers. Tissues were voltage clamped to zero, using an automatic voltage clamp (DVC-1000/EVC-4000, World Precision Instruments, Sarasota, USA). Once a stable baseline was achieved, capsaicin and veratridine were added to the serosal compartment of the Ussing chamber to determine neural activity of the tissues and *B. infantis* 35624 or *L. salivarus* UCC118 were added mucosally as tools to determine the tissue responsiveness to commensal organisms. Fifteen minutes later bethanechol and forskolin were added to the serosal side of the mounted tissues. All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using DataTrax software (World Precision Instruments). Transepithelial resistance (TER) was calculated using Ohm's law. The peak change in Isc was recorded and expressed as μA. cm^{-2}. GF and CONV tissues were paraffin wax embedded and processed for hematoxylin and eosin staining. ImageJ software was used to calculate mucosal thickness.

Data were analysed using GraphPad Prism 5 and are presented as the mean +/- standard error of the mean. A Student's unpaired t test was used for comparison between CONV and GF tissues and a $P<0.05$ was considered statistically significant.

3.4 Results

The I_{sc} response to forskolin, predominantly epithelial-mediated in mouse colon (data not shown), was significantly elevated in GF tissues (**Fig. 3.1**A-C, *n*=6-8). Baseline electrical parameters, I_{sc} and TER, were comparable between CONV and GF mouse colon (**Fig. 3.1**D, *n*=7-8). Calcium-mediated responses were comparable between GF and CONV animals (**Fig. 3.1**E, *n*=7-8). Similarly, neural stimulation of mucosa-submucosa preparations with veratridine or capsaicin resulted in a similar change in I_{sc} in GF and CONV mouse colon (**Fig. 3.1**F, *n*=5-8). Moreover, mucosal addition of the probiotics, *B. infantis* 35624 or *L. salivarus* UCC118, elicited a similar I_{sc} response in CONV and GF tissues (**Fig. 3.1**G, *n*=7-8). The thickness of the colonic mucosa in GF colon was significantly less than that observed in tissues from CONV animals (**Fig. 3.1**I and **3.1H**, *n*=4-5).

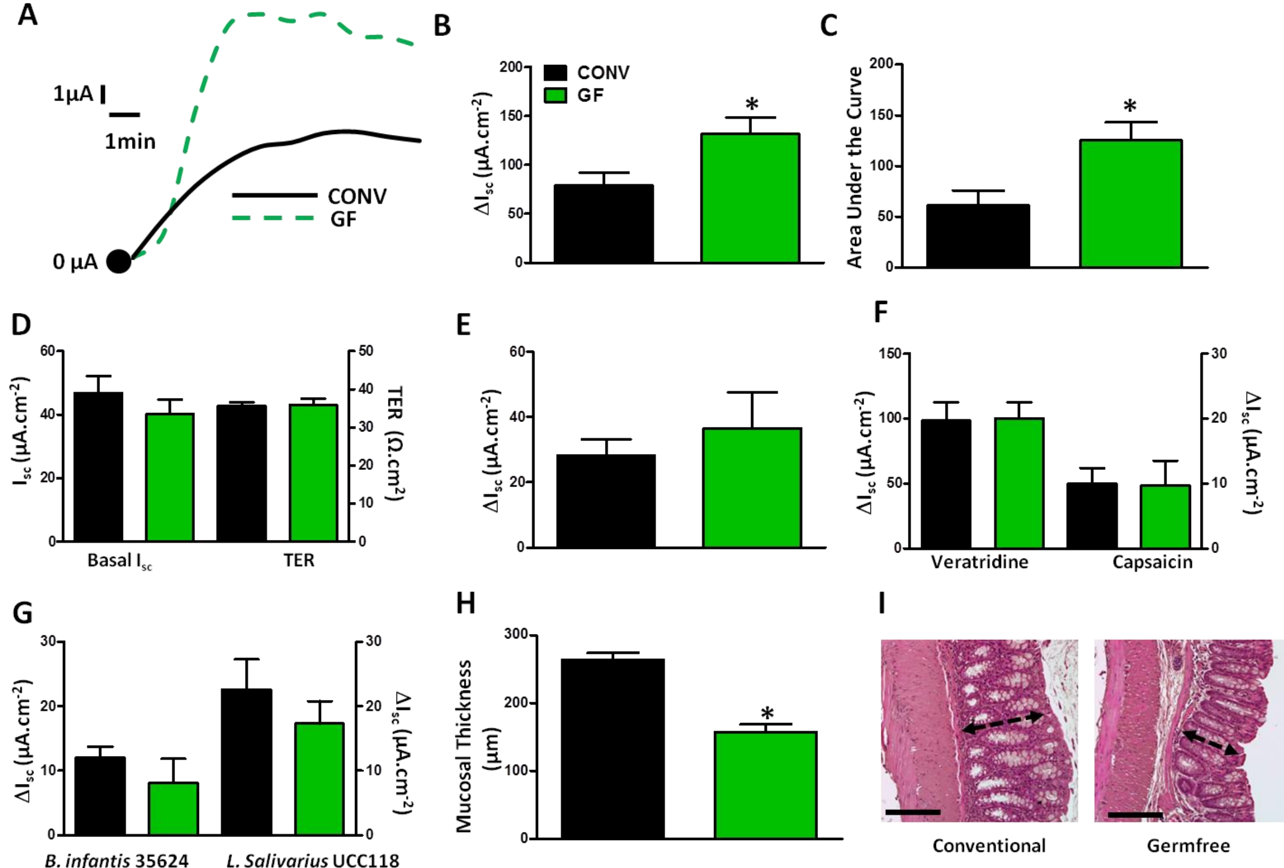

Figure 3.1. The change in short-circuit current (I_{sc}) elicited by forskolin (●, forskolin 10μM) was significantly elevated in germ free (GF) tissues compared to responses observed in conventional colon (CONV; A, representative trace; B, peak response; C, area under the curve (AUC)). Baseline electrical parameters, short circuit current (I_{sc}) and transepithelial resistance (TER), were comparable between CONV and GF mouse colon (D). Similarly, calcium-mediated responses were comparable between GF and CONV animals (E). Veratridine (F; left y axis) and capsaicin (F; right y axis) caused a similar change in Isc both in GF and CONV mouse colon. Moreover, mucosal addition of either *Bifidobacterium infantis* 35624 *or Lactobacillus salivarus* UCC118 elicited a similar I_{sc} response in CONV and GF tissues (F). Mucosal thickness of GF descending colon was significantly decreased relative to CONV tissues (H and I; 20X, scale bar 200μm). *n*=4-8. * P < 0.05.

3.5 Discussion

Our data suggests that in a coordinated physiological system mucosa-submucosa responses are largely maintained in GF animals. To the best of our knowledge, this is one of the first studies to examine the responsiveness of GF colon

to secretogogues *in vitro*. I observed no change in baseline I_{sc}, suggesting that basal electrogenic ion transport in GF tissues is maintained. However, in contrast to a suggested aldosterone-induced proabsorptive phenotype observed in GF rats (Rösel and von Engelhardt, 1996), GF mouse colon displayed a heightened response to forskolin stimulation. This is perhaps not unexpected given that a number of bacteria or probiotics have demonstrated inhibitory effects on cAMP-mediated responses (Lomasney *et al.*, 2013). However, such a heightened response may serve as a protective mechanism to prevent adhesion of pathogenic bacteria. For example, secretory diarrhea has long been considered a host defence mechanism based on the hypothesis that increased fluid and electrolyte movement into the gut lumen may inhibit the adherence of pathogenic organisms (Canny and McCormick, 2008). Therefore, a heightened secretory response may potentially act as a non-specific protective mechanism in GF colon. Noteworthy however, this heightened response to cAMP did not influence the tissue response to two probiotics, *B. infantis* 35624 or *L. salivarus* UCC118. Given that intrinsic primary afferent neurons from GF intestine display decreased excitability, I examined the responsiveness of GF mouse colon to veratridine and the sensory nerve activator, capsaicin. Colon from GF and CONV animals responded similarly to both stimulants, suggesting that secretomotor reflexes are unaltered in GF colon or have adapted to compensate for the lack of the microbiota. Moreover, the secretomotor response in our mucosa-submucosa preparations is likely to involve not only intrinsic primary afferent neurons, but also motor neurons as well as the epithelium which collectively determine the final I_{sc} response. Therefore, a level of redundancy may exist in a complex system, such as ours, to compensate for alterations at a single cell level which may be influenced by the microbiota. In addition to its well established role in the development and

maturation of the host immune system, recent evidence suggests that the microbiota may also play a role in the development of the enteric nervous system (McVey Neufeld *et al.*, 2013). Moreover, morphological studies have identified decreased mucosa and muscle thickness as a characteristic feature of GF animals. Here, I similarly observed decreased mucosal thickness in the colon of GF mice relative to CONV animals. While morphological changes in the intestinal musculature of GF animals may account, to some degree at least, for their altered motility, colonic secretomotor function appears to be largely maintained despite GF animals exhibiting a decrease in colonic mucosal thickness. This study provides further evidence that the microbiota is capable of mediating alterations in colonic ion transport, and specifically suggests that it can influence cAMP-mediated responses. Our data lends support to the hypothesis that an altered microbiota in disorders associated with secretomotor abnormalities, for example irritable bowel syndrome, may be responsible for symptom generation in such disorders, and further supports the utility of the GF mouse model as a tool to characterise the mechanisms by which specific microbes may influence secretomotor function.

Chapter 4:

Application of Ussing Chambers to Screen for the Functional Activity of the Commensal-Derived Neuroactive, GABA

4.1 Abstract

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the body. Both GABA and its receptors are widely expressed in the gastrointestinal tract (GI) where they regulate many physiological functions. An additional and increasingly recognised source of intestinal GABA is the host microbiota. However, there is a paucity of studies examining the effects of GABA on secretomotor function, and none, to date, in the colon. Therefore, I examined and characterised the *in vitro* effects of GABA and bacterial-derived GABA on colonic secretomotor function in the mouse. I assessed the influence of GABA and bacterial-derived GABA on baseline and stimulated short-circuit current (I_{sc}) in Ussing chambers. I further characterised the nature of the GABA-mediated effects using the $GABA_A$ receptor antagonist, bicuculline, the $GABA_B$ receptor antagonist, phaclofen and the neurotoxin, tetrodotoxin in colonic mucosa-submucosa preparations. GABA and bacterial-derived GABA significantly inhibited cholinergic-induced ion transport in mouse colon, though neither significantly altered baseline I_{sc}. The $GABA_A$ receptor antagonist did however significantly reduce baseline I_{sc} in mouse colon. $GABA_A$ receptor antagonism also reversed the inhibitory effect of GABA on cholinergic-induced ion transport, while phaclofen had no effect. Moreover, the $GABA_A$ receptor-mediated effect most likely occurs at the level of the enteric nervous system. GABA and bacterial-derived GABA exert similar effects on colonic ion transport in mouse colon, and this effect is mediated by $GABA_A$ receptors. GABA-producing bacteria, therefore, may prove to be potentially useful tools for delivery of GABA to the GI tract.

Keywords: Cholinergic; Colon; Gamma-aminobutyric acid; Lactobacillus; Microbial endocrinology Mouse; Secretomotor; Ussing chamber

4.2 Background

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the body and has been localised in both enteric neurones in addition to enterochromaffin cells in the gastrointestinal (GI) tract (Krantis, 2000). Hence GABA-mediated neurotransmission regulates many physiological functions, including those in the gut (Hyland *et al.*, 2010). In addition to host neuronal- and enterochromaffin cell-derived GABA, the host microbiota is another, and perhaps underappreciated, source of intestinal GABA (Li et al., 2010). The ability of microbes to produce neurotransmitters, including GABA, has given rise to the concept of microbial endocrinology (Lyte, 2011). Moreover, the biosynthetic pathway for the production of microbial GABA is identical, in some bacteria, to that in mammalian neuronal cells (Park et al., 2005; Mazzoli et al., 2010). GABA-producing bacteria, therefore, have potential as local delivery agents for GABA to the GI tract (Li et al., 2010). In this regard, the Ussing chamber offers a unique opportunity to asses the bioactivity of proposed neuroactive metabolites *ex-vivo*.

There are two major classes of GABA receptors, classified as either ionotropic $GABA_A$ and $GABA_C$ receptors, or metabotropic $GABA_B$ receptors (Watanabe *et al.*, 2002). Both $GABA_A$ and $GABA_C$ receptors are neurotransmitter-gated chloride ion channels and are hetero-oligomeric structures composed of a combination of α, β, γ, δ and ε subunits, while $GABA_C$ receptors are homo-oligomeric and composed of ρ1 or ρ2 subunits (Chebib *et al.*, 1999). Moreover, these two receptors produce distinctly different pharmacological and electrophysiological responses *in vitro* (Bormann, 2000). $GABA_B$ receptors on the other hand are seven transmembrane G-protein coupled receptors which activate second messengers and

subsequently regulate Ca^{2+} and K^+ ion channel activity (Gassmann *et al.*, 2012). Moreover, all three GABA receptors have been localised in the GI tract of several species (Krantis, 2000; Fletcher *et al.*, 2001).

Specifically, both GABA and its receptors are expressed by submucosal nerve cell bodies and mucosal nerve fibers (Krantis, 2000), implicating a role for GABA in the modulation of secretomotor function. Indeed, evidence for a tonic effect of GABA on intestinal ion transport was demonstrated by Hardcastle and colleagues (1991), whereby the $GABA_A$ receptor antagonist, bicuculline caused a decrease in baseline short-circuit current (I_{sc}) (Hardcastle *et al.*, 1991). This effect was absent following removal of the myenteric plexus and sensitive to inhibition with atropine suggesting that $GABA_A$ receptors on myenteric neurones contribute to GABAergic regulation of secretomotor function in the rat (Hardcastle *et al.*, 1991). In guinea-pig intestine, however, the submucosal plexus was implicated in mediating the $GABA_A$ receptor effects on intestinal ion transport, and, as in the rat, this effect was sensitive to cholinergic receptor blockade (MacNaughton *et al.*, 1996). However, *in vivo* studies, in mouse intestine, suggest that $GABA_A$ receptor-mediated fluid secretion occurs independent of an interaction with the enteric nervous system (Li *et al.*, 2012). This receptor expression pattern, and physiological effect of GABA and its receptors, suggests that mucosa-submucosa tissue preparations offer a platform for functional screening of putative GABA producing bacteria. I sought, therefore, to further investigate the influence of GABA, and its receptors, on secretomotor function in mouse colon (Casanova *et al.*, 2009) as well as the physiological effects of bacterial-derived GABA, obtained from an efficient GABA-producing strain, *Lactobacillus brevis* DPC6108 (Barrett *et al.*, 2012) on intestinal physiology.

4.3 Materials and Methods

4.3.1 *Animals*

Male Balb/c mice (22-27g) were obtained from Harlan, UK. All animals were kept on a strict 12-12h dark-light cycle with food and water available *ad libitum*. All experiments were conducted following institutional ethics guidelines and were in full accordance with the European Community Directive (86/609/EEC). To obtain distal colon, mice were euthanised by cervical dislocation, the distal colon was removed and immediately placed in chilled Krebs solution. The longitudinal and circular muscle layers, as well as the myenteric plexus, were removed by blunt dissection under a light microscope. The resulting mucosal-submucosal segments were mounted in Ussing chambers.

4.3.2 *Generation of GABA-containing bacterial supernatants*

To generate bacterial-derived GABA-containing supernatants, *Lactobacillus brevis* DPC 6108 (GABA-producing strain) and *Lactobacillus reuteri* DPC 6100 (non GABA-producing strain) were routinely propagated in mMRS medium at 37°C under anaerobic conditions, followed by inoculation (1% v/v) in mMRS medium supplemented with 30mg ml^{-1} monosodium glutamate (MSG) at 37°C for 72h anaerobically. After incubation, strains were separated from medium by centrifugation (4500 rpm, 30min, 4°C) and the supernatants were re-centrifuged (4500 rpm, 30min, 4°C). Supernatants and mMRS medium supplemented with 30mg ml^{-1} MSG were aliquoted in 1ml freeze drying vials, lyophilised and subsequently analysed for GABA content (**Table 4.1**).

Table 4.1 GABA content of lyophilised preparations obtained from *Lactobacillus reuteri* DPC6100 and *Lactobacillus brevis* DPC6108 culture media supplemented with monosodium glutamate.

	***Lactobacillus reuteri* DPC6100**	***Lactobacillus brevis* DPC6108**	**mMRS + MSG**
Fresh supernatant	0.014 ± 0.003 mg ml^{-1}	27.89 ± 0.13 mg ml^{-1}	NA
After freeze-drying	0.016 ± 0.002 mg ml^{-1}	23.87 ± 0.18 mg ml^{-1}	0.015 ± 0.001 mg ml^{-1}
GABA per powder	0.000 mg mg^{-1}	0.858 mg mg^{-1}	0.000 mg mg^{-1}

Lactobacillus reuteri DPC6100, non GABA-producer; *Lactobacillus brevis* DPC6108, GABA-producer. MSG, monosodium glutamate; NA, not applicable**.**

4.3.3 *Analysis of GABA content in bacterial-derived lyophilised preparations*

Briefly, to analyse the GABA content of bacterial-derived lyophilised preparations, samples were deproteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid. They were then allowed to stand for 10min before centrifuging. Supernatants were removed and diluted with 0.2M sodium citrate buffer, pH 2.2 to yield c. 250nM of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125nM. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser fitted with a Jeol Na^+ high-performance cation exchange column.

4.3.4 *Ussing chamber studies*

Distal colonic segments were placed between the two halves of the Ussing chamber, exposed tissue area of $0.12cm^2$, maintained at 37°C in Krebs solution and oxygenated with carbogen gas (95% O_2, 5% CO_2). Tissues were voltage clamped to zero, using an automatic voltage clamp (DVC-1000/EVC-4000, World Precision

Instruments, Sarasota, USA). All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using DataTrax software (World Precision Instruments).

4.3.5 *Experimental design*

Once a stable I_{sc} was achieved, either GABA (0.5µM - 100µM) (Li et al., 2012b), *Lactobacillus brevis* DPC 6108-derived GABA (1µM or 100µM) or reconstituted supernatants derived from *Lactobacillus reuteri* DPC 6100 (non-GABA control) were added to the serosal compartment of the Ussing chamber and the peak change in I_{sc} recorded. Bethanechol (100µM) (Hirota and McKay, 2006b) and forskolin (10µM) (Hyland and Cox, 2005) were subsequently added to the serosal reservoir 15mins and 30mins later, respectively. For antagonist studies, tissues were pre-treated for 15mins with the $GABA_A$ receptor antagonist, bicuculline (100µM) (Hardcastle et al., 1991; Parkman et al., 1993) or the $GABA_B$ receptor antagonist phaclofen (100µM)(Parkman et al., 1993). Similarly, tissues were pre-treated with tetrodotoxin (300nM) (Ikehara et al., 2010) for 15mins prior to addition of GABA (100µM), bethanechol and forskolin.

4.3.6 *Statistics*

Data were converted to $µA.cm^{-2}$, and are expressed as the mean +/- the standard error of the mean throughout. GraphPad Prism 5 was used for statistical analysis, and data were analysed using either a one-way ANOVA followed by Dunnett's post hoc analysis, two-way ANOVA followed by Bonferroni's post hoc analysis or by a Student's unpaired t-test. A P value less than 0.05 was considered significant.

4.3.7 *Materials*

All drugs were obtained from Sigma Aldrich Ltd. (Ireland). GABA, bethanechol, bicuculline and phaclofen were dissolved in dH_20. Forskolin was dissolved in dimethylsulfoxide and tetrodotoxin in sodium citrate buffer. Krebs buffer consisted of the following, 1.2mM NaH_2PO_4 , 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 11mM $CaCl_2$ and 10mM glucose.

4.4 Results

4.4.1 *Effect of GABA on baseline short circuit current*

Serosal addition of GABA (0.5µM-100µM) to mouse colonic mucosa-submucosa tissue preparations had no significant effect on baseline I_{sc} (**Table 4.2**).

4.4.2 *Effect of GABA on bethanechol- and forskolin-stimulated short circuit current*

Pre-treatment of tissues with GABA (0.5µM - 100µM) significantly influenced both the nature and magnitude of the subsequent tissue response to bethanechol (100µM; **Figure 4.1** and **Figure 4.2**). In the presence of GABA (1µM) a triphasic response to bethanechol was observed; and this was in contrast to the monophasic response in control tissues, and the monophasic, though blunted response, seen with 100µM GABA (**Figure 4.1**). Accordingly, I split the bethanechol-induced effect on I_{sc} into three phases; phase I, phase II and phase III, measured 30sec, 2mins and 3-5mins (peak response) after addition of bethanechol. Phase II and phase III of the bethanechol response were significantly inhibited by GABA at all the concentrations tested (0.5µM - 100µM; $P < 0.05 – P < 0.001$), and the phase I response by 0.5µM GABA ($P < 0.05$; **Figure 4.2**). For subsequent

experiments with GABA receptor antagonists and TTx, phase III of the bethanechol response was analysed, and tissues were pre-treated with GABA at a concentration of 100µM.

GABA (0.5µM - 100µM) had no significant effect on the tissue response to forskolin (10µM; **Table 4.3**).

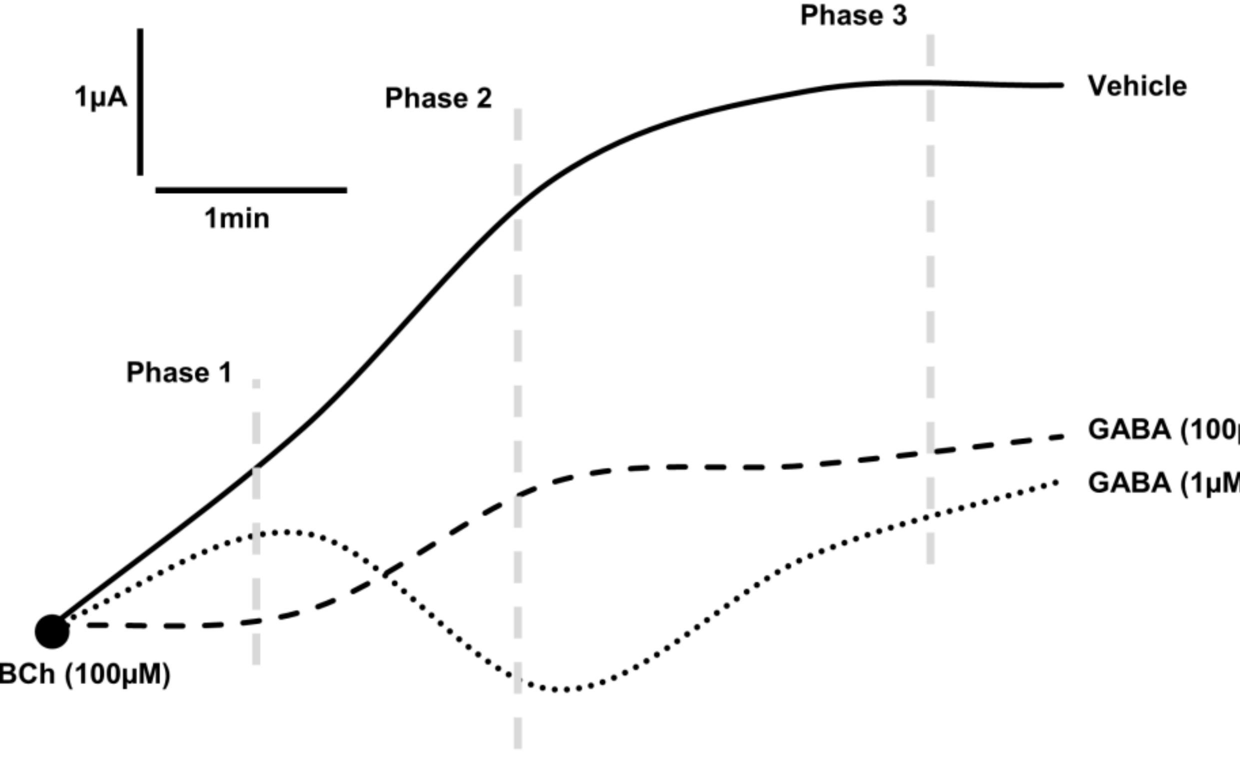

Figure 4.1. Representative response to bethanechol in the presence of GABA. In the presence of GABA (1µM) a triphasic response to bethanechol was observed; and this was in contrast to the monophasic response in control tissues, and the monophasic, though blunted response, seen with 100µM GABA. Phase I, phase II and phase III of the bethanechol response represent the short-circuit current response (I_{sc}) measured 30sec, 2mins and 3-5mins (peak response) after addition of bethanechol (●).

