***Limosilactobacillus reuteri* ATCC 6475 metabolites upregulate the serotonin transporter in the intestinal epithelium**

M. Engevik^{1,2*}, W. Ruan^{3,4}, C. Visuthranukul^{1,5}, Z. Shi^{1,6}, K.A. Engevik⁷, A.C. Engevik⁸, R. Fultz⁹, D.A. Schady^{1,6}, J.K. Spinler^{1,6} and J. Versalovic^{1,6}

¹Department of Pathology & Immunology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030, USA; ²Department of Regenerative Medicine & Cell Biology, Medical University of South Carolina, 173 Ashely Ave, BSB 626, Charleston, SC 29425, USA; ³Department of Pediatrics, Baylor College of Medicine, 6701 Fannin Street, Houston, TX 77030, USA; ⁴Section of Gastroenterology, Hepatology, and Nutrition, Texas Children's Hospital, 6701 Fannin St, Houston, TX 77030, USA; ⁵Pediatric Nutrition Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; ⁶Department of Pathology, Texas Children's Hospital, 6621 Fannin St, Houston, TX 77030, USA; ⁷Department of Molecular Virology & Microbiology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 7703, USA; ⁸Departments of Surgery, Vanderbilt University Medical Center, 1211 Medical Center Dr, Nashville, TN 37232, USA; ⁹Department of Neuroscience, Cell Biology, and Anatomy, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0625, USA; engevik@usc.edu

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Abstract

The serotonin transporter (SERT) readily takes up serotonin (5-HT), thereby regulating the availability of 5-HT within the intestine. In the absence of SERT, 5-HT remains in the interstitial space and has the potential to aberrantly activate the many 5-HT receptors distributed on the epithelium, immune cells and enteric neurons. Perturbation of SERT is common in many gastrointestinal disorders as well as mouse models of colitis. Select commensal microbes regulate intestinal SERT levels, but the mechanism of this regulation is poorly understood. Additionally, ethanol upregulates SERT in the brain and dendritic cells, but its effects in the intestine have never been examined. We report that the intestinal commensal microbe *Limosilactobacillus* (previously classified as *Lactobacillus*) *reuteri* ATCC PTA 6475 secretes 83.4 mM ethanol. Consistent with the activity of *L. reuteri* alcohol dehydrogenases, we found that *L. reuteri* tolerated various levels of ethanol. Application of *L. reuteri* conditioned media or exogenous ethanol to human colonic T84 cells was found to upregulate SERT at the level of mRNA. A 4-(4-(dimethylamino) phenyl)-1-methylpyridinium (APP+) uptake assay confirmed the functional activity of SERT. These findings were mirrored in mouse colonic organoids, where *L. reuteri* metabolites and ethanol were found to upregulate SERT at the apical membrane. Finally, in a trinitrobenzene sulphonic acid model of acute colitis, we observed that mice treated with *L. reuteri* maintained SERT at the colon membrane compared with mice receiving phosphate buffered saline vehicle control. These data suggest that *L. reuteri* metabolites, including ethanol, can upregulate SERT and may be beneficial for maintaining intestinal homeostasis with respect to serotonin signalling.

Keywords: serotonin, serotonin transporter, lactic acid bacteria, probiotics

1. Introduction

Various aspects of human health are influenced by the intestinal microbiota, including the modulation of the immune system, stimulation of the enteric and central nervous systems, production of vitamins and short chain

fatty acids, break-down of indigestible food components, and protection against pathogens (Martin *et al.*, 2013). Recent evidence has pointed to the role of the gut microbiota in regulating the serotonergic system (Akiba *et al.*, 2015; Cao *et al.*, 2018a,b; Engevik *et al.*, 2020; Esmaili *et al.*, 2009; Fukumoto *et al.*, 2003; Hata *et al.*,

2017; Nakaita *et al.*, 2013; Neufeld *et al.*, 2011; Nishino *et al.*, 2013; Nzakizwanayo *et al.*, 2015; O'Hara *et al.*, 2006; Reigstad *et al.*, 2015; Wang *et al.*, 2015; Yano *et al.*, 2015). The serotonergic system revolves around the important regulatory amine serotonin (5-HT) that is synthesised by intestinal enterochromaffin (EC) cells. Secreted 5-HT can elicit the activation of receptors on immune cells, goblet cells, epithelial cells, smooth muscle and neurons (Baganz and Blakely, 2013; Bellono *et al.*, 2017; Bertrand and Bertrand, 2010; Gershon and Tack, 2007; Hoffman *et al.*, 2012; Mawe and Hoffman, 2013). The actions of 5-HT are terminated by uptake via the highly selective sodium-chloride coupled serotonin transporter (SERT) SLC6A4 (Broer and Gether, 2012; Chen *et al.*, 1998; Esmaili *et al.*, 2009; Gill *et al.*, 2005; Kristensen *et al.*, 2011; Takayanagi *et al.*, 1995), which is expressed in the intestinal mucosa and the enteric and central nervous system. SERT-mediated uptake of 5-HT results in the degradation and termination of the 5-HT signal (Blakely *et al.*, 1991; Brownstein and Hoffman, 1994; Gershon and Sherman, 1982; Gershon *et al.*, 1990; Hoffman *et al.*, 1991; Kim and Khan, 2014; Martel *et al.*, 2003; Wade *et al.*, 1996). As a result, SERT is postulated to influence intestinal homeostasis by controlling 5-HT concentrations.

