

**Role of sphingolipids in gut immunology: effect in
Enterotoxigenic *E. coli* induced infectious diarrhoeal
model in mice**

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(Science) in Life Science and Biotechnology,
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By

SOHINI SIKDAR

(INDEX No. 52/20/LIFE SC/27)

(Registration No. SLSBT1205220)

**Department of Life Science and Biotechnology Jadavpur
University**

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Department of Health Research, Ministry of Health & Family Welfare, Govt. of India

WHO COLLABORATING CENTRE FOR RESEARCH AND TRAINING ON DIARRHOEAL DISEASES

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This is to certify that the thesis entitled “**Role of sphingolipids in gut immunology: effect in Enterotoxigenic *E. coli* induced infectious diarrhoeal model in mice**” Submitted by Smt. **Sohini Sikdar** who got her name registered on **04.11.2020** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Shanta Dutta (Former Director and Scientist- ‘G’, ICMR - National Institute for Research in Bacterial Infections)** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

Shanta Dutta 23/3/2025

डा: शान्ता दत्ता/Dr. Shanta Dutta
वैज्ञानिक-जि एवं निदेशक/Scientist-G & Director
राष्ट्रीय कॉलरा और आंत्र रोग संस्थान
National Institute of Cholera & Enteric Diseases

पी-३३, सी.आई.टी. रोड, स्किम-३०एम, बेलियाघाटा
P-33, C.I.T. Road, Scheme-XM, Beliaghata
(Signature of the Supervisor(s) date with official seal)



पी-३३, सी.आई.टी. रोड, स्किम - XM, बेलियाघाटा, कोलकाता - 700 010, पश्चिम बंगाल, भारत
P-33, C.I.T. Road, Scheme - XM, Beliaghata, Kolkata - 700 010, West Bengal, India



+91-33-2363 3373 (निदेशक/Director), +91-33-2370 1176, 5533 (प्रशासन/Administration)



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ABSTRACT

Sphingosine-1-phosphate (S1P) is a bioactive pleiotropic phospholipid produced in cells, where it gets phosphorylated by two isozymes of sphingosine kinases, i.e., SPHK1 and SPHK2. Where SPHK-1 derived S1P acts through autocrine and paracrine pathways, S1P generated by SPHK-2 is generally restricted to the nuclear compartment. By Snp2 transporter proteins, S1P is transported extracellularly to bind to its five G protein-coupled receptors (S1PR1-5). The expression of S1PRs is largely heterogeneous and thus regulates diverse roles according to cell type. S1P is known to regulate innate immune response, cell survivability, vascular barrier maintenance, epithelial cell proliferation and migration, angiogenesis, etc. In inflammatory conditions, residential cells increase tissue S1P levels, creating a higher concentration gradient that attracts lymphocytes to the inflammatory site. This phenomenon has led to the development of S1P agonists and antagonists as drugs. FTY720, a pro-drug similar to S1P, targets S1P receptors through phosphorylation by Sphingosine kinase. Its binding to S1PR leads to receptor internalization and degradation, preventing the S1P signaling cascade. This thesis investigated the impact of S1P signaling inhibition on gut barrier function and immune response.

To witness that, we treated HT-29 colon epithelial cells with SPHK-1 inhibitor N-N-Dimethyl Sphingosine and noticed a decrease in Trans Epithelial Electrical Resistance of HT-29 monolayer in a trans well system as well as a downregulation of the Tight junctional protein, Occludin expression. siSPHK-1 transfected HT-29 cells showed a decreased expression of Claudin-5, Occludin, and Junction Adhesion Molecule-A. For the in vivo model, S1P functional antagonist FTY720 (3mg/kg body weight) was given to C57BL/6 mice for 14 consecutive days. FTY720-treated mice showed decreased expression of Occludin and Claudin-4 and increased Claudin-2 in the colon. We also investigated that FTY720 targets the mTOR-Akt pathway to downregulate the tight junctional protein synthesis. Leading to this increased gut permeability, we found an increased bacterial translocation to the liver; however, the colonic expression of the antimicrobial peptide Cathelicidin and Hepcidin significantly reduced. To investigate a leaky gut-induced inflammatory response, we examined and found an increased expression of pro-inflammatory TNF- α , IFN- γ , CXCL-1, and CXCL-2. FTY720 blocks S1PR1 receptors on T cells, which is essential for T cell migration, induced T-cell homing in MLN and Spleen; thus, we found no significant T cell infiltration but an increased infiltration of Neutrophils, Natural Killer cells, B cells and macrophage at the FTY720 treated colonic tissue. Histological analysis of the colon revealed architectural damage of colonic crypts due to immune cell infiltration, crypt hyperplasia, and loss of goblet cells. While working with the enteric infection model with Enterotoxigenic *E. coli* (ETEC), we found that H10407 (LT+/ST+-ETEC) clinical isolate infected mice ileal tissues had an increased concentration of S1P. We chose probiotic and prebiotic-based therapy to suppress infection-induced inflammatory response over FTY720. The prophylactic administration of probiotics, prebiotics, and synbiotics (probiotic + prebiotic) was found to reduce ETEC colonization in the ileum and accelerate pathogen clearance. A reduced concentration of tissue S1P and low CD4+ T and CD8+ T cell population and reduced pro-inflammatory TNF- α , IFN- γ , IL-6, CXCL-1 and increased anti-inflammatory IL-10 was found in the treatment groups.

The study highlights that S1P signaling is vital for gut barrier maintenance, and its disruption leads to increased permeability, bacterial translocation, and inflammation. While FTY720 exacerbates gut dysfunction, probiotic/prebiotic therapy offers a promising alternative by restoring microbial balance, reducing inflammation, and enhancing pathogen clearance.

Sohini Sikdar
28/03/2025

Shanta Dutta

28/3/2025

डा: शान्ता दत्ता/Dr. Shanta Dutta
Ex- वैज्ञानिक-जि एवं निदेशक/Scientist-G & Director
राष्ट्रीय कॉलरा और आंत्र रोग संस्थान
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P-33, C.I.T. Road, Scheme-XM, Beliaghata
कोलकाता-७०० ०१०/Kolkata - 700 010

DECLARATION

I do, hereby declare that the work embodied in this thesis entitled "Role of sphingolipids in gut immunology: effect in Enterotoxigenic *E. coli* induced infectious diarrhoeal model in mice" submitted for the award of Doctorate of Philosophy (Science) in Life science and Biotechnology, is the completion of work carried out under the supervision of Dr. Shanta Dutta, former Director and Scientist- 'G', at Division of Bacteriology, ICMR-National Institute for Research in Bacterial Infections, Kolkata, India. Neither this thesis nor any part of it has been submitted for either any equivalent degree/diploma or any other academic award elsewhere.

Date: 28.03.2025

Place: ICMR - NIRBI



Signature of the candidate

Sohini Sikdar

*I dedicate this thesis to
my elder brother Sourav Sikdar*

It was a dream that he dreamt, and I lived

*I also dedicate this thesis to my parents and my sister,
Manali*

Their constant love and support kept me going...

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Appendix 6

Appendix: Abbreviations

μg- microgram (only with numbers)

μl- microliter (only with numbers)

μM- microMolar

AP- Alkaline phosphate

ApoM- Apolipoprotein M

B. bifidum- *Bifidobacterium bifidum*

b.w.- Body weight

BM- Bone marrow

BME- β- Mercaptoethanol

BSA- Bovine Serum Albumin

C57 BL/6- a mouse strain

CCL- Chemokine Ligand

CCR- Chemokine Receptor

CD- Cluster of Differentiation

cDNA – complementary DNA

Cer- Ceramide

CFU- Colony Forming Unit

CXCL- C-X-C motif ligand

DC- dendritic cell

DMEM- Dulbecco's modified Eagle's Medium

DMSO- Dimethyl sulfoxide

DNase- Deoxyribonuclease

DSS- - Dextran Sodium Sulfate

DTT- Dithiothreitol

E. coli- *Escherichia coli*

EDTA- Ethylene diamine tetraacetic acid

EGR- Early growth response protein

ELISA- Enzyme Linked Immune Sorbent Assay

ER- Endoplasmic Reticulum

ETEC- Entero toxigenic *Escherichia coli*

FACS- Fluorescence-activated cell sorter

FBS- Fetal Bovine Serum

FDA- Food and Drug Administration

FITC- Fluorescein isothiocyanate

gm- gram (only with numbers)

GPCR- G- protein-coupled receptor

HBSS- Hank's Balanced Salt Solution

HDAC- Histone deacetylase

HRP- Horseradish peroxide

hTERT- human Telomerase Reverse Transcriptase

IBD- Inflammatory Bowel Disease

IFN- γ - Interferon Gamma

Ig- Immunoglobulin

IL- Interleukin (i.e. IL-6)

IN- Inulin

JAM-A- Junction Adhesion Molecule-A

KDa- Kilo Dalton

kg- Kilogram

L. casei- *Lactobacillus casei*

L. lactis- *Lactococcus lactis*

LDL- Low-Density Lipoprotein

LN- Lymph node

LT- Heat Labile Toxin

MCP1- Monocyte chemoattractant protein-1

mg- milligram (only with numbers)

min- minute (only with numbers)

ml- milliliter (only with numbers)

MLN- Mesenteric lymph node

MPO- Myeloperoxidase

mRNA- messenger RNA

mTOR- Mammalian Target of Rapamycin

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide

nm- nanometer

ns- non-significant

OD- Optical density

P- probability

PAGE- Polyacrylamide gel electrophoresis

PBS- Phosphate- buffer saline

PCR- polymerase chain reaction

PE- phycoerythrin

PerCP- Peridinin-Chlorophyll-Protein

plt- paucity of lymph node T cells

PP- Peyer's Patch

rpm- revolutions per minute

S1P- Sphingosine-1-phosphate

S1PR- Sphingosine -1-phosphate receptor

SCFA- Short Chain Fatty Acids

Ser- Serine

SL- Sphingolipids

SP- Sphingosine

SPHK- Sphingosine kinase

SPNS- Spinster homolog

ST- Heat Stable Toxin

TBS- Tris Buffer Saline

TBST- Tris Buffer Saline with 0.1% Tween 20

TEER- Trans Epithelial Electrical Resistance

TGF- β - Transforming growth factor- beta

Thr- Threonine

TJ- Tight Junction

TNF- α - Tumor Necrosis Factor- α

Treg- Regulatory T cell

TRIS- tris (hydroxymethyl) aminomethane

ZO-1- Zonula Occludens

Chapter 1:

Introduction

Bioactive Sphingolipids including Sphingosine-1-phosphate (S1P) are an integral part of the physiological function of cells and various cellular signaling. S1P is a pleiotropic sphingolipid derivative derived from the phosphorylation of Sphingosine by the key isozymes Sphingosine kinase i.e.; SPHK1/SPHK2. S1P is vastly known for its regulatory function in innate and adaptive immune responses, autoimmunity, apoptosis, cell survivability, and vascular integrity. S1P in higher concentration in blood is necessary for lymphocyte trafficking at the site of inflammation and is a phenomenon evident in Inflammatory Bowel Disease, Ulcerative Colitis, as well as the Dextran Sodium Salt-induced colitis model in mice, depicting S1P as proinflammatory. Though adding some contrast to the previous concept, S1P has been proven to be critical for maintaining immune tolerance. S1P should be maintained at a physiological level for the immune cells to act before bacterial or viral pathogens, and for, endothelial cell layers, S1P helps them to retain their integrity. S1P functional antagonists like Fingolimod or FTY720 are used to treat multiple sclerosis and autoimmune diseases. Oral S1P blocker's continual dosing, affecting the gut epithelial layer and the local innate response underneath was never discussed earlier. All the experiments conducted in this thesis were to find out the role of S1P in gut barrier function and innate immune responses.

This thesis investigates the pathway for S1P synthesis and subsequently the S1P signaling in regulating and maintaining intestinal epithelial permeability. Earlier studies showed that S1P enhances the endothelial barrier and vascular barrier, even during sepsis S1P protects the vascular barrier. The Endothelial and epithelial cells form a physical barrier by regulating certain tight junctional proteins at the intercellular space. Tight junctional proteins such as ZO-1, Occludin, Claudins, and JAM-A are transmembrane proteins, located at the basolateral side of endothelial and epithelial cells, anchoring two adjacent cells. It is fascinating to understand the mechanism of the gut wall being a single cellular layer, forms the most critical barrier that separates trillions of bacteria from the host. We observed the fact that S1P inhibition could lead to the disruption of the Tight junction disruption by regulating the tight junctional protein synthesis.

Tight junctional disruption often leads to exposure of the gut commensal bacteria to the host, triggering an immune response. The mucosal innate immune cells such as Dendritic cells, macrophages and neutrophils play a key role in bacterial pattern recognition and initiating innate immune response. Whether the S1P receptor antagonists have a deleterious effect on the tight junction of epithelial cells and whether that could induce innate immune response was also discussed in this thesis. In mice, inducing lymphopenia by FTY720, we investigated whether it could worsen the gut inflammation when the gut barrier was compromised. An immune cell profiling for T-cells, B-cells, Dendritic cells, NK cells, and Macrophages was compared to control mice to conclude the effects. In order to determine the relationship between gut barrier disruption and gut microbiome, we also revealed the gut microbial pattern of control and FTY720 mice in this thesis, as gut inflammation frequently correlates with a changed microbiome, where a disproportionate firmicute to Bacteroidetes ratio results in a reduced

metabolism of short-chain fatty acids from dietary fiber, which is known to provide protection to the host.

A breach in the gut epithelium often leads to opportunistic pathogens to cause enteritis due to their exposure to the immune system. In infection-induced inflammation, S1P regulates lymphocyte trafficking by creating a concentration gradient that drives the lymphocytes to the inflamed site to combat pathogens. We have investigated the role of S1P in an enteric bacterial infectious model with Enterotoxigenic *Escherichia coli* and the inflammatory response followed by infection. We determined whether targeting S1P and its signaling inhibition could reduce the severity of the inflammation.

Chapter 2:

Review of Literature

2. Background

Despite decades of development, the idea holds "bioactive lipids," generally described as variations in lipid concentrations that have functional repercussions, just recently (20 years) have begun to gain traction and promises to take the front stage in research on cell biology during the 21st century. This belated recognition stems from previous presumptions about the exclusive roles of lipids in the construction of membranes and energy metabolism. It has prevented a more thorough analysis of the functional lipids of non-lipidologists. These difficulties are mostly the consequence of the many difficulties associated with dealing with lipids, their enzymes, and their targets. Sphingolipids (SLs) fall under the amphipathic lipids found in cell membranes that are important for signaling within cells. (Quinville et al. 2021).

2.1. Sphingolipid biology

Sphingolipids are made up of a hydrophilic headgroup at the main hydroxyl and a sphingoid base that is connected to a fatty acid via an amide bond. SLs may be categorized into three structural groups of increasing complexity based on these two functional categories: Ceramide, advanced SLs, and sphingoid bases and simple derivatives (Castillo et al. 2016), (Chen et al. 2010). The first class of SLs are long-chain amino alcohols that function as building blocks for other SLs, such as phenylalanine or sphinganine. After that, the headgroup's composition might vary depending on the class: the second class includes simple hydrogen (Cer), while the third class includes phospholipids (phosphosphingolipids) and sugars (glycosphingolipids), among other things. (Gai et al. 2023).

The pleiotropic substance sphingosine was the first to be identified for its function in controlling the actin cytoskeleton, apoptosis, endocytosis, and the cell cycle. Its pleiotropic action on kinases and other components is essential for preserving the cell's equilibrium (Smith et al. 2000). Along with S1P (sphingosine 1-phosphate), ceramide is one of the sphingolipids that has drawn attention. According to research by Obeid et al. (1993) and Venable et al. (1995), ceramide is critical for cell migration, survival, and inflammation (Hla 2004), and it plays a significant role in cell-stress responses by regulating apoptosis and senescence.

Apart from the multiple biological processes that sphingolipids are linked to, further intricacy in sphingolipid signaling are only now becoming comprehended. The metabolic interconnectedness of bioactive lipids causes this intricacy, as modifications to one lipid might have functional repercussions through metabolic "ripple" effects. Understanding Sphingolipids demands a minimum of two stages of research. First, it is fundamentally necessary to distinguish between the different particular processes that control sphingolipid metabolism and the molecular mechanisms that allow the bioactive lipid metabolites to send out their corresponding signals and second, it needs the knowledge of the subcellular and submembranous localization of lipid-mediated pathways, the metabolic transformation of bioactive

lipids, the integration or coordination of overall responses, and the metabolic organization of sphingolipid metabolism enzymes and their interrelationships are all necessary for such an understanding (Hannun and Obeid 2018).

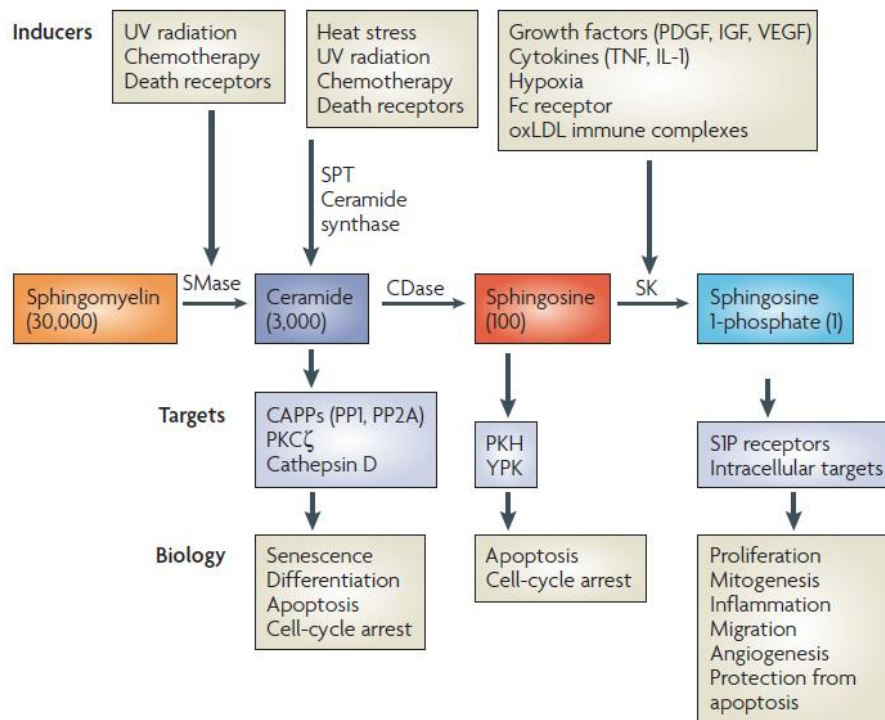


Figure 2.1A: sphingolipid biology. Diagrammatic illustration of the bioactive lipids sphingosine, ceramide, and sphingosine 1-phosphate (S1P) and how they may be involved in biological reactions in cells. (Hannun, 2008)

Sphingomyelinase initiates sphingolipid turnover, which results in the production of ceramide. Ceramidase then converts ceramide into sphingosine and could end up with one of its two outcomes. It is possible to recycle or "salvage" sphingosine into sphingolipid pathways (Figure 2.1A).