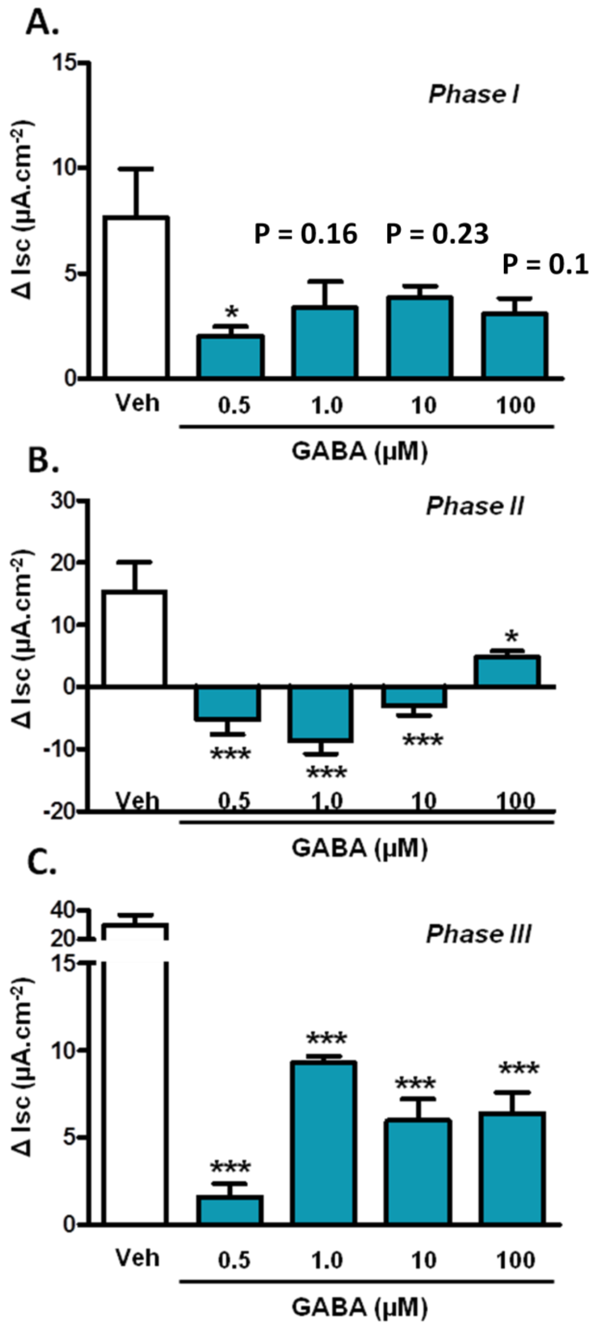

Figure 4.2. The influence of increasing concentrations of GABA on each phase of the bethanechol-induced response in mouse colon. Phase II (B) and phase III (C) of the bethanechol response was significantly inhibited by GABA; and the phase I response only by 0.5μM GABA (A). * vehicle *versus* GABA, *P < 0.05, ***P<0.001. *n*=4-6

4.4.3 *Effects of the $GABA_A$ receptor antagonist, bicuculline and $GABA_B$ receptor antagonist, phaclofen on baseline short circuit current*

The $GABA_A$ receptor antagonist, bicuculline (100µM) significantly decreased I_{sc} ($P < 0.001$) relative to vehicle (**Figure 4.3**A). In contrast, the $GABA_B$ receptor antagonist, phaclofen (100µM) did not significantly influence baseline I_{sc} in mouse colon (**Figure 4.3**B).

4.4.4 *Sensitivity of the GABA-mediated effect on bethanechol-induced ion transport to $GABA_A$ and $GABA_B$ receptor antagonism*

To determine whether the inhibitory effect of GABA (100 µM) on cholinergic-induced I_{sc} was mediated by either $GABA_A$ or $GABA_B$ receptors, tissues were pre-treated with either bicuculline (100µM) or phaclofen (100µM) prior to addition of GABA (100µM) and bethanechol (100µM). Pre-treatment with bicuculline significantly relieved the inhibitory effect of GABA on bethanechol-induced I_{sc} ($P < 0.001$; **Figure 4.4**A); in contrast phaclofen had no such effect (**Figure 4.4**B).

4.4.5 *Sensitivity of the GABA-mediated effect on bethanechol-induced ion transport to tetrodotoxin*

Tissues were pre-treated with TTx (300nM) to determine whether the inhibitory effect of GABA (100µM) on bethanechol-induced I_{sc} involved enteric neurones. Both TTx ($P < 0.001$) and GABA ($P < 0.001$) significantly inhibited the bethanechol response in mouse colon. Moreover, the combination of GABA and TTx together abolished the I_{sc} response to bethanechol (**Figure 4.5**).

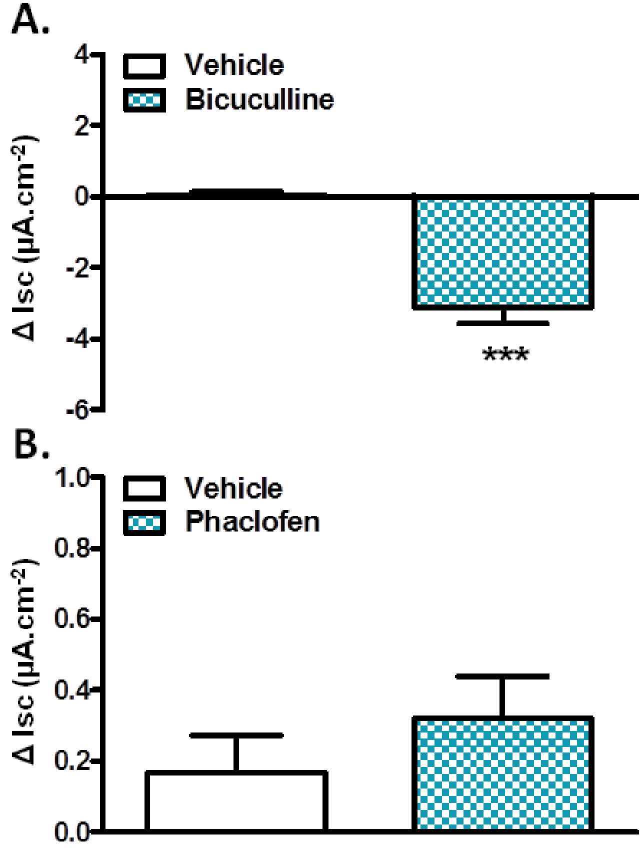

Figure 4.3. Effects of $GABA_A$ and $GABA_B$ receptor antagonism on baseline short-circuit current. Bicuculline (100µM; A) significantly reduced baseline short-circuit current (I_{sc}) in mouse colon. In contrast, phaclofen (100µM; B) did not significantly influence baseline I_{sc}. * vehicle *versus* GABA receptor antagonist, ***P<0.001. *n*=6.

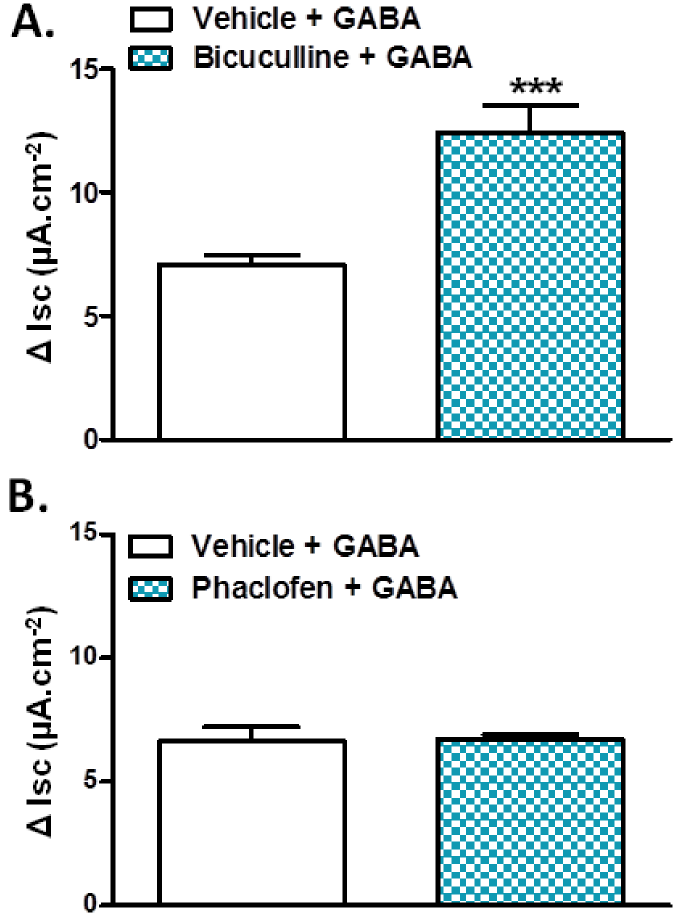

Figure 4.4. Effects of $GABA_A$ and $GABA_B$ receptor antagonism on GABA-induced inhibition of bethanechol responses in mouse colon. Pre-treatment of tissues with bicuculline (100µM; A) significantly relieved the inhibitory effect of GABA on bethanechol-induced I_{sc}, in contrast phaclofen (100µM; B) had no such effect. * GABA *versus* GABA receptor antagonist + GABA, ***P<0.001. *n*=4.

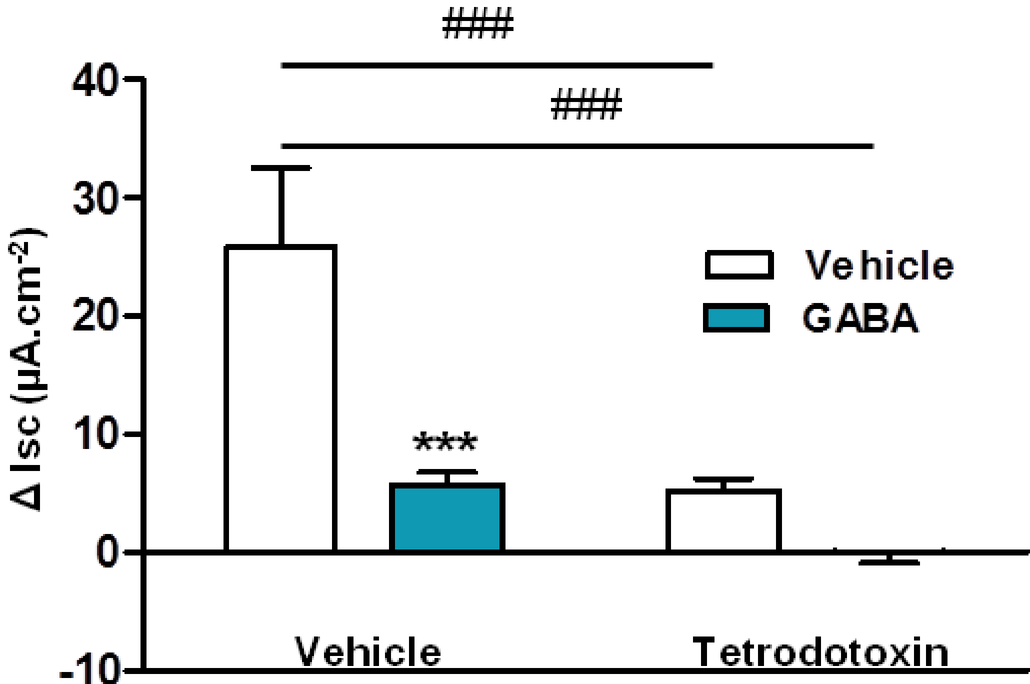

Figure 4.5. The effects of GABA and tetrodotoxin on bethanechol-induced I_{sc}. Both tetrodotoxin (TTx; 300nM) and GABA (100µM) significantly inhibited the bethanechol response in mouse colon, and the combination of GABA and TTx together abolished the I_{sc} response to bethanechol. * vehicle *versus* GABA, ***P<0.001; # vehicle *versus* TTx, ### P < 0.001. *n*=5-6.

4.4.6 *Effects of bacterial-derived, GABA-containing, supernatants on baseline and bethanechol-induced ion transport*

Lyophilised supernatants from *Lactobacillus brevis* DPC6108 were reconstituted and added to the serosal compartment of the Ussing chamber to yield a final GABA concentration of either 1µM or 100µM, and these were compared to supernatants prepared from the non GABA-producer, *Lactobacillus reuteri* DPC6100. Neither 1µM GABA-containing supernatants nor control supernatants had a significant effect on baseline I_{sc}, however 100µM GABA-containing supernatants significantly increased baseline I_{sc} (**Table 4.2**).

GABA-containing supernatants (1µM and 100µM) derived from *Lactobacillus brevis* DPC6108 significantly inhibited the tissue response to bethanechol ($P < 0.05$; **Figure 4.6** A and B). Forskolin responses were unaffected following pre-treatment with either GABA-containing or GABA-free supernatants (**Table 4.3**).

Table 4.2. Effect of exogenous GABA, GABA-containing and GABA-free bacterial supernatants on baseline short-circuit current.

	Concentration of GABA (µM)				
	0	**0.1**	**1**	**10**	**100**
Exogenous GABA (ΔI_{sc} µA.cm^{-2})	0.1±0.2	0.1±0.2	0.4±0.1	0.3±0.1	0.2±0.2
***Lactobacillus reuteri* DPC6100 (ΔI_{sc} µA.cm^{-2})**	NA	NA	0.2±0.03	NA	1.4±0.5
***Lactobacillus brevis* DPC6108 (ΔI_{sc} µA.cm^{-2})**	NA	NA	-1.5±2.0	NA	5.3±0.8 *

Lactobacillus reuteri DPC6100, non GABA-producer; *Lactobacillus brevis* DPC6108, GABA-producer. * $P < 0.05$, *Lactobacillus reuteri* DPC6100 *versus* *Lactobacillus brevis* DPC6108. NA, not applicable.

4.4.7 *Sensitivity of the bacterial-derived GABA-mediated effect on bethanechol-induced ion transport to $GABA_A$ and $GABA_B$ receptor antagonism*

To determine whether the inhibitory effect of bacterial-derived GABA (1μM) on cholinergic-induced I_{sc} was mediated by either $GABA_A$ or $GABA_B$ receptors, tissues were pre-treated with either bicuculline (100μM) or phaclofen (100μM) prior to addition of bacterial-derived supernatants and bethanechol (100μM). Supernatants from the non GABA-producer, *Lactobacillus reuteri* DPC6100 had no significant effect on bethanechol-induced I_{sc} (**Figure 4.7** A and B). Those obtained from *Lactobacillus brevis* DPC6108 significantly inhibited tissue responses to bethanechol (**Figure 4.7** A and B; $P < 0.05 – P < 0.01$), and this effect was reversed in the presence of the $GABA_A$ receptor antagonist, bicuculline (**Figure 4.7**A).

Table 4.3. Effect of exogenous GABA, GABA-containing and GABA-free bacterial supernatants on forskolin-stimulated short-circuit current.

	Concentration of GABA (μM)				
	0	**0.1**	**1**	**10**	**100**
Exogenous GABA (ΔI_{sc} μA.cm^{-2})	64.4±15.1	48.2.±12.2	80.0±.16.8	80.0±.05	73.4±34.2
***Lactobacillus reuteri* DPC6100 (ΔI_{sc} μA.cm^{-2})**	NA	NA	336.4±.44.0	NA	239.1±.23.2
***Lactobacillus brevis* DPC6108 (ΔI_{sc} μA.cm^{-2})**	NA	NA	247±58.5	NA	285.2±37.3

NA, not applicable. *Lactobacillus reuteri* DPC6100, non GABA-producer; *Lactobacillus brevis* DPC6108, GABA-producer. P=0.19 (*Lactobacillus reuteri* DPC6100 (1μM) vs. *Lactobacillus brevis* DPC6108 (1μM)), P=0.17 (*Lactobacillus reuteri* DPC6100 (100μM) vs. *Lactobacillus brevis* DPC6108 (100μM).

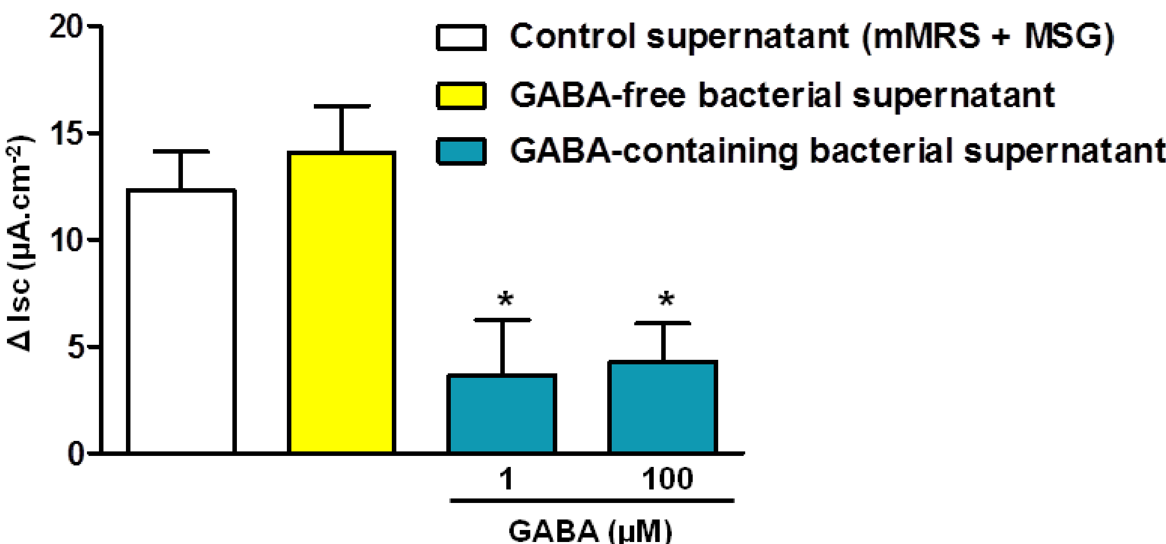

Figure 4.6. The influence of bacterial-derived GABA on bethanechol-induced short-circuit current in mouse colon. GABA-containing supernatants derived from *Lactobacillus brevis* DPC6108 (A, 1 μM and B, 100 μM) significantly decreased bethanechol-induced responses compared to supernatants prepared from media alone (mMRD + MSG) or the non-GABA-producer, *Lactobacillus reuteri* DPC6100. * GABA-containing bacterial supernatants *versus* GABA-free supernatants or control supernatants, *P<0.05. *n*=3-5.

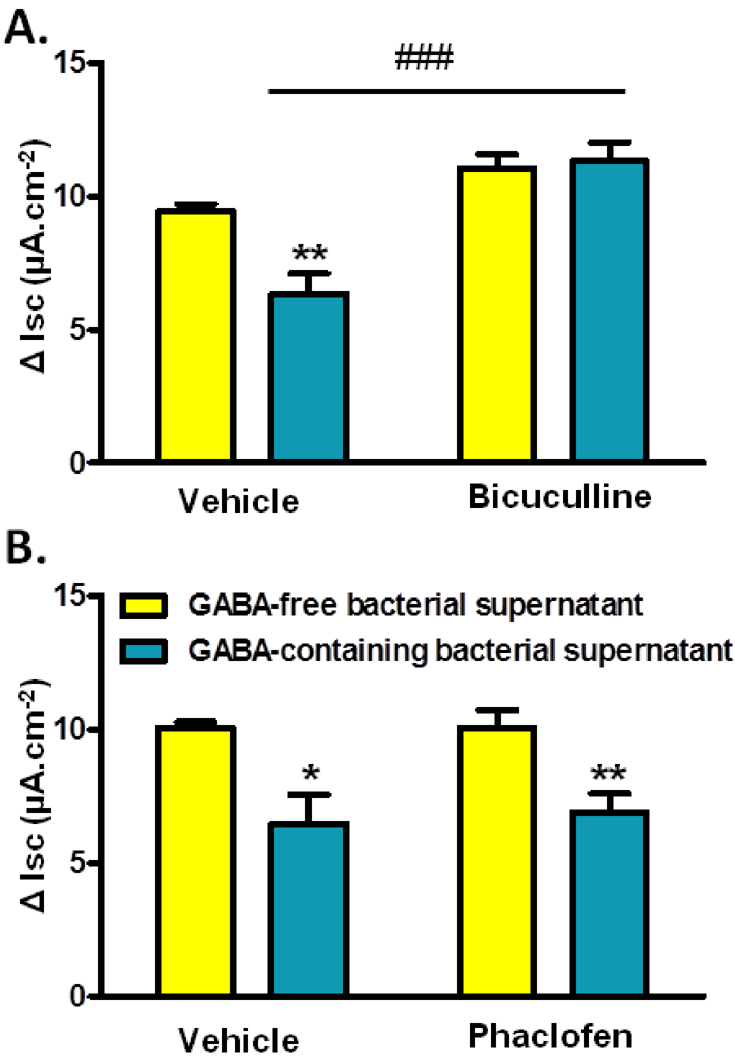

Figure 4.7. Effects of GABA$_A$ and GABA$_B$ receptor antagonism on bacterial-derived GABA-induced inhibition of bethanechol-induced short-circuit current in mouse colon. Supernatants from the non GABA-producer, *Lactobacillus reuteri* DPC6100 had no significant effect on bethanechol-induced short-circuit current (A and B). Those obtained from *Lactobacillus brevis* DPC6108 significantly inhibited tissue responses to bethanechol (A and B), and this effect was reversed in the presence of the GABA$_A$ receptor antagonist, bicuculline (A) and unaffected by the GABA$_B$ receptor antagonist, phaclofen (B). *GABA-containing bacterial supernatants *versus* GABA-free bacterial supernatants, *P<0.05, **P<0.01; # GABA-containing bacterial supernatant *versus* GABA receptor antagonist + GABA-containing bacterial supernatant; ### P < 0.001. *n*=5-7.

4.5 Discussion

Our data suggest that GABA significantly influences colonic secretomotor function in the mouse colon. This effect, however, does not appear to be concentration dependant. Similarly, GABA derived from an efficient GABA-producing commensal organism, *Lactobacillus brevis* DPC6108 influenced tissue responses to cholinergic stimulation in a similar manner to exogenous GABA. The inhibitory effect I observed for both exogenous and bacterial-derived GABA on cholinergic responses in mouse colon displayed sensitivity to the $GABA_A$ receptor antagonist, bicuculline, and were unaffected by the $GABA_B$ receptor antagonist, phaclofen. Moreover, the $GABA_B$ receptor agonist, baclofen did not significantly influence baseline I_{sc} in mouse colon (unpublished observation). The predominant role of $GABA_A$ receptors in the regulation of intestinal fluid and electrolyte transport in mouse colon is consistent with studies in the small intestine of other species (Hardcastle *et al.*, 1991; MacNaughton *et al.*, 1996). In the guinea-pig intestine, the $GABA_A$ receptor agonist, 3-amino-1-pro-panesulfonic acid (3-APS) stimulated an increase in I_{sc}, while baclofen had no effect (MacNaughton *et al.*, 1996), and in the rat small intestine bicuculline significantly reduced I_{sc} (Hardcastle *et al.*, 1991), further implicating a role for $GABA_A$ receptors in the regulation of intestinal ion transport.