Decreased SERT concentrations have been associated with intestinal pathogenesis. SERT is documented to be reduced in experimental models of gastrointestinal (GI) inflammation including dextran sulphate sodium (DSS) (Bertrand *et al.*, 2010) and trinitrobenzene sulphonate (TNBS) (Linden *et al.*, 2005) mouse models. Additionally, SERT concentrations are decreased in mouse models of enteric infection by enteropathogenic *Escherichia coli*, *Citrobacter rodentium*, *Listeria monocytogenes* and the parasite *Trichinella spiralis* (Esmaili *et al.*, 2009; Latorre *et al.*, 2016b; O'Hara *et al.*, 2006; Wheatcroft *et al.*, 2005). In patients, decreased SERT transcript levels have been documented in individuals with a history of diverticulitis or active inflammatory bowel disease (IBD) when compared with controls (Costedio *et al.*, 2008; Tada *et al.*, 2016). Consistent with the importance of SERT in homeostasis, SERT knockout mice exhibit exacerbated inflammation in experimental colitis (interleukin (IL)-10 deficiency and TNBS colitis) and fructose-induced hepatic steatosis in mice (Bischoff *et al.*, 2009; Haub *et al.*, 2010a,b). In parallel with animal studies, reduced SERT levels have been reported in patients with ulcerative colitis (Bellini *et al.*, 2003; Coates *et al.*, 2004). These findings implicate 5-HT signalling and SERT-mediated termination as critical factors in inflammatory conditions of the GI tract.

Commensal lactic acid bacteria (LAB) regulate SERT concentrations in the intestine. Studies have associated *Lactocaseibacillus* (previously classified as *Lactobacillus*) *rhamnosus*, *Bifidobacterium longum* and *Bifidobacterium dentium* with an increased abundance of SERT in the

intestine (Cao *et al.*, 2018a,b; Engevik *et al.*, 2020; Wang *et al.*, 2015). However, the exact mechanism by which these microbes influence SERT is unknown. Lactic acid bacteria are commonly found in the intestinal mucus layer where fermentation products like ethanol could easily be delivered to the host epithelium (Blomberg *et al.*, 1993; Bohle *et al.*, 2010; Carasi *et al.*, 2014; Coconnier *et al.*, 1992; Henriksson and Conway, 1996; Jacobsen *et al.*, 1999; Jensen *et al.*, 2014; Jonsson *et al.*, 2001; Ma *et al.*, 2006; Macias-Rodriguez *et al.*, 2009; Mackenzie *et al.*, 2010; Miyoshi *et al.*, 2006; Nishiyama *et al.*, 2015; Ouwehand and Conway, 1996; Ramiah *et al.*, 2007; 2009; Van Tassel and Miller, 2011). An inverse relationship between ethanol consumption and 5-HT concentrations has been demonstrated in the central nervous system linking ethanol concentrations to SERT activity (Kelai *et al.*, 2003). Mice treated with ethanol exhibit increased SERT expression in the cingulate cortex, nucleus accumbens, hippocampal CA1-CA3 layers, and mediodorsal nucleus of the thalamus (Shibasaki *et al.*, 2010). Babu and colleagues (2009) demonstrated that ethanol also upregulates SERT in dendritic cells. Interestingly, the microbes associated with a greater abundance of intestinal SERT also produce ethanol (Cao *et al.*, 2018a,b; Engevik *et al.*, 2020; Wang *et al.*, 2015), providing indirect evidence that bacterial generated ethanol may affect SERT activity. Despite these potential connections, little is known regarding bacterial produced ethanol on SERT levels in the intestinal epithelium. In this study, we aimed to identify whether ethanol producing *Limosilactobacillus* (previously classified as *Lactobacillus*) *reuteri* ATCC 6475 and endogenous ethanol could modulate intestinal SERT.

2. Materials and methods

Bacterial strains and microbiological culture conditions

L. reuteri ATCC-PTA 6475 was cultured anaerobically in De Man, Rogosa and Sharpe (MRS) medium (Difco, BD Biosciences, Franklin Lakes, NJ, USA) or a fully defined media, termed LDM4 as previously described (Engevik *et al.*, 2019). *L. reuteri* cultures were grown anaerobically in a workstation (AS-580; Anaerobe Systems, Morgan Hill, CA, USA) in a mixture of 5% CO₂, 5% H₂, and 90% N₂. Single colonies grown on MRS agar plates were used to inoculate overnight MRS cultures. From MRS, *L. reuteri* was sub-cultured into LDM4 (OD_{600nm} adjusted to 0.1) and cultured anaerobically at 37 °C for 24 h. Optical density (OD_{600nm}) was measured at 24 h on a Smartspec Plus spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA) or plate reader (Synergy H1; BioTek, Winooski, VT, USA). Cultures were centrifuged to remove bacterial cells (5,000×g, 5 min), supernatant pH was adjusted to pH 7.0 and sterile filtered through a 0.2 µm membrane filter (Millipore, San Diego, CA, USA). The resulting solution was termed conditioned medium (CM). As a negative control, uninoculated LDM4 bacterial medium was also

included. To generate heat killed bacteria, bacterial pellets were resuspended in 10 ml sterile phosphate buffered saline (PBS) and heated at 65 °C for 30 min. For growth curve experiments, *L. reuteri* was grown in LDM4 in 96-well plates and optical densities were examined at OD_{600nm} on a BioTek Synergy H1 plate reader.