The sphingosine kinase (Sphk) isoenzymes (SPHK-1 and SPHK-2) then phosphorylate sphingosine to give rise to S1P (Maceyka et al. 2005). The levels of S1P in cells are controlled by S1P production enzymes as well as degradative enzymes such as S1P phosphatases and S1P lyase (Blaho and Hla 2011), (Le Stunff, Milstien, and Spiegel 2004); (Saba and Hla 2004). Certain intracellular S1P phosphatases and potentially non-specific external lipid phosphate phosphatases can dephosphorylate S1P to replenish sphingosine. Alternatively, S1P lyase has the ability to permanently cleave S1P to produce hexadecenal and ethanolamine phosphate, which can then be reduced to palmitate and reintegrated into lipid metabolic pathways (de la Garza-Rodea et al. 2014) (Figure 2.1B). S1P binds to its intracellular targets or secretes extracellularly to initiate its physiological functions. Intracellular S1P stimulates

cellular proliferation, whereas plasma S1P modulates angiogenesis, assists in immune cell trafficking, and preserves vascular integrity. Thus, being amphipathic in nature, S1P must be actively carried across the cell membrane in

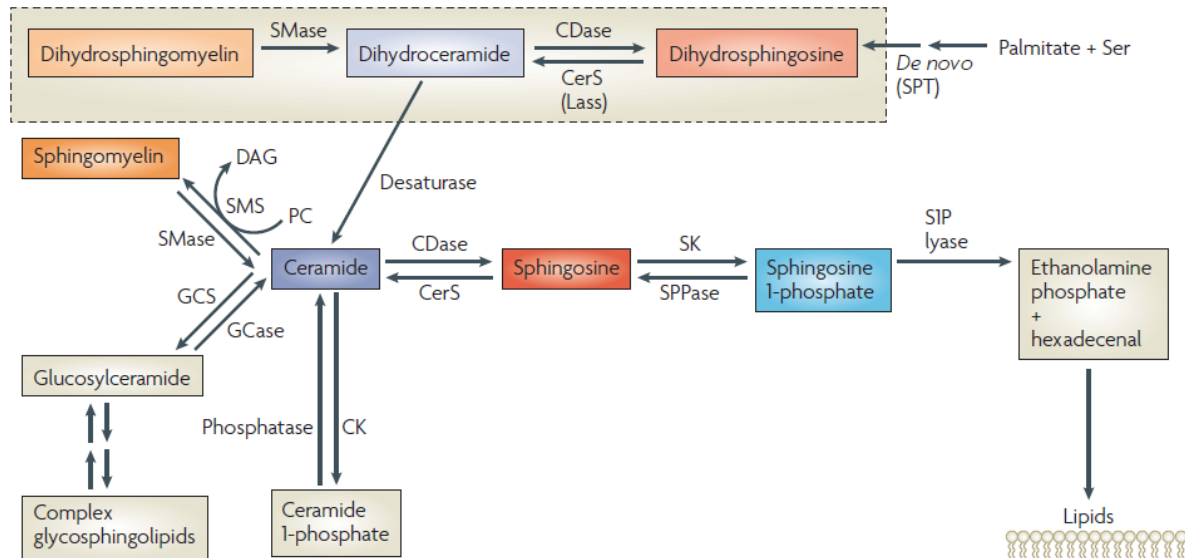


Figure 2.1B- Sphingolipid metabolic pathway and Sphingosine-1-phosphate biogenesis pathway. (Hannun, 2008)

order to cross it (Ren, R. et. al. 2021). Once produced inside the cell, certain transporter proteins, such as spinster homolog 2 (Spns2), export S1P (Kawahara et al. 2009). Following the discovery of a number of other S1P transporters, all transporters were categorized into two major facilitator superfamily (MFS) members: Mfsd2b and Spinster homolog 2 (Spns2), as well as additional ATP-binding cassette family transporters. Even though Spns2 has been explored the most (Kawahara et al. 2009). S1P, being a highly soluble sphingolipid, exists in cells at low nanomolar quantities but is linked to lipoproteins and albumin in serum at high nanomolar concentrations (Okajima, F. et. al. 2002). Sphingosine 1-phosphate is a bioactive sphingolipid found in high-density lipoprotein (HDL), and its main binding carrier is apoM. After binding to albumin and apoM in plasma, S1P is assisted in moving across the lipid bilayer by spinster homolog 2 (Spns2) and ATP-binding cassette (ABC) transporters (Mitra et al. 2006) (Figure 2.1C). In plasma, S1P typically binds 60% to apoM and 40% to albumin (Frej et al. 2015). S1P has to adhere to these carriers in order to pass through body fluids (Levkau 2015).

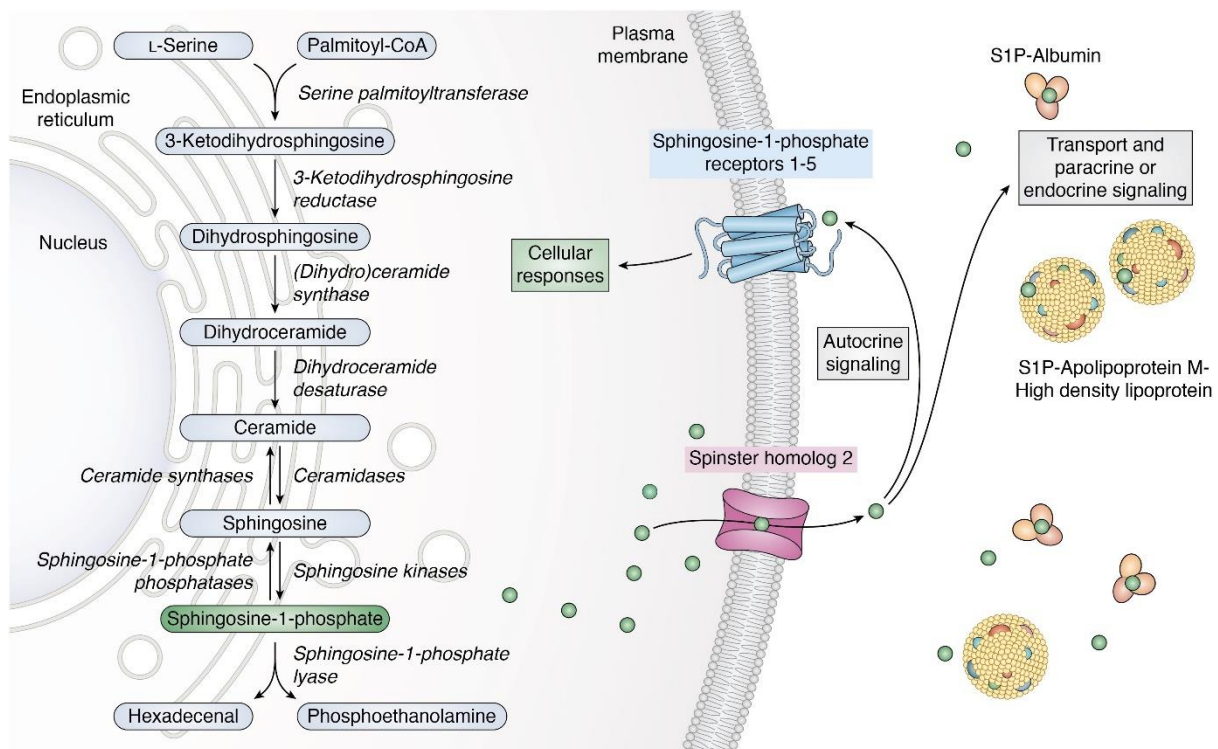


Figure 2.1C: Sphingolipid metabolism and transport: autocrine and paracrine pathway (Weigel, 2023)

The only known S1P receptors are high-affinity G protein-coupled receptors called S1PRs, with which the S1P binds. The biological activities of S1PRs, which use known intracellular signaling pathways, depend on selective tissue expression. Intracellular signaling pathways mediate the distinct actions of each of the five S1PRs (S1PR1–5) (Lee et al. 1998).

2.2. Sphingosine kinases and their function

More than 30 enzymes work together in a complex network in the sphingolipid metabolic pathway to produce hundreds of distinct species of sphingolipids (Heffernan-Stroud and Obeid 2013). There are two sphingosine kinases in mammalian cells. The cytoplasm contains SPHK1, while the nucleus and other organelles have SPHK2. Chromosomes 17 and 19 contain the human SPHK genes for SPHK1 and SPHK2, respectively. Three SPHK1 splice variants—SK1a, SK1b, and SK1c—have been found in humans (Venkataraman et al. 2006), and mouse and human SK1 enzyme variations are highly similar (Alemany et al. 2007). Mostly SPHK1 is found in the cytosol, but it can move to the plasma membrane when activated by certain stimuli (Johnson et al. 2002). The effects of S1P production largely depend on the cellular localization of SK1. When S1P is generated close to the plasma membrane, it can be exported before coming into contact with the ER-localized S1P lyase or S1P phosphatase (Ogawa et al. 2003), (Johnson et al. 2003). S1P biosynthesis by SPHK1 has a role in cell survivability. Proteolysis of SPHK1 leads to ceramide, a pro-death lipid accumulation in cells and cell death occurs. SPHK-1

downregulation is associated to various factors such as DNA damage (Taha et al. 2004), TNF- α (Taha et al. 2005), p53 tumor suppressor gene (Taha et al. 2004) and cathepsin-B (Taha et al. 2005), reactive oxygen species (Pchejetski et al. 2007). However, loss of SPHK1 is being considered to induce apoptosis indirectly by increasing Ceramide and autophagy, as has been proved by siRNA-mediated SPHK1 silencing (Taha et al. 2006). The standard treatment for IBD involves immunosuppression with corticosteroids and, more recently, anti-TNF treatments, similar to those for RA. It has been demonstrated that TNF- α activates SK1 in a variety of cell types (Billich et al. 2005), (Pettus et al. 2003), including rat intestinal epithelial cells (Kawamori et al. 2009) and HT29 colon cancer cells.

Mice lacking SphK1 or SphK2 survive, but deletion leads to embryonic lethality, indicating S1P's crucial role in survival (Mizugishi et al. 2005). As the name suggests SPHK2 should only phosphorylate Sphingosine (Sp), though SPHK2 efficiently catalyzes sphingadiene phosphorylation as it does to sphingosine (Jojima et al., 2020). Because of its increased substrate selectivity, SPHK2 is expected to be able to phosphorylate a greater variety of lipids that have a sphingoid base (Jojima et al. 20/20). Nuclear export and localization signals are present in SPHK2 and it travels back and forth between the cytoplasm and the nucleus (Diaz Escarcega, McCullough, and Tsvetkov 2021) (Figure 2.2A). During cellular stress, the pro-apoptotic protein SPHK2 is present in the endoplasmic reticulum of malignant cells and fibroblasts (Maceyka et al. 2005). It may also be detected in the mitochondria of cardiomyocytes and cancer cell lines (Strub et al. 2011); (Sivasubramanian et al. 2015).

Abnormal cytochrome c oxidase causes mitochondrial respiration to malfunction when SPHK2 levels fall (Strub et al. 2011), (Neubauer and Pitson 2013). Intracellular S1P produced by SK2 may bind to a number of substrates, including prohibitin 2 (Strub et al. 2011), human telomerase reverse transcriptase (TERT) (hTERT) (Panneer Selvam et al. 2015), and HDAC-1/2 (Hait et al. 2009), coupling the enzyme to epigenetic control, mitochondrial respiratory complex construction and aging, respectively. Overall multifunctional lipid kinase SPHK2 regulates several essential molecular processes. Even though S1P

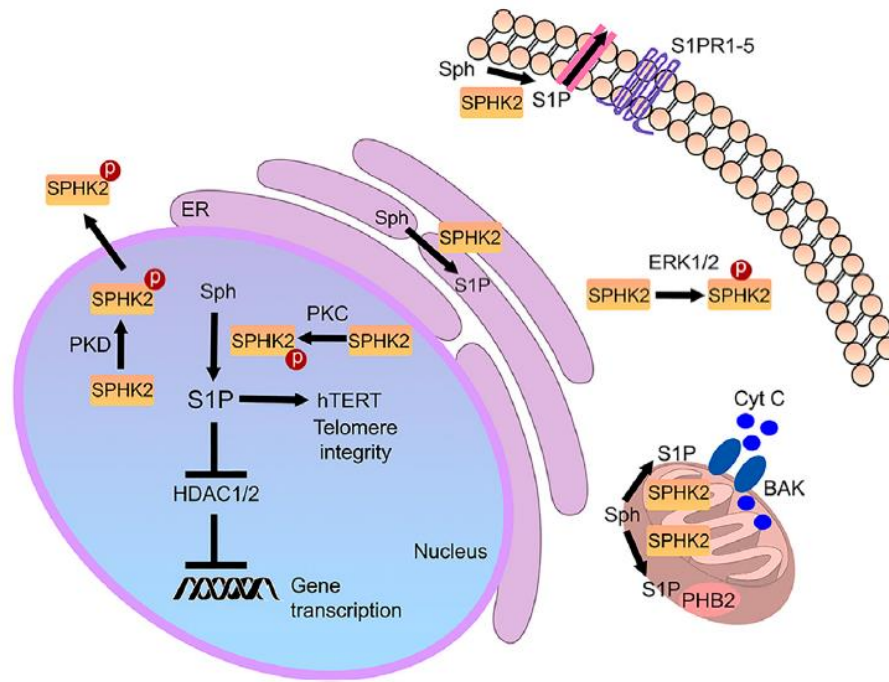


Figure 2.2A: Sphingosine kinase-2 or SPHK2 biogenesis and signaling. (Diaz Escarcega, 2021)

is thought to be pro-inflammatory, research over the past few decades has made it unclear whether S1P is pro- or anti-inflammatory. SPHK-2, previously by many proven data, has been believed to be anti-inflammatory as higher S1P levels have been reported in serum, lymphatic fluid, whole blood, and mesenteric lymph nodes of SPHK2^{-/-} mice; it has been proposed that this is because a compensatory SPHK1 expression has increased in response (Nagahashi et al. 2012). As the fact was discussed earlier that Sphk1^{-/-} mice are resistant to ulcerative colitis, it suggests that SPHK1 has a noticeable pro-inflammatory role in this condition (Snider et al. 2009). With selective SPHK2 inhibitors, the increase in SPHK1 expression levels observed in Sphk2^{+/+} mice is not replicated. These findings suggest a fundamental distinction between the persistent loss of SPHK2 protein caused by genetic deletion or knockdown and the small-scale suppression of SK2 activity have an impact on SPHK1 expression (Kharel et al. 2005).

2.3. Sphingosine-1-Phosphate signaling in immunity

The immune system relies on the continuous flow of cells between different anatomical locations since it is a systemic organ. Freshly developed lymphocytes need to travel through the bloodstream from the thymus or bone marrow (BM) to the lymph nodes (LNs), spleen, principal secondary lymphoid organ, Peyer's patches (PPs), and lymphoid tissues. It is a multi-step cascade that involves selectins for blood cell entry into the tissue. The initial rolling of cells along the endothelium is caused by chemokines and

the transmigration and adhesion of cells across the endothelium is mediated by firm integrins (von Andrian and Mempel 2003).

S1P affects cellular processes through its interactions with seven-helix GPCR receptors, or S1P receptors (S1PRs). Every member of this family of proteins has a high affinity for interacting with extracellular S1P.

When exposed to IL-2, CD4⁺ cells that have had their Sphk2 knockdown demonstrate a hyperactivated phenotype that includes considerably increased proliferation and cytokine secretion (IFN- γ) as well as decreased susceptibility to T(reg)-mediated repression *in vitro*. However, this effect seems to be independent of S1P receptors and S1P. This is in line with the idea that elevated SK1 expression would balance S1P levels and/or that SK2 may have non-catalytic effects on CD4⁺ cells, the latter of which SK2 inhibitors would not recapitulate. Consequently, the effects of Sphk2 knockdown on IL-2 signaling that have been seen in these cells may be SK1-mediated (Samy et al. 2007). SPHK1/2 might play a critical role in Th17 polarization (Liao, Huang, and Goetzl 2007), (Lee et al. 2010). Studies have shown that S1P promotes Th17 polarization by increasing STAT3 phosphorylation and IL-6 formation (Liang et al. 2013). Consequently, P-STAT3 increases the expression of ROR- γ t suggesting S1P acts in a ROR- γ t dependent Th17 polarization signaling pathway. Also. Some shreds of evidence showing the knockout of STAT3 or ROR- γ t fails Th17 polarization (Yang et al. 2007), (Ivanov et al. 2006).

Sphingosine kinase inhibitors now come into consideration as therapeutics against Th17-mediated disease models.

2.3.1. Sphingosine -1-phosphate receptor-1 (S1PR1) mediated immune response

In innate immunity, S1PR1 is a molecule that is extensively expressed and mediates activities in the majority of immune cells (Chae, Proia, and Hla 2004). It is associated with adhesion molecules (Reichardt et al. 2013) and is present in a variety of cell types and organs, such as immunological and endothelial cells (Chae, Proia, and Hla 2004). Adhesion molecules (Reichardt et al. 2013) and S1PR1 (Cyster and Schwab 2012) control the lymphatic arteries' ability to transport naïve T lymphocytes out of the LN. Macrophages express S1PR1, and experiments using an S1PR1/3 antagonist, VPC23019, and S1PR1 defective animals shown that migration toward S1P is reliant on S1PR1 expression (Weichand et al. 2013). The methods by which S1PR1 controls naïve T-cell egress have been examined in a number of recent investigations (Wei et al. 2005), (Rosen et al. 2007), (Grigorova et al. 2009), (Pham et al. 2008), (Zhi et al. 2011), (Thangada et al. 2010). FTY720, on the other hand, is a potent receptor agonist that functions as a functional antagonist by first activating the receptors and then triggering the internalization and destruction of the S1P receptors. S1PR1 and S1PR3 to -5 are two of the four different S1P receptors that it broadly modulates. Indeed, FTY720 inhibits effector T cell peripheral homing (Pinschewer et al. 2000) and egress of naïve T-cells from the LN and thymus (Mandala et al. 2002) in mice.

2.3.2. Sphingosine -1-phosphate receptor-2 (S1PR2) mediated immune response

S1PR2 is found on granulocytes, monocytes, and macrophages in innate cells (Blaho and Hla 2014). Alveolar macrophages with S1PR2 gene knockout as well as JTE-013 (S1PR2 antagonist) treatment in macrophages proved that S1P-S1PR2 interaction mediates clearance of the fungal pathogen *Cryptococcus neoformans* by increasing phagocytic action of immune cells. There are Phagocytic receptors such as FcγR I, II, and III were shown to be much less expressed in S1PR2 deletion macrophages (McQuiston, Luberto, and Del Poeta 2011). S1PR2^{-/-} macrophages displayed improved phagocytosis of *Escherichia coli* which is opsonin-independent since S1PR2 signaling inhibited IQGAP1-Rac1-dependent lamellipodial protrusion and RhoA-dependent cell contraction (Hou et al. 2015). Using JTE-013, an antagonist of S1P in the vaccinia viral infection model, a mast cell activation and increased antimicrobial peptide release were investigated (Wang et al. 2012).

2.3.3. Sphingosine -1-phosphate receptor-3 (S1PR 3) mediated immune response

S1PR3 In vitro, S1PR3 has been found to mediate macrophage chemotaxis, and in atherosclerosis models, it appears to contribute to the recruitment of monocytes and macrophages to plaques (Keul et al. 2011). In a rodent model of ischemia-reperfusion damage, it has more recently been discovered to also have a role in the maturation of DC or dendritic cells, Th1 response activation, and DC blockade of Treg responses (Bajwa et al. 2012), (Bajwa et al. 2016). Additionally, during hapten application-induced cutaneous reactions, S1PR3 is discovered to be strongly expressed on eosinophils and may be involved in recruitment mediation (Sugita et al. 2010). Similarly, upon isolation from pneumonia patients, neutrophils expressing S1PR3 exhibit increased chemotaxis in response to S1P, and S1PR3 is elevated in these cells (Rahaman et al. 2006). Research on S1PR3^{-/-} mice reveals less inflammation during noticed in bleomycin-induced lung damage; this inflammation could partially be mediated by neutrophils that express S1PR3 (Murakami et al. 2014). S1PR3 expression in endothelial cells increases P-selectin in a Gq, PLC-dependent manner, which promotes leukocyte rolling. According to Nussbaum et al. (2015) (Nussbaum et al. 2015), mast cell-released S1P interacts with S1PR3 on the leukocyte surface and helps in leukocyte rolling leading to recruitment at the inflammatory areas.

2.3.4. Sphingosine -1-phosphate receptor-4 (S1PR4) mediated immune response

It has been suggested that S1PR4 and the other receptors are crucial for innate immune cells. According to recent research, mice with S1PR4 deletion had less differentiation of plasmacytoid dendritic cells (Dillmann et al. 2015). Furthermore, human plasmacytoid activation and IFN-α production in response to tick-borne encephalitis vaccine were blocked by Cym50138, which targets S1PR4 but not other compounds that target S1PR1–3. By contrast, the S1P-induced reduction in interferon alpha was halted by the S1P4 antagonist Cym50358 (Dillmann et al. 2016). S1P builds up and produces neutrophilia in

mice lacking S1P lyase; this condition is somewhat alleviated by knocking down S1PR4 (Allende et al. 2011), indicating a role of S1PR4 in neutrophil recruitment. Furthermore, a connection between the Analysis of recent human genetic variants validated a phenotype in zebrafish and S1PR4 knockout mice where the S1PR4 missense variant was associated with decreased circulating neutrophil counts (Group 2016) (Pankratz et al., 2016). The receptor that human alveolar macrophages express the highest is S1PR4. According to recent research, there is a strong correlation between S1PR2 and S1PR4 expression (Barnawi et al. 2015). Cytokine production and migration in polarized macrophages are influenced by the expression profile of S1PR4, a receptor with distinct differences between M1 and M2-activated macrophages (Muller et al. 2017).