Though $GABA_A$ receptors have been implicated in the regulation of intestinal ion transport, the nature of the response they mediate appears to vary across species, or intestinal region. The observation of $GABA_A$-receptor mediated tone in the rat intestine (Hardcastle *et al.*, 1991) supports our findings of GABAergic tone in mouse colon, in which bicuculline significantly decreased baseline I_{sc}. These data, suggest that GABA, through $GABA_A$ receptors, tonically regulates colonic ion transport in

the mouse colon, which in the rat intestine was determined to be anti-secretory in nature (Hardcastle *et al.*, 1991). Nonetheless, I did observe a modest, albeit significant, increase in baseline I_{sc} in response to the supernatant derived from the GABA-producing bacterial strain, *Lactobacillus brevis* DPC6108. I did not, however, further characterise this response to determine whether it was GABA receptor mediated, though it is known that bacterial factors, independent of GABA, can influence intestinal ion transport *in vitro* (Lomasney *et al.*, 2013). In guinea-pig intestine both GABA and the $GABA_A$ receptor agonist, 3-APS significantly increased I_{sc}, and in the case of the later this effect was chloride ion dependent, and therefore most likely secretory in nature (MacNaughton *et al.*, 1996). Similarly, *in vivo* studies in mouse small intestine suggest that GABA, again via $GABA_A$ receptors, stimulates ileal fluid transport, and does so via an epithelial mechanism (Li *et al.*, 2012). Moreover, the same authors demonstrated that $GABA_A$ receptor antagonism in a mouse model of allergic diarrhoea decreased epithelial $GABA_A$ receptor expression, as well as the percentage of mice with diarrhoea (Li *et al.*, 2012). Conversely, the response I observed in mouse colon may be considered predominantly pro-absorptive, given the significant inhibition by GABA of cholinergic-induced ion transport. However, discrepancies between *in vivo* and *in vitro* studies are perhaps not unexpected, and may be further confounded by the nature of $GABA_A$ receptors, which have been demonstrated to regulate both excitatory and inhibitory responses in guinea pig distal colon (Minocha *et al.*, 1993). Therefore, the final response elicited by GABA is likely to depend on the balance of $GABA_A$ receptor activity on either inhibitory or excitatory interneurons, which may differ across species, tissue regions and experimental conditions, as well as on the neurochemistry of $GABA_A$ receptor expressing neurones.

The predominant mucosal effect of GABA on fluid transport in mouse ileum (Li *et al.*, 2012) is, however, in contrast to the effects observed in other species, namely rat (Hardcastle *et al.*, 1991) and guinea pig (MacNaughton *et al.*, 1996), in which the enteric nervous system has been implicated in mediating the GABAergic effects on intestinal ion transport. In the later, TTx abolished the I_{sc} response to the $GABA_A$ receptor agonist, 3-APS, and in rat small intestine the effects of the $GABA_A$ receptor antagonist, bicuculline on I_{sc} also displayed sensitivity to the neurotoxin (Hardcastle *et al.*, 1991). Moreover, 3-APS significantly inhibited capsaicin-stimulated I_{sc} responses. I cannot conclusively comment on the site of action, neural or epithelial, for GABAergic inhibition of bethanechol-induced ion transport in mouse colon. I can speculate however, that GABA may predominantly exert its effects at the level of the enteric nervous system, which is responsible, in the most part, for facilitating bethanechol-induced ion transport, as I demonstrated a similar inhibition of bethanechol-induced responses by either pretreatment with GABA or TTx. In mouse colon the bethanechol-induced effect on I_{sc} is predominantly muscarinic in nature, though appears to occur independent of M_1 receptors (Hirota *et al.*, 2006b). Whilst, others have demonstrated, in M_3 receptor knockout mouse colon, an appreciable decrease in carbachol-induced ion transport in the presence of TTx (Haberberger *et al.*, 2006), though there are a paucity of studies characterising cholinergic responses in the mouse intestine (Hirota *et al.*, 2006a). Nonetheless, in mouse colon both epithelial and neural muscarinic receptors are likely to be involved in mediating cholinergic induced ion transport, and as such GABA, indirectly, may influence cholinergic responses elicited by bethanechol at both sites. Moreover, nicotinic receptor blockade, in guinea pig intestine, did not influence the subsequent I_{sc} response to the $GABA_A$ receptor agonist, 3-APS (MacNaughton *et al.*, 1996). A

neural site of action for GABA in mouse colon is perhaps further supported by the depolarising effect of the $GABA_A$ receptor agonist, muscimol on intestinal epithelial cells (Li *et al.*, 2012), which one would expect to increase I_{sc}, inconsistent with the diminished secretory response I observed in the presence of GABA in mouse colon.

In the enteric nervous system $GABA_A$ receptors may be excitatory (Cherubini *et al.*, 1984), and therefore following activation have the potential to release both excitatory and inhibitory neurotransmitters. In rat colonic segments for example, the $GABA_A$ receptor antagonist bicuculline inhibited release of L-[3H]citrulline, a marker for nitric oxide (NO) biosynthesis (Grider, 1994), and may be particularly relevant given the demonstrated inhibitory effects of NO on I_{sc} in mouse intestine (Rao *et al.*, 1994), which functionally could dampen the subsequent I_{sc} response to secretogogues, such as bethanechol. Moreover, $GABA_A$ receptors can differentially regulate the release of enterochromaffin-cell derived serotonin (5-HT) from guinea pig small intestine, either by directly inhibiting 5-HT release, or, conversely, stimulating its release via a neural reflex (Schwörer *et al.*, 1989), which again could profoundly influence intestinal secretomotor function (Tuo *et al.*, 2004; Michel *et al.*, 2005; Yang *et al.*, 2008; Yang *et al.*, 2010). Therefore, further pharmacological characterisation of the GABA-induced inhibitory effect on cholinergic-induced ion transport in mouse colon is now warranted. Another striking effect of GABA on cholinergic-induced I_{sc} in mouse colon is the differential influence it has on the nature of the I_{sc} response at different concentrations. In control tissues, the I_{sc} response to bethanechol was monophasic in nature, comparable to that previously observed in mouse colon at the same concentration (Hirota *et al.*, 2009). However, at a comparably lower concentration, 1µM, the

nature of the I_{sc} response was significantly altered, and tri-phasic in nature. The mechanism underlying this differential effect of GABA on the nature of the bethanechol-evoked response in mouse colon is unclear, but may be due to differential effects of GABA, or GABA-mediated inhibition of cholinergic signaling, on either intracellular signaling molecules or ion channels known to contribute to cholinergic regulation of epithelial ion transport (Hirota *et al.*, 2006a).

Our data further support the concept of microbial endocrinology, a relatively new inter-disciplinary field bringing together microbiology and neuroscience, with the aim of gaining greater insight into host-microbial interactions (Lyte, 2011). In this study, I have demonstrated that GABA derived from *Lactobacillus brevis* DPC6108 influences colonic secretomotor function in a similar manner to exogenous GABA and by the same GABA receptor. Furthermore, though both forms of GABA were added serosally, GABA receptors are believed to be located primarily on enteric neurons (Takahashi et al., 2000b; Casanova et al., 2009a) while transepithelial transporters of GABA and GABA analogs (including muscimol) have been identified *in vitro* in Caco-2 cells (Thwaites et al., 2000; Chen et al., 2002; Larsen et al., 2008). Moreover, though further studies are warranted, in the context of functional GI disorders which often display a leaky epithelial barrier, it may be anticipated that high concentrations of luminal GABA may reach enteric nerves via a paracellular pathway. Indeed, cholinergic neurons are believed to play an important role in controlling epithelial permeability(Sharkey and Savidge, 2014), and enhanced neural cholinergic activity is associated with increased permeability (Cipriani et al., 2011). Therefore targeting neural GABA receptors may have potential therapeutic benefit at the level of the intestinal mucosa.

However, though I have been successful in ascribing physiological function to bacterial-derived GABA utilising *ex-vivo* tissue preparations, whether such bacteria generate sufficient quantities of GABA mucosally *in-vivo* to affect secretomotor function remains to be determined. Nevertheless, *Lactobacillus brevis* DPC6108, in the presence of other culturable microbes, produced 66.25µg/ml (~643µM) GABA after 4h, reaching a maximum of 70.72µg/ml (~686µM) after 9h in a pH-controlled faecal-based fermentation in the presence of MSG (Barrett et al., 2012). This would suggest that the microbiota, and GABA-producing microbes, generate luminal concentrations of GABA in excess of those demonstrated to exert physiological effects on colonic function in this study. Therefore, identifying commensal organisms, or probiotics, such as *Lactobacillus brevis* DPC6108, capable of producing physiologically high concentrations of GABA (Barrett *et al.*, 2012), may provide a novel and safe delivery mechanism of neuroactive compounds locally to the GI tract. In the context of our findings of $GABA_A$ receptor-mediated inhibition of cholinergic-stimulated ion transport, *Lactobacillus brevis* DPC6108, or $GABA_A$ receptor agonists, may be therapeutically relevant in the context of diarrhoea, with the *caveat* that sufficient quantities of GABA reach the secretomotor apparatus to affect colonic fluid and electrolyte transport.

Chapter 5:

General Discussion

5.1 A Summary of Results

In this thesis I present data that further supports the concept of host microbe interactions and the influence of probiotics, the host microbiota and commensal organisms on intestinal function, in particular colonic ion transport. In addition, I have identified strain-specific effects of *Bifidobacterium infantis 35624* and *Lactobacillus salivarius* UCC118 on intestinal ion transport in *ex-vivo* colonic tissue exposed to both microbes, acutely and following probiotic feeding for two weeks in the Ussing Chamber.

First, in **Chapter 2**, I examined the influence of the two different probiotic strains, *B.infantis* 35624 and *L.salivarius* UCC118, on I_{sc} responses in murine colon. *B.infantis* 35624 has demonstrated therapeutic potential clinically, in IBS (O'Mahony et al., 2005; Whorwell et al., 2006), while both have shown potential benefits in pre-clinical models of ulcerative colitis (O'Mahony et al., 2001; McCarthy et al., 2003; Ewaschuk et al., 2008; Feighery et al., 2008). *Ex-vivo* tissue studies allowed us to further characterise the influence of *B.infantis* 35624 and *L. salivarius* UCC118 on the GI function by examining their effects on colonic ion transport in particular. Our data suggest that *L. salivarius* UCC 118 mediates its effects on I_{sc} predominantly via a neurally driven pathway, with *B.infantis* 35624 exerting less of an influence on enteric neuron activity. Both probiotics also significantly attenuated cholinergic induced changes in I_{sc}, and may therefore be considered to have anti-secretory properties. Neither probiotic influenced cAMP-mediated ion transport. Additional pharmacological studies suggested that neither apical ENaC nor basolateral NKCC1channels were involved in mediating the effects of *B.infantis* 35624 and *L. salivarius* UCC118 on baseline I_{sc} following acute

addition to Ussing chambers. However, ion exclusion experiments did suggest a role for a Cl^--independent pathway as a potential mechanism by which *L. salivarius* UCC 118 induces changes in baseline ion transport. *In-vivo* administration of probiotics for two weeks similarly suggested that *L. salivarius* UCC118 preferentially influences enteric neural activity compared to *B. infantis* 35624. Both probiotics, however, similarly affected small intestinal transit.

In **Chapter 3**, I next examined the influence of the host microbiota on I_{sc} by taking an alternative approach. Rather than examining the effect of commensal organisms on colonic ion transport, I examined the effect of a germ-free environment on colonic secretomotor function. I found that the absence of the host microbiota significantly increased cAMP induced colonic ion secretion, while neurally- and cholinergic-driven ion transport remained unchanged. Moreover, in the absence of a host microbiota, baseline I_{sc} and TER were comparable to tissues examined from colonised mice. Tissues from germ free animals responded similarly to two commensal organisms, *B. infantis* 35624 and *L. salivarius* UCC118.

Finally, in **Chapter 4**, I utilised the Ussing chamber method to determine whether GABA derived from *L.brevis* DPC6108 influenced intestinal ion transport and compared these responses to those obtained with the effects of commercially obtained GABA on colonic ion transport. I demonstrated that GABA inhibits cholinergic-induced ion transport and that this effect is $GABA_A$ receptor mediated. Furthermore, I were able to show that GABA produced by a probiotic, *L.brevis* DPC6108 in the presence of other secreted bioactives, similarly influenced cholinergic-induced ion transport by a $GABA_A$ receptor dependent pathway.

Collectively, this thesis provides insight into host-microbe interactions and in particular their impact on colonic secretomotor function. Moreover, it demonstrates that distinct bacteria may differentially influence colonic ion transport. In this regard, *L. brevis* DPC6108 most likely does so as a result of its ability to generate GABA. However, with respect to the influence of specific commensal organisms, namely *B. infantis* 35624 and *L. salivarius* UCC118, as well as the host microbiota, on colonic ion transport, the specific bacterial components or metabolites which contribute to the effects observed in our studies remain, as yet, uncharacterised.

Background and current in-vivo observations

B. infantis 35624	*L. salivarius* UCC118	*L. brevis* DPC6108
Background • Immunomodulatory probiotic. • Targets epithelial and immune cells of the lamina propria. • Effective in treating global symtoms of IBS. ***In-vivo* observations** • Decrease in baseline I_{sc}. • Decrease in small intestinal transit.	**Background** • Produces secreted bioactives effective against pathogenic invasion. • Poor efficay in treating symptoms associated with IBS. ***In-vivo* observations** • Decrease in baseline I_{sc}. • Decrease in veratridine stimulated I_{sc}. • Decrease in small intestinal transit.	**Background** • Produces GABA at high a concentration in normal culture media. • Produces GABA in faecal fermentations in the presence of culturable gut-derived bacteria but at **significantly lower levels.**

Ex-vivo observations in Ussing Chamber and possible mechanisms

Conventional
Germ Free
B. infantis 35624
L. salivarius UCC118
L. brevis DPC6108

Mucous
Cl^- Cl^- HCO_3^-
↑↑↑cAMP
↑↑↑cAMP
???
Cl^-
CaCC
↑↑↑Ca^{2+}
???
TLR-9
TLR-2
TLR-6
Immune cells
BCh
???
IL-10
IL-10
IL-10
IL-10
T-reg (Foxp3)
HCO_3^-
???
↑↑↑Ca^{2+}
Cl^-
BCh
???
Somatostatin
???
BCh
Somatostatin
???
Cholinergic Secretomotor Neuron
GABAa Receptor
GABAergic Interneuron

Host microbiota and secretomotor function	The Ussing Chamber As a Screening Tool for Probiotics		Microbial Endocrinology
	In-vivo	***Ex-vivo***	
• Increased cAMP induced chloride and bicarbonate may prime the intestine for colonisation while protecting from invading pathogens. • Submucosal secretomotor neurons are not influenced by the host microbiota. • Secretomotor function in the presence of myenteric IPAN's should be examined in germ free mice.	*B. infantis* is anti-secretory via a non-neurally mediated mechanism.	*B. infantis* is anti-secretory via a non-neurally mediated mechanism.	• GABA succesfully identifed as an extracellular probiotic bioactive with the ability to influence ion transport function. • Further studies should now examine other neurotransmitters as putative probiotic bioactives
	L. salivarius is anti-secretory via a neurally mediated mechanism.	*L. salivarius* is anti-secretory via a neurally mediated mechanism.	
	Neither probiotic influences secretagogue induced ion transport.	Both probiotics attenuate cholinergic induced ion transport.	
	Both probiotics slowed small intestinal transit.	Small intesintal secretomotor and motility function were not assessed.	
	B.infantis may be usefull for correcting immune-related secretomotor dysfunction while *L. salivarius* may be beneficial in treating ENS mediated dysfunction.		

Figure 5.1. Summary of Key Findings - Germ Free mice displayed elevated cAMP induced ion transport. No effect on secretomotor neuron responses were observed. The elevated cAMP induced secretion may indicate an increase in bicarbonate which would prime the mucosa for colonisation and promote production of mucous. Chronic exposure to ***B. infantis* 35624**, previously characterised as an immunomodulatory probiotic effective in the treatment of IBS, caused a decrease in basal ion transport and small intestinal transit (SIT) *in-vivo* in healthy mice while no influence on neurally mediated ion transport was observed. Acute exposure to the same probiotic in *ex-vivo* distal colon submucosal preparation attenuated cholinergic secretory responses via a non-neurally mediated mechanism. Furthermore, the effects of ***B. infantis* 35624** on basal ion transport were mediated by a heat resistant secreted bioactive. Chronic exposure of healthy mice to ***L. salivarius* UCC118**, previously known to produce bacteriocin, targeting infectious microbes, demonstrated a similar attenuation of basal ion transport and SIT with the added effect of lowering neurally evoked ion transport. In line with this observation, basal responses following acute exposure *ex-vivo* were neurally mediated, while ***L. salivarius* UCC118** also caused a decrease in cholinergic ion transport, an effect which also appeared to be sensitive to neural blockade. With the caveat that not all functional effects can be predicted in-vivo, ex-vivo Ussing Chamber studies correlated with *in-vivo* studies and have the potential to predict possible uses in a clinical setting. ***L. salivarius* UCC118**, known to produce potent anti-microbials, may also be effective in treating pathogenic diarrhoea associated with the ENS e.g. rotavirus (Lundgren et al., 2000), while ***B. infantis* 35624** as previously demonstrated, would be more effective in diseases targeting the epithelium and immune function. Finally supernatant from ***L. brevis* DPC6108**, previously isolated from human intestine and show to produce high levels of GABA was shown to attenuate cholinergic induced ion transport in an manner analogous to exogenous GABA and in a similar manner to ***B. infantis* 35624** and ***L. salivarius* UCC118.** This identified GABA as probiotic bioactive with the ability to modulate ion transport function and may suggest GABA a possible bioactive involved in the effects on cholinergic ion transport mediated by ***B. infantis* 35624** and ***L. salivarius* UCC118.** Importantly, it also demonstrated the potential of screening previously identified bioactives, to understand the mechanisms of probiotics and to identify the bioactives involved.

5.2 Host-Microbe Interface and Secretomotor Function

Examining host-microbe interactions represents the study of complex and highly dynamic ecosystems, in which both the host and its inhabitants interact to ultimately determine the state of the environment at any particular time. The host environment will play a crucial role in the establishment of particular phylogenies, as well as determining subsequent activities executed in order for each organism to

adapt, survive and grow. In turn, the activity of these organisms can impact on the host environment - inextricably linking both. In particular, host factors should be taken into account when ascribing physiological function to the microbiota, in particular host species and segmental tissue differences along the intestine.

In the mouse, the neuronal composition of the submucosal plexus exclusively contains Dogiel Type I neurons which display S-type electrophysiology, while no Dogiel Type II neurons, or IPANs, have been identified thus far in any segment of the mouse intestine (Wong et al., 2008; Mongardi Fantaguzzi et al., 2009; Foong et al., 2014). This would suggest that the mouse may not be a particularly good model for examining submucosal sensory neurotransmission in the gut. However, it is possible that uni-axonal sensory neural function exists in the mouse, as previously observed in the guinea pig (Spencer and Smith, 2004; Mazzuoli and Schemann, 2009). Nevertheless, the innate uniformity of mouse submucosal neurons suggests a relatively incomplete secretomotor circuit in comparison to other species (Vanner and Macnaughton, 2004b). Moreover, McVey Neufeld et al., (2013) recently examined the influence of the microbiota on mouse enteric nerve activity, and found no effect of a germ free environment on the electrophysiological parameters of myenteric S-type neurons. Though this study focussed on myenteric rather than submucosal neurons, it is possible that the development of submucosal S-type neurons may not be influenced by enteric microbes, and may explain why I found no differences in neurally stimulated ion transport in **Chapter 3.** The same group did however, report abnormal myenteric plexus development corresponding with a decrease in the size of myenteric ganglia and an increase in the proportion of in nitrergic neurons (Collins et al., 2014). This study was carried out in early post-natal GF mice and neurochemistry should now be examined in adult GF mice.

Differences in the ENS have also been reported along the length of the intestine, in both the myenteric and submucosal plexus and therefore the response elicited by microbes in the small intestine may not mirror those in the colon and *vice versa* (See **Introduction**). The proportion of cholinergic neurons in mouse is significantly greater in the submucosal plexus of the small intestine compared to the large intestine (Mongardi Fantaguzzi et al., 2009; Foong et al., 2014). The same is true for the myenteric plexus (Sang and Young, 1998). Moreover, the neurochemistry of enteric neurones differs along the mouse intestine. In the ileal submucosal plexus, only 50% of cholinergic neurons are CGRP-IR (Mongardi Fantaguzzi et al., 2009), while all colonic cholinergic neurons are positive for this neuropeptide (Foong et al., 2014). In addition, cholinergic innervation of the colonic submucosal plexus increases from proximal to distal colon (Foong et al., 2014). Given the inhibitory effect I observed for *L. salivarius* UCC118 *in vivo* on enteric nerve activity in the mouse colon, and the TTx sensitivity of the I_{sc} response elicited by two commensal organisms following acute exposure in Ussing chambers, the functional consequences of the inhibitory effect of these microbes on enteric nerve activity is likely to differ depending on the tissue segment examined. Indeed, as discussed in **Chapter 2** studies have already demonstrated that the effects of probiotics on motility can vary depending on the intestinal region studied (Massi et al., 2006b; Wu et al., 2013).

On the other hand, the most significant effect of the GF state on active intestinal ion transport in rat, was observed in the distal segment of the distal colon in which baseline I_{sc} was over twice that observed in SPF animals, and represented 46% of the net absorptive sodium flux compared to 26% in SPF rats (Rösel and von Engelhardt, 1996). However, these dramatic changes in electrolyte transport were

not observed in the proximal segment of the distal colon. Therefore, commensal microbes may also contribute to segmental differences in the gut function. Indeed, the structural, immuno-histochemical and functional changes to myenteric plexus reported in GF mice were observed in jejunum and ileum, but not the duodenum (Collins et al., 2014) Furthermore, as discussed in **Chapter 2** several studies, including our own, have already demonstrated a divergence in the ability of specific commensal microbes to influence enteric nerve activity (Kunze et al., 2009a; Khoshdel et al., 2013; Mao et al., 2013b). The gradient in host commensal microbes along the intestinal tract varies from a low numbers of bacteria in stomach and duodenum [up to 10^3 colony-forming units (cfu) per millilitre (cfu x mL^{-1})], increasing numbers in jejunum and ileum (10^4–10^8 cfu x mL^{-1}) with the greatest number in the colon (10^9–10^{12} cfu x mL^{-1})(Blaut and Clavel, 2007). Given that neurally active factors, including acetylcholine, catecholamines, serotonin and GABA, among other neurotransmitters, are produced by specific strains of several species of bacteria common to the gastrointestinal tract, such as *Lactobacillus* and *Bifidobacteria* (**Table 5.1**) (Roshchina, 2010), differences in microbial populations could significantly impact on gut function and secretomotor activity. For example, free catecholamines in the gut lumen of mouse differed along the length of the intestine, with the highest levels of norepinephrine and dopamine being produced in the colon. Functionally, the higher levels of catecholamines are predicted to contribute to increased fluid and electrolyte absorption, as demonstrated by injection of colonic loops with high concentrations of dopamine (Asano et al., 2012). The effect may be mediated by epithelial dopamine receptor activation. It would be tempting to speculate that colonisation patterns of the gut by GABA-producing bacteria may similarly impact secretomotor function (**Table 1.2**).