Cell culture and cell treatment

The human colonic epithelial carcinoma cell line, T84 (American Type Culture Collection ATCC CCL-248), was grown in DMEM-F12 media supplemented with 10% foetal bovine serum at 37 °C, 5% CO₂. Additionally, the human colonic epithelial immortalised non-cancer NCM460D cell line was grown with enriched M3:10[™] medium (Incell Corporation LLC, San Antonio, TX 78249, USA). For both colon lines, approximately 1×10⁵ cells were seeded onto 24-well plates (Corning, Sommerville, MA, USA) with poly-D-Lysine coated coverslips, grown to confluence and various concentrations of uninoculated LDM4, *L. reuteri* CM or ethanol were applied to the apical membrane at 37 °C, 5% CO₂. Cells were incubated with treatment for 16 h and then monolayers were fixed in 4% PFA (ThermoFisher Scientific, Waltham, MA, USA) for 1 h and examined by immunofluorescent microscopy. Alternatively, monolayers were incubated for 6 h and processed in TRIZOL (Life Technologies, Grand Island, NY, USA) for mRNA extraction.

Trinitrobenzene sulphonic acid colitis model

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine (Houston, TX, USA) in accordance with all guidelines set forth by the U.S. National Institutes of Health. For acute colitis experiments, BALB/c mice (8-12 weeks old) were purchased from Taconic (Rensselaer, NY, USA) and housed at Baylor College of Medicine. After acclimation (1 week), mice were orally gavaged with 10⁹ cfu/ml *L. reuteri* in PBS or sterile PBS alone (vehicle control; ThermoFisher) daily for 1 week. Following pre-treatment with *L. reuteri* or PBS, mice were anaesthetised by isoflurane inhalation and 5% (w/v) 2,4,6-trinitrobenzenesulfonic acid (TNBS) (#P2297; Sigma, St. Louis, MO, USA) dissolved in an equal volume of absolute ethanol (100 mg/kg of body weight) was rectally delivered by catheter, 4 cm into the colon. Mice continued to receive daily oral gavage of treatment (*L. reuteri* in PBS or sterile PBS) until euthanasia (3 days post-TNBS). For histology, the colon was excised fixed in Carnoy's fixative (Sigma) and paraffin embedded. Haematoxylin and eosin stains of colon sections were used for histological scoring of colitis by a blinded board-certified anatomic pathologist.

Mouse organoid culture

C57BL/6 mice, 6-8 weeks of age, were euthanised with isoflurane followed by cervical dislocation. The mid to distal colon was excised and processed to generate organoids as previously described (Engevik *et al.*, 2013a; Fernando *et al.*, 2017). Briefly, the colon was opened lengthwise, washed with Ca²⁺/Mg²⁺-free PBS, and small sections were incubated with 3 mM EDTA (ThermoFisher), 0.5 mM DTT (Sigma) and 43.4 mM sucrose (Sigma) for 30 min at 4 °C. After crypt dissociation by shaking and pipetting, crypts were collected in chelation buffer (2% sorbitol, 1% sucrose, 1% bovine serum albumin). Intestinal crypts were then centrifuged at 300×g for 10 min and the crypt pellet was suspended in Matrigel (BD Biosciences). After Matrigel polymerisation, 400 µl CMGF+ (complete media with growth factors) containing 10 µM Y-27632 rock inhibitor was added to each well as previously described (Chang-Graham *et al.*, 2019). All organoid cultures were passaged >2 times to ensure the absence of cellular debris. To differentiate organoids, colonic organoids were grown for 48 h in CMGF+, then grown for 4-5 days in CMGF+ without Wnt, nicotinamide, and only 5% Noggin and 5% R-spondin. After differentiation for 4-5 days, 50% uninoculated LDM4, 50% *L. reuteri* CM and 5% ethanol was added to the differentiation media. Organoids were incubated overnight at 37 °C, 5% CO₂ for immunostaining or incubated for 6 h at 37 °C, 5% CO₂ for RNA isolation.

Immunostaining

Immunofluorescent staining was carried out on 7-µm paraffin sections of mouse organoids or mouse colon tissue. Slides were baked at 60 °C and deparaffinized in a series of xylene and alcohol washes. Antigen retrieval was performed using 0.1 M sodium citrate buffer, pH 6.0 for 20 min in a pressure cooker. Slides were washed with Tris-buffered saline containing Tween 20 (TBS-T), blocked with 10% donkey serum and incubated with a rabbit-anti-SERT antibody (diluted 1:100, Immunostar #24330) overnight at 4 °C. Sections were then stained with AlexaFluor donkey anti-rabbit 555 secondary antibody (diluted 1:1000, Life Technologies #A-31572) for 1 h at room temperature and counterstained with Hoechst 33342 (Invitrogen, Waltham, MA, USA) for 10 min. Coverslips were applied on slides using Fluoromount Aqueous Mounting Medium (Sigma #F4680). For T84 cells on coverslips, cells were permeabilised with 0.1% Triton-X in PBS for 30 min at room temperature, blocked in TBS-T containing 10% donkey serum and incubated with a rabbit-anti-SERT antibody (diluted 1:100; #24330; Immunostar, Hudson, WI, USA) overnight at 4 °C. After washing, cells were incubated with AlexaFluor donkey anti-rabbit 555 secondary antibody (diluted 1:1000) for 1 h at room temperature and counterstained with Hoechst 33342 for 10 min. Coverslips were mounted inverted on slides

using Fluoromount Aqueous Mounting Medium (Sigma #F4680). All slides were imaged on a Nikon Eclipse 90i (Nikon, Tokyo, Japan) microscope. FIJI (Formerly ImageJ, National Institutes of Health) software was used to semi-quantitatively quantify the fluorescence by tabulating mean pixel intensity as previously described (Engevik *et al.*, 2013a,b,c).