2.3.5. Sphingosine -1-phosphate receptor-5 (S1PR5) mediated immune response

It has recently been demonstrated that S1PR5 is crucial for natural killer cells and circulating monocytes despite the fact that it was previously believed to be limited to oligodendrocytes. According to Walder et al. (2007) (Walzer et al. 2007), S1PR5 is necessary for recruitment into inflammatory tissues, and animals lacking it have impaired natural killer cell homing. Mayol and colleagues (Mayol et al. 2011) claim that mature NK cells can leave LNs by expressing CXCR4 and S1PR5 in tandem. As they developed, a distinct and specific expression of these two proteins (CXCR4 and S1PR5) was observed on the cell surface. Unlike T cells, patrolling monocytes need interaction with S1PR5 to exit the bone marrow, however, this mechanism is independent of S1P gradients for trafficking, suggesting that S1PR5-mediated trafficking has a mechanism not similar to others (Debien et al. 2013).

2.4. Role of Sphingosine-1-Phosphate agonists and antagonists in cellular functions

The biological functions of S1P receptors include their importance in autoimmune illnesses, cancer, and conditions affecting the neurological, cardiovascular, immunological, and reproductive systems. In cells and tissues, they have distinct expression patterns (Kihara, Mizuno, and Chun 2015). Since S1P receptors have been identified, it is now easier to search for and produce S1P agonists and antagonists, which may lead to the discovery of novel treatment options (Im 2010).

2.4.1. Fingolimod (FTY720): a Sphingosine-1-Phosphate receptor (S1PR1-5) agonist yet functional antagonist

The immunosuppressive compound myriocin, found in the fungus *Isaria Sinclair*, was used to create the medication fingolimod, which was found in S1P research (Billich et al. 2003); (Im 2003); (Paugh et al. 2003). This metabolite was used in traditional Chinese herbal medicine, as a youth remedy (Adachi and Chiba 2007). Fingolimod is a special medication in two ways. Fingolimod is a prodrug, to start. Sphingosine kinases (SK1 and SK2) phosphorylate fingolimod in vivo, and subsequently phosphorylate. Four S1P receptor subtypes—S1P1, S1P3, S1P4, and S1P5—are agonistically mediated by fingolimod (Billich et al. 2003). Second, fingolimod's effects on lymphocyte egress include

functional antagonistic action against S1P1 rather than agonistic activity (Matloubian et al. 2004). According to reports, S1P and fingolimod phosphate both cause lymphopenia by agonistically activating S1P1 and causing S1P1 to internalize in the lymphocytes (Brinkmann, Cyster, and Hla 2004), (Thangada et al. 2010)). Internalized S1P1 is restored to the cell surface in a matter of hours when it came to S1P acting as an S1P1 agonist. However, when S1P1 is being modulated by fingolimod-phosphate as an antagonist, internalized S1P1 is degraded by proteases, which causes S1P1 to be silent for a long time until it is produced from the de novo synthetic pathway (Oo et al. 2007). This type of fingolimod functional antagonism results in a lengthy action time, which can occasionally be detrimental (Subei and Cohen 2015). Because FTY720 and sphingosine share structural similarities, there is conjecture that the medication may work through the sphingosine 1-phosphate (S1P) receptor S1P4 (previously Edg-6)2, which is known to be expressed by lymphocytes (Kohno et al. 2003). Furthermore, FTY720 was efficiently phosphorylated by lymphoid tissue, such as Peyer's patches, the spleen, and lymph nodes, but the heart, liver, and kidney had little amounts of the phosphorylated FTY720. The fascinating idea that phosphorylated FTY720 might be the active principle implies that FTY720/FTY720-P undergoes an in vivo alcohol/phosphate cycle, similar to that of sphingosine/S1P. In fact, FTY720 underwent substantial in vivo conversion to FTY720-P, leading to blood levels of FTY720-P that were up to four times greater than those of parent FTY720. Likewise, an in vivo mice study showed FTY720-P (0.1mg/kg) dephosphorylated and increased blood FTY720 level. Phospho-FTY720 holds greater receptor agonistic efficacy towards S1PR1, S1PR3, S1PR4, S1PR5 receptors except S1PR2 (Brinkmann et al. 2002).

Experimental autoimmune neuritis and spontaneous autoimmune polyneuropathy are two autoimmune disorders other than multiple sclerosis where Fingolimod's immune-modulatory activity has been noted (Kim et al. 2009; Nofer et al. 2007). It has been discovered that the medication fingolimod reduces neuroinflammation via controlling microglia activation and neuroprotective effects, mainly through S1P1 (Jackson, Giovannoni, and Baker 2011); (Noda et al. 2013); (Kolahdooz et al. 2015). Additionally, it prevents mice's airways from becoming inflamed and hyperreactive due to allergens (Ble et al. 2009); (Marsolais et al. 2011); (Trifilieff and Fozard 2012). (2-amino-2-[2-(4-octylphenyl)ethyl] is the chemical Propane-1,3-diol is a new, highly potent immune modifying drug that has shown remarkable efficacy in islet transplantation (Brinkmann et al. 2001) and other autoimmune and transplant models. It has also recently been shown to be successful in human renal transplantation (Saland et al. 2001). In contrast to the immunosuppressive drugs such as the calcineurin inhibitors tacrolimus and cyclosporin, the activation and proliferation of T cells are not restricted by FTY720, nor does it reduce immunity to systemic viral infection in mouse models (Pinschewer et al. 2000). It appears that FTY720 reduces some effector T cells in number that recirculate from the LN to inflammatory peripheral tissues (Pinschewer et al. 2000) and graft sites (Yanagawa et al. 1998) while also sequestering lymphocytes from circulation to secondary lymph tissue compartments (Chiba et al. 1998). Because the activation of FTY720 is seen

in both CCR-7-deficient and plt (paucity of lymph node T cells) animals, who lack CCR-7 ligands (CCL-19 and CCL-21), it does not work through the lymphocyte homing chemokine receptor CCR-7 (Henning et al. 2001).

Three phase 3 studies (FREEDOMS, FREEDOM II, and TRANSFORMS) have documented a number of fingolimod adverse effects (Kappos et al. 2010), (Calabresi et al. 2014), (Cohen, Khatri, et al. 2016). Macular edema, headache, cough, dyspnea, back discomfort, influenza, diarrhea, bradycardia as well as ventricular blockage at the first dose, and other adverse events were the most common (Subei and Cohen 2015). According to (Camm et al. 2014), first dosage Atrial myocytes' transient S1P1 activation is assumed to be the source of bradycardia; this would disappear if S1P1 were down-regulated. Bradycardia is produced by S1P3-containing atrial myocytes in mice, but not in humans (Forrest et al. 2004); (Sanna et al. 2004). Since fingolimod is a non-selective S1P agonist (S1P1, 3-5), additional side effects might result from off-target actions via other S1P receptors (Brinkmann et al. 2002). As a result, in the wake of fingolimod, a number of S1P1 selective agonists/modulators are presently being researched as potential medications (Figure 2.4.1A).

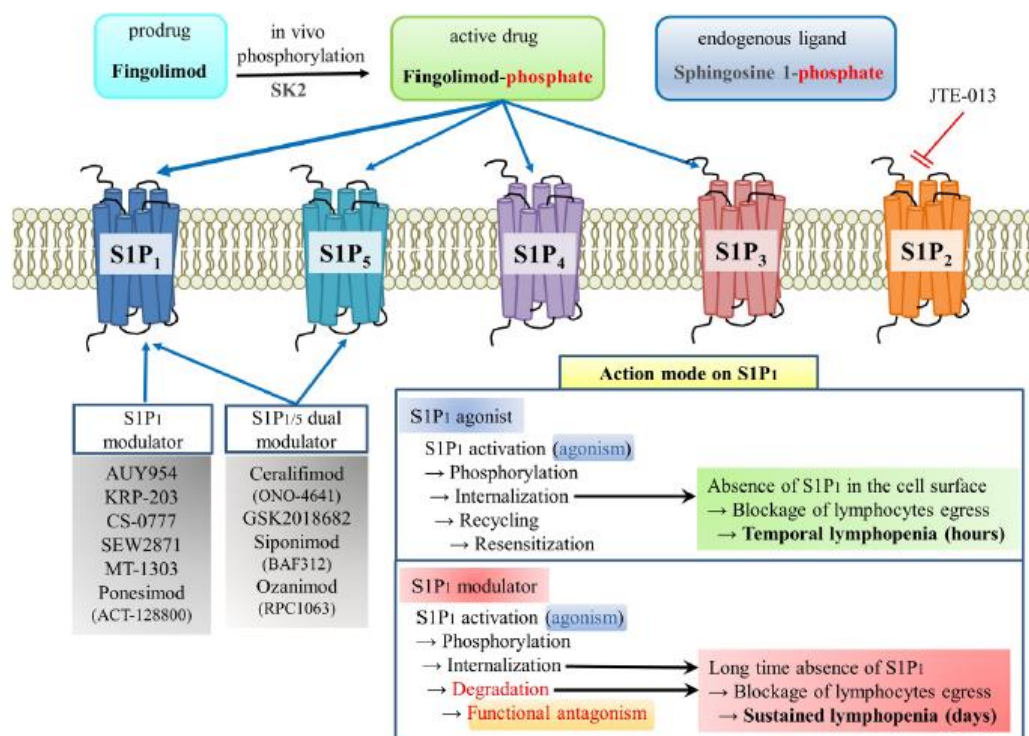


Figure 2.4.1.A: S1P receptor modulation by structural and functional agonists and antagonists. (Park, 2017)

A study with *Cryptococcus. neoformans* infection revealed that FTY720 impairs the phagocytosis property of macrophages (Bryan et al. 2020). The *Cryptococcus spp.* are fungal pathogens that cause

meningo-encephalitis in human which can be life-threatening for patients suffering from severe lymphopenia (Perfect and Casadevall 2002), (Casadevall, Coelho, and Alanio 2018). Prior research shown the significance of the S1P signaling pathway in the granulomatous reaction to a mouse model of attenuated *C. neoformans* strain (.gcs1), which generates pulmonary granulomas that remarkably resemble human granulomas (Rittershaus et al. 2006), (McQuiston, Luberto, and Del Poeta 2010), (Farnoud et al. 2015). Granuloma formation in reaction to *C. neoformans*. Sphingosine kinase 1 (SK1) was required for gcs1 infection, which was linked to increased S1P, Monocyte chemoattractant protein-1 (MCP1), and TNF- α levels in the bronchoalveolar lavage fluid of mice infected with *C. neoformans* (McQuiston, Luberto, and Del Poeta 2010), (Farnoud et al. 2015), (McQuiston, Luberto, and Del Poeta 2011). FTY720 increased 50% of *Cryptococcus. neoformans* infection burden in the brain and lung. Weight loss was one of the symptoms of disseminated cryptococcal illness in the FTY720-treated mice. Instead of forming a ring around the necrotic tissue, the large macrophages carrying *C. neoformans* were scattered throughout the granulomatous structure, indicating that the mice treated with FTY720 had an uncoordinated lung host response. It was discovered that FTY720 therapy promoted M1 and M2 polarization inside the fibrotic layer via CD38 and EGR2 (Jablonski et al. 2015). Prior studies shown that FTY720P suppresses signaling through G α q while also antagonistically interacting with S1PR3 pathways associated with G α i and G α 12/13 proteins (Jablonski et al. 2015), (Sensken et al. 2008). The effect of S1PR3-G α q signaling on phagocytosis may be mediated by substances that selectively agonistically (CYM5541), antagonistically (TY52156), or negatively alter G α q stream routes (phospholipase C, U73122). Phospholipase C inhibition with U73122 or receptor antagonism with TY52156 may similarly result in decreased phagocytosis, indicating that the reduced phagocytosis is connected to the suppression of S1PR3 coupling to G α q. The S1pr3^{-/-}-knockout macrophages exhibited compromised phagocytosis, which was not resolved by S1P addition (Bryan et al. 2020). Earlier research with SK1 mutant mice showed an elevated *Cryptococcus. neoformans* lung load in the absence of S1P signaling, which is consistent with the FTY720 treated reactivation mice model (McQuiston, Luberto, and Del Poeta 2010). The study investigates macrophage interactions with *C. neoformans* due to their significant role in controlling cryptococcosis, especially at the granuloma site (Kechichian, Shea, and Del Poeta 2007), (Goldman, Lee, and Casadevall 1994), (Brunet et al. 2018) and their mislocalization upon FTY720 treatment. The study focused on S1PR3, the S1P receptor, targeted by FTY720, as it is present on macrophages and linked to lung inflammation and bactericidal activity (Murakami et al. 2014), (Sanna et al. 2004), (Hou et al. 2017). S1PR4 is unlikely to play a role, as it is downregulated in macrophages. S1P is believed to stimulate M1 polarization in macrophages via S1PR3 (Gaire, Song, and Choi 2018), (Shen et al. 2019) while FTY720 may inhibit this polarization and promote M2 polarization (Gaire, Song, and Choi 2018), (Sun et al. 2018), (Qin et al. 2017) (Figure 2.4.1A).

2.4.2. SEW2871: a Sphingosine-1-phosphate Receptor-1(S1PR1) agonist

A selective S1P1 agonist called SEW2871 improves acute renal failure in mice following ischemia/reperfusion damage (Lien et al. 2006) and lowers blood lymphocyte counts (Wei et al. 2005), (Kim et al. 2009). Due to S1P1 activation, it also shields the tissues of the heart and liver following myocardial or hepatic damage (Hofmann et al. 2009), (Park et al. 2010). Preclinical research has demonstrated the potential therapeutic advantages of SEW2871 in treating inflammatory reactions, liver fibrosis, diabetes, and Alzheimer's disease. In a type 1 diabetes model in non-obese mice, SEW2871 inhibited monocyte/endothelial interactions and reduced monocyte adherence to diabetic aortas, suggesting that SEW2871-induced S1P receptor-1 signaling might be helpful in the vascular effects of type 1 diabetes treatment (Whetzel et al. 2006). Chronic administration of SEW2871 has been shown to inhibit b amyloid-induced spatial memory impairment and hippocampal neuronal loss in a rat model of Alzheimer's disease (Asle-Rousta et al. 2013). By controlling the migration of human hepatic myofibroblasts into injured regions, SEW2871 also modifies liver fibrosis (Li et al. 2011). SEW2871 raises the levels of procollagen α I and α III, smooth muscle α -actin, and total hydroxyproline in LX-2 cells (Liu et al. 2011). Furthermore, in inflammatory environments, it inhibits dendritic cell migration as well as chemotactic movement to the lymph nodes, promotes a transition to the M2 macrophage phenotype, and lowers proinflammatory cytokine levels (Gollmann et al. 2008), (Hughes et al. 2008)). SEW2871 administered intravenously reduces acute inflammation-induced lung damage caused by LPS, indicating a variety of uses for S1P1 agonists (Sammani et al. 2010).

2.4.3. KRP-203: a Sphingosine-1-phosphate Receptor-1 (S1PR1) agonist

Another immunosuppressive S1P1 agonist KRP-203 shares a molecular structure with fingolimod (Shimizu et al. 2005). It has immunosuppressive properties and controls lymphocyte homing (Shimizu et al. 2005). Its method of action includes functional antagonism of S1P1 and in vivo phosphorylation. S1P5 is agonistically affected by KRP-203-phosphate, however, S1P2-4 is not (Lukas et al. 2014). It was created for organ transplantation, and skin and heart allografts in rat models, it has been shown to improve graft survival and decrease chronic rejection and graft vasculopathy (Shimizu et al. 2005), (Takahashi et al. 2005). Additionally, following rat kidney transplantation, it was proposed as a possible immune modulator (Fujishiro et al. 2006). Numerous experimental models for inflammatory bowel illnesses and autoimmune disorders have made use of KRP-203. It decreased inflammation and prevented macrophage and CD4⁺ T cell infiltration in a rat model of autoimmune myocarditis (Ogawa et al. 2007). It lessened the CD4⁺ lymphocyte number in the liver and enhanced lymphocyte trapping in secondary LNs in a mouse model where induced autoimmune hepatitis was developed using concanavalin A (Kaneko et al. 2006). Additionally, KRP-203 was investigated for various inflammatory conditions, i.e., atherosclerosis and Crohn's disease. It decreased lymphocyte infiltration, avoided chronic colitis, and prevented weight loss and inhibited proinflammatory cytokine production in a model of chronic colitis (Song et al. 2008). It caused lymphopenia and inhibited the establishment of

atherosclerotic lesions in mice lacking the LDL receptor (Poti et al. 2013). A phase 1 trial to assess KRP-203's safety, tolerability, pharmacokinetics, and effectiveness in patients receiving stem cell transplantation for hematological malignancies is in underway ([NCT01830010](#)), as is a phase 2 clinical trial for subacute lupus erythematosus ([NCT01830010](#)).

2.4.4. AUY954: a selective Sphingosine-1-phosphate receptor-1 (S1PR1) agonist

A strong and selective S1P1 agonist is AUY954. It is an amino carboxylate analog of FTY720 (Pan et al. 2006) that has been exhibited to have positive potential for lung inflammation, experimental autoimmune neuritis, and rat heart transplantation. AUY954 extended the lifespan of cardiac allografts and reduced circulating lymphocytes in a rigorous rat transplantation model (Pan et al. 2006). AUY954 significantly lowered inflammatory demyelination, infiltration of immune cells, and metalloproteinase-9 and IL-17 expressions in an experimental sciatic nerve model of rat autoimmune neuritis. Additionally, lymphocytes were sequestered into secondary lymphoid tissues (Zhang et al. 2009). Its effects on respiratory conditions are complicated, though. AUY954 had no effect on eosinophils but suppressed lymphocyte accumulation in the model of allergen-induced inflammation in the airways (Ble et al. 2009). Long-term exposure exacerbated bleomycin-induced lung damage and vascular leak in a mouse model, whereas recurrent dosing exacerbated pulmonary fibrosis (Shea et al. 2010). As a result, it is important to carefully assess how S1P1 modulators affect respiratory conditions.

2.4.5. BAF312: an agonist of Sphingosine-1-Phosphate Receptor-1 (S1PR1) and Receptor-5 (S1PR5)

Siponimod, often referred to as BAF312, is an agonist of S1P1 and S1P5 and a new alkoxyimino derivative (Gergely et al. 2012). With regard to multiple sclerosis, it is being studied (Subei and Cohen 2015). Like FTY720, siponimod stops lymphocytes from leaving lymph nodes, which causes lymphopenia (Fryer et al. 2012). It was discovered to reduce demyelination induced by Lipopolysaccharide in the organotypic slice cultures and inhibit autoimmune encephalomyelitis in an experimental rat model (O'Sullivan et al. 2016). These results imply that similarly FTY720, siponimod could directly affect lymphopenia and brain cells (O'Sullivan et al. 2016), (Choi et al. 2011). Within four to six hours, siponimod decreased the amount of T lymphocytes and B lymphocyte cells in the blood of healthy people, and a week after treatment ended, the numbers returned to normal. Immune-mediated illnesses may benefit from siponimod therapy (Gergely et al. 2012). Initially and extending phase 2 clinical investigations for patients with a form of multiple sclerosis that recurs have been completed satisfactorily ([NCT 00879658](#), [NCT01185821](#)) (Selmaj et al. 2013), (Kappos et al. 2016). In addition to two phase 1 pharmacokinetic investigations for renal and hepatic impairments ([NCT01904214](#) and [NCT01565902](#)), siponimod is also being studied in a phase 3 effectiveness and safety clinical study in individuals with secondary progressive multiple ([NCT01665144](#)), polymyositis ([NCT01801917](#)), and dermatomyositis ([NCT02029274](#)).

2.4.6. 1CS-0777: a selective and orally active modulator of Sphingosine-1-Phosphate Receptor-1 (S1PR1)

Another selective S1P1 modulator called CS-0777 is being developed to treat autoimmune conditions like multiple sclerosis (Moberly et al. 2012). It inhibits experimental autoimmune encephalomyelitis and causes lymphopenia (Nishi et al. 2011). Oral CS-0777 reduced blood levels of CD4+ T cells and lymphocytes in a pilot phase 1 clinical study; after 4 weeks, the blood levels returned to normal. It has been studied with encouraging outcomes in multiple sclerosis ([NCT00616733](#)) (Moberly et al. 2012).

2.4.7. Ponesimod: a functional antagonist of Sphingosine-1-Phosphate Receptor-1 (S1PR1)

Ponesimod is also a selective S1P1 agonist that is taken orally and causes lymphocytes to sequester within lymphoid tissues (Bolli et al. 2010). After stopping, it goes away in a week and is easily reversible (D'Ambrosio, Freedman, and Prinz 2016). In an autoimmune model of mouse with non-diabetes, ponesimod has been demonstrated to prevent disease progression (You et al. 2013) and decrease inflammatory responses in lymphocyte-mediated inflammatory disorders (Piali et al. 2011). According to Vaclavkova et al. (2014), it has also been used to treat relapsing-remitting multiple sclerosis and chronic plaque psoriasis, a chronic autoimmune disease characterized by scaly, red, and itchy patches of abnormal skin. Furthermore, a phase 2 clinical trial is being conducted on Ponesimod to treat chronic graft-versus-host disease (GVHD), a side effect that happens after bone marrow or stem cell transplantation. GVHD involves both autoreactive and alloreactive T and B cells, and S1P1 modulators reduce the immune response by avoiding vascular damage and immune cell accumulation in the ([NCT02461134](#)) (Huu et al. 2013). One possible novel treatment approach for autoimmune diseases is ponesimod.