Table 5.1 Host commensal microbes known to produce neurally active metabolites - Adapted from (Lyte, 2011) **and reviewed in** (Roshchina, 2010)**.**

Genus	**Neurally Active Metabolite**
Lactobacillus, Bifidobacterium	GABA
Escherichia, Bacillus, Saccharomyces	Norepinephrine
Candida, Streptococcus, Escherichia, Enterococcus	Serotonin
Bacillus, Serratia	Dopamine
Lactobacillus	Acetylcholine

Noteworthy also is the expression of TLRs by enteric neurones which would suggest that the ENS has the capacity to respond to a host of bacterial components. TLRs have been are expressed by the ENS along the length of the GI tract (Barajon et al., 2009; Wang et al., 2010d; Brun et al., 2013). TLRs are known to play a crucial role in recognising and responding to luminal microbes and have been found on enteric neurons where they are vital to ENS development, regulation and function (Anitha et al., 2012; Brun et al., 2013). In the mouse, TLR-2,3,4 and 7 are expressed differentially within the ENS and mucosa of the small and large intestine (Barajon et al., 2009; Wang et al., 2010d; Anitha et al., 2012; Brun et al., 2013). Pertinent to our studies, TLR-4 is more highly expressed in the distal colonic submucosal plexus and mucosa of the mouse (Barajon et al., 2009; Wang et al., 2010d), while TLR-2 is more highly expressed proximally in the small intestinal ENS (Brun et al., 2013) and proximal colonic mucosa (Wang et al., 2010d). Therefore, the responses to probiotics which express ligands for TLR-2, such as EPS which may be produced by probiotics used in this thesis (Raftis et al., 2011; Fanning et al., 2012), could influence ENS function differentially in the small intestine compared with large intestine. Given that TLRs play such a key role in intestinal host-microbe communication, these segmental differences in the pattern of receptor expression, are also likely to influence the way in which particular populations of enteric neurones

respond to microbial components. In support of this, a recent study in mouse demonstrated that exposure of the distal colon to fecal slurry from the cecum and proximal colon induced a TLR-2 expression pattern similar to that normally observed in proximal intestinal regions (Wang et al., 2010d). Therefore, it is possible that differences in TLR expression patterns along the mouse intestine may reflect regional differences in the host microbiota.

From birth to old age, as we mature and grow our microbiome evolves with us and may even contribute to senescence itself (Biagi et al., 2010; Rampelli et al., 2013; Heintz and Mair, 2014). Age related neurodegeneration of the enteric nervous system has been well documented and is believed to contribute significantly to senescence driven GI dysfunction, such as constipation, due to alterations in motility as well as nutrient, fluid and electrolyte transport (Camilleri et al., 2008). In rodent models of healthy aging an increase in alpha synuclein and a loss loss in cholinergic neurons has been observed in both the myenteric and submucosal plexus (Phillips et al., 2003, 2007, 2013). Other studies have found a decrease in nitric oxide innervation and production (Takahashi et al., 2000b). Given that colonic constipation in elderly patients may be associated with a change in the expression of this neurotransmitter (Britton and McLaughlin, 2013), such a change in neurochemistry may underlie the altered GI function associated with aging. However, given that key neurotransmitters, such as noradrenaline, can influence the host microbiota (Lyte and Ernst, 1992), the possibility exists that an altered neurochemical profile of the ENS during aging may influence the microbiota.

Conversely, changes in microbially produced neuroactive metabolites (Table 5.1) could also impact on ENS function. Several studies have demonstrated changes in the ratios of certain phyla such as Bacteroidetes and Firmicutes (Mariat et al.,

2009; Rampelli et al., 2013) which, due to the their ability to produced important neuroactive metabolites, could significantly impact on ENS and secretomotor function. For example, a recent study reported an age-related reduction in the quantity of genes associated with SCFA production in fecal microbiota samples in humans (Rampelli et al., 2013). Such changes would be expected to influence epithelial fluid and electrolyte transport (Binder and Mehta, 1989; Vidyasagar et al., 2005; Zeissig et al., 2007) and motility (Soret et al., 2010; Suply et al., 2012).

Equally, during early life and ENS development, an altered microbiota may dictate long-term changes in the structure of function or the ENS into adulthood (Collins et al., 2014).

5.3 Influence of Secretory Activity on Host Microbiota

A major finding of this thesis was that forskolin-induced changes in I_{sc} were significantly enhanced in the germ free colon, suggesting that the microbiota can influence cAMP-stimulated ion transport, and that its absence results in a hyper-secretory response to forskolin. Moreover, this elevated forskolin response, along with bethanechol responses in GF mice, remained unchanged compared to conventional animals following acute exposure to either *B.infantis* 35624 or *L. salivarius* UCC118. Indeed forskolin responses to all probiotic treatments, including L. brevis DPC6108 supernatants remained unaltered throughout our studies. In contrast, a recent *in-vitro* study has demonstrated that both live pathogenic (E2348/69 and 86/24) and live non-pathogenic (C1, C28/2 and F18, Nissle 1917) *E. coli* strains attenuated forskolin induced changes in I_{sc} (Ohland et al., 2012). While

these influences may be related to the gram-negative cell wall of these bacteria, a similar outcome has been observed for *Lactobacillus rhamnosus* 10893 (Heuvelin et al., 2010).

I propose that the heightened response to forskolin stimulation exhibited by germ free colon may be a protective mechanism, possibly to prevent pathogen invasion or adherence. A number of studies have previously demonstrated the ability of enteric pathogens to cause an attenuation of cAMP mediated responses (Philpott et al., 1996; Hecht and Koutsouris, 1999; Li et al., 1999), and in one such study the effects were associated with increased bacterial adherence (Li et al., 1999). Of note, germ free colon mounted a similar I_{sc} response to two commensal organisms relative to conventional tissues. However , I did not assess the responsiveness of germ free tissue to pathogenic bacteria. Moreover, in NHE3 and NHE8 deficient mice, which display compromised secretory function, an increase in bacterial translocation was observed (Laubitz et al., 2008). Conversely, a recent study determined that cAMP-driven chloride secretion prevented the translocation of enteric pathogens across the intestinal epithelium (Keely et al., 2012). In particular, lubiprostone-stimulated I_{sc} was associated with a decrease in the translocation of both *E. coli* ATCC33694 and *S. typhimurium* ATCC700408, anaerobic and aerobic pathogens respectively (Keely et al., 2012). Elevated levels of cAMP in the absence of the host-microbiota could also reflect differences in the luminal levels of nutrients, neurotransmitters or atmospheric CO_2 levels, normally produced by host commensals, which could all potentially modify intracellular cAMP (Gancedo, 2013).

Another mechanism by which germ free intestine adapts to colonisation, here again, potentially as a protective mechanism, is the production of mucous. Stimulation of germ free intestine with common bacterial products, such as

lipopolysaccharide or peptidoglycan, stimulates establishment of a functional mucous layer (Petersson *et al.,* 2011). A characteristic feature of GF animals is a much thinner mucous layer, and fewer goblet cells compared to conventional mice/rats (Sharma et al., 1995). Therefore, the microbiota plays a role in establishing a functional mucous layer, which in turn decreases the translocation of bacteria across the epithelial barrier (Liong, 2008). Moreover, a balance exists between secretory activity and mucous formation, with CFTR deficient mice displaying an over accumulation of mucous (Garcia et al., 2009). Therefore, in germ free colon, in the absence of a functioning mucous layer, increased secretory function may act as a non selective protective mechanism.

Noteworthy, a recent study has demonstrated a role for epithelial ion transporters in microbial niche development (Engevik et al., 2013). Mice deficient for the apical anion exchanger, NHE3 (**Figure 1.1**), resulted in an increase in Bacteroidetes and decreased Firmicutes phyla both in the luminal and mucosa-associated bacterial populations (Engevik et al., 2013). Importantly, this study also demonstrated that the growth and proliferation of a specific commensal microbe *Bacteroidetes thetaiotaomicron* ATCC 29741 in the terminal ileum was directly dependent on the level of luminal Na^{+}. Keely et al., (2012) also determined that lubiprostone significantly influences the host microbiome, and observed alterations primarily within the Firmicutes and Bacteroides phyla, as well as an over representation of *Lactobacillus* in mouse faeces, indicating a direct effect of increased fluid secretion on microbial phylogeny. Together these results support the concept that ion transport may play an important and direct role in establishing a host microbial niche.

5.4 The Ussing Chamber as a Tool to Examine Host-Microbe Interactions.

Our increased understanding of the host microbiota as a complex organ, capable of shaping our development and maintaining our health, has led to a significant increase of microbiota-related research in a variety of disciplines, including physiology and neuroscience, which classically have not been associated with microbiology. While this is certainly exciting, a significant drawback is the lack of appropriate tools to tackle the enormous task of studying the impact of microbes on host physiology and function. Thus far, many of the most significant advances in our knowledge of host-microbe interactions , to some degree, have focused on the host immune system (Sommer and Bäckhed, 2013). With regards to ENS function, recent *ex-vivo* studies have provided some exciting insights into the functional and developmental impact of these interactions, demonstrating robust, strain dependant effects on sensory neural signalling (Kunze et al., 2009b; Mao et al., 2013a) and intestinal motility (Wang et al., 2010b) Furthermore, studies have now begun to investigate the influence of the microbiota and probiotics on intestinal secretory function, perhaps due to the availability of techniques which facilitate the examination of host-microbe interactions in this context, for example Ussing chambers (Rösel and von Engelhardt, 1996; Lomasney and Hyland, 2013).

The Ussing chamber is a particularly useful tool to examine the influence of the microbiota on host physiology, especially with regard to intestinal ion transport and barrier function. Ussing chambers are considered the gold standard for the study of epithelial ion transport (Hamilton, 2011). Since Ussing's original experiment examining the active transport properties of frog skin (Ussing and Zehran, 1951),

Ussing chambers have been continually used to better understand the molecular mechanisms underlying epithelial ion transport (Clarke, 2009a). Moreover, they have proven to be a useful tool in characterising the molecular mechanisms underlying pathogen-induced diarrheal diseases (Field, 2003). Perhaps the greatest advantage of Ussing Chambers is that they provide a relatively precise method of measuring electrical and transport parameters in polarised tissue (Clarke, 2009a), and can be adapted to examine *ex-vivo* tissue samples, including human biopsies (Larsen et al., 2001). In addition, they facilitate examination of tissues, such as the upper GI tract and small intestine, which are normally difficult to study *in-vivo* (Wu et al., 2013).

Thus far, the majority of studies examining the ability of probiotics to influence intestinal ion transport have utilised intestinal epithelial cell lines, such as T-84 and Caco-2 cells(Lomasney and Hyland, 2013). While representing a good model for studying epithelial ion transport (Dharmsathaphorn and Madara, 1990), and a practical and cost effective one (Cencic and Langerholc, 2010), how physiologically relevant such *in-vitro* approaches are remains open for discussion. This is primarily due to structural differences between epithelial mono-layers grown for *in-vitro* studies, notably the absence of other critical cell types in mono-culture systems such as enteric neurones and mucosal immune cells. *Ex vivo* tissue analysis, such as that employed in our studies, may overcome some of the limitations associated with *in vitro* cell line-based experiments, particularly with respect to tissue architecture and presence of tissue-associated immune and neural elements.

Short-circuit current, or I_{sc}, is a measurement of the sum of active ionic currents across an epithelium, and fluctuation in this current is considered an indicator that the net fluid transport in a tissue has changed (Clarke, 2009a). As a tool to screen probiotics for their effects on intestinal physiology, this technique

offers potential for determining the effects of putative probiotics to modify ion transport, thus giving an indication toward their application in particular disorders. However, I_{sc} alone is a relatively simple measurement and does not reflect the complexity of the physiological system. Indeed, most results require a certain amount of interpretation, even with regard to the most basic nature of the response measured. For example, I_{sc} alone does not provide definitive information on the direction of ion flux (Larsen, 2011). To truly understand the ionic nature of a response, further experimentation, such as ion substitution experiments and pharmacological inhibition of transporters are often required. Moreover, stripped tissue, lacking the myenteric plexus and associated muscle layers, while influencing tissue viability, may also result in an under estimation of I_{sc} responses to certain stimulants, particularly those involving a myenteric reflex, such as GABA-stimulated ion transport (Hardcastle and Hardcastle, 1996). The physical consequences of tissue preparation and dissection may also activate stretch receptors and subsequent release of compounds including eicosanoids, potentially confounding subsequent I_{sc} responses.

In addition, although voltage clamping is the accepted method in many laboratories for studying active ion transport, given that probiotics can alter electroneutral ion exchange (Lomasney and Hyland, 2013), it is also important to measure passive transport processes. In this regard, measuring the potential difference (PD) may be of more benefit as in general, PD reflects the voltage gradient generated by the tissue, therefore, any alterations in ion channel or exchanger activity will be picked up. Moreover, measuring the flux of radioactive isotopes, such as $^{22}Na^{+}$, or ion substitution experiments can to help determine the nature of changes in PD (Rösel and von Engelhardt, 1996; Clarke, 2009). Used in

conjunction with I_{sc}, these approaches may give more insight with regard to the influence of host-microbe interactions on intestinal fluid and electrolyte transport (Rösel and von Engelhardt, 1996). However, whether such techniques can be successfully applied in the presence of bacteria and their supernatants remains to be determined.

In addition to recording I_{sc}, the Ussing chamber facilitates measurement of TER. TER has been used in numerous studies, including those presented in this thesis, to assess the impact of microbes on intestinal barrier integrity. However, TER represents an overall measure of the whole exposed area of the experimental chamber and consists of a para-cellular and a trans-cellular resistance in parallel. Therefore, while TER is useful when used in conjunction with I_{sc}, as a direct indicator for the physical "tightness" of the para-cellular seal, it is not always a dependable indicator of actual para-cellular permeability and barrier function. Indeed, molecular differences in the TJ complexes responsible for para-cellular permeability to ions, solutes and water, do not always result in a change in TER and barrier integrity, though they may affect transport function (Schulzke et al., 2005). Equally, as discussed in **Chapter 2**, changes in TER do not always manifest as a change in either para-cellular permeability or TJ protein gene expression. .This can be due to the physiochemical properties and thus selective permeability of TJ proteins, which in some cases, may be altered without modifying the integrity of other protein complexes involved in cell-cell adhesion, such as desmosomes and apical junctional complexes (Suzuki, 2013). This is likely because TJ formation has to be dynamic to accommodate homeostatic intestinal epithelial cell turnover.

Furthermore, in *ex-vivo* intestinal tissue, TER and I_{sc} may also be influenced by the resistance of underlying subepithelial tissues, such as blood vessels, muscle

and other non-epithelial tissue (Gitter et al., 1998). Indeed, in leaky epithelial tissues, such as those found in the intestine, the subepithelial resistance can exceed the TER (Fromm et al., 1985; Hemlin et al., 1988). Therefore, TER measurements in *ex-vivo* tissue, containing subepithelial structures, may not accurately represent changes in epithelial barrier permeability and transport alone.

Moreover, intestinal enterocytes can adapt both morphologically and functionally to compensate for fluctuations in fluid and ion transport capacity, for example, following intestinal resection (Whang et al., 1996; Rubin et al., 1998; Gillingham et al., 2000; Healey et al., 2010). Therefore, physical changes such as the decreased mucosal thickness of GF mice described in **Chapter 3**, may represent an adaption to compensate for the increased secretory capacity in the absence of a functioning microbiota. Though morphological changes to the mucosa do not necessarily indicate a change in active function of ion transporters or indeed barrier permeability (Gillingham et al., 2000), nevertheless, these parameters were not accounted for and may have impacted on the results observed in this study.

A useful method to measure barrier permeability which is commonly used is para-cellular flux measurements. This involves the addition of labelled hydrophilic solutes, such as mannitol, horseradish peroxidase or dextran-FITC, to one reservoir and subsequent measurement of their flux across the tissue. These molecules do not typically undergo trans-cellular transport and in addition, molecules of different sizes can be used to further probe para-cellular permeability (Clarke, 2009)

Limitations in measuring TER may also be overcome by employing a conductance scanning method, which uses a horizontal Ussing Chamber and scanning electrodes to measure voltage drops both at the apical membrane of the

epithelial cells and at the TJ. (Gitter et al., 1997), or impedance spectroscopy (IS) (Günzel et al., 2012). Overall, when assessing the influence of the microbiota or putative probiotics on secretomotor or barrier function, multiple experimental approaches are warranted to accurately draw conclusions with respect to the influence of the microbiota on host secretomotor and barrier function.

5.5 Application of the Ussing Chamber a Tool to Ascribe Physiological Function to Bacterial Factors

Recent developments in our understanding of the influence of the microbiota on host physiology and function has led to the advent of numerous new concepts, including Microbial Endocrinology. Microbial Endocrinology represents the intersection of host neurophysiology with the microbiome in which neuroendocrine-bacterial interactions are a governing mechanism (Lyte, 2013)

In Chapter 4 I applied the Ussing chamber technique to determine whether the bioactive metabolite, and neurotransmitter, GABA, derived from *Lactobacillus brevis* DPC6108, could influence colonic physiology in the same way as commercially sourced GABA. A similar approach has been taken by others to draw conclusions about which bacterial components or metabolites influence enteric nerve activity (Kunze et al., 2009b; Mao et al., 2013b). For example, Tram-34 and clotrimazole, antagonists of IK_{Ca} channels, mimicked the effects of *Lactobacillus reuteri* on sensory nerve activity (Kunze et al., 2009b) Mao et al., 2013 took a similar approach, and demonstrated that polysaccharide A mirrored the effects of *Bacteroides fragilis* on sensory nerve activity. Both approaches have provided key functional insights of how commensal microbes can communicate with the ENS.

Despite such studies, screening for, and identifying, probiotics with the potential to produce significant levels of neuroactive metabolites *in-vivo* is challenging. However, Ussing chambers at least offer the opportunity to determine whether putative neuroactive-producing probiotcs exert similar effects on intestinal physiology compared to endogenous neurotransmitters.

The growing availability of genome sequences as well as the corresponding molecular tools allows us to pursue more knowledge-driven approaches. The growing availability of genome sequences and annotations provides information for the encoded gene functions, including their metabolic and biosynthetic pathways, stress responses, cell-wall associated proteins, and potential host interaction factors. Such methods may be applied in an effort to identify potential probiotics with the ability to produce known metabolites. Meta-genomics, for example, is the study of collective genomes within an eco-system. Large scale meta-genomic approaches such as shot-gun sequencing or pyrosequencing in combination with bioinformatics can be applied to assemble genetic based infomation on functional communities within a microbiome (Fraher et al., 2012). Such approaches could be used to examine the expression of genes associated with the production of bacterial metabolites with known, or hypothesised, effects on intestinal physiology. Another approach, metabolomics, may be used to identify particular metabolites, or metabolite foot prints, associated with particular microbes or more complex populations by examining the metabolite mass:charge ratios, which can then be compared to pre-existing data bases (Reigstad and Kashyap 2013). This approach has already proven useful in identifying commensal-derived metabolites which influence intestinal transit in mice (Kashyap et al., 2013a). This study determined that diet influenced changes in the microbiome, and microbial metabolite production,

thereby altering altered GI transit time. This effect was accompanied by lower levels of the serotonin metabolite, 5-HIAA (Kashyap et al., 2013a).

Finally, in the interest of fully elucidating host-microbe relationships in the intestine, it is important to also understand host mucosal responses at the genomic level. One study has already successfully examined host transcriptomal responses in human intestinal tissue to three specific Lactobacillus strains (van Baarlen et al., 2011). This study not only observed differential induction of gene-regulatory pathways by each probiotic, but intriguingly it also found that host-transcriptomal response profiles to each probiotic strain were notably comparable to the response profiles observed for specific drugs and bioactive molecules. Indeed, several gene-regulatory pathways identified were thought to be involved in water retention and ion homeostasis, including angiogenin and oxytocin (van Baarlen et al., 2011)

Therefore, studies using this approach, in conjunction with tools to examine functional changes in physiology, such as the Ussing Chamber, will likely provide the best means to ascribing and understanding the physiological impact of probiotics and their bioactives.

5.6 Utility of Germ-Free Animals as a Tool to Investigate Host-Microbe Interactions

Germ free animals offer a unique opportunity to investigate host-microbe interactions. With regard to studying individual bacterial strains, mono-colonisation studies have provided some unique insights as to how individual microbes can influence intestinal health and development (Hooper et al., 2001). Some of the first

evidence demonstrating a role for specific bacterial strains in modulating MMC of the intestine took such a mono-colonisation approach (Husebye et al., 2001) Mono-colonisation with *L. acidophilus* A10 or colonisation in combination with *B.bifidum* B11, *Clostridium tabificum* VP 04 promoted MMC activity, while *E. coli* X7 was without effect and *Micrococcus luteus* suppressed MMC activity. These experiments provided some of the first evidence that specific bacterial strains may modulate intestinal physiology. In addition, the activity of bacteria depends very much on the host, and may be influenced by *in-situ* microbial populations (Sonnenburg et al., 2006; Goodman et al., 2009). Such microbe-microbe interactions are difficult to predict using *ex-vivo* or indeed *in-vitro* approaches. Studies using GF mice to look at specific combinations of commensal microbes can help further our understanding of the complex interactions between commensals and the host *in vivo,* in an integrative system.

However, GF animals are not without their limitations, in particular the influence of species specific development in the host microbiota. To address this limitation, a novel approach has been developed, that of humanised mouse models of GI microbiota. These have already been proven very useful for predicting the impact of diet on the intestinal microbiome (Faith et al., 2011). An investigation of species abundance and microbial gene expression in a model community of ten sequenced human gut-derived bacteria introduced into GF mice, examined responses to randomised perturbations of four specific dietary components. This allowed the subsequent development of a statistical algorithm capable of predicting over 60% of the variation in microbial species abundance in response to precise dietary changes (Faith et al., 2011). In addition, a more recent study employing humanised mice was able to demonstrate an inextricable link between intestinal transit, diet as well as the

host-microbiota and its metabolites and they could point to 5-HT as an important mediator of these interactions (Kashyap et al., 2013). Collectively, these studies provide strong evidence that such an approach could be employed with great effect to predict and understand the effects of probiotic bioactives such as GABA, on the intestinal microbiome and GI function.

Indeed, with regard to the use of humanised mice in the context of studying disease, this approach has already helped characterise the role of the microbiota in obesity (Turnbaugh et al., 2009). However, humanised mice may be particularly useful for unravelling the complex relationships between the host and its microbiota in disorders such as IBS, which display diverse symptom heterogeneity, including polarised alterations in fluid and electrolyte transport, associated with distinct microbial populations (Parkes et al., 2012). Mice containing specific microbial "phenotypes" of disease could be used not only to understand disease pathology, but indeed how to approach treatment, for example, which probiotic to use and why.

One further limitation of GF animals however is that they exhibit altered physiology and immune function in adulthood (Smith et al., 2007). Though colonisation reverses many of the effects, the timing of colonisation on GI function is also critical, as only colonisation early in life can completely reverse many of the differences in immune function observed in GF animals (Smith et al., 2007). A recent study, however, has provided an interesting and innovative approach to the use of GF mice to study host-microbe interactions, utilising reversible colonisation (Hapfelmeier et al., 2010). The reversible colonisation was carried out using an m-DAP auxotrophic Δasd mutant *E. coli* K-12 strain JM83 derivative χ6096, with a requirement for essential nutrients that could not be satisfied by any mammalian host metabolites. Therefore, animals recolonised with these mutants returned to their GF

status 72 hours after the last exposure (Hapfelmeier et al., 2010). Importantly, GF animals which had been transiently mono-colonised, and examined 14 days after the last dose of *E. coli* K-12 JM83, demonstrated similar immunological changes to permanently recolonised mice. More studies are now required to determine whether such an approach can fully reverse all the immune alterations observed in GF mice, allowing for subsequent and more physiologically relevant GF studies.