Serotonin transporter activity assay

T84 cells were treated with bacterial conditioned media or ethanol overnight. Following incubation, cells were washed and then treated with 10 μ M of 4-(4-(dimethylamino)phenyl)-1-methylpyridinium (APP⁺, Sigma #SML0756). APP⁺ is a fluorescent substrate for organic cation transporters which has previously been used to calculate SERT activity (Solis *et al.*, 2012; Wilson *et al.*, 2014). T84 cells were incubated with APP⁺ at 37 °C, 5% CO₂ for 30 min and washed three times with Krebs Ringers bicarbonate buffer. Intracellular APP⁺ was quantified by measuring fluorescence intensity at λ_{ex} = 505 nm, λ_{em} = 550 nm on a plate reader (Biotek Syngery H1).

Alcohol dehydrogenase ELISA

An alcohol dehydrogenase ELISA kit (#ab102533; Abcam, Cambridge, UK) was used to assess the concentrations of alcohol dehydrogenase protein in *L. reuteri*. The assay is based on the inter-conversion between alcohols and aldehydes or ketones with the reduction of NAD⁺ to NADH. In Abcam's Alcohol Dehydrogenase Assay Alcohol NADH will utilise isopropanol as a substrate leading to a proportional colour development, which is measured colorimetrically (λ = 450 nm).

Alcohol measurements in *Limosilactobacillus reuteri* supernatant

Ethanol production by *L. reuteri* was determined in LDM4 using an ethanol kit (#10176290035; R-Biopharm, Darmstadt, Germany) according to manufacturer's details as previously described (Oh *et al.*, 2019). Briefly, *L. reuteri* was sub-cultured into LDM4 (OD_{600nm} adjusted to 0.1) and cultured anaerobically at 37 °C for 24 h. Cultures were centrifuged to remove bacterial cells (5,000×g, 5 min), the supernatant was sterile filtered through a 0.2 μ m membrane filter (Millipore) and then the supernatant was used for colorimetric ethanol analysis.

Quantitative real time PCR (qPCR)

RNA was collected from T84, NCM460, and mouse organoids using TRIZOL reagent (Invitrogen) according to the manufacturer's details. One microgram of RNA was reverse transcribed to single-stranded cDNA using the SensiFAST cDNA kit (Bioline, Taunton, MA, USA).

qPCR was performed using the Quantstudio 3 qPCR machine (Applied Biosystems, Foster City, CA, USA) using FAST SYBR Green PCR master mix (ThermoFisher Scientific) with the following primers: human *SERT* forward: AATGGGTACTCAGCAGTTCC; human *SERT* reverse: CCAAGCATAGCCAATCAC; human *18S* forward: GATATGCTCATGTGGTGTG; human *18S* reverse: AATCTTCTTCAGTCGCTCCA; mouse *Sert* forward: TGGGCGCTCTACTACCTCAT; mouse *Sert* reverse: ATGTTGTCCTGGGCGAAGTA; mouse *18S* forward: CTTAGAGGGACAAGTGCG; and mouse *18S* reverse: ACGCTGAGCCAGTCAGTGTA. Relative mRNA levels of target genes were normalised to the housekeeping gene 18S and analysed by the $\Delta\Delta$ CT method. All values are presented as fold changes relative to untreated media controls as previously described (Engevik *et al.*, 2013a, 2015).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6) software (GraphPad Inc., La Jolla, CA, USA). Data were examined using a student's T-test or One-way analysis of variance (ANOVA). Differences between the groups were considered significant at $P < 0.05$.

3. Results

Limosilactobacillus reuteri secretes ethanol

L. reuteri ATCC 6475 is a human commensal gut microbe with a characteristic rod shape (Supplementary Figure S1A). *L. reuteri* belongs to the heterofermentative group III class of bacteria, which converts glucose to lactate, CO₂, and ethanol (Arskold *et al.*, 2008; Elshagabee *et al.*, 2016; Zaunmuller *et al.*, 2006). Ethanol measurements of 24 h supernatant revealed that *L. reuteri* produced 83.4±9.0 mM ethanol in our fully-defined LDM4 medium, which equates to 3.8±0.42% of ethanol per volume (Supplementary Figure S1B). These data support previous work which demonstrates that *L. reuteri* secretes ethanol *in vitro* (Oh *et al.*, 2019). Bacteria manage ethanol tolerance through alcohol dehydrogenase enzymes, which facilitate the interconversion between alcohols and aldehydes with the reduction of NAD⁺ to NADH. We measured alcohol dehydrogenase activity in *L. reuteri* by measuring the conversion of isopropanol to NADH. Using this assay, we identified elevated alcohol dehydrogenase activity in *L. reuteri* (4.82±0.24 mU/ml), suggesting that *L. reuteri* is equipped to generate and tolerate ethanol in the environment. We challenged *L. reuteri* with increasing concentrations of ethanol and observed that *L. reuteri* was capable of growing in the presence of 1% ethanol without any loss of viability (Supplementary Figure S1C). *L. reuteri* exhibited a slight decrease in growth at 5% ethanol (0% ethanol: 0.93±0.04 vs 5% ethanol: 0.78±0.04 at 16 h). However, addition of 10% ethanol significantly inhibited

L. reuteri growth (10% ethanol: 0.25 ± 0.02). These data indicate that *L. reuteri* secretes ethanol and possesses the necessary enzyme activity to tolerate ethanol.