2.4.8. Ozanimod: an agonist of Sphingosine-1-Phosphate Receptor-1 (S1PR1) and Receptor-5 (S1PR5)

The oral selective S1P1/5 dual modulator ozanimod controls the immune response and causes lymphopenia (Scott et al. 2016). It has been demonstrated to reduce inflammatory parameters in three autoimmune disease models: colitis model with CD4+ CD45RBhi lymphocyte adoptive transfer, TNBS-induced colitis, and experimental autoimmune encephalitis (Cohen, Arnold, et al. 2016), (Scott et al. 2016). IBD, often known as Crohn's disease (CD) or ulcerative colitis (UC), and multiple sclerosis are other conditions for which Ozanimod is being researched (Rivera-Nieves 2015). In UC, ozanimod produced a noticeably greater incidence of clinical remission than a placebo in a double-blind, placebo-controlled phase II study ([NCT01647516](#)) (Sandborn et al. 2016). Phase III studies are now being

conducted on patients with UC of moderate to severe ulceration ([NCT02435992](#)), and relapsing multiple sclerosis ([NCT02047734](#)).

2.4.9. JTE-013: an antagonist of the Sphingosine-1-Phosphate Receptor-2 (S1PR2)

A specific S1P2 antagonist, JTE-013 has been utilized to investigate its functions in a range of cell types and illnesses (Osada et al. 2002). Numerous biological responses in endothelial cells, including cytoskeletal re-structuring and cell-extracellular/intracellular matrix interactions, are influenced by S1P2. In vitro, S1P2 inhibition controlled endothelial tight junctions and barrier function, and in vivo, it dramatically reduced microvascular permeability in an animal model (Lee et al. 2009). In a preclinical investigation, JTE-013 inhibited cerebrovascular permeability, intracerebral hemorrhage, and neurovascular damage in a stroke model, therefore modulating responses of the brain endothelium (Kim et al. 2015). JTE-013 therapy decreased myogenin and Akt levels and postponed muscle regeneration, suggesting that S1P2 signaling is essential for both microvascular permeability and muscle regeneration (Germinario et al. 2012). Histamine and heparin-rich granules seen in lung mast cells are essential for allergic disorders and anaphylaxis. S1P2 has a role in mast cell migration (Yokoo et al. 2004) and suppresses allergic reactions and ovalbumin-induced mast cell activation (Trifilieff, Baur, and Fozard 2009). Mice treated with JTE-013 have reduced pulmonary edema and IgE-stimulated anaphylactic reactions (Oskeritzian et al. 2010). JTE-013 decreases airway infiltration, mast cell activation, and serum histamine and cytokine levels, according to both in vitro and in vivo research (Oskeritzian et al. 2015). Additionally, it prevents the chemotactic movement of fibroblast cells, Rho signaling activation, and focal adhesion kinase (FAK) phosphorylation that are triggered by S1P and impact tissue healing following injury (Hashimoto et al. 2008). In human bronchial smooth muscle cells, JTE-013 also inhibits the Rac1-dependent signaling pathway and migration caused by S1P (Kawata et al. 2005). Insulin resistance and type-II diabetes are associated with pancreatic β (beta) cell defection. In mouse models, JTE-013 has been demonstrated to inhibit pancreatic β cell apoptosis, diabetes incidence, and streptozotocin-induced blood glucose rises (Imasawa et al. 2010). Additionally, obese diabetic mice had a diet of high-fat content and preserved pancreatic β A cells (Japtok et al. 2015). An inhibitor of Plasminogen activator, PAI-1 rises in mouse adipocytes were inhibited by JTE-013, and animals lacking S1P2 displayed smaller epididymal adipose tissue cells (Ito et al. 2013) and improved glucose/insulin tolerance test results (Kitada et al. 2016). ApoE^{-/-}-deficient animals treated with JTE-013 had lower levels of inflammatory cytokines, and atherosclerosis was decreased by S1P2 gene deficiency (Skoura et al. 2011). Additionally, it controlled inflammatory reactions and vascular endothelial integrity during endotoxemia in blood (Zhang et al. 2013). In macrophages, S1P2 is an essential receptor that influences the phagocytic activity of macrophages and antimicrobial actions throughout the pathophysiology of septic condition (Hou et al. 2015). According to these results, JTE-013 may be used to treat inflammatory diseases.

Follicle helper T-cell retention and mast cell degranulation depend on S1PR2 activation (Michaud, Im, and Hla 2010), (Oskeritzian et al. 2010), (Moriyama et al. 2014). On macrophages, it is the most prevalent S1P receptor (McQuiston, Luberto, and Del Poeta 2011), (Hughes et al. 2008). A lack of S1PR2 protects against lung damage and polymicrobial sepsis (Hou et al. 2015). In one investigation, Bone marrow transplantation between WT and S1pr2^{-/-} mice led to higher rates of bacterial engulfment and a survival rate of 66.7%. Additionally, S1pr2^{-/-} AMs showed higher rates of bacterial engulfment (Hou et al. 2015). According to McQuiston's in vitro research, extracellular S1P up-regulated the expression of phagocytic Fc receptors, which in turn enhanced opsonin-mediated phagocytosis of *Cryptococcus neoformans* through S1PR2 (Hou et al. 2015).

The bidirectional interaction between gut microbes and non-antibiotic drugs such as Lipid-lowering Statins, PPI, antidepressants, Antihistamine, Paracetamol, anti-cancer drugs and many others can alter the gut microbiome, leading to health issues. Also, in a reverse manner, gut probiotics enhance drug metabolism and efficacy (Weersma, Zhernakova, and Fu 2020). Precision medicine projects, which employ genomic expression, metabolomic, and proteomic data to make advancements in patient therapy and care, are driven by advanced throughput sequencing and analytical chemistry (Kuntz and Gilbert 2017). However, there is still work to be done in order to systematically maximize therapeutic efficacy and decrease toxicity using these varied data types. Pharmacogenomics and pharmacometabolomics, two subdisciplines of pharmacology, are working to address this difficulty by determining how human metabolism and genetics affect patient drug reactions (Aziz R.K. 2012). Antibiotics, lipid-lowering medications, antidiabetic medications, and analgesics are among the most commonly given medications in the U.S. (Martin et al. 2019). Many factors, such as genetics, environment, and diet, influence the makeup of the gut flora, yet it was proposed that that drug might have the largest overall effect magnitude (Falony et al. 2016).

2.5. The importance of dietary sphingolipids on Intestinal microflora

With an emphasis on the immune system and gut, researchers have shown that Western lifestyles are associated with a higher occurrence of illness. Because low-grade inflammation modifies the immune response, it may lead to disease progression instead of an acute defensive reaction (Bennett et al. 2018), (Ribaldone, Pellicano, and Actis 2019). This can result in long-term health issues such as depression, obesity, insulin resistance, arteriosclerosis, autoimmune, and allergy illnesses. Knowing how diet affects the gut-associated lymphoid tissues (GALT) is essential. It has been discovered that high-fat diets increase intestinal inflammation, change gut flora, and promote gut permeability (Samadi, Klems, and Untersmayr 2018), (Weidmann et al. 2018)). Nutritional lipids, which are included in dietary items, have the ability to alter immune responses and aggravate conditions linked to inflammation (Simopoulos 2002), (Roduit et al. 2019). With more than 4000 subtypes (Norris and Blesso 2017), SLs are a varied lipid class that is mostly present in eukaryotic species but is sometimes discovered in

viruses, prokaryotes, and archaea (Vesper et al. 1999). SLs were first identified as parts of cell membranes, but because of Westernized eating patterns, they are now connected to the rising incidence of illnesses in affluent nations. It has been proposed that high intakes of protein, fat, sugar, salt, and cholesterol might lead to chronic inflammation in the gastrointestinal lumen, which can impact immunological response and subsequently cause disorders in many organ systems (Vesper et al. 1999). The amount of SL in different nutritional components varies a lot. Adults following a Western diet consume 0.3 to 0.4 g of SLs daily, mostly from SM. There are fewer SMs and more cerebroside in Asian diets (Yunoki et al. 2008). The structure of dietary SLs varies according to their origin and necessitates a lumen wall breakdown to sphingoid backbones prior to intestinal absorption (Deevska and Nikolova-Karakashian 2011), (Kunisawa and Kiyono 2016). The majority of SLs found in dietary products from mammals are of different kinds, and SL homeostasis is proposed as a common stress reaction.

Members of the phylum Bacteroidetes and some Proteobacteria are the first known to produce SL prokaryotically (Stoffel, Dittmar, and Wilmes 1975), (Ogawa, Tachimoto, and Kaga 2010). A tight symbiotic connection is shown by the fact that several known bacterial SL producers are linked to eukaryotic hosts (Human Microbiome Project 2012). The microbiome of the digestive tract is a very intricate ecosystem that strongly impacts host health. It is yet unclear how much bacterial SLs affect immunological homeostasis, metabolic functions, and eukaryotic physiology. The variety of bacterial SL structures has been shown by recent research; certain species produce sulfonolipids in the mouse cecum, while others produce various kinds of SLs (Walker et al. 2017). These bacterially produced SLs have the ability to penetrate the gut's epithelial barrier and get into the metabolic pathways of their hosts (Johnson et al. 2020). The gut flora is shaped in large part by diet, which also affects how particular foods affect blood sugar levels. It is crucial to comprehend the fundamental metabolic functions and dynamic interactions of microbes related to diet and nutrition. Using a click-chemistry-based method, recent data on dietary sphinganine's microbial absorption in the mouse small intestine has been demonstrated. Understanding the close relationships between Sphingolipid metabolism, the gut microbial population, and host immunological homeostatic stability requires looking into how dietary Sphingolipids affect the gut microbiota (Figure 2.5A).

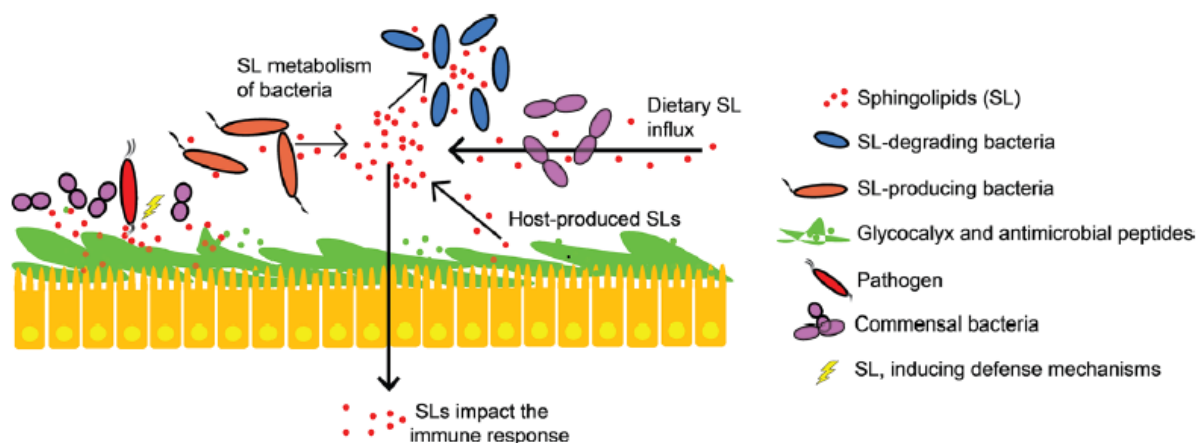


Figure 2.5A: Impact of Sphingolipid (SL), derived from gut bacterial metabolism, host's metabolic activity and dietary SL on host's immune system. (Rohrhofer et al. 2021)

The gut microbiome and bioactive substances interact with intestinal cells, including intestinal cells (IECs). Pathogenic bacteria cannot infiltrate the mucosal location because to the protective properties of synthetic lipids (SLs) like SM (Sprong, Hulstein, and Van der Meer 2001). For repurposing, enterocytes absorb SM and break down its lipid backbone. Glycosphingolipids and SL degradation by-products interact with the host's immune compartments. Gram-positive bacteria and gram-negative harmful bacteria are susceptible to the antibacterial effects of lyso-SLs and sph (Sprong, Hulstein, and Van der Meer 2001), (Pewzner-Jung et al. 2014), (Tavakoli Tabazavareh et al. 2016). While lower Sph levels encourage bacterial infections, high Sph expression in cells of human nasal epithelium is linked to protective barrier function (LaBauve and Wargo 2014). In eukaryotic cells, glycosphingolipids serve as mediators of cell adherence, antigens, and receptors for poisons and microbial metabolites. In eukaryotic cells, glycosphingolipids serve as mediators of cell adhesion, antigens, and receptors for poisons and microbial metabolites. A glycosphingolipid called lactosylceramide (LacCer) has a unique purpose in identifying PAMPs (pathogen-associated chemical patterns) and triggering phagocytic activity (Iwabuchi et al. 2008), (Yoshizaki et al. 2008). Antimicrobial peptides (AMPs), which are essential for the operation of the antimicrobial barrier and innate immunity, are produced in tandem with an increase in cellular Cer accumulation. When the epidermis is stressed, elevated S1P levels promote the production of cathelicidin antimicrobial peptide (Park, Lee, and Lee 2013). LacCer is essential for pathogen binding since it makes about 70% of the glycosphingolipids in the host neutrophil plasma membrane (Chiricozzi et al. 2018), (Chiricozzi et al. 2015).

2.6. Sphingosine-1-phosphate in gut inflammation

The epithelial barrier, gut flora, and the innate and adaptive compartment of cellular immunity make up the majority of the intestinal mucosal microenvironment (Ohman, Tornblom, and Simren 2015). Intestinal homeostasis depends heavily on bacteria to the host cells and crosstalk among host cells in the epithelium. Intestinal dysbacteriosis, increased intestinal permeability, and immunological dysfunction are all known characteristics of the pathogenesis of Inflammatory Bowel Disease, and disruption in that interaction results in an abnormal microenvironment that causes these symptoms (Zaidi et al. 2020). Chronic, nonspecific inflammation in the gastrointestinal tract is one of the hallmarks of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). In addition to being common in Western nations, IBD is also becoming increasingly common in recently industrialized nations in Asia, Africa, and America. It has also emerged as a worldwide health concern that puts an increasing financial as well as medical burden on individuals (Ng et al. 2017). S1P has been shown in several studies to play a crucial part in inflammation and endothelial barrier function (Kunisawa and Kiyono 2012), (Oskouian and Saba 2007). S1P signaling pathway activation has been related to colorectal cancer, IBD, and colitis. S1P involves its five G protein-coupled receptors (S1PR1–5) and influencing important intracellular molecules that control gene transcription, such as the inhibition of histone deacetylases (HDACs), which are epigenetic regulators of gene expression, and the activation of the pro-inflammatory transcription factor NF- κ B. Preclinical models of colitis have shown abnormal S1P signaling, indicating that focusing on S1P synthesis or interacting with S1PRs may help treat colitis and lessen its severity. It has been demonstrated that S1P has a protective role in a number of tissues, including the heart, lung, brain, and kidney, therefore not all S1P activation is harmful. In animal models, S1P reduces acute lung damage and improves lung endothelial function (Hofmann et al. 2009), (Couttas et al. 2014), (Peng et al. 2004), (Perry et al. 2016), (Mehaffey et al. 2018). S1P has also been demonstrated to shield the heart from ischemia-reperfusion damage (Morel et al. 2016). The significance of Sphingosine-1-Phosphate in the protective function of the intestinal epithelial barrier has just lately been clarified, despite the abundance of research on the role of S1P contributing to the integrity of endothelium. Given its function in T cell development and lymphocyte egress, S1P may prove to be a useful therapeutic target in this respect (Garris et al. 2014). Furthermore, it has been proposed that S1PR2, S1PR3, and S1PR5 are involved in the trafficking of natural killer cells and macrophages (Aoki et al. 2016).

The connection between S1P and SphK1 and TNF α has led to their involvement in colorectal cancer. In order to generate S1P, TNF α facilitates SphK1's translocation to the plasma membrane. Additionally, it is proposed that Sphingosine kinase1 and intracellular Sphingosine Kinase- 1 can increase TRAF2's E3 ligase functionality, which can activate the NF κ B pathway and produce anti-apoptotic and inflammatory signals (Pyne and Pyne 2013). This idea was corroborated by genetic research when SphK1-deficient animals showed some protection against colitis induced on by dextran sulfate sodium

(DSS) (Snider et al. 2009). A juvenile case study that looks at the genomic levels of proteins involved in the Sphingosine-1-Phosphate metabolic process provides more evidence for the importance of S1P in IBD. When compared to normal or remission people, colon samples from IBD patients with moderate to severe symptoms showed higher levels of SphK1, SphK2, signaling receptors (S1PR1, 2, and 4), and degradation enzymes (Sphingosine-1-phosphate Lyase-1 or SGPL-1). Furthermore, compared to controls, IBD patients had significantly greater levels of Ceramide and Ceramide-1-phosphate expression (Suh et al. 2018).

Normal and inflamed intestines also express S1PP and S1PL differently. According to Degagné et al. (2014) (Degagne et al. 2014) and Karuppuchamy et al. (2017) (Karuppuchamy et al. 2017), S1PL is downregulated in colitis model mice and robustly expressed in colon tissues in a homeostatic environment. In a mouse model of colitis caused by DSS showed that the intestinal tissues of Ulcerative Colitic individual had downregulated expression of S1PP2 mRNA. A DSS-induced colitic murine model similarly showed downregulated S1PP1 mRNA expression (Karuppuchamy et al., 2017) (Karuppuchamy et al. 2017). According to Karuppuchamy et al. (2017) (Karuppuchamy et al. 2017) Patients with UC and CD also have higher intestinal mucosal expression of the S1P transporter Spinster Homolog-2, which may be a factor in the elevated S1P levels in the inflammatory bowel.

2.7. Sphingosine-1-phosphate in Intestinal Barrier regulation

A microbiome of about 100 trillion bacterial species resides in the gut, the biggest immunological barrier organ in the human body. The gut ecosystem remains balanced under normal physiological conditions because the bacteria and the host are symbiotic and mutually helpful. The body can be shielded from poisons and intestinal microorganisms via the intestinal barrier (Sender, Fuchs, and Milo 2016), (Haak, Prescott, and Wiersinga 2018). The Epithelial Cells that cover the vascular surface are in charge of facilitating the movement of solutes and water from the interstitium to the blood. Cells, that are connected to the basement membranes, linked by focal adhesion (FA) to the extracellular matrix (ECM) (Rodrigues and Granger 2015). There are three interendothelial junctions i.e.; tight junctions (TJ), adherent junctions (AJ), and gap junctions (GJ)—connect adjacent ECs (Jernigan et al. 2015). According to current research, S1P-S1PR1 signaling changes actin rearrangements in ECs to enhance cellular AJ, which leads to vascular endothelial cadherin and other molecules' expression (Ziegler and Graler 2021). During sepsis, the degree of endothelial function impairment rises as the plasma concentration of S1P decreases (Fan et al. 2020).