Nevertheless, despite changes in GI physiology, this is likely an adaption to the absence of host-microbes and certain functional aspects do remain unchanged in GF mice. For example, although GF mice display an enlarged caecum and increased fluid content, *ex-vivo* studies demonstrated that the absorptive capacity of the tissue remains almost unchanged (Gordon and Pesti, 1971; Grover and Kashyap, 2014). Our studies promote this concept and emphasise that colonic secretomotor function is predominantly maintained in GF mouse making it a useful model for future studies examining the influence of host-microbe interactions on intestinal fluid and electrolyte transport.

5.7 Therapeutic Application of Probiotics in Disorders Associated with Altered Secretory Function.

The efficacy of probiotics for the treatment of GI disorders has long been under debate, and a growing number of studies are now beginning to demonstrate their therapeutic potential (Lee and Bak, 2011). However, identifying specific probiotics for the treatment of a particular disorder remains uncertain at best, and reliable predictions may be hindered by the lack of mechanistic characterisation of putative probiotics. Pre-clinical findings indicate that the selection of strain, or strain

combinations, as well as disease pathology, is of the utmost importance when considering probiotics as a therapeutic approach to treat specific GI disorders.

Both *B. infantis* 35624 and *L. salivarius* UCC 118, in both preclinical and clinical studies, display differential effects on particular symptoms and disease severity (McCarthy et al., 2003; O'Mahony et al., 2005; Whorwell et al., 2006). The differential effects of these two organisms on host physiology is further supported by our current work in the context of secretomotor function. Therefore, future therapeutic application of probiotics should consider the influence of strain when selecting treatment options. In past years, many clinical trials testing the efficacy of probiotics in GI disorders have failed to specify the exact strain used, thus making it more difficult to replicate any positive therapeutic effects described(Ritchie *et al.*, 2012). For example, two separate clinical trials using strains of *Lactobacillus acidophilus* display differential clinical efficacy for the treatment of Travellers diarrhea (TD) (Boulloche et al., 1994; Katelaris et al., 1995). Katelaris et al., (1995) did not specify the specific strain of *Lactobacillus acidophilus* tested, therefore, while the discrepancy between studies is likely strain related, a number of other confounding factors account for the discrepancy between these studies in the context of TD. Consideration of disease aetiology is critical to the design of most in considering therapeutic strategies and probiotic therapy is no exception to this rule. Indeed, a recent meta-analysis demonstrated no overall efficacy of probiotic treatment for TD (Ritchie and Romanuk, 2012). Therefore, despite the similar functional consequences of infections with certain enteric pathogens (i.e. acute diarrhoea), it is important that future studies provide specific information on the original causative agents of disease, which may determine susceptibility to treatment with particular probiotic strains or combinations.

In the context of anti-biotic associated diarrhea (AAD), which occurs shortly after the administration of antibiotics, and is believed to be due to a loss in host-microbial populations and diversity, fermented milk containing a multi-strain probiotic combination, demonstrated an 80% prevention rate (Wenus et al., 2008). Therefore, certain disorders associated with a significant overall loss in host-microbiota, may be more amenable to multi-strain probiotic therapy aimed at restoring the host microbiota rather than targeting specific pathogen-induced changes in host function. This is further supported by the recent success of fecal microbiota transplantation for the treatment of diarrhea caused by *Clostridium difficile* (CDAD) (Shankar et al., 2014). Certain single strains such as *Lactobacillus rhamnosus* GG ATCC 53103 (Floch et al., 2011) and *L. plantarum 299v* (Wullt et al., 2003) have shown efficacy in the treatment of and prevention of CDAD, however the choice between single and multi-strain use remains controversial (Chapman et al., 2011). The success of such treatment is believed to be due to a restoration in host-microbe population and diversity rather than specific anti-microbial activity, the restoration of which is also likely to influence secretomotor pathways.

On the other hand, the ability of certain single strains to provide global relief to conditions such as IBS, which display remarkable symptom heterogeneity, particularly with regard to fluid and electrolyte transport, would suggest that certain strains may be better equipped to act as pivotal regulators of intestinal function (Whorwell et al., 2006; Ducrotté et al., 2012). For example, the mechanism by which *B. infantis* 35624 exerts such beneficial effects in IBS likely occurs through modulation of both intestinal and systemic immune function (Konieczna et al., 2012) Groeger et al., 2013), indirect effects of which may also beneficially influence host physiology. Such multi-facetted strains may represent an opportunity to gain insight

into a plethora of beneficial host-microbe interactions relatively easily by focusing extensively on one strain. However, such an approach is unlikely to provide a true understanding of the role these microbes play in health and disease.

5.8 Conclusions and future perspectives

It is clear that there is much to learn with respect to pin pointing the precise mechanisms which underlie host-microbe communication, particularly with respect to the effects of microbes on host GI physiology. However, significant strides forward have been made in the last decade and have opened up countless avenues of novel approaches to research which will undoubtedly shed further light on the true extent and influence of these interactions. Future research into novel probiotics will likely be focussed on finding appropriate species, or mix of species, which can re-establish a certain microbial niche, depending on the individual. The emergence of fecal microbiota transplantation for the successful treatment of reoccurring *Clostridium difficile* induced diarrhoea, as well as inflammatory bowel diseases is also promising for host-microbe targeted therapies. However, short and long-term health consequences of this type of treatment remain relatively unknown, and given the importance of the host microbiota for maintaining long-term health, it is still necessary to elucidate the precise functional roles that specific microbes play in developing and maintaining host microbial communities, and subsequently their influence on the maintenance and protection of host health and physiological function. It is only by understanding these interactions completely that the full potential of probiotic and microbial based therapeutics for the treatment of disorder in the gut and indeed beyond can be realised. To aid in the screening and identification of functionally relevant probiotics and effecter molecules, future

studies should employ multi-omics approaches in combination with robust physiological experimentation *ex-vivo.* GF studies should be used to aid in the understanding of applying these identified microbes *in-vivo*. Mice containing humanised microbiota will help to further improve the translational value of these pre-clinical studies.

In conclusion, this thesis has furthered our understanding of the functional impact of host-microbe interactions on colonic secretomotor function. It has not only provided insight into the strain specific mechanisms through which individual probiotics can influence fluid and electrolyte transport, but has provided an important step in understanding the role of the host-microbiota on intestinal fluid and electrolyte transport.

Bibliography

Abrams, G.D., and Bishop, J.E. (1967). Effect of the normal microbial flora on gastrointestinal motility. Proc. Soc. Exp. Biol. Med. *126*: 301–4.

Alasbahi, R.H., and Melzig, M.F. (2012). Forskolin and derivatives as tools for studying the role of cAMP. 5–13.

Amasheh, S., Barmeyer, C., Koch, C.S., Tavalali, S., Mankertz, J., Epple, H.J., et al. (2004). Cytokine-dependent transcriptional down-regulation of epithelial sodium channel in ulcerative colitis. Gastroenterology *126*: 1711–1720.

Anderson, R.C., Cookson, A.L., McNabb, W.C., Park, Z., McCann, M.J., Kelly, W.J., et al. (2010). Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. *10*: 316.

Anitha, M., Vijay-Kumar, M., Sitaraman, S. V, Gewirtz, A.T., and Srinivasan, S. (2012). Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology *143*: 1006–16.e4.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., et al. (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am. J. Physiol. Gastrointest. Liver Physiol. *303*: G1288–95.

Baarlen, P. van, Troost, F., Meer, C. van der, Hooiveld, G., Boekschoten, M., Brummer, R.J.M., et al. (2011). Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proc. Natl. Acad. Sci. U. S. A. *108 Suppl* : 4562–9.

Bachmann, O., and Seidler, U. (2011). News from the end of the gut--how the highly segmental pattern of colonic HCO_3^- transport relates to absorptive function and mucosal integrity. Biol Pharm Bull *34*: 794–802.

Baker, D.E. (2007). Loperamide: a pharmacological review. Rev. Gastroenterol. Disord. *7 Suppl 3*: S11–8.

Bär, F., Koschitzky, H. Von, Roblick, U., Bruch, H.P., Schulze, L., Sonnenborn, U., et al. (2009). Cell-free supernatants of Escherichia coli Nissle 1917 modulate human colonic motility: evidence from an in vitro organ bath study. Neurogastroenterol. Motil. *21*: 559–66, e16–7.

Barajon, I., Serrao, G., Arnaboldi, F., Opizzi, E., Ripamonti, G., Balsari, A., et al. (2009). Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J. Histochem. Cytochem. *57*: 1013–23.

Barrett, E., Ross, R.P., O'Toole, P.W., Fitzgerald, G.F., and Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. J. Appl. Microbiol. *113*: 411–7.

Barrett, K.E., and Keely, S.J. (2000). Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. Annu. Rev. Physiol. *62*: 535–72.

Bercik, P., Park, A.J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., et al. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol. Motil. *23*: 1132–9.

Bergmann, K.R., Liu, S.X.L., Tian, R., Kushnir, A., Turner, J.R., Li, H.-L., et al. (2013). Bifidobacteria stabilize claudins at tight junctions and prevent intestinal barrier dysfunction in mouse necrotizing enterocolitis. Am. J. Pathol. *182*: 1595–606.

Bertelsen, L.S., Eckmann, L., and Barrett, K.E. (2004). Prolonged interferon-gamma exposure decreases ion transport, NKCC1, and Na+-K+-ATPase expression in human intestinal xenografts in vivo. Am J Physiol Gastrointest Liver Physiol *286*: G157–65.

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., et al. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One *5*: e10667.

Binder, H.J. (2009). Mechanisms of diarrhea in inflammatory bowel diseases. Ann N Y Acad Sci *1165*: 285–293.

Binder, H.J., and Mehta, P. (1989). Short-chain fatty acids stimulate active sodium and chloride absorption in vitro in the rat distal colon. Gastroenterology *96*: 989–996.

Binder, H.J., Rajendran, V., Sadasivan, V., and Geibel, J.P. (2005). Bicarbonate secretion: a neglected aspect of colonic ion transport. J Clin Gastroenterol *39*: S53–8.

Blaut, M., and Clavel, T. (2007). Metabolic diversity of the intestinal microbiota: implications for health and disease. J. Nutr. *137*: 751S–5S.

Borthakur, A., Gill, R.K., Hodges, K., Ramaswamy, K., Hecht, G., and Dudeja, P.K. (2006). Enteropathogenic Escherichia coli inhibits butyrate uptake in Caco-2 cells by altering the apical membrane MCT1 level. Am J Physiol Gastrointest Liver Physiol *290*: G30–5.

Borthakur, A., Gill, R.K., Tyagi, S., Koutsouris, A., Alrefai, W.A., Hecht, G.A., et al. (2008). The probiotic Lactobacillus acidophilus stimulates chloride/hydroxyl exchange activity in human intestinal epithelial cells. J Nutr *138*: 1355–1359.

Boulloche, J., Mouterde, O., and Mallet, E. (1994). Management of Acute Diarrhea in Infants and Toddlers - Controlled Study of the Antidiarrheal efficacy of killed

Lactobacillus-acidophilus (LB strain) versus a Placebo and a Reference Agent (Loperamide). *41*: 457–463.

Britton, E., and McLaughlin, J.T. (2013). Ageing and the gut. *72*: 173–177.

Bron, P.A., Baarlen, P. van, and Kleerebezem, M. (2012). Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat. Rev. Microbiol. *10*: 66–78.

Brown, D. a, Melvin, J.E., and Yule, D.I. (2003). Critical role for NHE1 in intracellular pH regulation in pancreatic acinar cells. Am. J. Physiol. Gastrointest. Liver Physiol. *285*: G804–12.

Brun, P., Giron, M.C., Qesari, M., Porzionato, A., Caputi, V., Zoppellaro, C., et al. (2013). Toll-like receptor 2 regulates intestinal inflammation by controlling integrity of the enteric nervous system. Gastroenterology *145*: 1323–33.

Caenepeel, P., Janssens, J., Vantrappen, G., Eyssen, H., and Coremans, G. (1989). Interdigestive myoelectric complex in germ-free rats. Dig Dis Sci *34*: 1180–1184.

Cameron, H.L., and Perdue, M.H. (2007). Muscarinic acetylcholine receptor activation increases transcellular transport of macromolecules across mouse and human intestinal epithelium in vitro. Neurogastroenterol. Motil. *19*: 47–56.

Camilleri, M., Cowen, T., and Koch, T.R. (2008). Enteric neurodegeneration in ageing. Neurogastroenterol. Motil. *20*: 418–29.

Canny, G.O., and McCormick, B.A. (2008). Bacteria in the intestine, helpful residents or enemies from within? Infect. Immun. *76*: 3360–73.

Carew, M. a, and Thorn, P. (2000). Carbachol-stimulated chloride secretion in mouse colon: evidence of a role for autocrine prostaglandin E2 release. Exp. Physiol. *85*: 67–72.

Casanova, E., Guetg, N., Vigot, R., Seddik, R., Julio-Pieper, M., Hyland, N., et al. (2009a). A mouse model for visualization of GABA(B) receptors. Genesis *47*: 595–602.

Casanova, E., Guetg, N., Vigot, R., Seddik, R., Julio-Pieper, M., Hyland, N.P., et al. (2009b). A mouse model for visualization of GABA(B) receptors. Genesis *47*: 595–602.

Catalioto, R.-M., Maggi, C.A., and Giuliani, S. (2011). Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions. Curr. Med. Chem. *18*: 398–426.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*: 816–24.

Catterall, W.A. (1975a). Activation of the action potential Na+ ionophore of cultured neuroblastoma cells by veratridine and batrachotoxin. J. Biol. Chem. *250*: 4053–9.

Catterall, W.A. (1975b). Cooperative activation of action potential Na+ ionophore by neurotoxins. Proc. Natl. Acad. Sci. U. S. A. *72*: 1782–6.

Cencic, A., and Langerholc, T. (2010). Functional cell models of the gut and their applications in food microbiology--a review. Int. J. Food Microbiol. *141 Suppl* : S4–14.

Chaflin, D., Cooperstein, I.L., and Hogben, C.A. (1958). Fluid and electrolyte movement across intestinal wall of bullfrog. Proc Soc Exp Biol Med *99*: 746–748.

Chapman, C.M.C., Gibson, G.R., and Rowland, I. (2011). Health benefits of probiotics: are mixtures more effective than single strains? Eur. J. Nutr. *50*: 1–17.

Chebib, M., and Johnston, G.A. (1999). The “ABC” of GABA receptors: a brief review. Clin Exp Pharmacol Physiol *26*: 937–940.

Chen, Z., Fei, Y.-J., Anderson, C.M.H., Wake, K.A., Miyauchi, S., Huang, W., et al. (2002). Structure, function and immunolocalization of a proton-coupled amino acid transporter (hPAT1) in the human intestinal cell line Caco-2. J. Physiol. *546*: 349–361.

Cheng, A.C., McDonald, J.R., and Thielman, N.M. (2005). Infectious diarrhea in developed and developing countries. J Clin Gastroenterol *39*: 757–773.

Cheng, C.A., Hwang, D.F., Tsai, Y.H., Chen, H.C., Jeng, S.S., Noguchi, T., et al. (1995). Microflora and tetrodotoxin-producing bacteria in a gastropod, Niotha clathrata. Food Chem. Toxicol. *33*: 929–34.

Cipriani, S., Mencarelli, A., Chini, M.G., Distrutti, E., Renga, B., Bifulco, G., et al. (2011). The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. PLoS One *6*: e25637.

Clarke, L.L. (2009a). A guide to Ussing chamber studies of mouse intestine. Am. J. Physiol. Gastrointest. Liver Physiol. *296*: G1151–66.

Clarke, L.L. (2009b). A guide to Ussing chamber studies of mouse intestine. Am. J. Physiol. Gastrointest. Liver Physiol. *296*: G1151–66.

Colgan, S.P., Resnick, M.B., Parkos, C.A., Delp-Archer, C., McGuirk, D., Bacarra, A.E., et al. (1994). IL-4 directly modulates function of a model human intestinal epithelium. J. Immunol. *153*: 2122–9.

Collins, J., Borojevic, R., Verdu, E.F., Huizinga, J.D., and Ratcliffe, E.M. (2014). Intestinal microbiota influence the early postnatal development of the enteric nervous system. Neurogastroenterol. Motil. *26*: 98–107.

Cooperstein, I.L., and Hogben, C.A. (1959). Ionic transfer across the isolated frog large intestine. J Gen Physiol *42*: 461–473.

Corr, S.C., Li, Y., Riedel, C.U., O'Toole, P.W., Hill, C., and Gahan, C.G.M. (2007). Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. Proc. Natl. Acad. Sci. U. S. A. *104*: 7617–21.

Couvineau, A., and Laburthe, M. (2012). VPAC receptors: structure, molecular pharmacology and interaction with accessory proteins. Br. J. Pharmacol. *166*: 42–50.

Cox, H.M. (2007). Neuropeptide Y receptors; antisecretory control of intestinal epithelial function. Auton. Neurosci. *133*: 76–85.

Cox, H.M., Rudolph, A., and Gschmeissner, S. (1994). Ultrastructural co-localization of neuropeptide Y and vasoactive intestinal polypeptide in neurosecretory vesicles of submucous neurons in the rat jejunum. Neuroscience *59*: 469–76.

Cuthbert, A.W., Hickman, M.E., Thorn, P., and MacVinish, L.J. (1999). Activation of Ca2+- and cAMP-sensitive K+ channels in murine colonic epithelia by 1-ethyl-2-benzimidazolone. Am J Physiol Cell Physiol *277*: C111–120.

Czerucka, D., Roux, I., and Rampal, P. (1994). Saccharomyces boulardii inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. Gastroenterology *106*: 65–72.

Dai, C., Guandalini, S., Zhao, D.-H., and Jiang, M. (2012). Antinociceptive effect of VSL#3 on visceral hypersensitivity in a rat model of irritable bowel syndrome: a possible action through nitric oxide pathway and enhance barrier function. Mol. Cell. Biochem. *362*: 43–53.

Daly, J.W., Padgett, W.L., Saunders, R.L., and Cover, J.F. (1997). Absence of tetrodotoxins in a captive-raised riparian frog, Atelopus varius. Toxicon *35*: 705–9.

Dean, P., Maresca, M., Schüller, S., Phillips, A.D., and Kenny, B. (2006). Potent diarrheagenic mechanism mediated by the cooperative action of three enteropathogenic Escherichia coli-injected effector proteins. Proc. Natl. Acad. Sci. U. S. A. *103*: 1876–81.

Dharmsathaphorn, K., and Madara, J.L. (1990). Established intestinal cell lines as model systems for electrolyte transport studies. Methods Enzymol. *192*: 354–89.

Dobson, A., Cotter, P.D., Ross, R.P., and Hill, C. (2012). Bacteriocin production: a probiotic trait? Appl. Environ. Microbiol. *78*: 1–6.

Ducrotté, P., Sawant, P., and Jayanthi, V. (2012). Clinical trial: Lactobacillus plantarum 299v (DSM 9843) improves symptoms of irritable bowel syndrome. World J. Gastroenterol. *18*: 4012–8.

DuPont, A.W., and DuPont, H.L. (2011). The intestinal microbiota and chronic disorders of the gut. Nat. Rev. Gastroenterol. Hepatol. *8*: 523–31.

Dupont, J.R., Jervis, H.R., and Sprinz, H. (1965). Auerbach's plexus of the rat cecum in relation to the germfree state. J. Comp. Neurol. *125*: 11–8.

E. Husebye (1999). The patterns of small bowel motility: physiology and implications in organic disease and functional disorders. Neurogastroenterol. Motil. *11*: 141–161.

Effler, P., Ieong, M.C., Kimura, A., Nakata, M., Burr, R., Cremer, E., et al. (2001). Sporadic Campylobacter jejuni infections in Hawaii: associations with prior antibiotic use and commercially prepared chicken. J. Infect. Dis. *183*: 1152–5.

Eldere, J. Van, Robben, J., Caenepeel, P., and Eyssen, H. (1988). Influence of a cecal volume-reducing intestinal microflora on the excretion and entero-hepatic circulation of steroids and bile acids. J. Steroid Biochem. *29*: 33–9.

Engevik, M. a, Aihara, E., Montrose, M.H., Shull, G.E., Hassett, D.J., and Worrell, R.T. (2013). Loss of NHE3 alters gut microbiota composition and influences Bacteroides thetaiotaomicron growth. Am. J. Physiol. Gastrointest. Liver Physiol. *305*: G697–711.

Escamilla, J., Lane, M.A., and Maitin, V. (2012). Cell-free supernatants from probiotic Lactobacillus casei and Lactobacillus rhamnosus GG decrease colon cancer cell invasion in vitro. Nutr. Cancer *64*: 871–8.

Evans, D.G., Miles, A.A., and Niven, J.S. (1948). The enhancement of bacterial infections by adrenaline. Br J Exp Pathol *29*: 20–39.

Ewaschuk, J.B., Diaz, H., Meddings, L., Diederichs, B., Dmytrash, A., Backer, J., et al. (2008). Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. Am. J. Physiol. Gastrointest. Liver Physiol. *295*: G1025–34.

Faith, J.J., McNulty, N.P., Rey, F.E., and Gordon, J.I. (2011). Predicting a human gut microbiota's response to diet in gnotobiotic mice. Science *333*: 101–4.

Fanning, S., Hall, L.J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., et al. (2012). Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc. Natl. Acad. Sci. U. S. A. *109*: 2108–13.

Feighery, L.M., Smith, P., O'Mahony, L., Fallon, P.G., and Brayden, D.J. (2008). Effects of Lactobacillus salivarius 433118 on intestinal inflammation, immunity status and in vitro colon function in two mouse models of inflammatory bowel disease. Dig. Dis. Sci. *53*: 2495–506.

Field, M. (2003). Intestinal ion transport and the pathophysiology of diarrhea. J. Clin. Invest. *111*: 931–43.

Floch, M.H., Walker, W.A., Madsen, K., Sanders, M.E., Macfarlane, G.T., Flint, H.J., et al. (2011). Recommendations for probiotic use-2011 update. J. Clin. Gastroenterol. *45 Suppl*: S168–71.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Muñoz-Quezada, S., and Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. Br J Nutr *109 Suppl* : S35–50.

Fontgalland, D. de, Brookes, S.J., Gibbins, I., Sia, T.C., and Wattchow, D.A. (2014). The neurochemical changes in the innervation of human colonic mesenteric and submucosal blood vessels in ulcerative colitis and Crohn's disease. Neurogastroenterol. Motil. *26*: 731–44.

Foong, J.P., Tough, I.R., Cox, H.M., and Bornstein, J.C. (2014). Properties of cholinergic and non-cholinergic submucosal neurons along the mouse colon. J. Physiol.

Foong, J.P.P., and Bornstein, J.C. (2013). Properties of cholinergic and non-cholinergic submucosal neurons along the mouse colon. Auton. Neurosci. *177*: 59–60.

Fraher, M.H., O'Toole, P.W., and Quigley, E.M.M. (2012). Techniques used to characterize the gut microbiota: a guide for the clinician. Nat. Rev. Gastroenterol. Hepatol. *9*: 312–22.

Fromm, M., Schulzke, J.D., and Hegel, U. (1985). Epithelial and subepithelial contributions to transmural electrical resistance of intact rat jejunum, in vitro. Pflugers Arch. *405*: 400–2.

Furness, J. (2008). The enteric nervous system.

Furness, J.B. (2012). The enteric nervous system and neurogastroenterology. Nat. Rev. Gastroenterol. Hepatol. *9*: 286–94.

Furness, J.B., Alex, G., Clark, M.J., and Lal, V. V (2003). Morphologies and projections of defined classes of neurons in the submucosa of the guinea-pig small intestine. Anat. Rec. A. Discov. Mol. Cell. Evol. Biol. *272*: 475–83.