Limosilactobacillus reuteri metabolites, including ethanol, upregulate epithelial serotonin transporter

Ethanol has been shown to upregulate SERT in the central nervous system and in dendritic cells (Babu *et al.*, 2009; Daws *et al.*, 2006; Shibasaki *et al.*, 2010), but little information exists on the effects of ethanol on SERT in the intestine. To address this question, we tested the effects of

L. reuteri metabolites and ethanol on human colonic T84 cells. *L. reuteri* was cultured in LDM4 for 24 h and varying concentrations of cell-free supernatants were used to treat T84 cells for 6 h. Uninoculated LDM4 and 5% ethanol were used as negative and positive controls, respectively. qPCR analysis revealed that *L. reuteri* conditioned media (25 and 50%) or 5% ethanol significantly enhanced the expression of *SERT* in T84 cells (Figure 1A). Of note, heat killed *L. reuteri* had no effect on SERT mRNA levels (data not shown), suggesting that secreted factors were responsible for SERT upregulation. To test the functional activity of SERT, T84 cells were incubated with APP+, a fluorescent

Figure 1. *Limosilactobacillus reuteri* conditioned LDM4 and exogenous ethanol upregulate serotonin transporter (SERT) in human colonic T84 cells. (A) SERT mRNA expression analysis of T84 cells treated with media (DMEM), 50% uninoculated LDM4, 10% *L. reuteri* conditioned LDM4, 25% *L. reuteri* conditioned LDM4, 50% *L. reuteri* conditioned LDM4, or 5% ethanol. Cells were incubated for 6 h and examined for SERT expression by qPCR. * $P < 0.05$; ANOVA. $n = 4$ replicates/experiment, repeated 3 $\times$ independent times. (B) SERT functional assay as assessed by APP+ cell fluorescence via plate reader (em:505/ex:550). (C) Quantification of T84 immunostaining for SERT in media controls (DMEM), 50% uninoculated LDM4, 50% *L. reuteri* conditioned LDM4, or 5% ethanol after 16 h treatment using FIJI software. (D) Representative images of T84 cells incubated with the same conditions as (C) with SERT staining in pink and nuclei in blue. Scale bar = 50 μ m.

substrate for organic cation transporters commonly used to measure uptake by SERT (Solis *et al.*, 2012; Wilson *et al.*, 2014). Increased intracellular APP+ was observed in *L. reuteri* conditioned media and ethanol-treated T84 cells, compared to untreated or 50% uninoculated LDM4 controls (Figure 1B). Consistent with mRNA expression and functional activity, SERT upregulation was also seen at the protein level (Figure 1C,D). Immunostaining of SERT in T84 cells on coverslips revealed that *L. reuteri* conditioned media and 5% ethanol were able to increase the abundance of SERT.

Although cancer-derived cell lines are commonly used to model the intestine, these cells do not entirely recapitulate the functions and signalling pathways of the normal human epithelium. Multiple groups have begun to use organoids as a more physiologically relevant model for the gut. Organoids are derived from intestinal stem cells and mirror the native intestinal epithelium (Sato *et al.*, 2009, 2011). They contain enterocytes, goblet cells, Paneth cells, stem cells and enteroendocrine cells and they retain their segment specificity. We first compared baseline SERT expression in T84 cells and NCM460 cells (an immortalised human colon cell line) to mouse and human colon-derived organoids. Examination of SERT message in these various models revealed that human and mouse organoids expressed greater concentrations of *SERT* mRNA when compared to either the immortalised human NCM460 cells and cancer-derived human T84 cells (Figure 2A). To query this further, we examined the effects of *L. reuteri* metabolites and ethanol on mouse colonic organoids. Incubation of 3D colonic organoids with 50% LDM4, 50% *L. reuteri* conditioned media or 5% ethanol revealed that *L. reuteri* metabolites or ethanol could increase *Sert* at the level of mRNA (Figure 2B). Moreover, immunostaining showed *L. reuteri* metabolites or ethanol were able to increase the abundance of SERT protein by >2 fold at the apical membrane of mouse colonic organoids compared to untreated organoid controls (Figure 2C,D). Together these data demonstrate that *L. reuteri* secreted products such as ethanol can upregulate cellular SERT quantities.

***Limosilactobacillus reuteri* promotes epithelial serotonin transporter in a TNBS-model of colitis**

TNBS acute colitis is characterised by decreased levels of SERT in both mice and guinea pigs as compared to healthy controls (Linden *et al.*, 2003, 2005; O'Hara *et al.*, 2004). To identify whether *L. reuteri* can regulate SERT levels during colitis, BALB/c mice were rectally administered TNBS and orally gavaged with PBS (vehicle control) or 10^9 cfu *L. reuteri*. As a control, BALB/c mice which did not receive TNBS were also used for comparison. Consistent with the literature (Linden *et al.*, 2003, 2005; O'Hara *et al.*, 2004), we observed decreased levels of SERT by

immunostaining at the apical membrane of the mid-colon in TNBS mice compared to control mice which did not receive TNBS (Figure 3A). In mice that received TNBS and oral gavage of *L. reuteri*, we observed retention of SERT at the apical membrane, resulting in a higher abundance of SERT in the *L. reuteri* treated animals compared to PBS treated mice. Quantification of the immunostaining using the FIJI software confirmed that these findings were statistically significant (Figure 3B). Additionally, retention of SERT in the *L. reuteri* group mirrored colitis scores (Figure 3C). Compared to untreated mice, mice receiving TNBS and PBS exhibited greater histologic scores, which reflect disruption of crypt architecture, inflammatory cell infiltration, muscle thickening, goblet cell depletion, crypt abscesses and necrosis. In contrast, mice receiving TNBS with *L. reuteri* exhibited ~7-fold lower histological scores than TNBS mice supplemented with PBS. Consistent with our histological scores, we observed decreased colonic levels of pro-inflammatory cytokine expression (tumour necrosis factor (TNF), chemokine (C-X-C motif) ligand 1 (KC) and interferon (IFN)- γ) in our TNBS mice treated with *L. reuteri* compared to our TNBS mice supplemented with PBS (Figure 3D); indicating that *L. reuteri* treatment reduced colitis. Collectively, these results indicate that *L. reuteri* metabolites, including ethanol, regulate intestinal SERT levels and ameliorate colitis (Figure 4).