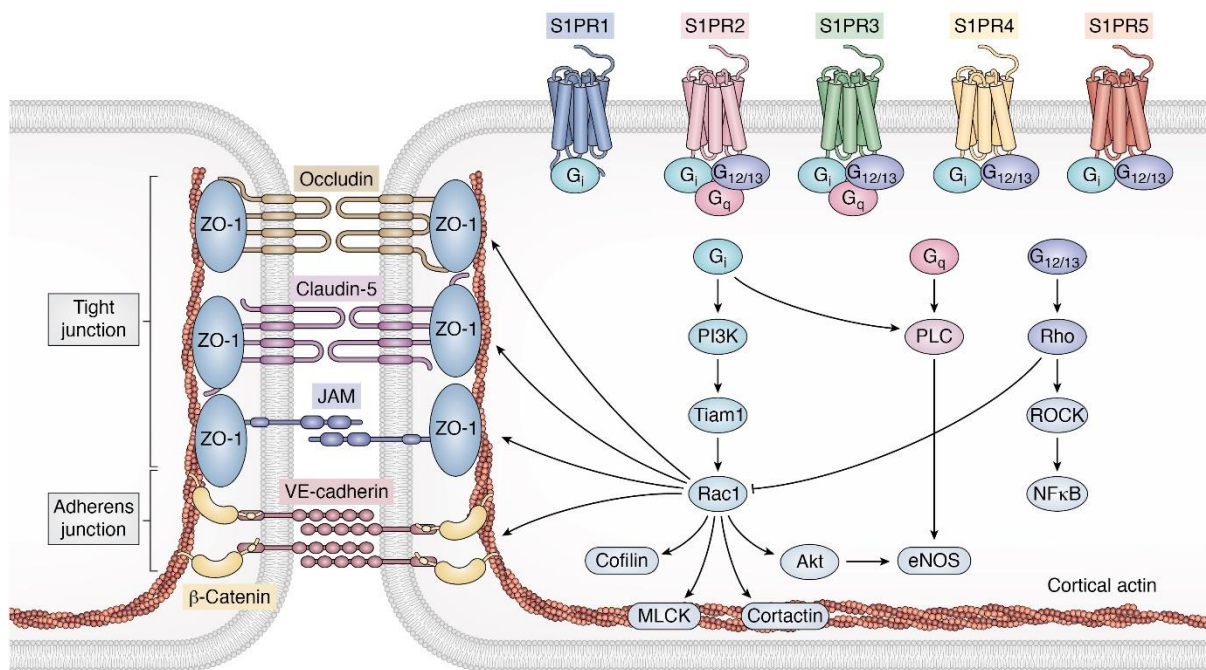


Figure 2.7A: S1P signaling and S1P receptors in epithelial tight junctional protein regulation.
(Weigel, 2023)

A number of pathologic diseases, including infection, trauma, inflammation, and cancer, are associated with intestinal barrier failure (Carneiro-Filho et al. 2004), (Lima et al. 1997). Specialized structures like as desmosomes, adherens junctions, and tight junctions are necessary for the integrity of this layer (Cereijido, Shoshani, and Contreras 2000), (Collares-Buzato et al. 1994), (Gumbiner 1996), (Mitic and Anderson 1998), (Takeichi 1990), (Yap, Briher, and Gumbiner 1997). The commencement and maintenance of cell-to-cell contact depend on cadherins, including E-cadherin (Gumbiner 1996), (Mitic and Anderson 1998), (Takeichi 1990)).

Through homodimerization, E-cadherin-mediated adhesions are strong with adjacent cells, and its cytoplasmic conserved domain interacts with the cytoskeleton via actin-binding proteins (Takeichi 1990), (Angst, Marcozzi, and Magee 2001), (Rimm et al. 1995), (Troxell et al. 1999), (Weiss et al. 1998). By raising TEER along with lowering ¹⁴C-paracellular permeability, S1P markedly improved the epithelial barrier. At a dosage of 0.5 μ M, this impact peaked and persisted for up to 24 hours. The impact peaked at 31% and was observed as early as 30 minutes (Greenspon et al. 2011). E-cadherin levels were significantly impacted by S1P within an hour, reaching their maximum at three hours before plateauing. The intestinal barrier's integrity is modulated by S1P, which may have an impact on pathological situations when the barrier is compromised. EDG-1 and EDG-3 in cell surface receptors are two examples of the particular receptor-mediated interactions that mostly cause S1P (Dudek and Garcia 2001). This shift in the barrier is caused by modifications in paracellular transport rather than

transcellular transport. Additionally, S1P is essential for the integrity of endothelial junctions by rearranging the cytoskeleton of proteins that anchors to actin filaments i.e.; myosin light-chain kinase (MLCK) and cortactin (Dudek et al. 2004), (McVerry and Garcia 2004). Interaction with the EDG-1 receptor, activation of the downstream PI3K, and actin filament rearrangements are all necessary for S1P-regulated barrier function. Silencing at any of these stages eliminates S1P's protective function (Singleton et al. 2005), (McVerry and Garcia 2005). A protein called VE-cadherin, which localizes to regions of cell-cell contact, is peripherally enhanced by S1P (McVerry and Garcia 2005). Through the integration of information from many signaling transduction pathways, the adherens junction controls the function of the gastroepithelial barrier. The junction's calcium-dependent cell-surface component, e-cadherin, interacts with the catenin protein family to produce a cadherin-catenin complex that strengthens cell-cell adhesion and controls the intestinal epithelial barrier (McVerry and Garcia 2005). When certain antibodies are used to silence E-cadherin, the junctions are disrupted and permeability increases (Corada et al. 2001), (Corada et al. 1999). It has been shown that S1P elaborates permeability alterations through pathways involving E-cadherin, which is essential for intestinal permeability (Guo et al. 2003). The study also shows that S1P, through the S1P1/Akt/Rac small GTPase pathway, alters barrier function in endothelium and the subcellular localization of E-cadherin in differentiated Cdx2L1 cells. A functional role in S1P-induced cell-cell contacts and barrier integrity is further suggested by the co-localization of a number of proteins in cellular junctional sites upon treatment with S1P, including VE-cadherin and JAM-A (Greenspon et al. 2011) (Figure 2.7A).

Claudin family proteins, Occludins, family of Junctional Adhesion Molecules (JAMs), and adhesion molecules of the endothelium are among the protein components that make up tight junctions (Bhat et al. 2018). In ECs, mostly claudins are prevalent TJ proteins. Claudin-5 is essential for limiting the passage of big molecules across paracellular space (Cerutti and Ridley 2017). Trans membranous Claudins are composed of four domains extracellularly, one of which dimerizes with the surface of neighboring cells. Because of the PDZ domain in their cytoplasmic tail, they can attach to Zonula Occludens-1, that links the claudins with the actin cytoskeleton (Cerutti and Ridley 2017). Extracellularly possessing 4 domains, occludins dimerize with other TJ protein molecules under occludin family on nearby cells to preserve barrier integrity (Bhat et al. 2018). To stabilize tight junctions, JAMs—singly-spanning transmembrane proteins—interact with Junction Adhesion Molecules on neighboring cells (Bhat et al. 2018). Junction Adhesion Molecules come in three different varieties: Junction Adhesion Molecule-A, B, and C. While JAM-B is exclusively present on ECs, JAM-A and -C are expressed by endothelial cells as well as epithelial cells. Complexes known as adherens junctions are mostly made up of VE-cadherin on ECs (Cerutti and Ridley 2017). Transmembrane proteins called cadherins have five extracellular repeats that resemble cadherins. whereby, on the surface of nearby cells, they can homodimerize with other cadherin molecules (Mehta and Malik 2006). Two domains are present in the cytoplasmic tail of Vascular Endothelial-cadherin: a C-terminal domain that binds to plakoglobin and p120 site of catenin, and a juxta membrane domain that binds to β -catenin

(Mehta and Malik 2006). N-cadherin, generally found throughout the cell membrane instead of restricting only at the adherens junction complexes, is also expressed by ECs but not LECs. Negatively charged phosphate groups like S1P need a transporter to get exported intercellular space from the cells since they are unable to pass through the plasma membrane on their own. S1P is primarily transported by spinster homolog 2 (SPNS) in both VECs and LECs (Spiegel et al. 2019). S1PR1 is highly expressed in VECs, while S1PR2 and S1PR3 are expressed at comparatively modest levels (Lee et al. 1999). Blood S1P, carried by the high-density lipoproteins having albumin and apoM or apolipoprotein M within them, stimulates S1P receptor-1 on Vascular Endothelial Cells to sustain the function of the endothelium barrier by encouraging cell-cell contact (Maceyka and Spiegel 2014). Proia's group was the first to describe how S1P regulates the vascular endothelial barrier. They discovered that due to leakage in the blood vessels and a lack of vascular maturity, S1PR1 knockout is fatal (Liu, Wada, et al. 2000). A reciprocal connection among these receptors is suggested by the fact that mice accelerated these traits throughout embryonic development (Kono et al. 2004).

In response to S1P stimulating a monolayer of Endothelial Cells, S1PR1 facilitated the recruitment of the actin filament regulatory protein, cofilin, and the activation of Rac, which increased the Trans-Epithelial Electrical Resistance (Garcia et al. 2001). Additionally, S1P increased PI3 kinase activation, PIP3 synthesis, and the recruitment of S1P receptor-1 into microdomains, enriched with caveoli (Singleton et al. 2005). Additionally, myosin light chain kinase MLCK and myosin light chain MLC, and cortactin phosphorylation are linked to Rac activation, which results in cortical actin polymerization and tight junction remodeling (Dudek et al. 2004). In VECs, S1PR1 signaling reduces leukocyte adhesion molecules, increases endothelial nitric oxide synthase-derived NO generation (Cantalupo et al. 2017) and stabilizes adherens junctions to reduce vascular inflammation (Galvani et al. 2015), (Burg et al. 2018). Additionally, it inhibits downstream signaling and VEGFR2 phosphorylation, stabilizes junctional VE-cadherin, and decreases VEGF-induced vascular sprouting (Jung et al. 2012). S1PR1 protects growing blood vessels against abnormal angiogenesis by acting as a vascular-intrinsic stability mechanism. It promotes angiogenic signaling during tumor development that is mediated by VEGFR2 (Gaengel et al. 2012). In mouse models, it is essential for tumor vascular normalization, blood circulation improvement, and anticancer treatment enhancement (Gaengel et al. 2012). Increased expression of Sphk1 and Spns2 results from the EGR1 and STAT3 activation, which are transcription factors that act upon ECs damage. This restores vascular integrity by raising S1P, which intensifies the programming of ECs that positively express S1PR1 (Akhter et al. 2021). High-density lipoprotein penetrates endothelial barriers via particular mechanisms, as evidenced by the fact that S1PR1 activation also increases SR-BI expression. Which in turn, facilitates the protein binding and its trans-endothelial transport by aortic Endothelial Cells in humans (Velagapudi et al. 2021). S1P's ligation of S1PR2 triggers a RhoA/ROCK-dependent signaling path, which increases the permeability of the endothelial barrier and decreases actin polymerization and VE-Cadherin trafficking to adherens junctions (Velagapudi et al. 2021). In mice, S1PR2 causes endothelial inflammation by triggering NF-

$\kappa\beta$ and stress-activated kinase p38 (Zhang et al. 2013). Mice that have S1PR2 deleted are less susceptible to neutrophil infiltration and LPS-induced alterations of the BBB and barrier (Zhang et al. 2013), (Sammani et al. 2010), (Xiang et al. 2021). The role of S1P receptor-3 in the endothelial layer and its barrier activities is more debatable. It may interact with Gq, causing calcium signaling to activate eNOS and the RhoA/ROCK pathway. Adherens junctions are disrupted by NO production through the nitrosylation of p190RhoGAP, a GTPase that inhibits RhoA activation (Anwar and Mehta 2020). After ligation damage, mice with the deletion of S1PR3 had larger carotid artery lesions (Keul et al. 2011). On the other hand, P-selectin is mobilized by S1PR3 on the Endothelial cell surface, which encourages leukocyte rolling (Nussbaum et al. 2015). A boost in the adherens and the tight junctions are achieved by activating Rac through S1PR4 activation on ECs, which in turn supports BBB integrity under homeostatic settings (Hansen et al. 2022). However, increased abluminal S1P reduces S1PR4 expression under pathological circumstances and plasma S1P leakage, increasing BBB disruption linked to NF- $\kappa\beta$ and RhoA/ROCK signaling (Hansen et al. 2022). Functional antagonist of Sphingosine-1-phosphate receptor 1 & 5, Siponimod (BAF-312) increases the expression and activity of endothelial tight junctional proteins, therefore attenuating the breakdown of the blood-brain barrier caused by inflammatory circumstances (Spampinato, 2021). High amounts of SphK1, SphK2, Spns2, and, to a lesser degree, S1PR1 and S1PR3 are expressed by the lymphatic endothelium (LECs) (Pham et al. 2010). The shape of the first lymphatic vessels in non-lymphoid organs was changed in mice with a conditional deletion of lymphatic SphK1, which resulted in a loss of S1P in lymph but not in plasma. This implies that S1P is involved in the development of lymphatic vessels and that S1P may control LECs differentially in the lymph node and in the vasculature of non-lymphoid tissues (Pham et al. 2010). Lymph S1P and circulating lymphocytes are significantly reduced in Spns2 knockout mice (Mendoza et al. 2012). S1P generated from high-endothelial cells is necessary for promoting contact between dendritic cells and HEV and preserving HEV integrity via autocrine modulation of S1PR1-Gi signaling (Szandor Simmons, 2019). When C-type lectin-like receptor 2 (CLEC-2) on extravasated platelets is engaged by fibroblastic reticular cells, S1P is released locally, which increases the expression of VE-cadherin on HEV, which is essential for immunological responses (Herzog et al. 2013). To migrate CD4⁺T lymphocytes over lymphatic endothelial cells and into lymphatic arteries and draining LNs, S1PR2 on LECs—rather than S1PR1—is necessary (Xiong et al. 2019). S1PR2 inhibition alters VCAM-1 and increases the expression of VE-cadherin in buttons, as well as the tight junction-forming proteins ZO-1 and Occludin. Through ERK, S1PR2 signals control lymphatic permeability, the quantity and position of button and zipper endothelial junctions, and the interaction between CD4 T cells and LECs during translymphatic endothelial trafficking (Xiong et al. 2019).

2.8. Sphingosine-1-phosphate in gut infection

Intestinal barrier disruption has been identified as a prevalent consequence of sepsis by extensive investigation (Assimakopoulos et al. 2018). The onset of multiple organ failure and systemic

inflammation is preceded by intestinal damage (Subramanian, Geng, and Tan 2020). However, research has indicated that sepsis is linked to a greater death risk from injuries in the gastrointestinal tract (Lelubre and Vincent 2018). S1P preserves vascular endothelial cells' (ECs') integrity throughout the pathogenic phase of sepsis, encourages lymphocytic circulation, lowers host response, as well as safeguards the intestinal barrier's integrity (Rohrhofer et al. 2021), (Ziegler and Graler 2021). Clinical investigations now available have shown that patients with sepsis had lower plasma concentrations of S1P than healthy individuals (Winkler et al. 2017) and have suggested that when the severity of sepsis increases, S1P levels are further reduced (Frej et al. 2016), (Winkler et al. 2015). S1P preserves the colonic mucosal barrier, reduces intestinal epithelial cell damage brought on by LPS, and guards against intestinal damage during sepsis, according to recent research (Wang et al. 2023). During sepsis, a variety of triggers control important enzymes, which impacts sphingolipid metabolism and biosynthesis and limits the generation of S1P (Maceyka and Spiegel 2014). A study on human and baboon sepsis showed that most septic groups had S1P levels that were almost lower than those of normal controls. There was a uniform distribution of S1P between apoM and albumin, and 46% in acute septic shock. During sepsis, a strong relationship between apoM and S1P decrease was noted (Frej et al. 2016). Since hepatocytes are the main source of apoM and albumin, low S1P levels may play a greater role in the onset of multiple organ dysfunction syndrome (MODS). Additionally, it has been discovered that serum S1P levels predict early mortality in individuals with chronic liver disease and drastically decline with advanced liver disease (Mucke et al. 2020).

A noninvasive enteropathogen called *Citrobacter rodentium* has been used to simulate human Escherichia coli infections. Brush border microvilli are destroyed and actin rearrangements occur as a result of its attachment to the gut epithelium (Kenny et al. 1997). Immunocompetent mice that have an oral infection experience temporary colonization and inflammation, which peak after one week and gradually clear in two to three weeks (Mundy et al. 2005). In line with earlier findings, FTY720 resulted in peripheral blood lymphocyte and T cell entrapment in MLNs (Brinkmann et al. 2010), (Brinkmann and Lynch 2002). Mice treated with FTY720 showed more widespread pathogen distribution and decreased bacterial clearance, making them more vulnerable to colonic and systemic infection with *C. rodentium*. Unlike earlier animal models of colitis representing inflammatory bowel disease, they also showed larger colonic damage and higher disease activity during peak infection (Daniel, Sartory, Zahn, Geisslinger, et al. 2007), (Daniel, Sartory, Zahn, Schmidt, et al. 2007), (Deguchi et al. 2006), (Fujii et al. 2006), (Mizushima et al. 2004). In comparison to vehicle-treated controls, the study discovered that FTY720 inhibited lymphocyte migration to intestines infected with *C. rodentium*, resulting in a decrease in colonic T lymphocyte cells, Dendritic cells, B lymphocyte cells, and macrophages but an increase in neutrophils in two weeks of post-infection. These outcomes are comparable to those observed in animals lacking RAG1 (Simmons et al. 2003). A mostly granulocytic colonic infiltration and increased bacterial presence in peripheral organs and colons are the results of *C. rodentium* infection in these mice (Maaser

et al. 2004), (Simmons et al. 2003), (Vallance, Deng, Knodler, et al. 2002). Increased neutrophil influx is linked to upregulated expression of innate immunity and the related cytokines IL-17a, IL-6, IL-1, iNOS, and TNF- α post-infection (Higgins et al. 1999), (Smith et al. 2011). These cytokines are important in early infection for host defense and clearance of *C. rodentium* (Dann et al. 2008), (Gobert et al. 2004), (Ishigame et al. 2009), (Vallance, Deng, De Grado, et al. 2002). In order to stop the spread of germs like *C. rodentium* and regulate the bacterial burden, neutrophils are crucial for the early innate immune response (Lebeis et al. 2007), (Spehlmann et al. 2009) but they can also contribute to host tissue pathology during chronic inflammatory diseases (Ito et al. 2008), (Sartor 1994), (Southey et al. 1997). According to the study, when FTY720 is administered to infected colons on day 14, the innate immune response may make up for the reduced adaptive response. According to earlier research, FTY720 may inhibit immunological response in viral and antigen-specific virus-induced inflammation (Horga, Castillo, and Montalban 2010). With the exception of a greater frequency of lung and lower respiratory tract infections in individuals treated with fingolimod as opposed to those treated with a placebo, clinical investigations of MS patients revealed no significant side effects (Kappos et al. 2010), (Kappos et al. 2014).

It has been demonstrated that fingolimod (FTY720), which alters viral persistence and T cell activities, is useful in treating HIV remission (Li et al. 2021), (Vesper et al. 1999). Based on the research of team Monick et al., the respiratory syncytial virus-induced lung epithelial cells' neutral ceramidase and SphK activities, which resulted in the accumulation of S1P, which is in charge of the Akt/ERK survival pathways. By blocking these routes, SphK inhibition hastened cell death. SphK1 activity has been seen to increase after infection with influenza virus A, bovine viral diarrhoea virus, measles virus, and human cytomegalovirus (Monick et al. 2004), (Machesky et al. 2008), (Seo et al. 2010), (Seo et al. 2013), (Yager and Konan 2019), (Xia et al. 2018). Viral ribonucleoprotein translocation and RNA synthesis were both hampered by SphK1 inhibition (Seo et al. 2013). Inhibitors of SphK and acid ceramidase efficiently prevented the spread of the measles virus (Graffen et al. 2019) and shielded animals infected with influenza A (Xia et al. 2018). These results confirm the importance of Sphingosine Kinase-1 activation in the spread of viruses and the efficacy of SphK inhibitors in avoiding infection. The chikungunya virus host factor nuclear-localized SphK2 facilitates influenza A virus replication and sustains viral latency (Xia et al. 2018), (Reid et al. 2015), whereas SphK1 activity declines in bovine viral diarrhoea and dengue virus infections (Wati et al. 2011). Tests have been conducted on sphingosine analogs that modulate S1PR1,3-5, including AAL-R and FTY720. AALR is ineffective in simian-human with immunodeficiency and human lymphocytes infected with choriomeningitis virus, but it restricts the infiltration of T-cells and reduces pro-inflammatory responses in animal models infected with influenza viruses (Matheu et al. 2013). The human cytomegalovirus's ability to replicate is reduced by the S1PR2 antagonist JTE-013 (Kersh et al. 2009), (Walsh et al. 2010). These findings point to a

Virus	Enzyme/S1PR Modulator	Effect
S1P metabolism		
RSV infection	↑ SphK ↑ ceramidase	↑ infected lung epithelial cell survival
HCMV * infection	↑ SphK prot/mRNA	↑ virus replication
IAV infection	↑ SphK1	↑ RNA virus synthesis
	SphK1 overexpression	↑ infection susceptibility
	S1P lyase overexpression	↓ virus replication
	↓ SphK	protection of infected mice
MV infection	↓ SphK	↓ replication
BVDV infection	↑ SphK	↑ replication
DENV infection	↓ SphK1	↓ cell survival ↑ replication
CHIKV infection	↑ SphK2	↑ replication
KSV * infection	↑ SphK2	maintenance latency
Signaling		
IAV	AAL-R (S1PR1,3-5 modulator)	↓ inflammatory response
HIV	FTY720	↓ circulating T and B cells
LCMV *	FTY720	no lymphopenia, no effect on replication
HCMV *	JTE-013 (S1PR2 antagonist)	↓ replication

Table 2.8A: Effect of Sphingolipid and sphingolipid modulators in infectious disease. (Meacci, Garcia-Gil, and Pierucci 2020)

possible involvement of specific S1PR signaling in some viral infections. SARS-CoV-2 immunology and cell response to infections depend on the S1P/S1P signaling pathway, which can be modulated by inhibitors, agonists, or antagonists to viral entrance blockage, replication, release, or virus-induced inflammatory reaction. SL analogs, like Fingolimod (FTY720), may be helpful for COVID-19 patients by protecting the central nervous system and improving lung endothelial cell integrity, but the risks and side effects of FTY720 treatment may limit its use (Vestri et al. 2017), (Gold et al. 2014); newer analogs with minor side effects are now available (Grassi et al. 2019). Clinical trials targeting S1PRs and S1P metabolism have been carried out for a variety of pathologies, including inflammatory diseases. Additional research and experimental confirmation of the significance of Sphingosine-1-phosphate signaling and metabolic process in infection in other tissues are required (Table 2.8A).