Furness, J.B., Robbins, H.L., Xiao, J., Stebbing, M.J., and Nurgali, K. (2004). Projections and chemistry of Dogiel type II neurons in the mouse colon. Cell Tissue Res. *317*: 1–12.

Gancedo, J.M. (2013). Biological roles of cAMP: variations on a theme in the different kingdoms of life. Biol. Rev. Camb. Philos. Soc. *88*: 645–68.

Garcia, M.A.S., Yang, N., and Quinton, P.M. (2009). Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. J. Clin. Invest. *119*: 2613–22.

Gareau, M.G., Jury, J., and Perdue, M.H. (2007). Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. Am. J. Physiol. Gastrointest. Liver Physiol. *293*: G198–203.

Gareau, M.G., Jury, J., Yang, P.C., MacQueen, G., and Perdue, M.H. (2006). Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance. Pediatr. Res. *59*: 83–8.

Gareau, M.G., Silva, M.A., and Perdue, M.H. (2008). Pathophysiological mechanisms of stress-induced intestinal damage. Curr. Mol. Med. *8*: 274–81.

Gawenis, L.R., Bradford, E.M., Alper, S.L., Prasad, V., and Shull, G.E. (2010). AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon. Am. J. Physiol. Gastrointest. Liver Physiol. *298*: G493–G503.

Gawenis, L.R., Bradford, E.M., Prasad, V., Lorenz, J.N., Simpson, J.E., Clarke, L.L., et al. (2007). Colonic anion secretory defects and metabolic acidosis in mice lacking the NBC1 Na+/HCO3- cotransporter. J. Biol. Chem. *282*: 9042–9052.

Gawenis, L.R., Stien, X., Shull, G.E., Schultheis, P.J., Woo, A.L., Walker, N.M., et al. (2002). Intestinal NaCl transport in NHE2 and NHE3 knockout mice. Am. J. Physiol. Gastrointest. Liver Physiol. *282*: G776–84.

Giancamillo, a di, Vitari, F., Bosi, G., Savoini, G., and Domeneghini, C. (2010). The chemical code of porcine enteric neurons and the number of enteric glial cells are altered by dietary probiotics. Neurogastroenterol Motil *22*: e271–8.

Gill, R.K., Borthakur, A., Hodges, K., Turner, J.R., Clayburgh, D.R., Saksena, S., et al. (2007). Mechanism underlying inhibition of intestinal apical Cl/OH exchange following infection with enteropathogenic E. coli. J. Clin. Invest. *117*: 428–37.

Gillingham, M.B., Dahly, E.M., Carey, H. V, Clark, M.D., Kritsch, K.R., and Ney, D.M. (2000). Differential jejunal and colonic adaptation due to resection and IGF-I in parenterally fed rats. Am. J. Physiol. Gastrointest. Liver Physiol. *278*: G700–9.

Gitter, A., Bendfeldt, K., Schmitz, H., Schulzke, J., Bentzel, C., and Fromm, M. (2000). Epithelial barrier defects in HT-29/B6 colonic cell monolayers induced by tumor necrosis factor-alpha. Ann. N. Y. Acad. Sci. *915*: 193–203.

Gitter, A.H., Bertog, M., Schulzke, J., and Fromm, M. (1997). Measurement of paracellular epithelial conductivity by conductance scanning. Pflugers Arch. *434*: 830–40.

Gitter, A.H., Fromm, M., and Schulzke, J.-D. (1998). Impedance analysis for the determination of epithelial and subepithelial resistance in intestinal tissues. J. Biochem. Biophys. Methods *37*: 35–46.

Goodman, A.L., McNulty, N.P., Zhao, Y., Leip, D., Mitra, R.D., Lozupone, C. a, et al. (2009). Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe *6*: 279–89.

Gordon, H., and Pesti, L. (1971). Gnotobiotic Animal as a tool in study of host microbial relationships. *35*:

Greenwood-Van Meerveld, B., Tyler, K., and Keith, J.C. (2000). Recombinant human interleukin-11 modulates ion transport and mucosal inflammation in the small intestine and colon. Lab. Invest. *80*: 1269–80.

Groeger, D., O'Mahony, L., Murphy, E.F., Bourke, J.F., Dinan, T.G., Kiely, B., et al. (2013). Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. Gut Microbes *4*: 325–39.

Grover, M., and Kashyap, P.C. (2014). Germ-free mice as a model to study effect of gut microbiota on host physiology. Neurogastroenterol. Motil. *26*: 745–748.

Grubb, B.. R. (1997). Ion Transport Across the Murine Intestine in the Absence and Presence of CFTR. Comp. Biochem. Physiol. Part A Physiol. *118*: 277–282.

Günzel, D., Zakrzewski, S.S., Schmid, T., Pangalos, M., Wiedenhoeft, J., Blasse, C., et al. (2012). From TER to trans- and paracellular resistance: lessons from impedance spectroscopy. Ann. N. Y. Acad. Sci. *1257*: 142–51.

Gustafsson, B.E., and Norman, A. (1969). Influence of the diet on the turnover of bile acids in germ-free and conventional rats. Br. J. Nutr. *23*: 429–42.

Haanpää, M., and Treede, R.-D. (2012). Capsaicin for neuropathic pain: linking traditional medicine and molecular biology. Eur. Neurol. *68*: 264–75.

Haberberger, R., Schultheiss, G., and Diener, M. (2006). Epithelial muscarinic M1 receptors contribute to carbachol-induced ion secretion in mouse colon. Eur. J. Pharmacol. *530*: 229–33.

Hamilton, K.L. (2011). Ussing's "Little Chamber": 60 Years+ Old and Counting. Front. Physiol. *2*: 6.

Hanauer, S.B. (2008). The role of loperamide in gastrointestinal disorders. Rev. Gastroenterol. Disord. *8*: 15–20.

Hapfelmeier, S., Lawson, M.A.E., Slack, E., Kirundi, J.K., Stoel, M., Heikenwalder, M., et al. (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science *328*: 1705–9.

Hardcastle, J., and Hardcastle, P.T. (1996). gamma-Aminobutyric acid and intestinal secretion. Gastroenterology *111*: 1163–1164.

Hardcastle, J., Hardcastle, P.T., and Mathias, W.J. (1991). The influence of the gamma-amino butyric acid (GABA) antagonist bicuculline on transport processes in rat small intestine. J Pharm Pharmacol *43*: 128–130.

Healey, K.L., Bines, J.E., Thomas, S.L., Wilson, G., Taylor, R.G., Sourial, M., et al. (2010). Morphological and functional changes in the colon after massive small bowel resection. J. Pediatr. Surg. *45*: 1581–90.

Hecht, G., and Koutsouris, A. (1999). Enteropathogenic E. coli attenuates secretagogue-induced net intestinal ion transport but not Cl- secretion. Am. J. Physiol. *276*: G781–8.

Heintz, C., and Mair, W. (2014). You are what you host: microbiome modulation of the aging process. Cell *156*: 408–11.

Hemlin, M., Jodal, M., Lundgren, O., Sjövall, H., and Stage, L. (1988). The importance of the subepithelial resistance for the electrical properties of the rat jejunum in vitro. Acta Physiol. Scand. *134*: 79–88.

Hering, N.A., Richter, J.F., Fromm, A., Wieser, A., Hartmann, S., Günzel, D., et al. (2013). TcpC protein from E. coli Nissle improves epithelial barrier function involving PKCζ and ERK1/2 signaling in HT-29/B6 cells. Mucosal Immunol.

Heuvelin, E., Lebreton, C., Bichara, M., Cerf-Bensussan, N., and Heyman, M. (2010). A Bifidobacterium probiotic strain and its soluble factors alleviate chloride secretion by human intestinal epithelial cells. J Nutr *140*: 7–11.

Hirota, C.L., and McKay, D.M. (2006a). Cholinergic regulation of epithelial ion transport in the mammalian intestine. Br. J. Pharmacol. *149*: 463–79.

Hirota, C.L., and McKay, D.M. (2006b). M3 muscarinic receptor-deficient mice retain bethanechol-mediated intestinal ion transport and are more sensitive to colitis. Can. J. Physiol. Pharmacol. *84*: 1153–61.

Hodges, K., Alto, N.M., Ramaswamy, K., Dudeja, P.K., and Hecht, G. (2008). The enteropathogenic Escherichia coli effector protein EspF decreases sodium hydrogen exchanger 3 activity. Cell. Microbiol. *10*: 1735–45.

Höglund, P., Haila, S., Socha, J., Tomaszewski, L., Saarialho-Kere, U., Karjalainen-Lindsberg, M.L., et al. (1996). Mutations of the Down-regulated in adenoma (DRA) gene cause congenital chloride diarrhoea. Nat. Genet. *14*: 316–9.

Holliday, N.D., Pollock, E.L., Tough, I.R., and Cox, H.M. (2000). PYY preference is a common characteristic of neuropeptide Y receptors expressed in human, rat, and mouse gastrointestinal epithelia. Can. J. Physiol. Pharmacol. *78*: 126–33.

Holzer, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. Pharmacol. Rev. *43*: 143–201.

Hooper, L., Wong, M., and Thelin, A. (2001). Molecular analysis of commensal host-microbial relations hips in the intestine. *291*: 881–884.

Howlett, M., Chalinor, H. V, Buzzelli, J.N., Nguyen, N., Driel, I.R. van, Bell, K.M., et al. (2012). IL-11 is a parietal cell cytokine that induces atrophic gastritis. Gut *61*: 1398–409.

Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell *155*: 1451–63.

Hummel, S., Veltman, K., Cichon, C., Sonnenborn, U., and Schmidt, M.A. (2012). Differential targeting of the E-Cadherin/β-Catenin complex by gram-positive probiotic lactobacilli improves epithelial barrier function. Appl. Environ. Microbiol. *78*: 1140–7.

Husebye, E., Hellström, P.M., and Midtvedt, T. (1994). Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. Dig Dis Sci *39*: 946–956.

Husebye, E., Hellström, P.M., Sundler, F., Chen, J., and Midtvedt, T. (2001). Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. Am J Physiol Gastrointest Liver Physiol *280*: G368–80.

Hyland, N.P., and Cox, H.M. (2005). The regulation of veratridine-stimulated electrogenic ion transport in mouse colon by neuropeptide Y (NPY), Y1 and Y2 receptors. Br. J. Pharmacol. *146*: 712–722.

Hyland, N.P., and Cryan, J.F. (2010). A Gut Feeling about GABA: Focus on GABA(B) Receptors. Front. Pharmacol. *1*: 124.

Hyland, N.P., Sjöberg, F., Tough, I.R., Herzog, H., and Cox, H.M. (2003). Functional consequences of neuropeptide Y Y 2 receptor knockout and Y2 antagonism in mouse and human colonic tissues. Br. J. Pharmacol. *139*: 863–871.

Ikehara, O., Hayashi, H., Watanabe, Y., Yamamoto, H., Mochizuki, T., Hoshino, M., et al. (2010). Proteinase-activated receptors-1 and 2 induce electrogenic Cl Ϫ secretion in the mouse cecum by distinct mechanisms. 115–125.

Indrio, F., Riezzo, G., Raimondi, F., Bisceglia, M., Cavallo, L., and Francavilla, R. (2009). Effects of probiotic and prebiotic on gastrointestinal motility in newborns. J. Physiol. Pharmacol. *60 Suppl 6*: 27–31.

Indrio, F., Riezzo, G., Raimondi, F., Bisceglia, M., Filannino, A., Cavallo, L., et al. (2011). Lactobacillus reuteri accelerates gastric emptying and improves regurgitation in infants. Eur. J. Clin. Invest. *41*: 417–22.

Jacob, P., Rossmann, H., Lamprecht, G., Kretz, A., Neff, C., Lin-Wu, E., et al. (2002). Down-regulated in adenoma mediates apical Cl-/HCO3- exchange in rabbit, rat, and human duodenum. Gastroenterology *122*: 709–724.

Jacobi, C.A., and Malfertheiner, P. (2011). Escherichia coli Nissle 1917 (Mutaflor): new insights into an old probiotic bacterium. Dig. Dis. *29*: 600–7.

Jiang, Q., Sheldon, R.J., and Porreca, F. (1990). Opioid modulation of basal intestinal fluid transport in the mouse: actions at central, but not intestinal, sites. J. Pharmacol. Exp. Ther. *253*: 784–90.

Jijon, H., Backer, J., Diaz, H., Yeung, H., Thiel, D., McKaigney, C., et al. (2004). DNA from probiotic bacteria modulates murine and human epithelial and immune function. Gastroenterology *126*: 1358–73.

Jodal, M., Holmgren, S., Lundgren, O., and Sjöqvist, A. (1993). Involvement of the myenteric plexus in the cholera toxin-induced net fluid secretion in the rat small intestine. Gastroenterology *105*: 1286–1293.

Johansson, M.E. V, Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J.K., Ermund, A., et al. (2011a). Composition and functional role of the mucus layers in the intestine. Cell. Mol. Life Sci. *68*: 3635–41.

Johansson, M.E. V, Larsson, J.M.H., and Hansson, G.C. (2011b). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc. Natl. Acad. Sci. U. S. A. *108 Suppl* : 4659–65.

Johns, A. (1980). Ouabain-sensitive thallium fluxes in smooth muscle of rabbit uterus. J. Physiol. *309*: 391–403.

Jones, S.L., and Blikslager, A.T. (2002). Role of the Enteric Nervous System in the Pathophysiology of Secretory Diarrhea. 222–228.

Kandil, H., Berschneider, H., and Argenzio, R. (1994). Tumour necrosis factor alpha changes porcine intestinal ion transport through a paracrine mechanism involving prostaglandins. Gut VN - Readcube.com.

Karczewski, J., Troost, F.J., Konings, I., Dekker, J., Kleerebezem, M., Brummer, R.-J.M., et al. (2010). Regulation of human epithelial tight junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier. Am. J. Physiol. Gastrointest. Liver Physiol. *298*: G851–9.

Kashyap, P.C., Marcobal, A., Ursell, L.K., Larauche, M., Duboc, H., Earle, K. a, et al. (2013a). Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology *144*: 967–77.

Kashyap, P.C., Marcobal, A., Ursell, L.K., Larauche, M., Duboc, H., Earle, K.A., et al. (2013b). Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology *144*: 967–77.

Katelaris, P.H., Salam, I., and Farthing, M.J. (1995). Lactobacilli to prevent traveler's diarrhea? N. Engl. J. Med. *333*: 1360–1.

Keely, S., Kelly, C.J., Weissmueller, T., Burgess, A., Wagner, B.D., Robertson, C.E., et al. (2012). Activated fluid transport regulates bacterial-epithelial interactions and significantly shifts the murine colonic microbiome. Gut Microbes *3*: 250–60.

Khoruts, A. (2014). Faecal microbiota transplantation in 2013: Developing human gut microbiota as a class of therapeutics. Nat. Rev. Gastroenterol. Hepatol. *11*: 79–80.

Khoshdel, a, Verdu, E.F., Kunze, W., McLean, P., Bergonzelli, G., and Huizinga, J.D. (2013). Bifidobacterium longum NCC3001 inhibits AH neuron excitability. Neurogastroenterol. Motil. *25*: e478–84.

Kiela, P.R., Kuscuoglu, N., Midura, A.J., Midura-Kiela, M.T., Larmonier, C.B., Lipko, M., et al. (2007). Molecular mechanism of rat NHE3 gene promoter regulation by sodium butyrate. Am J Physiol Cell Physiol *293*: C64–74.

Kimberg, D. V, Field, M., Johnson, J., Henderson, a, and Gershon, E. (1971). Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. J. Clin. Invest. *50*: 1218–30.

Konieczna, P., Akdis, C.A., Quigley, E.M.M., Shanahan, F., and O'Mahony, L. (2012). Portrait of an immunoregulatory Bifidobacterium. Gut Microbes *3*: 261–6.

Krantis, A. (2000). GABA in the Mammalian Enteric Nervous System. News Physiol Sci *15*: 284–290.

Kunze, W. a, Mao, Y.-K.K., Wang, B., Huizinga, J.D., Ma, X., Forsythe, P., et al. (2009a). Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. J Cell Mol Med *13*: 2261–2270.

Kunze, W.A., Mao, Y.K., Wang, B., Huizinga, J.D., Ma, X., Forsythe, P., et al. (2009b). Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. J Cell Mol Med *13*: 2261–2270.

Kunzelmann, K., and Mall, M. (2002). Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol. Rev. *82*: 245–89.

Larsen, E.H. (2002). Hans H. Ussing--scientific work: contemporary significance and perspectives. Biochim Biophys Acta *1566*: 2–15.

Larsen, E.H. (2011). Reconciling the Krogh and Ussing interpretations of epithelial chloride transport - presenting a novel hypothesis for the physiological significance of the passive cellular chloride uptake. Acta Physiol. (Oxf). *202*: 435–64.

Larsen, M., Larsen, B.B., Frølund, B., and Nielsen, C.U. (2008). Transport of amino acids and GABA analogues via the human proton-coupled amino acid transporter, hPAT1: characterization of conditions for affinity and transport experiments in Caco-2 cells. Eur. J. Pharm. Sci. *35*: 86–95.

Larsen, R., Mertz-Nielsen, A., and Hansen, M. (2001). Novel modified Ussing chamber for the study of absorption and secretion in human endoscopic biopsies. *173*: 213–222.

Laubitz, D., Larmonier, C.B., and Bai, A. (2008). Colonic gene expression profile in NHE3-deficient mice: evidence for spontaneous distal colitis. *295*:

Leach, M.W., Davidson, N.J., Fort, M.M., Powrie, F., and Rennick, D.M. (1999). The role of IL-10 in inflammatory bowel disease: "of mice and men."Toxicol Pathol *27*: 123–133.

Lebeer, S., Claes, I., Tytgat, H.L.P., Verhoeven, T.L.A., Marien, E., Ossowski, I. von, et al. (2012). Functional analysis of Lactobacillus rhamnosus GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. Appl. Environ. Microbiol. *78*: 185–93.

Lee, B.J., and Bak, Y.-T. (2011). Irritable bowel syndrome, gut microbiota and probiotics. J. Neurogastroenterol. Motil. *17*: 252–66.

Li, Y., Xiang, Y.-Y., Lu, W.-Y., Liu, C., and Li, J. (2012a). A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion. Am. J. Physiol. Gastrointest. Liver Physiol. *303*: G453–60.

Li, Y., Xiang, Y.-Y., Lu, W.-Y., Liu, C., and Li, J. (2012b). A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion. Am. J. Physiol. Gastrointest. Liver Physiol. *303*: G453–60.

Li, Z., Elliott, E., Payne, J., Isaacs, J., Gunning, P., and O'loughlin, E. V (1999). Shiga toxin-producing Escherichia coli can impair T84 cell structure and function without inducing attaching/effacing lesions. Infect. Immun. *67*: 5938–45.

Liong, M.-T. (2008). Safety of probiotics: translocation and infection. Nutr. Rev. *66*: 192–202.

Liu, C., Xu, H., Zhang, B., Johansson, M.E. V, Li, J., Hansson, G.C., et al. (2013). NHE8 plays an important role in mucosal protection via its effect on bacterial adhesion. Am. J. Physiol. Cell Physiol. *305*: C121–8.

Lomasney, K.W., Houston, A., Shanahan, F., Dinan, T.G., Cryan, J.F., and Hyland, N.P. (2014). Selective influence of host microbiota on cAMP-mediated ion transport in mouse colon. Neurogastroenterol. Motil. *26*: 887–890.

Lomasney, K.W., and Hyland, N.P. (2013). The application of Ussing chambers for determining the impact of microbes and probiotics on intestinal ion transport. Can. J. Physiol. Pharmacol. *91*: 663–70.

Lu, J., Philpott, D.J., Saunders, P.R., Perdue, M.H., Yang, P.C., and McKay, D.M. (1998). Epithelial ion transport and barrier abnormalities evoked by superantigen-activated immune cells are inhibited by interleukin-10 but not interleukin-4. J. Pharmacol. Exp. Ther. *287*: 128–36.

Lu, R., Fasano, S., Madayiputhiya, N., Morin, N.P., Nataro, J., and Fasano, A. (2009). Isolation, identification, and characterization of small bioactive peptides from Lactobacillus GG conditional media that exert both anti-Gram-negative and Gram-positive bactericidal activity. J. Pediatr. Gastroenterol. Nutr. *49*: 23–30.

Lundgren, O. (2000). Role of the Enteric Nervous System in the Fluid and Electrolyte Secretion of Rotavirus Diarrhea. Science (80-.). *287*: 491–495.

Lutgendorff, F., Akkermans, L.M.A., and Söderholm, J.D. (2008). The role of microbiota and probiotics in stress-induced gastro-intestinal damage. Curr. Mol. Med. *8*: 282–98.

Lyte, M. (2004). Microbial endocrinology and infectious disease in the 21st century. Trends Microbiol *12*: 14–20.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays *33*: 574–81.

Lyte, M. (2013). Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. PLoS Pathog. *9*: e1003726.

Lyte, M., and Ernst, S. (1992). Catecholamine induced growth of gram negative bacteria. Life Sci *50*: 203–212.

MacNaughton, W.K., Pineau, B.C., and Krantis, a (1996). gamma-Aminobutyric acid stimulates electrolyte transport in the guinea pig ileum in vitro. Gastroenterology *110*: 498–507.

Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., et al. (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology *121*: 580–591.

Madsen, K.L. (2001). Inflammatory bowel disease: lessons from the IL-10 gene-deficient mouse. Clin. Invest. Med. *24*: 250–7.

Madsen, K.L., Malfair, D., Gray, D., Doyle, J.S., Jewell, L.D., and Fedorak, R.N. (1999). Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. Inflamm. Bowel Dis. *5*: 262–70.

Madsen, K.L., Tavernini, M.M., Mosmann, T.R., and Fedorak, R.N. (1996). Interleukin 10 modulates ion transport in rat small intestine. Gastroenterology *111*: 936–44.

Mao, Y.-K., Kasper, D.L., Wang, B., Forsythe, P., Bienenstock, J., and Kunze, W. a (2013a). Bacteroides fragilis polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. Nat. Commun. *4*: 1465.

Mao, Y.-K., Kasper, D.L., Wang, B., Forsythe, P., Bienenstock, J., and Kunze, W.A. (2013b). Bacteroides fragilis polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. Nat. Commun. *4*: 1465.

Mariat, D., Firmesse, O., Levenez, F., Guimarăes, V., Sokol, H., Doré, J., et al. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. *9*: 123.

Massi, M., Ioan, P., Budriesi, R., Chiarini, A., Vitali, B., Lammers, K.M., et al. (2006a). Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue. World J. Gastroenterol. *12*: 5987–94.

Massi, M., Ioan, P., Budriesi, R., Chiarini, A., Vitali, B., Lammers, K.M., et al. (2006b). Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue. World J Gastroenterol *12*: 5987–5994.

Matos, J.E., Sausbier, M., Beranek, G., Sausbier, U., Ruth, P., and Leipziger, J. (2007). Role of cholinergic-activated KCa1.1 (BK), KCa3.1 (SK4) and KV7.1 (KCNQ1) channels in mouse colonic Cl- secretion. Acta Physiol *189*: 251–258.

Matsumoto, K., Hosoya, T., Tashima, K., Namiki, T., Murayama, T., and Horie, S. (2011). Distribution of transient receptor potential vanilloid 1 channel-expressing nerve fibers in mouse rectal and colonic enteric nervous system: relationship to peptidergic and nitrergic neurons. Neuroscience *172*: 518–34.

Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature *453*: 620–5.

Mazzuoli, G., and Schemann, M. (2009). Multifunctional rapidly adapting mechanosensitive enteric neurons (RAMEN) in the myenteric plexus of the guinea pig ileum. J. Physiol. *587*: 4681–94.

McCarthy, J., O'Mahony, L., O'Callaghan, L., Sheil, B., Vaughan, E.E., Fitzsimons, N., et al. (2003). Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut *52*: 975–980.