4. Discussion

Our data demonstrate that the human gut commensal *L. reuteri* is capable of secreting ethanol which can upregulate SERT in human colon cell lines as well as mouse organoids. We confirmed the functional activity of SERT using the APP+ assay in our human colon lines. Moreover, we identified that *L. reuteri* was able to modulate cellular quantities of SERT in the setting of colitis. Based on the pivotal role of the gut microbiome in gut homeostasis, these findings may have practical applications for human health. We speculate that modulation of intestinal SERT by commensal bacteria has the potential to be harnessed for the management of gastrointestinal disorders.

Commensal microbes and particularly probiotic organisms are being examined for their ability to modulate specific GI targets (Yano *et al.*, 2015). Currently, several probiotic strains have clinical efficacy in the treatment or management of GI disorders (Clarke *et al.*, 2012; Committee *et al.*, 2006; Goldin and Gorbach, 2008; Kopp-Hoolihan, 2001; O'Sullivan *et al.*, 2005; Shanahan, 2009). As a result, these beneficial microbes (or probiotics) and their metabolites have garnered great interest with respect to their therapeutic potential to promote health. Probiotic strains, particularly *Lactobacillus* (now reclassified as *Lacticaeibacillus*, *Lactiplantibacillus*, *Limosilactobacillus*, etc.) and *Bifidobacterium*, have been hypothesised to beneficially influence diseases associated with the serotonergic system (Guglielmetti *et al.*, 2011;

Figure 2. *Limosilactobacillus reuteri* conditioned LMD4 and exogenous ethanol upregulate serotonin transporter (SERT) in mouse colonic organoids. (A) SERT mRNA expression analysis of human colonic T84 cells (cancer-derived), human colonic NCM460 cells (immortalised), human colonic organoids (colonoids) and mouse colonic organoids by qPCR. (B) *Sert* mRNA expression analysis by qPCR of mouse colonic organoids after incubation with organoid media, 50% uninoculated LDM4, 50% *L. reuteri* conditioned LDM4, or 5% ethanol for 6 h. * $P < 0.05$; ANOVA. $n = 4$ replicates/experiment, repeated 2 $\times$ independent times. (C) Quantification of mouse colonic organoid immunostaining for SERT in media controls, 50% *L. reuteri* conditioned LDM4, or 5% ethanol after 16 h treatment using FIJI software. (D) Representative images of mouse colonic organoids incubated with the same conditions as (C) with SERT staining in pink and nuclei in blue. Scale bar = 50 μ m.

Kato *et al.*, 2004; O'Mahony *et al.*, 2005; O'Sullivan and O'Morain, 2000; Oliva *et al.*, 2012; Zocco *et al.*, 2006). Since these regulatory functions of lactic acid bacteria are highly strain-specific, further characterisation is required to identify specific microbes with SERT modulating functions. Our work has identified a common theme among LAB that regulate SERT: the ability to secrete ethanol.

Ethanol production by human microbes is well characterised. One study demonstrated steady ethanol

levels (~15 mmol/l) in infant faecal suspensions over ~150 days (Wolin *et al.*, 1998). Additionally, using C^{13} -labeled glucose, the authors identified that ethanol could be produced by fermentation (Wolin *et al.*, 1998). This is consistent with another study that found ethanol in the faeces of infants (Stansbridge *et al.*, 1993). The authors also found that the levels of ethanol were higher in stool from infants fed a formula containing lactobacilli compared to infants receiving a formula without lactobacilli. Select lactobacilli can generate ethanol. It has been

Figure 3. *Limosilactobacillus reuteri* promotes serotonin transporter (SERT) at the apical membrane in the setting of TNBS colitis. (A) Representative immunostaining of mouse colon 3 days following TNBS administration in mice treated with either phosphate buffered saline (PBS) (control) or 10^9 live *L. reuteri*. SERT is identified in pink, nuclei in blue, and SERT apical localisation is outlined in white. Scale bar = 50 μ m. (B) Quantification of mouse colon immunostaining for SERT in TNBS + PBS or TNBS + *L. reuteri* treated mice using FIJI software. (C) Histological scoring performed by a blinded board-certified pathologist. (D) qPCR analysis of pro-inflammatory cytokines tumour necrosis factor (TNF), chemokine (C-X-C motif) ligand 1 (KC; interleukin-8 homologue), and interferon (IFN)- γ in TNBS + PBS or TNBS + *L. reuteri* treated mice. * $P < 0.05$, ANOVA. $n = 6$ mice/group.

demonstrated that *Lactocaseibacillus paracasei*, *Lactocaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* and *L. reuteri* can generate ethanol (Elshaghabee *et al.*, 2016; Narendranath *et al.*, 1997; Oh *et al.*, 2019). Interestingly, the ability of lactobacilli to produce ethanol depends on the carbohydrate source.

For example, *L. fermentum* produced high amounts of ethanol (80 mM) from glucose, but no ethanol from other carbohydrates (fructose, arabinose, ribose, lactose, lactulose, or inulin) (Elshaghabee *et al.*, 2016). Similarly, Oh and colleagues identified that *L. reuteri* generated high ethanol levels (100 mM) with glucose, but no ethanol

Figure 4. Proposed model for *Limosilactobacillus reuteri* serotonin transporter (SERT) modulation. Our data suggest that *L. reuteri* secretes ethanol and we predict that ethanol drives the transcription of SERT at the level of mRNA. We speculate SERT is trafficked to the apical membrane where it can uptake enterochromaffin (EC) released serotonin (5-HT), thereby aiding intestinal homeostasis.

with a mixture of glucose and fructose (20:80) or fructose alone (Oh *et al.*, 2019). These data suggest that diet could potentially regulate the ability of *lactobacilli* to produce ethanol and thus beneficially modulate SERT.