Enterotoxigenic *E. coli* is a pivotal pathogen for studying enteric infections due to its significant global health impact, well-characterized mechanisms of pathogenesis, and relevance to broader issues such as antibiotic resistance and vaccine development. Research on ETEC not only advances our understanding of diarrheal diseases but also contributes to the development of effective prevention and treatment strategies, ultimately improving public health outcomes. Enteric infections, particularly caused by enterotoxigenic *Escherichia coli* (ETEC), are a major cause of diarrheal diseases, especially in developing countries. ETEC produces heat-labile (LT) and heat-stable (ST) enterotoxins, which disrupt intestinal epithelial cell function, leading to fluid secretion and diarrhea. The interplay between S1P signaling and ETEC infection is an emerging area of research with several potential connections. ETEC

serves as an excellent model for studying host-pathogen interactions in the gut. It interacts with intestinal epithelial cells, immune cells, and the gut microbiota, providing a comprehensive system to explore these complex relationships.

2.9. Enterotoxigenic *Escherichia coli*: Discovery and pathogenesis

Rice-water diarrhoea and collapse were often caused by *Vibrio cholerae* infection. However, non-cholera vibrios can also cause it (McIntyre et al. 1965). A significant percentage of patients had no known etiologic agent (Carpenter et al. 1965). Despite advanced stool culture methods, this group remains a mystery. In 1968, 17 hospitalized patients at the Infectious Diseases Hospital, Calcutta, India, who were diagnosed with severe diarrhoea without enteric pathogens were studied for small bowel perfusion and intubation (Gorbach et al. 1971), (Banwell et al. 1971). It was discovered that the majority of these individuals had either one or both of these *Escherichia coli* infections in their small intestines. In 1973, a study with Twenty-five enterotoxigenic (tox +) and sixteen non-enterotoxigenic (tox -) *E. coli* strains, collected from India, Bangladesh, and Chicago, U.S.A, established a relationship existing between the vascular permeability factor activity of *E. coli* and the diarrhoeagenic component. Anti-H10407 antisera, raised in rabbits by immunization, showed the neutralizing effect of the *E. coli* enterotoxin (ECT) of those strains (Evans, Evans, and Gorbach 1973). In an epidemiologic study conducted in Bangladesh, before 1973 (Sharif et al. 2023), the documented ETEC O78:H11:K80 strain, was isolated from an infected adult exhibiting signs similar to cholera. It was then demonstrated to cause diarrhoea in adult volunteers (Coster et al. 2007), (Evans et al. 1978). Lot 0519 of the Walter Reed Army Institute of Research (WRAIR) cGMP stock, which was produced in February 1998, included the *E. coli* H10407 strain that was sequenced. The whole genome was sequenced using small-insert libraries of pUC19 (insert size: 2.8 to 5 kb) and pMAQ1b (insert size: 5.5 to 10 kb) to a depth of 8× coverage (Iguchi et al. 2009).

According to the Department of Immunization, Vaccines and Biologicals (IVB) of the World Health Organization (WHO) on 31st May 2021, among several causes, it was reported that the main cause of morbidity and death rate in the globe is diarrhoeal illness. It is predicted that diarrhoea among children under the age of five is accountable for around 446,000 deaths, primarily in South Asia and sub-Saharan Africa (390 894–504 613). ETEC, or enterotoxigenic, is one of the *Escherichia coli* major causes of diarrhoea caused by bacteria, particularly in children living in lower- middle-income countries (LMIC), tourists, and military personnel from wealthy nations. ETEC is classified into three genotypes according to the presence of the toxin genes that produce heat-labile toxin or LT-ETEC, heat-stable toxin or ST ETEC, or ST/LT-ETEC both. Geographically, patients with traveller's diarrhoea or asymptomatic carriers have different proportional proportions of heat labile (LT), heat stable (ST), and LT/ST toxin-generating ETEC. Since ETEC strains that express solely LT are more commonly isolated from healthy

individuals than from sick ones, they may be viewed as less significant pathogens in particular communities (Isidean et al. 2011).

ETEC is thought to be responsible for 220 million diarrhoea episodes worldwide, of which 75 million occur in children below five. Based on projections from Maternal Child Epidemiology and the Institute for Health Metrics and Evaluation, these episodes cause between 18,700 and 42,000 deaths in children under five years old. A strategy that can successfully lower the mortality and morbidity burden of ETEC diarrhoea in children aged 0 to 5 will influence the long-term effects of infection linked to malnutrition, which raises the chance of dying from other infectious diseases and causes poor physical and cognitive development. By promoting better social and economic development, such an intervention could prevent 4.2–6.0% of fatalities from diarrhoea in children under five (Khalil et al. 2018) and provide older children, adolescents, and adults with important, albeit currently underappreciated, public health benefits. Though the ETEC and other pathogen diarrhoeal death rates are decreasing as a result of advancements in economic development and access to clean water and sanitary facilities, these decreases have not been accompanied by appreciable drops in diarrhoea-associated morbidity, which still has a detrimental effect on the infants and children health of in many LMICs.

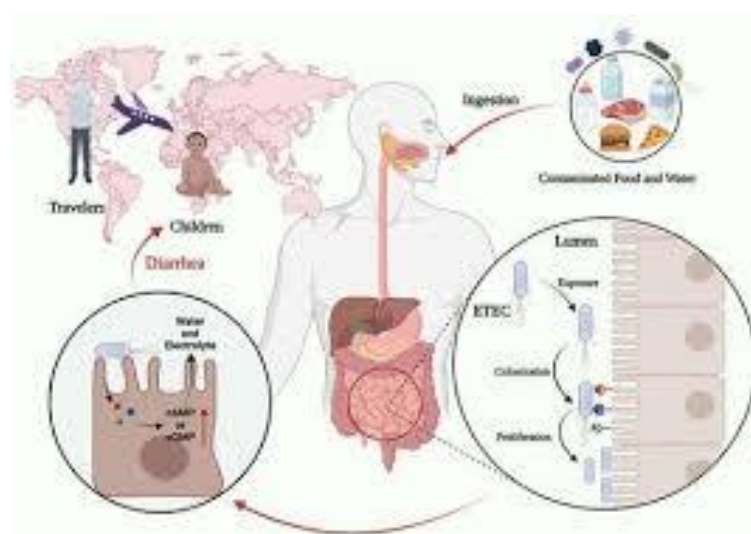


Figure 2.9A: Mode of ETEC infection by contamination and outbreak. ETEC toxins in disrupting mucosal homeostasis and induce water loss creating diarrhoea phenotype. (Zhang, 2022)

Children at the age of 9 months to 24 months are frequently encounter enteric illness caused by ETEC. There are alternatives for prevention and treatment of ETEC-related diarrhoeal sickness, which are crucial for preventing and lowering the high disease burden; However, in environments with limited resources, their sustainability and execution are not always feasible. Antibiotics are widely used, frequently recommended empirically to treat diarrhoea, or used over-the-counter in Antimicrobial resistant (AMR) strains of ETEC and other bacteria are more likely to proliferate in certain LMICs. An ETEC vaccine is expected to have important indirect effects and possibly direct, individual effects on

ETEC mortality and morbidity. These include lowering antibiotic use and AMR bacterial prevalence, enhancing community-level herd protection, reducing healthcare costs by preventing malnutrition, and enhancing the physical and cognitive development of children. A global stakeholder approach to "Leave No One Behind" for the years 2021–2030 is the Immunization Agenda 2030 (IA2030) (Global Vaccination and Immunization Strategy (2021-2030)). It contains the main objectives. to: 1) lower disease burden by expanding access to and adoption of novel vaccinations; and 2) lower death rate and morbidity due to vaccine-avoidable diseases throughout the life. For the past 20 years, WHO has prioritized the development of ETEC vaccines. The children in LMICs who encounter ETEC infections generally show symptomatic or non-symptomatic diarrhoeal episodes maybe because of the multiple pathotypes of ETEC with combinations of enterotoxin and colonization factor. A protective immunity has been shown to be developed alongside the increased exposure and age which therefore decreases the symptomatic illness of ETEC diarrhoea (Nataro and Kaper 1998), (Isidean et al. 2011), (Dutta S, 2014). ETEC is also prevalent among the travelers who are visiting LMICs (Barrett and Brown 2016), (Riddle et al. 2006), (Olson et al. 2019) consisting of about 30% of the global travelers travelling to the high prevalence states, such as the Middle East, Caribbean, and Latin America (Riddle et al. 2006) (Figure 2.9A).

Chad K. Portera, et. al. (Porter et al. 2011) mentioned in their systemic review of infection models with enterotoxigenic *Escherichia. coli* or ETEC that human isolates of the diarrhoea-causing ETEC just like the porcine isolates were capable of inducing small intestinal tissue fluid efflux in rabbit ileal loop model, however only human clinical isolates were able of inducing watery diarrhoea in human due to its species-specific tropism. The three most commonly administered LT and ST enterotoxin-positive strains are H10407, E24377A and B7A. The diarrhoea attack rate was shown to be dose-dependent, with greater attack rates corresponding to larger dosages for these three strains. Among several symptoms associated with ETEC diarrhoea, nausea, headaches, and stomach cramps/pain were somewhat prevalent with all dosages and strains; however, vomiting was most commonly observed after the introduction of the strains LSN03-016011/A, B7A, and H10407. Notably, fever rates were quite low for all strains except B7A and H10407. The H10407 strain had much more severe symptoms than other strains, which showed a range of severity in these ETEC-associated symptoms. Adult ETEC-associated diarrhea has always been thought to be a minor, self-limiting sickness, but recent studies have raised the possibility that there may be a connection to post-infectious consequences. Distinct meta-analyses data are showing (Halvorson, Schlett, and Riddle 2006; Thabane, Kottachchi, and Marshall 2007), ETEC commonly causes acute infectious gastroenteritis (IGE), which increases the incidence of post-infectious irritable bowel syndrome by seven times. Furthermore, two investigations (Okhuysen et al. 2004; Tuteja et al. 2008) have discovered this connection in regions where ETEC prevalence is high.

2.10. Animal models in acute watery diarrhoea caused by ETEC and other pathogens

Since there are now no alternative techniques for developing treatment methods, animal models remain essential (Table 2.10A). Evaluating immunological reactions and guaranteeing efficacy and safety prior to human studies. Generally speaking, there are three types of animal models: those used to assess immune responses, those that mimic natural or surrogate disease models, and those intended for experimental or surgical procedures. Back in 2022 a group of scientists (Tew et al. 2024) (Hui Xian T, et. al. 2022) used a model of adult female BALB/c mice to assess MyChol, the proto- vaccine's cross-protection effectiveness against ETEC H10407. ETEC produces a heat-labile enterotoxin that is comparable to *V. cholerae*. Two copies of CT-B are also present in this prototype vaccine, MyChol, which overexpresses the CT's B component. Antibodies that can successfully cross-react with and neutralize the LT-B from ETEC (Xian et al. 2019), (Xian et al. 2020). Another vaccine Dukoral, containing the Inactivated *V. cholerae* and a recombinant CT-B component both present in it, offers temporary defense against travelers' diarrhoea due to the CT generated by *V. cholerae* and got pre-qualified by WHO (Hui Xian et al. 2022). However later, one of the biggest obstacles in the development and assessment of potential vaccines was the fact that only humans are inherently susceptible to cholera infection with *V. cholerae* (Nygren et al. 2009). According to (Hui Xian T, et. al. 2022), in BALB/c mice, 1×10^9 CFU/200 μ L of ETEC H10407 constituted a 100% fatal dose (LD100), and these findings are comparable to the previously documented studies. Following both the initial and booster doses of the MyChol vaccination, the mice showed excellent tolerance against ETEC immunization with a dose of LD100. For a maximum of 28 days, no negative responses or fatalities were noted. When the immunized animals were exposed to CT, their systemic IgG and IgA immune responses rose, which may have been caused by cross-reactivity with the LT toxin generated by ETEC. The vaccinated mice had a 100% survival rate in the ETEC H10407 challenge tests and shown no symptoms of weight loss or diarrhoea, and following a 24-hour ETEC challenge, their intestinal fluid accumulation ratio was lower than that of the unimmunized mice. The intestinal histological sections of the immunized animals showed no signs of villi loss or injury.

These findings suggest that the immunized mice produced anti-LT and anti-LT-B antibodies. It prevented LT from being endocytosed into the cell by blocking the LT and LT-B components of ETEC from attaching to the GM1 ganglioside receptors on the intestinal epithelial cells. These findings are consistent with earlier reports (Liu, Zhang, et al. 2020), (Zhao et al. 2022).

Animal models	Areas of research	Advantages	Disadvantages
Adult mice	• Reactogenicity • Immunogenicity • Protective efficacy	• Cost-effective • Easily handling • Easily accessible • Short reproductive cycle • Genetic engineering accessible for study Immune system of mice resembles humans and is well characterised • Beneficial for early vaccine screening and basic research	• Small animal • Difficult to collect and analyse the samples • Require antibiotics • No disease symptoms
Rat	• Safety • Toxicity	• Cost-effective • Easily handling • Easily accessible • Short reproductive cycle	• Not susceptible to <i>Vibrio cholerae</i>
Infant rabbit	• Colonisation potential • Immunogenicity • Protective efficacy	• Cost-effective • Susceptible to cholera toxin	• Immature immune system • No disease symptoms
Adult rabbit	• Reactogenicity • Immunogenicity • Protective efficacy	• Cost-effective • Susceptible to cholera toxin • Can perform surgical	• Require antibiotics • Require surgical technician

Table 2.10A: Table represents suitable animals for ETEC infection and their advantages and disadvantages and their utility in various research area. (Tew et al. 2024)

2.11. ETEC infection- induced immune response

In decades of research in ETEC infection and the disease etiology, scientists across the world have looked at the host immunity and the type of immune reaction triggered when ETEC infects. Several virulence factors are associated with ETEC. The overproduction of cyclic adenosine monophosphate (cAMP) is induced by LT and ST. and, in turn, cyclic guanosine monophosphate (cGMP) also increases. Protein kinase A or PKA and cyclic GMP-dependent protein kinase II-regulated pathways are activated by elevated cAMP and cGMP levels, which ultimately result in diarrhoea, chloride secretion, and activation of the cystic fibrosis transmembrane conductance regulator (CFTR) (Fleckenstein et al. 2010). Numerous intestinal processes, including tight junctions (TJ) (Guttman et al. 2006), (Guttman and Finlay 2009), aquaporins (Guttman et al. 2007), and the inflammatory response (Sham et al. 2011), are interfered with by enteric pathogens and their enterotoxins. It may cause diarrhoea in an indirect way (Guttman and Finlay 2008). The vast majority are conscious that ETEC infection connects with the NF-kB pathway and MAPKinase to induce pro-inflammatory responses in IPEC-1 and IPEC J2 cells of the pig intestinal epithelium (Sargeant, Miller, and Shaw 2011), (Zanello et al. 2011). However, given the large number of genes implicated in the NF-kB or MAPK pathway, it is still unknown which gene the ETEC infection intends to modify. NF-kB or MAPK pathway. Goblet cell mucus layer, paneth cell antimicrobial peptides, and secretory IgA all affect mucosal defense and homeostasis. The NF-kB

pathway was activated in mice infected with ETEC strain SEC470, according to Ren et al., as a pro-inflammatory response to the infection. This is an ETEC isolate from a piglet of 39 days old with diarrhoea in Jingxi Province from China. (Tang et al. 2014), (Tang et al. 2015). They also found an increased small intestinal expression of proinflammatory Interleukin-6, 17, 18, 1 β , and TNF- α upon ETEC infection. Antimicrobial peptides, which are released by intestinal paneth cells, are essential for maintaining mucosal defense and homeostasis (Bevins and Salzman 2011). These antimicrobial peptides include lysozyme C, secretory group IIA phospholipase A2 (Spla2), α -defensins, CRS, also known as cryptdin-related sequence peptides, Reg3g, a C-type lectin and Ang4 or RNase angiogenin 4 in mice (Bevins and Salzman 2011). Wenkai Ren et. al. 2014 found that ETEC dramatically reduced the expression of Lyzs (lysozyme), but had minimal impact on other jejunum genes and markedly elevated cysteine-rich sequence 4C expression. Overall, the researchers found that the ETEC infection increases intestinal production of many proinflammatory cytokines, including IL-1 β . TNF- α , IL-6, IL-17, and IL-18. ETEC infection raises Rel expression and causes p38 and ERK1/2 protein phosphorylation, which is not surprising given the critical roles that NF- κ B and MAPK pathways play in bacterially driven inflammatory reactions. The intestine TLR4, which interacts with the lipopolysaccharides from SEC470 bacteria, may mediate these controls. Additionally, a prior study showed that the activation of the NF- κ B and MPK pathways results from ETEC adhesion to intestinal epithelial cells (Wang and Hardwidge 2012) (Figure 2.11A).

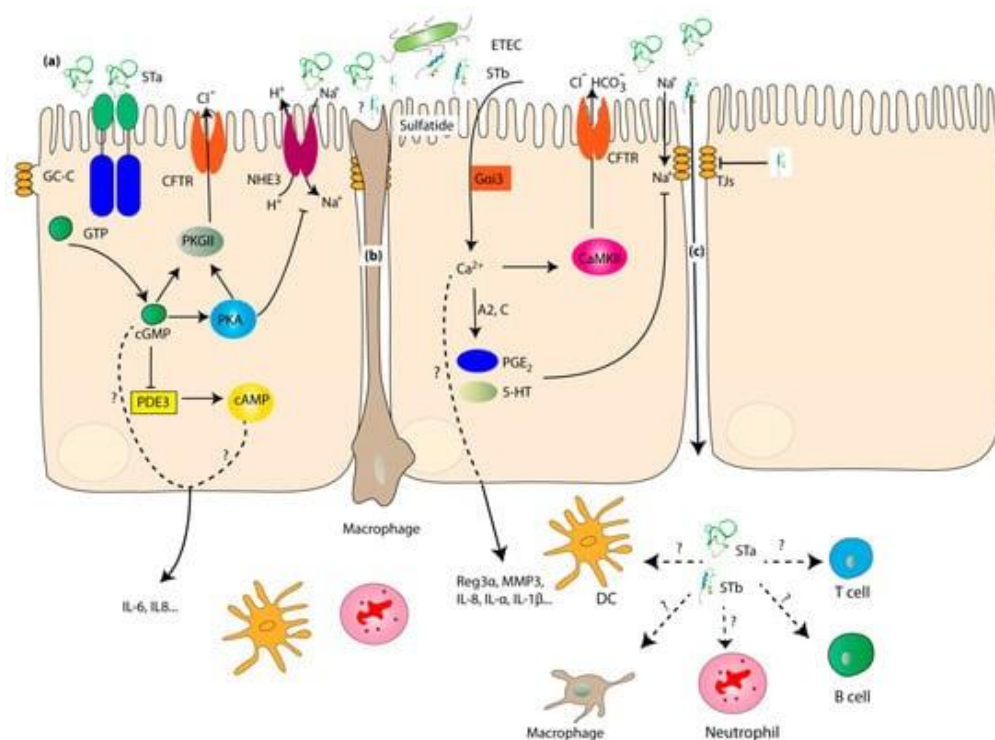


Figure 2.11A: ETEC toxins cause inflammation in the mucosa, and STs impact paracellular transport, transepithelial dendrites, and apical membranes. Proteins such as GC-C, CFTR, PKA,

NHE3, PDE3, PKGII, Gai3, PGE2, 5-HT, A2, C, CaMKII, TJs, and MMP1 are important players. These toxins also impact paracellular trafficking, transepithelial dendrites, and apical membranes. The study emphasizes how critical it is to comprehend these toxins for the health of mucosa (Wang, 2019).