McGuckin, M. a, Eri, R., Simms, L. a, Florin, T.H.J., and Radford-Smith, G. (2009). Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm. Bowel Dis. *15*: 100–13.

McKernan, D.P., Fitzgerald, P., Dinan, T.G., and Cryan, J.F. (2010). The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. Neurogastroenterol Motil *22*: 1029–35, e268.

McVey Neufeld, K. a, Mao, Y.K., Bienenstock, J., Foster, J. a, and Kunze, W. a (2013). The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol. Motil. *25*: 183–e88.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., et al. (1998). XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. *50*: 143–50.

Miranda-Morales, M., Ochoa-Cortes, F., Stern, E., Lomax, A.E., and Vanner, S. (2010). Axon reflexes evoked by transient receptor potential vanilloid 1 activation are mediated by tetrodotoxin-resistant voltage-gated Na+ channels in intestinal afferent nerves. J. Pharmacol. Exp. Ther. *334*: 566–75.

Miyauchi, E., O'Callaghan, J., Butto, L.F., Hurley, G., Melgar, S., Tanabe, S., et al. (2012a). Mechanism of protection of transepithelial barrier function by Lactobacillus salivarius: strain dependence and attenuation by bacteriocin production. AJP Gastrointest. Liver Physiol. *303*: G1029–G1041.

Miyauchi, E., O'Callaghan, J., Buttó, L.F., Hurley, G., Melgar, S., Tanabe, S., et al. (2012b). Mechanism of protection of transepithelial barrier function by Lactobacillus salivarius: strain dependence and attenuation by bacteriocin production. Am. J. Physiol. Gastrointest. Liver Physiol. *303*: G1029–41.

Mongardi Fantaguzzi, C., Thacker, M., Chiocchetti, R., and Furness, J.B. (2009). Identification of neuron types in the submucosal ganglia of the mouse ileum. Cell Tissue Res. *336*: 179–89.

Narahashi, T. Pharmacology of tetrodotoxin. J. Toxicol. Rev. *20*: 67–84.

Nieto, F.R., Cobos, E.J., Tejada, M.Á., Sánchez-Fernández, C., González-Cano, R., and Cendán, C.M. (2012). Tetrodotoxin (TTX) as a therapeutic agent for pain. Mar. Drugs *10*: 281–305.

Nissle (2009). Ueber die Grundlagen einer neuen ursächlichen Bekämpfung der pathologischen Darmflora 1). DMW - Dtsch. Medizinische Wochenschrift *42*: 1181–1184.

Nurgali, K., Stebbing, M.J., and Furness, J.B. (2004). Correlation of electrophysiological and morphological characteristics of enteric neurons in the mouse colon. J. Comp. Neurol. *468*: 112–24.

O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., et al. (2001). Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. Aliment Pharmacol Ther *15*: 1219–1225.

O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., et al. (2005). Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology *128*: 541–51.

O'Malley, D., Dinan, T.G., and Cryan, J.F. (2012). Interleukin-6 modulates colonic transepithelial ion transport in the stress-sensitive wistar kyoto rat. Front. Pharmacol. *3*: 190.

O'Malley, D., Liston, M., Hyland, N.P., Dinan, T.G., Cryan, J.F., and Malley, D.O. (2011). Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism. Am. J. Physiol. Gastrointest. Liver Physiol. *300*: G241–52.

O'Neill (Ed.), M.J. (2013). The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals. 15th ed. New Jersey: Merck, 2013.

O'Neill, J., Brock, C., Olesen, A.E., Andresen, T., Nilsson, M., and Dickenson, A.H. (2012). Unravelling the mystery of capsaicin: a tool to understand and treat pain. Pharmacol. Rev. *64*: 939–71.

Ohland, C.L., DeVinney, R., and MacNaughton, W.K. (2012). Escherichia coli-induced epithelial hyporesponsiveness to secretagogues is associated with altered CFTR localization. Cell. Microbiol. *14*: 447–59.

Ohland, C.L., and Macnaughton, W.K. (2010a). Probiotic bacteria and intestinal epithelial barrier function. Am. J. Physiol. Gastrointest. Liver Physiol. *298*: G807–19.

Ohland, C.L., and Macnaughton, W.K. (2010b). Probiotic bacteria and intestinal epithelial barrier function. Am. J. Physiol. Gastrointest. Liver Physiol. *298*: G807–19.

Palmer, L.G., and Andersen, O.S. (2008). The two-membrane model of epithelial transport: Koefoed-Johnsen and Ussing (1958). J. Gen. Physiol. *132*: 607–12.

Parkes, G.C., Rayment, N.B., Hudspith, B.N., Petrovska, L., Lomer, M.C., Brostoff, J., et al. (2012). Distinct microbial populations exist in the mucosa-associated microbiota of sub-groups of irritable bowel syndrome. Neurogastroenterol. Motil. *24*: 31–9.

Parkman, H.P., Stapelfeldt, W.H., Williams, C.L., Lennon, V.A., and Szurszewski, J.H. (1993). Enteric GABA-containing nerves projecting to the guinea-pig inferior mesenteric ganglion modulate acetylcholine release. J. Physiol. *471*: 191–207.

Patel, R.M., Myers, L.S., Kurundkar, A.R., Maheshwari, A., Nusrat, A., and Lin, P.W. (2012). Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am. J. Pathol. *180*: 626–35.

Patel, Y.C., Greenwood, M.T., Warszynska, A., Panetta, R., and Srikant, C.B. (1994). All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenylyl cyclase. Biochem. Biophys. Res. Commun. *198*: 605–12.

Pepe, G., Moncayo, R., Bombardieri, E., and Chiti, A. (2012). Somatostatin receptor SPECT. Eur. J. Nucl. Med. Mol. Imaging *39 Suppl 1*: S41–51.

Petrof, E.O., and Khoruts, A. (2014). From Stool Transplants to Next-Generation Microbiota Therapeutics. Gastroenterology *146*: 1573–1582.

Phillips, R.J., Kieffer, E.J., and Powley, T.L. (2003). Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. Auton. Neurosci. *106*: 69–83.

Phillips, R.J., Martin, F.N., Billingsley, C.N., and Powley, T.L. (2013). Alpha-synuclein expression patterns in the colonic submucosal plexus of the aging Fischer 344 rat: implications for biopsies in aging and neurodegenerative disorders? Neurogastroenterol. Motil. *25*: e621–33.

Phillips, R.J., Pairitz, J.C., and Powley, T.L. (2007). Age-related neuronal loss in the submucosal plexus of the colon of Fischer 344 rats. *28*: 1124–1137.

Philpott, D.J., McKay, D.M., Sherman, P.M., and Perdue, M.H. (1996). Infection of T84 cells with enteropathogenic Escherichia coli alters barrier and transport functions. Am. J. Physiol. *270*: G634–45.

Piche, T. (2014). Tight junctions and IBS--the link between epithelial permeability, low-grade inflammation, and symptom generation? Neurogastroenterol. Motil. *26*: 296–302.

Piche, T., Barbara, G., Aubert, P., Bruley des Varannes, S., Dainese, R., Nano, J.L., et al. (2009). Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. Gut *58*: 196–201.

Pol, O., Alameda, F., and Puig, M.M. (2001). Inflammation enhances mu-opioid receptor transcription and expression in mice intestine. Mol. Pharmacol. *60*: 894–9.

Putaala, H., Salusjärvi, T., Nordström, M., Saarinen, M., Ouwehand, A.C., Bech Hansen, E., et al. (2008). Effect of four probiotic strains and Escherichia coli O157:H7 on tight junction integrity and cyclo-oxygenase expression. Res. Microbiol. *159*: 692–8.

Qu, Z.-D., Thacker, M., Castelucci, P., Bagyánszki, M., Epstein, M.L., and Furness, J.B. (2008). Immunohistochemical analysis of neuron types in the mouse small intestine. Cell Tissue Res. *334*: 147–61.

Raftis, E.J., Salvetti, E., Torriani, S., Felis, G.E., and O'Toole, P.W. (2011). Genomic diversity of Lactobacillus salivarius. Appl. Environ. Microbiol. *77*: 954–65.

Raheja, G., Singh, V., Ma, K., Boumendjel, R., Borthakur, A., Gill, R.K., et al. (2010). Lactobacillus acidophilus stimulates the expression of SLC26A3 via a transcriptional mechanism. Am. J. Physiol. Gastrointest. Liver Physiol. *298*: G395–401.

Rampelli, S., Candela, M., Turroni, S., Biagi, E., Collino, S., Franceschi, C., et al. (2013). Functional metagenomic profiling of intestinal microbiome in extreme ageing. Aging (Albany. NY). *5*: 902–12.

Resta-Lenert, S., and Barrett, K.E. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut *52*: 988–97.

Resta-Lenert, S., and Barrett, K.E. (2006). Probiotics and commensals reverse TNF-alpha- and IFN-gamma-induced dysfunction in human intestinal epithelial cells. Gastroenterology *130*: 731–46.

Resta-Lenert, S.C., and Barrett, K.E. (2009). Modulation of intestinal barrier properties by probiotics: role in reversing colitis. Ann. N. Y. Acad. Sci. *1165*: 175–82.

Ritchie, M.L., and Romanuk, T.N. (2012). A meta-analysis of probiotic efficacy for gastrointestinal diseases. PLoS One *7*: e34938.

Rocha, F., Musch, M.W., Lishanskiy, L., Bookstein, C., Sugi, K., Xie, Y., et al. (2001). IFN-gamma downregulates expression of Na(+)/H(+) exchangers NHE2 and NHE3 in rat intestine and human Caco-2/bbe cells. Am J Physiol Cell Physiol *280*: C1224–32.

Rösel, E., and Engelhardt, W. von (1996). Sodium transport across the caecal and colonic epithelium of germfree and specific-pathogen free rats. Reprod Nutr Dev *36*: 289–299.

Roshchina, V. V. (2010). Evolutionary Considerations of Neurotransmitters in Microbial, Plant, and Animal Cells. 17–52.

Rubin, D.C., Swietlicki, E.A., Wang, J.L., and Levin, M.S. (1998). Regulation of PC4/TIS7 expression in adapting remnant intestine after resection. Am. J. Physiol. *275*: G506–13.

Sagmanligil, V., and Levin, R.J. (1993). Electrogenic ion secretion in proximal, mid and distal colon from fed and starved mice. Comp. Biochem. Physiol. C. *106*: 449–56.

Samson, A., Hamilton, K.L., and Butt, A.G. (2000). Effect of somatostatin on electrogenic ion transport in the duodenum and colon of the mouse, Mus domesticus. Comp Biochem Physiol A Mol Integr Physiol *125*: 459–468.

Sánchez, B., Urdaci, M.C., and Margolles, A. (2010). Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiology *156*: 3232–3242.

Sang, Q., Williamson, S., and Young, H.M. (1997). Projections of chemically identified myenteric neurons of the small and large intestine of the mouse. J. Anat. *190 (Pt 2*: 209–22.

Sang, Q., and Young, H.M. (1996). Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. Cell Tissue Res. *284*: 39–53.

Sang, Q., and Young, H.M. (1998). The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. Anat. Rec. *251*: 185–199.

Sanger, G.J., Munonyara, M.L., Dass, N., Prosser, H., Pangalos, M.N., and Parsons, M.E. (2002). GABA B receptor function in the ileum and urinary bladder of wildtype and GABA B1 subunit null mice. 147–154.

Sassone-corsi, P. (2012). The Cyclic AMP Pathway. 3–5.

Sayer, B., Lu, J., Green, C., Söderholm, J.D., Akhtar, M., and McKay, D.M. (2002). Dextran sodium sulphate-induced colitis perturbs muscarinic cholinergic control of colonic epithelial ion transport. Br. J. Pharmacol. *135*: 1794–800.

Schlee, M., Harder, J., Köten, B., Stange, E.F., Wehkamp, J., and Fellermann, K. (2008). Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin. Exp. Immunol. *151*: 528–35.

Schmitz, H., Fromm, M., Bentzel, C., Scholz, P., Detjen, K., Mankertz, J., et al. (1999). Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. J. Cell Sci. *112 (Pt 1*: 137–146.

Schultheis, P.J., Clarke, L.L., Meneton, P., Harline, M., Boivin, G.P., Stemmermann, G., et al. (1998). Targeted disruption of the murine Na+/H+ exchanger isoform 2 gene causes reduced viability of gastric parietal cells and loss of net acid secretion. J. Clin. Invest. *101*: 1243–53.

Schulzke, J.D., Gitter, A.H., Mankertz, J., Spiegel, S., Seidler, U., Amasheh, S., et al. (2005). Epithelial transport and barrier function in occludin-deficient mice. Biochim. Biophys. Acta *1669*: 34–42.

Schwartz, C.J., Kimberg, D. V, Sheerin, H.E., Field, M., and Said, S.I. (1974). Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. J. Clin. Invest. *54*: 536–44.

Seidler, U., Lenzen, H., Cinar, A., Tessema, T., Bleich, A., and Riederer, B. (2006). Molecular mechanisms of disturbed electrolyte transport in intestinal inflammation. Ann. N. Y. Acad. Sci. *1072*: 262–75.

Seth, A., Yan, F., Polk, D.B., and Rao, R.K. (2008). Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. Am. J. Physiol. Gastrointest. Liver Physiol. *294*: G1060–9.

Shanahan, F., and Quigley, E.M.M. (2014). Manipulation of the Microbiota for Treatment of IBS and IBD-Challenges and Controversies. Gastroenterology *146*: 1554–1563.

Shankar, V., Hamilton, M.J., Khoruts, A., Kilburn, A., Unno, T., Paliy, O., et al. (2014). Species and genus level resolution analysis of gut microbiota in Clostridium difficile patients following fecal microbiota transplantation. Microbiome *2*: 13.

Sharkey, K.A., and Savidge, T.C. (2014). Role of enteric neurotransmission in host defense and protection of the gastrointestinal tract. Auton. Neurosci. *181*: 94–106.

Sharma, R., Schumacher, U., Ronaasen, V., and Coates, M. (1995). Rat intestinal mucosal responses to a microbial flora and different diets. Gut *36*: 209–214.

Sharp, G.W., and Hynie, S. (1971). Stimulation of intestinal adenyl cyclase by cholera toxin. Nature *229*: 266–269.

Shcheynikov, N., Kim, K.H., Kim, K.-M., Dorwart, M.R., Ko, S.B.H., Goto, H., et al. (2004). Dynamic control of cystic fibrosis transmembrane conductance regulator Cl(-)/HCO3(-) selectivity by external Cl(-). J. Biol. Chem. *279*: 21857–65.

Sheldon, R.J., Malarchik, M.E., Burks, T.F., and Porreca, F. (1990a). Effects of nerve stimulation on ion transport in mouse jejunum: responses to Veratrum alkaloids. J. Pharmacol. Exp. Ther. *252*: 636–42.

Sheldon, R.J., Malarchik, M.E., Fox, D.A., Burks, T.F., and Porreca, F. (1989). Pharmacological characterization of neural mechanisms regulating mucosal ion transport in mouse jejunum. J. Pharmacol. Exp. Ther. *249*: 572–82.

Sheldon, R.J., Rivière, P.J., Malarchik, M.E., Moseberg, H.I., Burks, T.F., and Porreca, F. (1990b). Opioid regulation of mucosal ion transport in the mouse isolated jejunum. J. Pharmacol. Exp. Ther. *253*: 144–51.

Simpson, J.E., Schweinfest, C.W., Shull, G.E., Gawenis, L.R., Walker, N.M., Boyle, K.T., et al. (2007). PAT-1 (Slc26a6) is the predominant apical membrane Cl-/HCO3- exchanger in the upper villous epithelium of the murine duodenum. Am J Physiol Gastrointest Liver Physiol *292*: G1079–88.

Singh, A.K., Riederer, B., Chen, M., Xiao, F., Krabbenhöft, A., Engelhardt, R., et al. (2010). The switch of intestinal Slc26 exchangers from anion absorptive to HCOFormula secretory mode is dependent on CFTR anion channel function. Am J Physiol Cell Physiol *298*: C1057–65.

Singh, V., Raheja, G., Borthakur, a., Kumar, a., Gill, R.K., Alakkam, a., et al. (2012). Lactobacillus acidophilus Upregulates Intestinal NHE3 Expression and Function. AJP Gastrointest. Liver Physiol. *303*: G1393–401.

Smith, K., McCoy, K.D., and Macpherson, A.J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin. Immunol. *19*: 59–69.

Sommer, F., and Bäckhed, F. (2013). The gut microbiota--masters of host development and physiology. Nat. Rev. Microbiol. *11*: 227–38.

sommerfield, D., O'Brien, N., Kiely, B., Collins, J.K., Shanahan, F., O'Mahony, L., et al. (2003). Influence of bifidobacterium infantis feeding on secretagogue response and gut barrier function in rats recovering from colitis. Gastroenterology *124*: A311.

Sonnenburg, J.L., Chen, C.T.L., and Gordon, J.I. (2006). Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. PLoS Biol. *4*: e413.

Soret, R., Chevalier, J., Coppet, P. De, Poupeau, G., Derkinderen, P., Segain, J.P., et al. (2010). Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. Gastroenterology *138*: 1772–82.

Spencer, N.J., and Smith, T.K. (2004). Mechanosensory S-neurons rather than AH-neurons appear to generate a rhythmic motor pattern in guinea-pig distal colon. J. Physiol. *558*: 577–96.

Subramanya, S.B., Rajendran, V.M., Srinivasan, P., Nanda Kumar, N.S., Ramakrishna, B.S., and Binder, H.J. (2007). Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum. Am. J. Physiol. Gastrointest. Liver Physiol. *293*: G857–63.

Sugi, K., Musch, M.W., Field, M., and Chang, E.B. (2001). Inhibition of Na+,K+-ATPase by interferon gamma down-regulates intestinal epithelial transport and barrier function. Gastroenterology *120*: 1393–1403.

Sultana, R., McBain, A.J., and O'Neill, C.A. (2013). Strain-dependent augmentation of tight-junction barrier function in human primary epidermal keratinocytes by Lactobacillus and Bifidobacterium lysates. Appl. Environ. Microbiol. *79*: 4887–94.

Suply, E., Vries, P. de, Soret, R., Cossais, F., and Neunlist, M. (2012). Butyrate enemas enhance both cholinergic and nitrergic phenotype of myenteric neurons and neuromuscular transmission in newborn rat colon. Am. J. Physiol. Gastrointest. Liver Physiol. *302*: G1373–80.

Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions. Cell. Mol. Life Sci. *70*: 631–59.

Takahashi, A., Sato, Y., Shiomi, Y., Cantarelli, V. V, Iida, T., Lee, M., et al. (2000a). Mechanisms of chloride secretion induced by thermostable direct haemolysin of Vibrio parahaemolyticus in human colonic tissue and a human intestinal epithelial cell line. J Med Microbiol *49*: 801–810.

Takahashi, T., Qoubaitary, A., and Chung, O. (2000b). Decreased expression of nitric oxide synthase in the colonic myenteric plexus of aged rats. *883*: 15–21.

Tao, Y., Drabik, K.A., Waypa, T.S., Musch, M.W., Alverdy, J.C., Schneewind, O., et al. (2006). Soluble factors from Lactobacillus GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *60637*: 1018–1030.

Thwaites, D.T., Basterfield, L., McCleave, P.M., Carter, S.M., and Simmons, N.L. (2000). Gamma-Aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers. Br. J. Pharmacol. *129*: 457–64.

Tough, I.R., Forbes, S., Tolhurst, R., Ellis, M., Herzog, H., Bornstein, J.C., et al. (2011). Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y_1 and Y_2 receptors. Br. J. Pharmacol. *164*: 471–84.

Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. Nat. Rev. Mol. Cell Biol. *2*: 285–93.

Turnbaugh, P.J., Ridaura, V.K., Faith, J.J., Rey, F.E., Knight, R., and Gordon, J.I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci. Transl. Med. *1*: 6ra14.

Turner, J.R. (2009). Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. *9*: 799–809.

Ukena, S.N., Singh, A., Dringenberg, U., Engelhardt, R., Seidler, U., Hansen, W., et al. (2007). Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One *2*: e1308.

Ussing, H.H., and Zehran, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol Scand *23*: 110–127.

Vanner, S., and Macnaughton, W.K. (2004a). Submucosal secretomotor and vasodilator reflexes. Neurogastroenterol. Motil. *16 Suppl 1*: 39–43.

Vanner, S., and Macnaughton, W.K. (2004b). Submucosal secretomotor and vasodilator reflexes. Neurogastroenterol. Motil. *16 Suppl 1*: 39–43.

Varma, J.K., Marcus, R., Stenzel, S.A., Hanna, S.S., Gettner, S., Anderson, B.J., et al. (2006). Highly resistant Salmonella Newport-MDRAmpC transmitted through the domestic US food supply: a FoodNet case-control study of sporadic Salmonella Newport infections, 2002-2003. J. Infect. Dis. *194*: 222–30.

Veltman, K., Hummel, S., Cichon, C., Sonnenborn, U., and Schmidt, M.A. (2012). Identification of specific miRNAs targeting proteins of the apical junctional complex that simulate the probiotic effect of E. coli Nissle 1917 on T84 epithelial cells. Int. J. Biochem. Cell Biol. *44*: 341–9.

Verdu, E.F., Bercik, P., Huang, X.X., Lu, J., Al-Mutawaly, N., Sakai, H., et al. (2008). The role of luminal factors in the recovery of gastric function and behavioral changes after chronic Helicobacter pylori infection. Am. J. Physiol. Gastrointest. Liver Physiol. *295*: G664–70.

Vidyasagar, S., Barmeyer, C., Geibel, J., Binder, H.J., and Rajendran, V.M. (2005). Role of short-chain fatty acids in colonic HCO(3) secretion. Am. J. Physiol. Gastrointest. Liver Physiol. *288*: G1217–26.

Vidyasagar, S., Rajendran, V.M., and Binder, H.J. (2004). Three distinct mechanisms of HCO3- secretion in rat distal colon. Am. J. Physiol. Cell Physiol. *287*: C612–21.

Wang, B., Mao, Y.-K., Diorio, C., Wang, L., Huizinga, J.D., Bienenstock, J., et al. (2010a). Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. Neurogastroenterol. Motil. *22*: 98–107, e33.

Wang, B., Mao, Y.-K.K., Diorio, C., Pasyk, M., Wu, R.Y., Bienenstock, J., et al. (2010b). Luminal administration ex vivo of a live Lactobacillus species moderates mouse jejunal motility within minutes. FASEB J *24*: 4078–4088.

Wang, B., Mao, Y.-K.K., Diorio, C., Wang, L., Huizinga, J.D., Bienenstock, J., et al. (2010c). Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. Neurogastroenterol Motil *22*: 98–107, e33.

Wang, C., Xu, H., Chen, H., Li, J., Zhang, B., Tang, C., et al. (2011). Somatostatin stimulates intestinal NHE8 expression via p38 MAPK pathway. Am. J. Physiol. Cell Physiol. *300*: C375–82.

Wang, Y., Devkota, S., Musch, M.W., Jabri, B., Nagler, C., Antonopoulos, D.A., et al. (2010d). Regional mucosa-associated microbiota determine physiological expression of TLR2 and TLR4 in murine colon. PLoS One *5*: e13607.

Wang, Z., Petrovic, S., Mann, E., and Soleimani, M. (2002). Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine. Am. J. Physiol. Gastrointest. Liver Physiol. *282*: G573–9.

Wenus, C., Goll, R., Loken, E.B., Biong, A.S., Halvorsen, D.S., and Florholmen, J. (2008). Prevention of antibiotic-associated diarrhoea by a fermented probiotic milk drink. Eur. J. Clin. Nutr. *62*: 299–301.