Although we have found that ethanol can upregulate SERT, ethanol has also been shown to interfere with the intestinal barrier. Studies have found that long-term ethanol abusers, defined as individuals with a consumption of more than four drinks (>80 g alcohol) per day, have increased intestinal permeability (Elamin *et al.*, 2013; Holford, 1987; Parlesak *et al.*, 2000). Similarly, rodent studies have demonstrated that both short- and long-term ethanol administration can increase intestinal permeability and activate intestinal inflammation (Bode *et al.*, 1991; Keshavarzian *et al.*, 2001, 2009; Lambert *et al.*, 2003). Despite these findings, administration of *lactobacilli*, which can produce ethanol locally, attenuates ethanol-induced injury (Fang *et al.*, 2019; Forsyth *et al.*, 2009; Gu *et al.*, 2020; Li *et al.*, 2016; Liu *et al.*, 2004). We speculate that ethanol-producing *lactobacilli* are also actively secreting anti-inflammatory compounds which prevent ethanol-induced injury. For example, we have shown that *L. reuteri* secretes metabolites which stimulate anti-inflammatory IL-10 production from dendritic cells (Engevik *et al.*, 2021). We have also found that *L. reuteri* generates small compounds such as γ -glutamyl-cysteine which can suppress epithelial oxidative stress. As a result, we hypothesise that anti-oxidative stress and anti-inflammatory compounds may suppress the deleterious effects of ethanol and allow for upregulation of SERT without intestinal damage.

Multiple pathways could be responsible for the effects of ethanol on SERT. Ethanol enhances intracellular cyclic AMP (cAMP) levels in a number of cell types (Atkinson *et al.*, 1977; Rabe *et al.*, 1990; Uhlemann *et al.*, 1979). cAMP in turn has been shown to regulate SERT (Yammamoto *et al.*, 2013). Thus, it is possible that ethanol-induced cAMP could be promoting increased SERT transcription and thus protein quantities. Alternatively, Szabo and colleagues reported that ethanol was effective in inducing transforming growth factor (TGF)- β (Szabo *et al.*, 1992) and in a separate study, TGF- β was found to upregulate SERT protein levels in Caco-2 cells (Nazir *et al.*, 2015). Application of *L. reuteri* conditioned media to HT29 cells, which have also been used for SERT studies, was found to increase phosphorylated p38 (data not shown). Previous studies have demonstrated that p38 MAPK signalling pathways were involved in rapid SERT up-regulation (Zhu *et al.*, 2005); suggesting that p38 signalling may be involved in *L. reuteri*-mediated SERT upregulation. It is also possible that ethanol enhances the activity of other *L. reuteri* metabolites through unknown pathways or that other *L. reuteri* metabolites are primarily responsible for SERT regulation and ethanol is expendable.

In our TNBS mouse model, we speculate that multiple signalling pathways are involved in *L. reuteri*-mediated retention of SERT at the apical membrane. *L. reuteri* has been shown to shift the intestinal microbiome (He *et al.*, 2017, 2019; Romani Vestman *et al.*, 2015), increasing 'beneficial' genera *Bifidobacterium*, *Prevotella*, and *Lactobacillus*, while decreasing *Anaeroplasma*, *Rikenellaceae*, and *Clostridium*. Shifts in the microbiome, particularly

gram-negative microbes, may affect TLR signalling. In Caco-2 cells, TLR2 activation reduced SERT protein levels at the apical membrane (Latorre *et al.*, 2016a). Tlr2^{-/-} mice were also found to have augmented intestinal SERT expression, providing evidence of the roles of microbial pattern recognition receptors in modulating intestinal SERT (Latorre *et al.*, 2016a). Interestingly, we did not observe any effect of heat-killed *L. reuteri* on SERT levels in our T84 cell model, indicating that *L. reuteri* activation of TLR2 may not be responsible for our observed SERT regulation. However, *L. reuteri*-driven shifts in the microbiome may indirectly affect SERT levels via TLR2 activation by other microbes. *L. reuteri* is also known to be immune-modulatory and suppress pro-inflammatory cytokines. Our data demonstrate that *L. reuteri* reduced levels of pro-inflammatory TNF, KC (IL-8 homologue) and IFN- γ . In Caco-2 cells, application of the pro-inflammatory cytokines TNF and IFN- γ decreased SERT function and expression (Foley *et al.*, 2007). These findings suggest that by regulating inflammation, *L. reuteri* may also contribute to the maintenance of SERT at the membrane. Although the exact mechanism is unknown, our work demonstrates that *L. reuteri* regulates SERT *in vitro* and *in vivo*.

An important consideration for future work is whether *L. reuteri* can regulate SERT levels once SERT has been diminished; such as in diverticulitis or IBD patients. In our studies, we pre-treated mice with *L. reuteri* and then administered TNBS. While this may be beneficial for prevention, it doesn't indicate the effectiveness of this strategy for treatment. Additionally, it is unclear whether ETOH alone before or after colitis would beneficially regulate SERT. Based on animal models with ethanol alone (Bode *et al.*, 1991; Keshavarzian *et al.*, 2001, 2009; Lambert *et al.*, 2003) which demonstrate adverse intestinal affects, we speculate that microbial-produced ethanol, complemented with anti-inflammatory compounds, would be necessary for ethanol-mediated upregulation of SERT *in vivo*. Further studies are necessary to identify the ability of *L. reuteri* to upregulate diminished SERT and the ability of ethanol to directly modulate SERT levels *in vivo*.