2.12. ETEC infection and intestinal barrier disruption

ETEC strains have been found to impact the epithelial permeability barrier by reducing the number of TJ strands without lowering the TJ depth, keeping TJ's sealing capabilities unaltered (Dubreuil 2017). However, Roselli and colleagues demonstrated that a pig ETEC strain (K88) decreased abundance of occludin in swine IPEC-1 intestinal cells (Roselli et al. 2007), and mislocalization of ZO-1 in Caco-2 cells human large intestinal cells (Roselli et al. 2003), and TJ permeability as determined by Trans Epithelial Electrical Resistance (TEER). Gao et al. (Gao et al. 2013) found that the ETEC K88 challenge significantly impacted claudin-1 expression in the ileum whereas only ZO-2 was significantly decreased in the duodenum. Concerning the impact of probiotics, Yu et al. (2012) discovered that *Lactobacillus amylophilus* D14 might lessen the harm that *E. coli* K88 caused to cell integrity, as seen by decreased dextran permeability and increased TEER (Yu, Wang, and Yang 2012). Assessed on Caco-2 cells, this strain's protective properties showed that coculturing with *E. coli* K88 enhanced the expression and improved the distribution of Tight Junction maintaining proteins (Zonula Occludens type-1, claudin-1, and E-cadherin). Following the ETEC K88 challenge, pigs' duodenum and jejunum showed stunted villi and deeper crypt in addition to decreased expression of Zonula Occludens-1 and occludin (Yang et al. 2014). *Lactobacillus plantarum*'s preventive impact on diarrhea showed a connection between dietary *L. plantarum* and Enterotoxigenic *E. coli* because it enhanced barrier function by maintaining gastrointestinal permeability and shape, and more specifically, the expression of ZO-1 and occludin genes. The IPEC-J2 cell model was also pre-treated with *L. plantarum* for 6 hours, which stopped the ETEC-induced decrease in claudin-1, occludin, and ZO-1 protein production as well as the decline in TEER (Wu et al. 2016). Like guanylin, STa enterotoxin decreases ter in T84 epithelium polarized cell monolayers at doses greater than 4 μ M. While paracellular permeability is increased by both substances, STa-induced decrease for ETEC bacteria to attach and elaborate STa toxin takes place at specific epithelial locations. This implies that STa is the cause of mild intestinal barrier failure and triggers water secretion by activating guanylate cyclase C (Nakashima, Kamata, and Nishikawa 2013). Johnson et al. (2010) found that LT enterotoxin-independent reduction of host TEER occurs without TJ protein mislocalization (Johnson, Kaushik, and Hardwidge 2010). While ETEC strains have been shown to reduce TEER in infected IPEC-J2 cells, there have been no discernible alterations in the expression of the TJ protein. According to a different research, polarized T84 epithelial cell monolayers exhibited a decrease in TEER that was reversible up to 48 hours after infection. With a defect ranging from 4 to 67 kDa, LT enterotoxin results in intestinal barrier dysfunction by generating barrier

permeability for big molecules. Through cAMP, LT enterotoxin independently induces barrier permeability for big molecules (Kreisberg et al. 2011).

While the role of S1P in ETEC infection is not fully understood, emerging evidence suggests that S1P signaling could be a key player in host-pathogen interactions. Future research should focus on:

- Elucidating the specific mechanisms by which S1P influences ETEC-induced diarrhea and inflammation.
- Investigating the potential of S1P-based therapies to treat or prevent ETEC infections.
- Exploring the interplay between S1P and other signaling pathways activated by ETEC enterotoxins.

In summary, S1P signaling is a promising area of research in the context of ETEC infections, with potential implications for understanding pathogenesis and developing novel therapeutic strategies. All these decades of research contributed in building our research objectives and helped in constructing experiments.

Chapter 3:

Objectives of the

thesis

3. Objectives

I attempted to study the following three objectives in this thesis:

Objective 1 : The function of S1P in intestinal barrier regulation.

Objective 2 : The role of S1P in gut immunology

Objective 3 : The role of S1P in ETEC induced infection model and its prevention

Chapter 4:

Materials and

Methods

4. Materials used in this study

4.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific) and antibiotics (streptomycin, penicillin) were purchased from Sigma-Aldrich. For tissue culture, T-25 tissue culture flasks and 96 well flat bottom, 96 well flat bottom assay plates, and Transwell system were used. For mouse colon organoid culture Mouse OGM kit and gentle cell dissociation reagent (GCDR) were purchased from Stem cell systems. Matrigel matrix was purchased from Corning.

Sphingosine-1-phosphate, Dimethyl sulfoxide (DMS), FTY720 as a functional inhibitor of S1P, and Dextran Sodium Sulfate (DSS), FITC-Dextran 4kD were purchased from Sigma-Aldrich. The m-siSPHK-1 and h-siSPHK-1. Lipofectamin 2000 was purchased from Invitrogen. Other general chemicals used in the present study were of analytical grade and were purchased from Sigma-Aldrich. For cytokine and chemokine analysis for TNF- α , IFN- γ , IL-6, IL-10, CXCL-1, and CXCL-2 was used.

All the polyclonal antibodies used are- anti-GAPDH, anti-Occludin, anti-ZO-1, anti-Claudin-4, anti-Claudin-1, 2, and anti-JAM-A. Secondary antibodies used were HRP-conjugated anti-IgG, AP-conjugated IgG, and FITC-conjugated IgG purchased from Abcam. HRP substrate and NBT-BCIP were used for western blot development. Trizol, cDNA kit, and SYBR green super mix were purchased from TaKaRa. Antibodies for Flow cytometry and immune fluorescence were: mouse FITC-CD3 (cat no. 100204, BioLegend), mouse anti-CD4, mouse anti-CD8, Mouse anti-FITC-CD11b (cat no. 11-0112-85, e Biosciences), mouse anti-A700-Ly-6G (cat no. 127621, BioLegend), mouse anti-NK1.1, mouse anti-PerCP-CD19 (cat no. 115532, BioLegend), mouse anti-CD25, anti-FoxP3, and anti-MPO antibody and Fc blocker was used. The following antibodies were used in western blotting: p- Akt 1/2/3 (Ser 473) (cat no. AF0016, Affinity), Akt (cat no. GTX121937, Gene 99Tex), P-mTOR (Ser 2448) (cat no. 5536T, Cell Signaling Technology), and mTOR (7C10).

De Man, Rogosa and Sharp (MRS) broth (BD 288130) and agar (M641, Himedia), Luria Bertini broth (BD 244620), Bifidobacterium broth (M1395, Himedia) and Macckonkey agar (BD 211387) were purchased from BD biosciences.

Probiotic strains were Lactobacillus casei ATCC 393, Lactococcus lactis ATCC 49032, Bifidobacterium bifidum ATCC 11863. Diarrhea was induced with ETEC H10407 strain which is both LT and ST positive.

4.1.1. Primer sets used in this study-

	Gene	Accession no.	Sequences	Product length
1	m-iNOS	>NM_001313922 .1	5'CAGCTGGGCTGTACAAACCTT3' (Tm- 57.8) 5'CATTGGAAGTGAAGCGGTTCG3' (Tm- 56.7)	95
2	m-Mucin-2	>NM_023566.4	5'ATGTCCTGACCAAGAGCGAA3' (T- 56.2) 5'GATTTGAAGGCCACCACGTT3' (Tm- 56.0)	142
3	m-Cathelicidin	>NM_009921.2	5'-GGCAGCTACCTGAGCAATGT-3' (Tm- 59.6) 5'-CTGTGCACCAGGCTCGTTA-3' (Tm - 59.8)	122
4	m-Hepcidin	>NM_032541.2	5'AGGGCAGACATTGCGATACC3' (Tm- 57.5) 5'GCAACAGATACCACACTGGGA3' (Tm- 57)	111
5	mClaudin-4	>NM_009903.2	5'TCGTGGGTGCTCTGGGGATGCTT3' (Tm- 65.0) 5'GCGGATGACGTTGTGAGCGGTC3' (Tm- 62.8)	170
6	m-Occludin	>NM_001360538 .1	5'TCACTTTTCTGCGGTGACT3' (Tm- 59.53) 5'GGGAACGTGGCCGATATAATG3' (Tm - 58.93)	138
7	m-Claudin-2	>NM_001410421 .1	5'TATGTTGGTGCCAGCATTGT3' (Tm- 58.08) 5'TCATGCCCACCACAGAGATA3' (Tm - 58.12)	205
8	m-GAPDH	>NM_001411843. 1	5'ACCCAGAAGACTGTGGATGG3' (Tm- 59.01) 5'CACATTGGGGTAGGAACAC3' (Tm - 55.49)	170

9	mClaudin-1	>NG_021418.1	5'CTGGGAGGTGCCCTACTTTG3' (Tm- 60.04) 5'GCTGGAAGGTGCAGGTTTTG3' (Tm- 59.97)	90
10	m-ZO-1	>XM_006540779 .5	5'TCTTCCATCATTTTCGCTGTGT3' 5'TCTGAAACCATCAAGTCCACA3'	107
11	m-Claudin-5	>NM_013805.4	5'CTTTGTTACCTTGACCGGCG3' 5'CCCAGCTCGTACTTCTGTGA3'	198
12	m-JAM-A	>NM_172647.2	5'CTGATCTTTGACCCCGTGAC3' 5'ACCAGACGCCAAAAATCAAG3'	187
13	m-TNF- α	>NM_013693.3	5'CATCTTCTCAAAATTCGAGTGACA A3' (Tm- 55.9) 5'TGGGAGTAGACAAGGTACAACCC3 ' (Tm- 59.4)	175
14	m-IFN- γ	>NM_008337.4	5'TCAAGTGGCATAGATGTGGAAGAA 3' (Tm- 56.1) 5'TGGCTCTGCAGGATTTTCATG3' (Tm- 55.7)	92
15	m-IL-6		5'TGCTTCCCCATCTCTCATGC3' (Tm- 59.82) 5'CTGTCTGGAAAAAGTGCCGC3' (Tm- 60.04)	75
16	m-IL-10	>NM_010548.2	5'GGTTGCCAAGCCTTATCGGA3' (Tm- 57.7) 5'ACCTGCTCCACTGCCTTGCT3' (Tm- 61.9)	191
17	m-TGF- β	>NM_001429648 .1	5'CCTCGAGACAGGCCATTTGT3' (Tm- 57.4) 5'GCCAGCTGACTGCTTTTCTG3' (Tm- 56.8)	125
18	Hu IL-8	>NM_001354 840.3	5'CTGGCCGTGGCTCTCTTG3' 5'CCTTGGCAAAACTGCACCTT3'	69
19	HuCXCL-1	>NM_001511.4	5'AGTGGCACTGCTGCTCCT3' 5'TGGATGTTCTTGGGGTGAAT3'	123
20	HuCXCL-2	>NM_002089.4	5'CTGCTCCTGCTCCTGGTG3' 5'AGGGTCTGCAAGCACTGG3'	86

21	Firm934 (F) Firm1060 (R)		5'GGACGATGTGGTTTAATTCGAAGC A3' (Tm- 58) 5'AGCTGACGACAACCATGCAC3' (Tm- 58.2)	
22	Bac960 (F) Bac1100 (R)		5'GTTTAATTCGATGATACGCGAG3' (Tm- 51.7) 5'TTAACCCGACACCTCACGG3' (Tm- 57)	
23	Bacterial 16sRNA27 (F) Bacterial 16SrRNA1525 (R)		5'AGAGTTTGATCCTGGCTCAG3' (Tm- 54.3) 5'AAGGAGGTGWTCCARCC3' (Tm- 52.9)	
24	Eubacteria		5'AGAGTTTGATCCTGGCTCAG3' (Tm- 54.3) 5'GCCTTAAACCCTRCGCTT3' (Tm- 54.2)	
25	Hu Cadherin- 1	>NM_001317185 .2	5'GAAGGTGACAGAGCCTCTGGAT3' (Tm 61.48) 5'GATCGGTTACCGTGATCAAAAT3' (Tm 56.86)	122
26	Hu Claudin-2	>XM_054328085 .1	5'GAGG AGGGACTGCTCCCTTGTCTT3' 5'GATTAGAGGTGTTCAAGG3'	60
26	Hu GAPDH	>NM_002046.7	5'GATTTGGTCGTATTGGGCGC3' (Tm- 56.9) 5'TTCCCGTTCTCAGCCTTGAC3' (Tm- 57.2)	169
27	Hu SphK1	>NM_001355139 .2	5'CTGGCAGCTTCCTTGAACCAT3' (Tm- 58) 5'TGTGCAGAGACAGCAGGTTCA3' (Tm- 57.4)	124
28	Hu-Claudin-4	>NM_001305.5	5'CATCGGCAGCAACATTGTCAC3' (Tm- 57.2) 5'GCACCACGCAGTTCATCCATAG3' (Tm- 58.4)	65

29	Hu-Occludin	>NM_021101.5	5'GCACATACCTTCATGTGGCTCAG3' (Tm- 58.1) 5'TGGAACAGAGCACAAACATGTCA3' (Tm-57.1)	91
30	Hu-Claudin-1	>NM_021101.5	5'CTGGGAGGTGCCCTACTTTG3' (Tm- 57.7) 5'GCTGGAAGGTGCAGGTTTTG3' (Tm- 57.1)	89
31	Butyryl-CoA Co transferase		5'GCIGAICATTTCACITGGAAYWSITG GCAYATG3' (Tm- 66.3) 5'CCTGCCTTTGCAATRTCIACRAANG C3' (Tm- 61.5)	

4.2. Methodology

4.2.1. Cell culture

In vitro experiments were carried away with human colorectal adenocarcinoma (HT29) cell line, murine macrophage RAW264.7 cell line and human embryonic intestinal Int 407 cell line. Cells were cultured in DMEM high glucose medium or RPMI-1640 medium supplemented with 10% FBS with 100 U/ml penicillin and 100 µg/ml streptomycin in humidified air/CO₂ (5% CO₂) at 37 °C within T25 flasks.

4.2.2. Cell viability assay or MTT assay for FTY720

The cytotoxicity was measured using a Tetrazolium-based MTT reagent. Cells upon treatment at different concentrations and time introduced to MTT (0.5mg/ml) for 4-5 hours. The purple formazan that was produced by the live cells was then dissolved in DMSO after discarding the cell sup. OD of the solution was measured in spectrophotometer in 590 nm. The optical density or OD of control cells were considered as 100% viability and compared to that viability of cells from each and all other groups were calculated.

4.2.3. Trans Epithelial Electrical Resistance in vitro analysis

Transwell inserts provide a reliable model once the integrity of the cell monolayer is verified. HT-29 cells are originally human adenocarcinoma cell lines that have a structural resemblance to gut epithelial cells. These HT-29 cells were seeded with the concentration of 2×10^5 cells per Transwell insert and cultured with DMEM and 10% FBS. Cells were allowed to grow and differentiate for 14 every day. Cells having an initial TEER value of $\sim 250 \Omega\text{cm}^2$ were treated with FTY720 at different concentrations, dissolved in complete medium and added to the apical chambers of the transwell system.

While the basal chambers were filled only with the complete medium. Cells are maintained at the 37 °C in a 5 % CO₂ humidified atmosphere. TEER was measured repeatedly at several time intervals.

4.2.4. FITC-Dextran paracellular permeability assay in vitro

Complete DMEM media supplemented with FITC -Dextran (1mg/ml) and was added to the apical chamber of a transwell system where HT-29 cells were allowed to form uniform monolayer and incubated with and with FTY720 at different concentration. Upon FTY720 administration, the paracellular influx of FITC-Dextran from the apical chamber to the basal chamber was measured at different time intervals. A small portion of the basal media was drawn out of the system at every 2 hours of the treatment. A standard curve of FITC-Dextran was generated using serial dilutions of FD 4 from 1000 µg/ml – 0. Medium containing FITC-Dextran was determined using a multimode reader at an excitation and emission wavelength of 485 nm and 520 nm.

4.2.5. Gene silencing using siRNA

HT-29, with a concentration of 2×10^6 cells per well, was seeded in 6 well plates and cultured with DMEM with 10% FBS. Cells were incubated with serum free DMEM for 1 hour prior to transfection. (conc) siSPHK-1, negative scrambled RNA (conc) and lipofectamine-2000 was added separately to 50 µl aliquots of serum free media and kept aside for 5-10 minutes. After 10 minutes, 50 ul lipofectamine and 50 ul siSPHK-1 were mixed and introduced to cell. After 4 hrs medium with lipofectamine was changed with fresh growth medium. After 24hrs cells were used to check expressions of various genes and proteins.

4.2.6. Mice and Animal Ethics

C57BL/6 mice (6 weeks old) were procured from the ICMR-National Institute for the Research in Bacterial Infections' animal facility. All the protocol for the study was approved by the Institutional Animal Ethics Committee of ICMR-NIRBI, Kolkata, India (PRO/151/July 2018–June2021). Experiments have been carried out in accordance with the guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India.

4.2.7. Treatment with FTY720

All mice were housed in cages containing straw bedding held in pathogen-free facilities maintained at 24°C with a 50% relative humidity and 12-h light: dark cycle. All mice had ad libitum access to standard rodent chow. The mice were divided into two groups (i.e.- Control and FTY720; n=5/ group). Mice in the FTY720 group were given 3 mg/kg body weight (Choi et al. 2011), (Rau et al. 2011) of FTY720 orally for 14 days, while mice from the control group were given normal saline. Food and water

consumption was monitored throughout the experimental period. The body weights of the mice were also measured at regular time intervals. The mice were sacrificed on day.

4.2.8. Interference of ETEC growth with probiotic species in Co-culture

To investigate the effect of probiotic species on the growth kinetics of ETEC H10407, we performed a Co-culture assay. For this experiment, 50 ml of single-strength Tryptic Soy Broth (TSB; BD, USA) and De Man-Rogosa-Sharpe (MRS; HiMedia) broth were inoculated with 1×10^8 CFU of ETEC and probiotic species (*Lactobacillus spp.*, *Lactococcus spp.* and *Bifidobacterium spp.*) respectively, and were incubated at 37°C with a constant shaking of 150 rpm. Similarly, 50 ml of co-culture medium containing 25 ml of each of double strength TSB and MRS broth (TSB: MRS-1:1) were inoculated with 1×10^8 CFU of both ETEC and probiotics species simultaneously. 100 µl mono-cultures of both ETEC and probiotic as well as 100 µl of co-cultures were collected at 0, 2, 4, 8, 10, 12, 24 h and lawn cultured in appropriate agar plates to determine the viable cell count. The plates were incubated overnight at 37°C, and the colonies were counted. The tests were performed in triplicates.

4.2.9. Cellular cytotoxicity assay

HT-29 and Raw-264.7 cells were seeded in separate 96-well plates (1×10^5 cells/well) and incubated at 37°C with 5% CO₂ for 24 h. RPMI medium was removed. Before beginning the experiment, both ETEC and probiotic species were adjusted to 0.5 McFarland (1×10^8 CFU) and were centrifuged at 5000 rpm for 5 mins. The supernatant was removed and the cultures were resuspended in 1X PBS. 200 µl of 1X PBS was added both in HT-29 and Raw- 264.7 in triplicate and used as control. 200 µl of ETEC and *Lactobacillus casei* mono-culture and 100 µl of each of *Lactobacillus casei* + ETEC together were plated in triplicate, followed by an incubation of 4 hr at 37°C in darkness. Bacterial cultures were then removed and 200 µl of RPMI + 10 % FBS + 0.5 % of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added into each well. Incubate at 37°C for 2 hrs in darkness. MTT solution was removed gently and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The absorbance (λ) was measured at 540 and 690 nm. The correction value was calculated by subtracting the OD₆₉₀ from OD₅₄₀. The percentage of cell viability was calculated by: % Cell viability = (Correction value of treated cells / Correction value of cell control) $\times$ 100. The experiment was repeated with different concentrations (10^8 , 10^6 and 10^4) to determine and standardize the cytotoxic concentration of both ETEC and *Lactobacillus casei*. in HT-29 and Raw 264.7 cell lines. Same procedure was followed for *Lactococcus lactis* and *Bifidobacterium bifidum*.