Whang, E.E., Dunn, J.C., Joffe, H., Mahanty, H., Zinner, M.J., McFadden, D.W., et al. (1996). Enterocyte functional adaptation following intestinal resection. J. Surg. Res. *60*: 370–4.

Whorwell, P.J., Altringer, L., Morel, J., Bond, Y., Charbonneau, D., O'Mahony, L., et al. (2006). Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol *101*: 1581–1590.

Wong, V., Blennerhassett, M., and Vanner, S. (2008). Electrophysiological and morphological properties of submucosal neurons in the mouse distal colon. Neurogastroenterol. Motil. *20*: 725–34.

Wood, J.D., and Galligan, J.J. (2004). Function of opioids in the enteric nervous system. Neurogastroenterol. Motil. *16 Suppl 2*: 17–28.

Wu, R.Y., Pasyk, M., Wang, B., Forsythe, P., Bienenstock, J., Mao, Y.-K., et al. (2013). Spatiotemporal maps reveal regional differences in the effects on gut motility for Lactobacillus reuteri and rhamnosus strains. Neurogastroenterol. Motil. *25*: e205–14.

Wullt, M., Hagslatt, M., and Odenholt, I. (2003). Lactobacillus plantarum 299v for the treatment of recurrent clostridium difficile-associated diarrhoea: A double-blind, placebo-controlled trial. *35*: 365–367.

Yan, F., Cao, H., Cover, T.L., Washington, M.K., Shi, Y., Liu, L., et al. (2011). Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism. J. Clin. Invest. *121*: 2242–53.

Yoo, D., Lo, W., Goodman, S., Ali, W., Semrad, C., and Field, M. (2000). Interferon-gamma downregulates ion transport in murine small intestine cultured in vitro. Am. J. Physiol. Gastrointest. Liver Physiol. *279*: G1323–32.

Yun, C.H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C.M., et al. (1997). cAMP-mediated inhibition of the epithelial brush border Na □H , 3EHN , regnahcxe □ requires an associated regulatory protein. Proc Natl Acad Sci U S A *94*: 3010–3015.

Zeissig, S., Bergann, T., Fromm, A., Bojarski, C., Heller, F., Guenther, U., et al. (2008). Altered ENaC Expression Leads to Impaired Sodium Absorption in the Noninflamed Intestine in Crohn's Disease. Gastroenterology *134*: 1436–1447.

Zeissig, S., Fromm, A., Mankertz, J., Weiske, J., Zeitz, M., Fromm, M., et al. (2007). Butyrate induces intestinal sodium absorption via Sp3-mediated transcriptional up-regulation of epithelial sodium channels. Gastroenterology *132*: 236–48.

Zyrek, A.A., Cichon, C., Helms, S., Enders, C., Sonnenborn, U., and Schmidt, M.A. (2007). Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. Cell. Microbiol. *9*: 804–16.

Appendix A
List of Abbreviations

5-HT Serotonin

ACh Acetylcholine :

AC Adenylyl Cyclase

AHP: Afterhyperpolarising potential

ATP: Adenosine triphosphate

BCh Bethanechol:

CaCC Calcium Activated Chloride Channel

cAMP: Cyclic Adenosine Monophosphate

CalB: Calbindin

CalR: Calretnin

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CGRP: Calcitionin Gene Related Peptide

CM: Conditioned Medium

CT: Cholera Toxin

DAG: Diacylglycerol

DIDS: 4,4'-diisothiocyano-2,2'-stilbene disulphonic acid

DRA Down-regulated in Adenoma

EHEC: enterohaemorragic *Escherichia coli*

EIEC: Enteroinvasive *Escherichia coli*

ENaC Epithelial Sodium Channel:

ENS: Enteric Nervous System

EPEC: Entero-pathogenic *Escherichia coli*

ERK: Extracellular Signal-Regulated Kinases

FSK Forskolin:

GABA γ-amino butyric acid:

GF: Germ Free

IFN: Interferon

IL: Interleukin

IP_3: Inositol 1,4,5 triphosphate

IP_4:Inositol 3,4,5,6-tetrakisphosphate

IPAN Intrinsic Primary Afferent Neuron

I_{sc} Short Circuit Current:

mAChR: Muscarinic Acetylcholine Receptor

nAChR: Nicotinic Acetylcholine Receptor

MAPK Mitogen-activated protein kinase

NKCC1: Sodium Potassium Chloride Symporter

NPY: Neuropeptide Y:

NHE: Sodium Hydrogen Exchanger

NOS: Nitric Oxide Synthase

$K_{Ca}3.1$ Small Conductance K^+ channel

$K_V7.1$ Voltage Gated K^+ channels

MMC: Migrating Motor Complexes

PAT-1: Putative Anion Transporter-1

PGE_2: Prostaglandin E_2

PI3K: Phosphatidyl-inositol 3 kinase

PIP_2: Phosphatidylinositol 4-5 bisphosphate

PKA: Protein Kinase A

PKC: Phospholipase C

SCFA : Short Chain Fatty Acid

SGLT1:Sodium glucose transporter 1

SOM:Somatostatin

SP: Substance P

SPF: Specific Pathogen Free

SSTR: Somatostatin Receptor

T3SS: Type 3 Secretion System

TER Transepithelial Electrical Resistance

TH: Tyrosine Hydroxylase

TJ: Tight Junction

TLR: Toll-Like Receptor

TNF: Tumor Necrosis Factor

TRPV-1: Transient Receptor Potential Cation Channel Subfamily V member 1

TTX: Tetrodotoxin

VIP: Vasoactive Intestinal Peptide

VGSC: Voltage Gated Sodium Channels

Y: NPY receptor

ZO: Zonula occludens

Appendix B:

The I_{sc} response to a commensal *Bifidobacterium* was sustained in the presence of loperamide

Introduction

Loperamide (4-[4-chlorophenyl]-4-hydroxy-N-dimethyl-alpha, alpha-diphenyl-1-piperidine-butanamide hydrochloride) is a phenylpiperidine derivative, a potent μ-opioid receptor (μ-OR) agonist and the most common over-the-counter medication approved for the control of diarrheal symptoms in numerous GI disorders, including travellers' diarrhoea and irritable bowel syndrome (Baker, 2007; Hanauer, 2008). Electrophysiological recordings in enteric neurons have demonstrated that opiates and opioid peptides mediate their effects through suppression of neuronal excitability (Wood and Galligan, 2004). Early studies *in-vivo* demonstrated the ability of the μ-OR specific agonist, DAMGO, to significantly increase intestinal absorption of an isotonic solution following intracerebroventricular injection (Jiang et al., 1990). In contrast, a study carried out by the same group, in isolated jejunal segments mounted in Ussing Chamber, demonstrated a local effect, observing a decrease in I_{sc} following serosal addition of DAMGO (Sheldon et al., 1990b). Furthermore, this effect appeared to be neurally mediated as tissues stripped of the enteric neural plexi did not not display such an effect (Sheldon et al., 1990b). This has been supported recently by immuno-histochemical studies which demonstrated μ-OR expression on enteric nerve endings in the myenteric plexus as well as the submucosal plexus (Pol et al., 2001).

Therefore I sought to determine whether a commensal *Bifidibactetrium* could influence I_{sc} in the presence of loperamide.

Materials and Methods

Materials

Bethanechol and forskolin were obtained from Sigma Aldrich (Tallaght, Dublin) while loperamide was obtained from Tocris (Bristol, United Kingdom).

Methods

Mucosal-submucosal preparations of descending colon from male Swiss Webster mice (25-35g) were mounted in Ussing chambers, voltage clamped at 0mV and were super fused with Krebs buffer solution bubbled with carbogen (95% O2, 5% CO2) and maintained at 37°C. Changes in short circuit current (Isc) were continuously recorded using DataTrax software. Bacteria were added to the mucosal compartment at $1x10^9$ CFU/ml in Krebs). Loperamide (0.1-0.5µg/ml in DMSO), bethanechol (BCh; 100µM in dH_2O) and forskolin (FSK; 10µM), were added serosally.

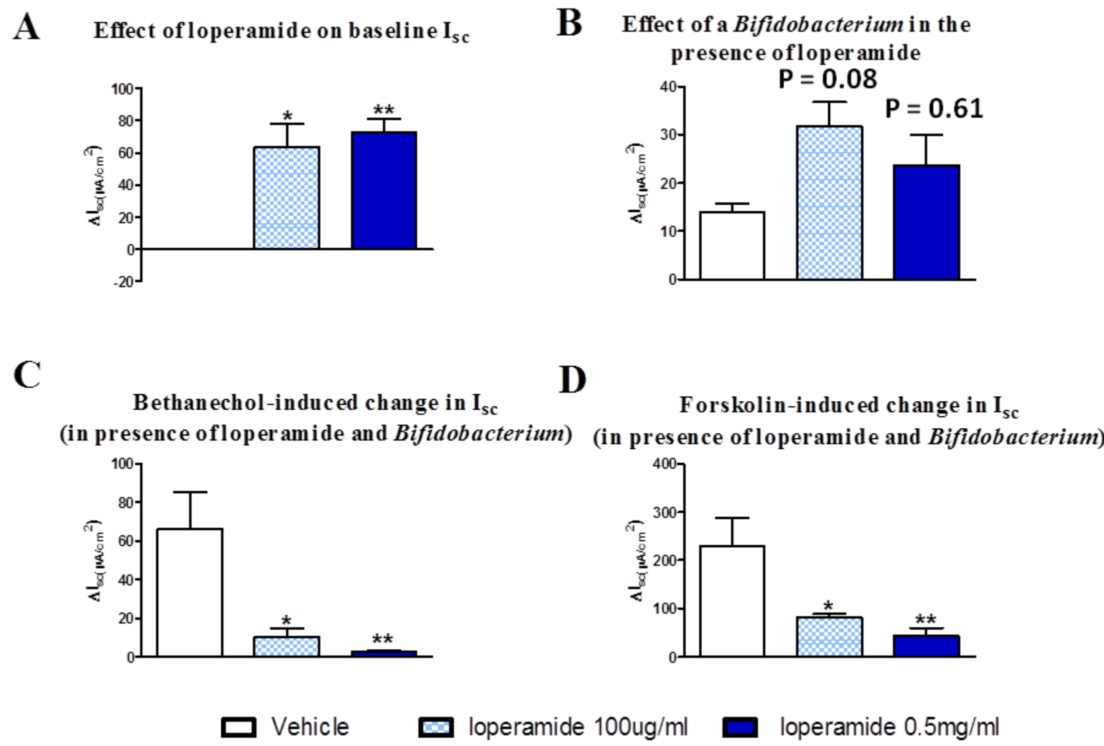

Figure B1. (A) Serosal addition of loperamide caused a significant increase in I_{sc} compared to vehicle. The increase was independent of dose. (B) In the presence of loperamide, a *Bifidobacterium* elicited an increase in I_{sc}. (C) The combination of loperamide and a *Bifidobacterium* significantly decreased BCh and (D) forskolin-induced $I_{sc.}$ n=5-6, *P<0.05,**P<0.01.

Appendix C

Supplementary Material for Chapter 2

Materials

All drugs were obtained from Sigma-Aldrich Ltd. (Ireland) unless otherwise stated. The following compounds were used, with the final concentration and diluent in parenthesis, amiloride (100µM dissolved in dH_2O), bethanechol (100µM; dissolved in dH_2O), forskolin (10µM dissolved in DMSO), furosemide (100µM dissolved in DMSO), Chromanol 293b (100µM dissolved in DMSO) tetrodotoxin (300nM dissolved in sodium citrate buffer), BPIPP (10µM dissolved in DMSO),veratridine (30µM; dissolved in 70% ethanol) *Bordetella pertussis* toxin Wortmannin (10µM dissolved in DMSO), PD98059 (10µM dissolved in DMSO) Krebs buffer was prepared as follows, 1.2mM NaH_2PO_4 , 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 11mM $CaCl_2$ and 10mM glucose. Chloride-free buffer consisted of 116mM $NaNO_3$, 5.4mM KNO_3, 1.2mM NaH_2PO_4, 2.5mM $Ca(NO_3)_2$, 1.2mM $Mg(NO_3)_2$, 22mM $NaHCO_3$ and 11.2mM glucose. Calcium-free buffer consisted of 1.2mM NaH_2PO_4 , 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, and 10mM glucose. *B. infantis* 35624 and *L. salivarius* UCC 118 were from Alimentary Health Ltd. (Cork, Ireland).

Results

To further determine the mechanisms involved in the modulation of ion transport by *B. infantis* 35624 and *L. salivarius* UCC 118 observed in **Chapter 2**,

additional pharmacological manipulation and ion substitution was carried out. Neither, Chromanol 293b ($K_V7.1$ blocker) or low Ca^{2+} Krebs buffer significantly affected the baseline response to either probiotic. Similarly, *Bordetella pertussis* ($G_{\alpha i}$ - protein coupled receptor signalling inhibition) Wortmannin (PI3K inhibition), PD98059 (MAPK inhibition) had no effect, suggesting intra-cellular signalling mechanisms may not be involved. Importantly, the cGMP inhibitor, BPIPP did not block the response to either probiotic, indicating that this pathway did not mediate the change in baseline I_{sc} (**Table C1**).

Table C1 Pharmacological Manipulations and Ion Substitution Experiments

Pharacological Manipulation	Baseline Responses (μA.cm^{-2}) to:						
	B.infantis 35624	*L. salivarius* UCC 118	Bethanechol (following *B.infantis* 35624)	Bethanechol (following *L.salivarius* UCC118)	Forskolin (following *B.infantis* 35624)	Forskolin (following *L.salivarius* UCC118)	8-Bromo cGMP
Amiloride	20.74±5.18 (n=6)	25.52±3.32(n=5)	22.50±39.4 (n=6)	24.4±8.6 (n=5)	188.6±26.4 (n=5)	163.3±49.7 (n=5)	
Vehicle (dH2O)	26.07±1.31 (n=10)	27.47±4.47 (n=5)	39.44±8.82 (n=10)	16.71±5.184 (n=5)	185.7±33.6 (n=5)	149.2±50.9 (n=5)	
Furosemide	25.24±3.71 (n=6)	28.81±0.71 (n=6)	8.6±2.6* (n=6)	21.5±6.2 (n=5)	108.4±19.55 (n=5) **P=0.11**	192.3±46.9 (n=5)	
Vehicle (DMSO)	21.77±7.94 (n=7)	23.12±3.04 (n=6)	61.98±21.09 (n=6)	29.4±0.6 (n=5)	228.7±58.63(n=5)	261.3±28.4 (n=5)	
Chromanol 293b	20.11±3.32 (n=15)	27.66±5.51 (n=9) **P=0.19**	47.30±17.6 (n=15) **P=0.18**	23.7±5.4 (n=9)	138.4±14.1 (n=15)**	142.0±26.0 (n=15) **P=0.40**	
Vehicle (DMSO)	27.66±5.51 (n=10)	40.39±7.62(n=9)	19.86±9.2 (n=10)	19.6±3.2 (n=9)	236.7±27.91 (n=10)	181.9±37.31 (n=10)	
Wortmannin	11.67±3.2 (n=4)		36.88±20.4 (n=4)		240.6±90.64 (n=5)		
Vehicle (DMSO)	16.45±4.2 (n=4)		29.82±16.4 (n=5)		245.6±12.96 (n=4)		
PD98059	17.91±4.8 (n=5)		23.41±11.90 (n=4)		338.0±19.51 (n=5)**		
Vehicle (DMSO)	16.47±4.2 (n=4)		32.52±8.7 (n=5)		245.6±12.96 (n=4)		
BPIPP	33.62±6.17 (n=6)		34.06±10.56 (n=6) **P=0.14**		171.4±68.98 (n=6)		16.49±8.9 (n=6)
Vehicle (DMSO)	26.08±3.0 (n=6)		70.0±18.11 (n=6)		134.7±68.98 (n=6)		23.41±8.4 (n=6)
Bordetella pertussis toxin	10.91±3.2 (n=6)	16.45±5.91 (n=6)	19.14±5.88 (n=5)	16.84±7.1 (n=6)	216.5±20.41(n=6)	198.5±35.16 (n=5)	
Vehicle (dH2O)	9.41±2.0 (n=6)	20.08±6.642 (n=5)	46.63±11.0 (n=6)	25.92±15.57 (n=6)	202.1±31.5 (n=6)	175.7±36.64 (n=5)	
Chloride-free Krebs	17.30±3.24 (n=6)	52.70±12.00 *(n=6)	30.52±3.5 (n=5)	25.72±3.8 (n=5)	163±19.24 (n=5)	172.2±26.4 (n=5)	
Krebs	18.91±4.21 (n=6)	22.53±7.45 (n=6)	45.12±8.3 (n=5)	41.52±6.3 (n=5)	300.34±15.2 (n=5)*	280±21.14 (n=5)*	
Calcium-free Krebs	21.12±3.13 (n=5)	38.28±8.62 (n=4)	30.09±6.3 (n=5)	20.91±4.9 (n=5)*	157.7±39.6 (n=5)	185.7±23.09 (n=5)	
Normal Krebs	21.77±7.94 (n=6)	29.57±4.727 (n=5)	61.9±21.1 (n=5)	37.12±6.2 (n=5)	228.7±58.6 (n=5)	240.5±48.9 (n=5)	

Appendix D:

Supplementary Material for Chapter 3

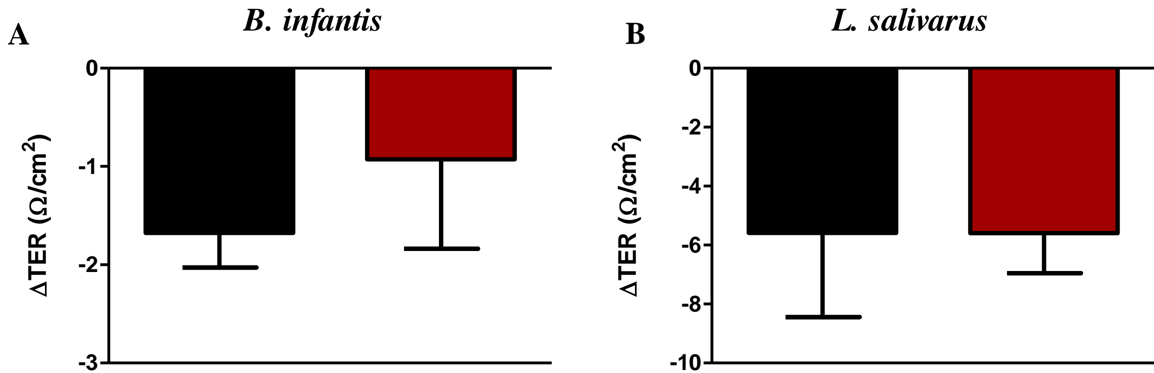

Figure D1. Effect of *B.infantis* 35624 or *L. salivarius* UCC118 on the transepithelial resistance (TER) exhibited by GF colon following acute addition to Ussing chambers. Neither organism significantly influenced TER after 30 mins. n=7-8.

Appendix E:

Supplementary Material for Chapter 4

Results

The $GABA_A$ agonist, muscimol, and positive allosteric modulation of $GABA_{A\alpha1}$, CDP, induces a decrease in baseline I_{sc} implying a role for this receptor, and receptor subtype, in regulating colonic secretomotor function.

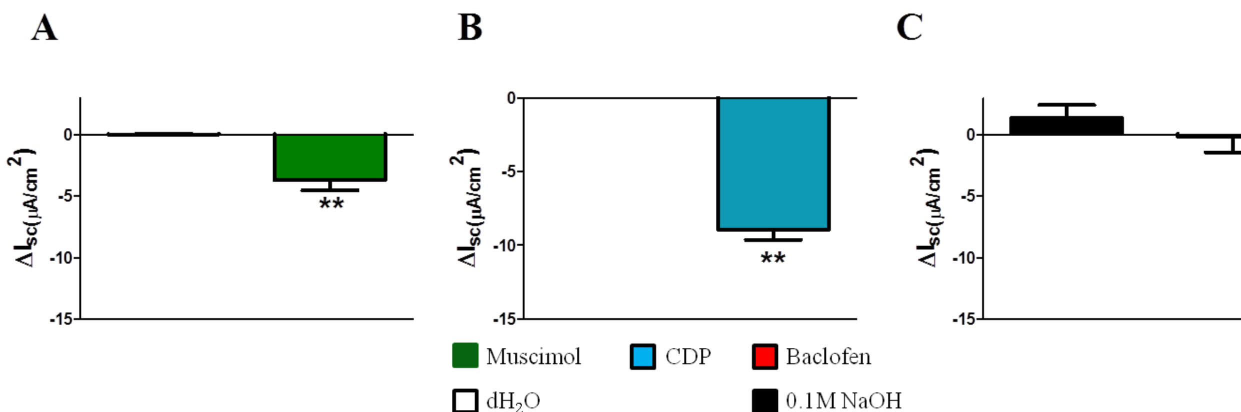

Figure E1. Baseline I_{sc} response to Muscimol, CDP and Baclofen: Both muscimol (A) and CDP (B) caused a decrease in I_{sc} with CDP inducing a significant response at 100μm (Data for 0.1μM and 1μM not shown (N=4).(C) The $GABA_B$ agonist Baclofen had no significant effect on baseline I_{sc} (N=6) **P<0.005.

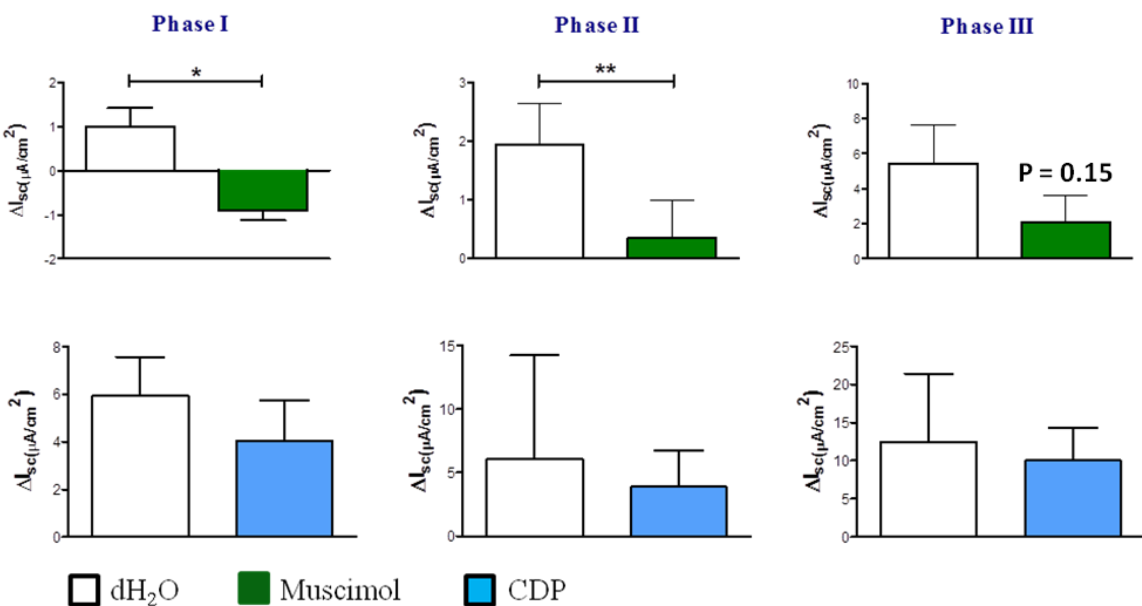

Figure E2. Cholinergic Responses following Muscimol and CDP: (A) $GABA_A$ agonist Muscimol (100µM) induced a pattern of decrease in cholinergic induced responses similar to that of exogenous GABA (N=6) *P<0.05, **P<0.005. (B) CDP (0.1-100µM), a positive modulator of $GABA_{A\alpha 1}$ receptors did not mimic this effect (N=3). Data for 0.1µM and 1µM not shown (N=4).