Another interesting finding in our work was the strong apical expression of SERT in our mouse colonic organoids and mouse colon. We have previously shown that serotonin is secreted both apically and basolaterally in response to stimuli using human enteroids/organoids (Chang-Graham *et al.*, 2019). In this study, we observed that serotonin levels were elevated in both the apical and basal chambers in response to rotavirus infection, suggesting that apically released serotonin may be physiologically relevant. Consistent with our findings, Gill *et al.* (2008) observed a similar high apical SERT staining and low basolateral staining in human surgical resections that were paraffin embedded. When frozen sections were stained, the authors still observed strong apical localisation of SERT,

but observed less positive staining of goblet cell mucin vacuoles; suggesting that some signal was an artifact of staining paraffin-embedded tissue. Since our organoids and colon were paraffin embedded we recognise that our positive staining of goblet cells and the mucus layer above the epithelium is likely an artifact. However, we believe that the prominent apical SERT staining and low basolateral SERT in mouse colons are valid. In the future, we are interested in examining SERT in frozen sections and think that tissue preparation is an important consideration for SERT related research.

Serotonin (5-hydroxytryptamine; 5-HT) is actively produced by enterochromaffin cells in the small and large intestine. Serotonin released into the lamina propria can interact with neighbouring epithelial cells, goblet cells smooth muscle, enteric nerves or immune cells (Chen *et al.*, 1998, 2001; Gershon, 2013; Ghia *et al.*, 2009; Karsenty and Gershon, 2011; Li *et al.*, 2011b; Wade *et al.*, 1996; Yadav *et al.*, 2010). Serotonin release in response to damage appears to be a conserved mechanism. Mice treated with dinitrobenzenesulfonic acid (DNBS) (Khan *et al.*, 2006), TNBS (Linden *et al.*, 2005), or DSS (Bertrand *et al.*, 2010) exhibit increased serotonin after damage. Likewise, in guinea pigs, TNBS-induced colitis (Linden *et al.*, 2003) and ileitis (O'Hara *et al.*, 2004) both exhibited increased serotonin. In addition to colitis models, increased serotonin has been observed in response to acetic acid induced gastric ulcers (Amirov and Trubitsyna, 1981), indomethacin induced colonic wounds (Kato *et al.*, 2012) and enteric infection by *Nippostrongylus brasiliensis* (Farmer and Lanionu, 1984). The effects of serotonin are largely dependent on the receptor they activate. Spohn and colleagues demonstrated that activation of 5-HTR4 by the agonist tegaserod minimised DSS and TNBS driven colitis in mice (Spohn *et al.*, 2016). Consistent with the protective role of 5-HTR4, Kato *et al.* (2012) demonstrated that a 5-HTR4 receptor agonist, mosapride, attenuated indomethacin-induced intestinal lesions in mice. In contrast to the protective role of 5-HTR4, blockade of 5-HTR3 receptor with antagonists ondansetron and ramosetron dose-dependently reduced damage, indicating that 5-HTR3 drives inflammation. These findings suggest that serotonin exerts a dual role in colitis. This is supported by the fact that aberrant upregulation of serotonin by loss of the transporter SERT or supplementation of endogenous serotonin exacerbates colitis (Chen *et al.*, 2016; Ghia *et al.*, 2009; Khan *et al.*, 2006; Li *et al.*, 2011a; Margolis and Pothoulakis, 2009; Regmi *et al.*, 2014). It is likely that high levels of serotonin, not necessarily localised to damaged sites, may activate pro-inflammatory receptors on immune cells (Li *et al.*, 2011a). We speculate that aberrantly high serotonin levels due to the loss of SERT, which is seen in colitis models and IBD patients, activates several pro-inflammatory cytokines on immune cells, thereby promoting a cycle of inflammation. We believe that *L. reuteri* upregulates SERT and suppresses

inflammation, thereby restoring normal serotonin levels and aiding the transition back to intestinal homeostasis. In healthy mice with a complete gut microbiota, we did not observe upregulation of SERT with *L. reuteri* administration (data not shown). Since mice are normally colonised with *L. reuteri* strains, additional *L. reuteri* supplementation (particularly by a human-derived *L. reuteri* strain) is likely unable to further upregulate SERT. Alternatively, it is possible that enterocytes in the healthy setting already harbour maximal SERT levels which don't allow for further upregulation. Using tissue-derived organoids in a reductionist approach, we have demonstrated that *L. reuteri* secreted products are capable of upregulating SERT levels. However, it is difficult to distinguish whether the SERT levels we observe in untreated organoids are representative of the gut, since normally microbes and microbial products are present. In the future, we believe gnotobiotic animals and additional organoid experiments will shed light into the precise role of microbial-induced SERT regulation.

With this work, we have now identified another pathway by which commensal microbes can modulate the host. Our work suggests that *L. reuteri* can secrete ethanol and regulate SERT. Given the importance of serotonin in host health, we speculate that this regulation of SERT may have important implications in both GI and systemic homeostasis.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0216>.

Figure S1. *Limosilactobacillus reuteri* ATCC PTA 6475 secretes ethanol and is ethanol tolerant.

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Conflict of interest

JV receives unrestricted research support from the Swedish Probiotic Company BioGaia AB. JV also serves on the scientific advisory boards of Seed (USA-based probiotics/prebiotics company), Biomica (Israeli informatics enterprise) and Plexus Worldwide (USA-based nutrition company). All other authors have no financial disclosures.

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