4.2.10. Bacterial adhesion assay

For ETEC adhesion assay with INT407 cells were seeded at a density of 10^6 cells/well. The day before the experiment the cell culture medium was replaced with antibiotic free medium. From each fresh batch of the culture the ETEC H10407, *L. casei*, *L. lactis* and *B. bifidum* were harvested by centrifugation at 10,000 rpm for 8 min at 4°C and introduced to cultured cells at MOI 50. In this assay,

we assessed the adhesion inhibition and competitive inhibition of ETEC H10407 by different probiotic strains. For the adhesion inhibition assay, probiotics were introduced 2 hours before ETEC addition to the cells, and for the competitive inhibition assay, the probiotics and ETEC were introduced at the same time to the cells (PMID: 23586634). After ETEC introduction, cells were incubated for 2 hours. Cells were then washed 5 times with cold 1X PBS buffer. Cells were lysed using Triton-X-100 and spread on. The viable colonies from each cultured organism were counted from plates of serial diluents on MRS and Luria agar and used accordingly in the assay.

4.2.11. Experimental design for developing ETEC-induced diarrhoea in mice and treatment method

Four to six weeks old Balb/c mice were grouped ($n \geq 5$) into- (a) PBS control; (b) ETEC; (c) IN; (d) *L. casei*; (e) *L. lactis*; (f) *L. casei* + IN; (g) *L. lactis* + IN and (h) *B. bifidum* + IN. Mice were fed a standard chow diet ad libitum and autoclaved drinking water during the experimental period and reared in a 12-hour light-dark cycle condition and temperature ($25^{\circ} \text{C} \pm 2$). Mice in IN, *L. casei* + IN, and *L. lactis* + IN groups were fed Inulin (2.5g/ kg body wt.) from day 0 to day 14. Mice from *L. casei*, *L. lactis*, *L. casei* + IN, and *L. lactis* + IN were orally administrated with 10^8 CFU/ml of respective probiotic strains from day 7 to day 14. All the groups except for the control were infected with 10^9 CFU/ml of ETEC strains on day 14.

4.2.12. ETEC colonisation in ileum

All mice were sacrificed after 20 hours of infection. Portions of ileum from mice from all the groups were taken and homogenized in PBS and plated onto the MacConkey agar plate. Colonies were further validated by real-time PCR using specific primers against both ST and LT gene of ETC H10407 strain.

4.2.13. Histology for ileal architectural analysis

To investigate the severity of intestinal inflammation in the different groups, small sections of the distal colon were first washed with phosphate buffer saline (PBS) and were fixed with 10% Formaldehyde solution and kept for 48 hrs. The tissues were dehydrated in alcohol and embedded in paraffin. Ultra-microtome was used to cut sections of 5 to 6 μm thickness and stained with Eosin and Haematoxylin dyes, which were observed under the microscope. Tissue sections were graded concerning the below-mentioned parameters and the values of each parameter were added together to form the final score.

(i) degree of inflammation along with damage to colonic architecture:

0: normal morphology; 1: lost goblet cells, 2: lost goblet cells in large areas; 3: lost crypts; 4: lost crypts in large areas.

(ii) infiltration of leukocyte:

0: no infiltration, 1: infiltrating crypt basis, 2: infiltrating muscularis mucosa; 3: extensively infiltrating muscularis mucosa with thickened mucosa and obvious swelling; 4: infiltrating

4.2.14. Gut permeability assay

On the day of sacrifice mice from both the control and the treated group were force-fed with 44 mg/100 g body weight FITC labelled 4 kDa dextran (FD 4; MW, 4,000 Da; Sigma Aldrich; Merck KGaA) via gavage following fasting overnight (Liu, 2020), (Chakraborty, 2023), (Kang, 2017), (Sikdar, 2024). After 4 hours of FD 4 administration, blood was collected and centrifuged at 2,500 x g for 10 min at 4°C to collect the serum. The fluorescence intensity of FD-4 in the serum was determined using a multimode reader at an excitation and emission wavelength of 485 nm and 520 nm. Subsequently, a standard curve was generated using serial dilutions of FD 4 from 1000 µg/ml – 0.

4.2.15. Bacterial translocation assay

A portion of each liver was collected under aseptic conditions after the mice were sacrificed. Liver samples were washed in 200 µl PBS containing gentamicin (50 µg/mL) and then homogenized in ice-cold PBS. 100 microlitre of each tissue homogenates were spread onto the Nutrient agar and incubated at 37°C for 24 h. The number of bacterial colonies was counted based on the dilution factor and expressed as colony forming unit (CFU). CFU/g of tissue denoted the amount of bacterial translocation.

4.2.16. Flow cytometric analysis for T cell population

Mesenteric lymph nodes and spleens were collected from each individual and homogenized in PBS. Both spleen and MLN lysates were then passed through 70µm cell strainers to obtain a cell suspension devoid of any debris. For MLN the cell suspensions were centrifuged at 2000 rpm for 5 min to obtain the cell pellets. The cell pellets were then resuspended in FACS buffer (PBS with 10% FBS). On the other hand, Splenic filtrates were centrifuged along with ficol paque plus at a ratio of 1:3 for density gradient centrifugation at 2000 rpm for 30 min at zero breaks. The white cloudy phase of PMNs was then collected leaving both the aqueous and ficol layers. Further centrifugation was done to obtain the PMN pellets and resuspended them in FACS buffer. Cell suspensions were then divided into batches to stain with respective FACS antibodies which were FITC conjugated anti-CD3, PerCP cy5.5 conjugated anti-CD4, and PE-conjugated anti-CD8 antibodies. The cell population was analyzed using FACS (BD Aria II).

4.2.17. Real-time PCR

RNA extraction was done by TRIZOL reagent (Invitrogen, Massachusetts, USA) from each colon sample and quantified with nanodrop (NanoDrop 8000 Spectrophotometer, ThermoFisher Scientific, USA). 0.5 µg of RNA was taken for reverse transcription with oligo dT primers, dNTPs, RNase inhibitor, and MuLV reverse transcriptase according to the kit manual (PrimeScript 1st strand cDNA Synthesis Kit- TaKaRa Bio) to synthesize cDNA. Quantitative- PCR was conducted using SYBR green master mix (TaKaRa TB Green Premix Ex Taq- II) and ROX reagent in a real-time PCR system and ΔC_t values were calculated to determine relative changes of target genes.

4.2.18. Enzyme-linked immune sorbent assay for cytokine and chemokine profiling

To measure the inflammatory markers i.e., cytokines and chemokine, colon tissues were homogenized in PBS and centrifuged at 10000g for 1 min to collect the supernatants devoid of any tissue debris. The protein concentration of the lysates was quantified using the BCA Protein Assay Kit (Thermo Scientific™ Pierce™, USA). 50 µg lysate from each animal sample was used to perform ELISA for CXCL-1(R&D Systems DY453-05), CXCL-2 (R&D Systems DY452-05), TNF- α (BD OptEIA 555268), IL-6 (BD OptEIA 555240), IFN- γ (BD OptEIA 555138), and IL-10 (BD OptEIA 555252) according to the manufacturer's instructions.

4.2.19. Analysis of Gut microbiota

DNA was extracted with Qiagen's power fecal pro kit using Qiacube from the fecal fractions collected from the Control and FTY720 group. The DNA yield was checked using Qiaexpert. The DNA yield for Control and FTY720 was found to be 113.01 ng/µl and 104.76 ng/µl. respectively. The samples were processed on Illumina Novaseq 6000 which gave a paired-end read of 150x2 bp and the samples were processed by downstream analysis. The de novo assembly of the adapter trimmed fastq files is carried out using MetaSPAdes (v 3.10.1). The contigs obtained from the assembly were used as input to MetaGeneAnnotator (MGA) for the prediction of open reading frames (ORFs). The predicted ORFs are searched against the non-redundant (NCBI-nr) database using DIAMOND (v0.7.9.58). The alignment file along with the filtered ORFs was used as input to the functional annotation using MEGAN6 (MetaGenome Analyzer) software. Further Fastq quality checks and base quality score distribution were carried out. Denovo metagenome assembly was carried out for the sample by assembling contigs from the reads using the MetaSPAdes program. Further contigs were linked by the assembly algorithm to create scaffolds. Assembly was performed with default Kmer sizes 21, 33, and 55 using the de-bruijn graph method. PERL and Python code were used to parse the fastq files for the downstream analysis. As a result of de novo assembly, contigs in FASTA format were generated for all samples. The FASTA files were analyzed on CLC workbench (Qiagen) and OUT analysis and the QC tool was used to obtain the taxonomic abundance within samples. Further, the taxonomic abundance table was used as input to calculate the alpha and Beta diversity. (Sankarasubramanian et al.2020), (Mitra et al. 2023).

4.2.20. GC-MS for mouse fecal Short Chain Fatty Acids

GC/MS was used to quantify the butyrate concentrations in the feces, as previously reported (Li et al., 2020). To put it briefly, 200 µl of distilled water was used to homogenize 50 mg of fecal samples from both sets. The samples were then centrifuged for five minutes at 4000 rpm, and the outcome It was collected as supernatant. To release the gas created during the reaction, the tube lids were left open for one minute after 200 µl of a benzyl alcohol–pyridine combination (3:2) and 100 µl of DMSO were added to the supernatant. The mixture was then vortexed for five seconds before 100 µl of the

derivatizing agent benzyl chloroformate was carefully added. The resultant mixture was vortexed for three minutes after the tube lids were shut. Following derivatization, 200 µl of cyclohexane was added, and the mixture was vortexed for one minute before being centrifuged. 5 minutes of gation at 4000 rpm. For further analysis, 1 µl of the separated 100 µl of the resultant derivative extract (upper cyclohexane layer) was added to a GC-MS device. With an AOC-20i auto injector, the Shimadzu GCMS-QP2020 (Shimadzu Corporation, Kyoto, Japan) was used to analyze the samples. The separation was carried out using an InertCap WAX capillary column (30 m × 0.32 mm × 0.25 µm; GL Sciences Inc., Tokyo, Japan). With a flow rate of 1 milliliter per minute, helium was employed as a carrier gas. The front inlet temperature was set at 250 °C, while the ion source and transfer line temperatures were set at 280 °C and 230 °C, respectively. Following three minutes of holding the starting column temperature at 70 °C, the temperature was raised to 200 °C at a rate of 10 °C per minute, then to 290 °C at a rate of 35 °C per minute, and ultimately kept at this temperature for seven minutes. One run took 25.5 minutes. 6.7 minutes was chosen as the solvent delay time. With a gain factor of 2.0, the electron energy was -70 eV. (Chakraborty M, 2022).

Chemical/material	Calibration series sample	Biological sample		
		Feces	Cecum content	Plasma/serum
Biological sample	–	±50 mg	±10 mg	10 µL
Water	250 µL	550 µL	690 µL	250 µL
Acetone	250 µL	250 µL	250 µL	250 µL
1 µg/mL IS solution	10 µL	10 µL	10 µL	10 µL
Standards in EtOH	10 µL ^a	–	–	–
EtOH	–	10 µL	10 µL	10 µL
172 mM PFBBBr	100 µL	100 µL	100 µL	100 µL
<i>n</i> -hexane	500 µL	500 µL	500 µL	500 µL
1.5 mL plastic tubes	–	2	3	–
Clean steel beads	–	2	2	–
Glass autosampler vials	2	2	2	2
Glass autosampler inserts	1	1	1	1
Glass autosampler caps	2	2	2	2

Table 4.2.20A- Materials and chemicals needed for each sample in order to quantify SCFAs. (Hoving, 2018)

FA	Name	RT (min)	Monitored m/z (M^-)	LLOQ (μM) ^a	IS
FA 2:0-d4	Acetic acid-d4	7.19	62	N/A	N/A
FA 2:0	Acetic acid	7.22	59	20	FA 2:0-d4
FA 3:0-d6	Propionic acid-d6	7.86	78	N/A	N/A
FA 3:0	Propionic acid	7.89	73	5	FA 3:0-d6
FA 4:0-d8	Butyric acid-d8	8.41	94	N/A	N/A
FA 4:0	Butyric acid	8.44	87	2	FA 4:0-d8
FA 5:0	Valeric acid	8.99	101	1	FA 4:0-d8
FA 6:0	Hexanoic acid	9.51	115	5	FA 4:0-d8
FA 7:0	Heptanoic acid	9.99	129	1	FA 4:0-d8

Table 4.2.20B- An overview of FAs. For every SCFA, the IS to be utilized, the m/z -value, the retention time (RT), and an indicator of the LLOQ are displayed. (Hoving, 2018)

4.2.21. Interference of *Lactobacillus* with ETEC growth in Co-culture

To investigate the effect of probiotic bacteria on the growth kinetics of ETEC H10407, we performed a Co-culture assay. For this experiment, 50 ml of single strength Tryptic Soy broth (TSB; BD, USA) and De Man-Rogosa-Sharpe (MRS; HiMedia) broth were inoculated with 1×10^8 CFU of ETEC and *Lactobacillus spp.*, *Lactococcus spp.*, and *Bifidobacterium spp.* respectively, and were incubated at 37°C with a constant shaking of 150 rpm. Similarly, 50 ml of co-culture medium containing 25 ml of each of double strength TSB and MRS broth (TSB: MRS-1:1) were inoculated with 1×10^8 CFU of both ETEC and probiotics simultaneously. 100 μ l mono-cultures of both ETEC and probiotics, as well as 100 μ l of co-cultures, were collected at 0, 2, 4, 8, 10, 12, and 24 h and lawn cultured in appropriate agar plates to determine the viable cell count. Plates were incubated overnight at 37°C and the colonies were counted. The tests were performed in triplicates.

4.2.22. Cellular cytotoxicity assay

HT-29 and Raw-264.7 cells were seeded in separate 96-well plates (1×10^5 cells/well) and incubated at 37°C with 5% CO₂ for 24 h. RPMI medium was removed. Before beginning the experiment, both ETEC and probiotic strains' OD were adjusted to 0.5 McFarland (1×10^8 CFU) and were centrifuged at 5000 rpm for 5 mins. The supernatant was removed, and the cultures were resuspended in 1X PBS. 200 μ l of 1X PBS was added both in HT-29 and Raw- 264.7 in triplicate and used as control. 200 μ l of ETEC and probiotic mono-culture and 100 μ l of each of the probiotic species + ETEC together were plated in triplicate, followed by an incubation of 4 hr at 37°C in darkness. Bacterial cultures were then removed and 200 μ l of RPMI + 10 % FBS + 0.5 % of 3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added into each well. Incubate at 37°C for 2 hrs in darkness. MTT solution was removed gently and 100 μ l of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The absorbance (λ) was measured at 540 and 690 nm. The correction value was

calculated by subtracting the OD₆₉₀ from OD₅₄₀. The percentage of cell viability was calculated by: % Cell viability = (Correction value of treated cells / Correction value of cell control) × 100. The experiment was repeated with different concentrations (10⁸, 10⁶ and 10⁴) to determine and standardize the cytotoxic concentration of both ETEC and Lactobacillus spp. in HT-29 and Raw 264.7 cell lines.

4.2.23. Immunofluorescence Staining and imaging

Colon samples from each group were collected and fixed in 4% paraformaldehyde. Paraffin embedded 5µm thick sections were generated. Sections were then de-paraffinized in xylene and were rehydrated with graded ethanol, followed by distilled water. 1 mM EDTA buffer (pH 8.0) was used for antigen retrieval. The sections were then permeabilized with 0.1% sodium citrate and 0.5% Triton-X in TBST. The sections were then blocked with 5% animal sera in TBST at room temperature. The primary antibodies were added to the sections and incubated overnight at 4°C. The sections were washed and then incubated with Alexa Fluor 488 conjugated secondary antibody for 2 hours at room temperature, and counterstained with Hoechst 33342 stain. Sections were mounted and visualized using Carl Zeiss microscope equipped with a CCD camera and images processed by Zen software (Gumber, Nusrat, and Villinger 2014). The fluorescence intensity of the target proteins was measured using ImageJ software.

4.2.24. Histopathologic examination of proximal colon

The proximal colon samples were fixed in 4% paraformaldehyde for 48 h at 4°C. The fixed tissues were then dehydrated through graded alcohols, embedded in paraffin, and routine microtomy then carried out to generate 5 µm sections. The sections, in turn, were stained with hematoxylin and eosin for later microscopic examination. (Chakraborty and Bhaumik 2020)

4.2.25. Detection of Neutrophil infiltration by MPO activity

Paraffin-embedded ileal tissue sections were deparaffinized with Xylene and rehydrated with graded ethanol. 0.5% Triton X-100 was used as a permeabilization buffer. Sections were boiled with 0.1% Sodium citrate solution for antigen retrieval. Tissues were incubated for 1 hour in 5% sera in TBST as a blocking reagent. After that, tissue sections were incubated with the specific primary antibodies (1: 200) overnight at 4° C. Sections were washed with an order of PBS-PBST-PBS for 5 min at each step and incubated with HRP tagged secondary antibody (1: 200) for 2 hours at room temperature and developed by using DAB substrate solution (0.5mg/ ml of 1% H₂O₂ in PBS 1X). Tissues were counter-stained with Hematoxylin and then mounted with DPX.

4.2.26. Western blot analysis

HT-29 cells were seeded with a density of 10⁶ cells/ well and grouped into control, FTY720 (20µM), and Rapamycin (500nM) and incubated for 48 hours. After that cells were lysed with RIPA lysis buffer (5M NaCl, 0.5M EDTA, 1M Tris-HCl (pH 8.0), 0.5% Tween-20, 10% SDS, PMSF, dH₂O) followed by

sonication and centrifugation. The total protein concentration of lysates was determined using Thermo Scientific™ Pierce™ BCA Protein Assay Kit (23225). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate components of cell lysates. Proteins were transferred onto the PVDF membrane (IPVH00010, Millipore). 5% BSA in TBST was used for blocking and the membrane was incubated overnight with specific primary antibodies at 4° C. Membranes were incubated with AP/HRP conjugated secondary antibody for 2 hours at room temperature and detected using NBT BCIP substrate solution (Thermo scientific) / Chemiluminescent HRP substrate (Immobilon Western, Millipore).

4.2.27. Fecal Microbiome Transplant (FMT) and pathogen clearance

Balb/c mice (n=3) were divided into three groups and each group received any of the three probiotic strains (*L. casei*, *L. lactis* and *B. bifidum*) at a concentration of 10^8 CFU/ml. Another three sets of Balb/c mice (n=3) were infected with ETEC (10^8 CFU/ml). After 24 hours, the fecal pellets were collected from individuals of each probiotic group and homogenized in a sterile PBS buffer to prepare microbial soup separately. Each ETEC-infected mice group then received any of the three fecal microbial soup, thus named as *L. casei* FMT, *L. lactis* FMT and *B. bifidum* FMT. The clearance of ETEC infection was monitored daily by ETEC CFU in the fecal matter of the FMT-grouped mice for 10 days. Fecal pellets were collected and weighed and homogenized and diluted in PBS and then spread onto the MacConkey agar plate for counting.

4.2.28. Statistical analysis

All experiments were conducted in triplicate and data are expressed as mean $\pm$ SEM, Student's T- test and one-way analysis of variance (ANOVA) was conducted followed by Tukey's multiple comparison test using Graph Pad Prism 8 software (Graph Pad Software Inc., San Deigo, CA, USA) and difference with $P < 0.05$ was considered significant. ($P < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$).

Chapter 5:

Results &

Discussions

Objective One:

“Investigating the function of Sphingosine-1-phosphate (S1P) in intestinal barrier regulation”

5.1. Introduction

With a physical barrier on the outside and an immunological barrier within, the mammalian gastrointestinal system is a multilayered system that allows the gut microbiota to keep the body in balance. It is necessary to take into account the wall of the gut that preserves these luminal chemicals. With its pleiotropic qualities, the Sphingosine 1 phosphate (S1P) helps sustain cell-cell integration, which is dependent on the gut wall, which accumulates luminal chemicals (Santacreu et al. 2019), the regulation of the epithelial barrier (Weigel, Bellaci, and Spiegel 2023), cell division (Chen et al. 2017), migration (Chen et al. 2017), survival (Van Brocklyn and Williams 2012), and more. Maceyka and Spiegel (2014), Le Stunff, Milstien, and Spiegel (2004), Schmidt et al. (2019), and Imamura et al. (2001) have reported that S1P is widely distributed in cells and is generated by the important enzyme sphingosine kinase (SPHK). SPHK1 and SPHK2 are protein isoforms that catalyze the ATP-dependent phosphorylation of sphingosine (Sph) (Hatoum et al. 2017; Alemany et al. 2007; Liu, Sugiura, et al. 2000).

The gastrointestinal epithelial layer makes the largest physical and chemical barrier, interacting with the outside world effectively forms a protecting barrier that is responsible for the mucosa's selective ability toward the absorption of luminal toxins and foreign antigenic substance, permitting nutrient absorption and water influx and efflux. According to Laksitorini et al. (2014), epithelial cells create this particular barrier by utilizing both the paracellular and transepithelial transport channels. Because it regulates gastrointestinal permeability in both healthy and diseased settings, the tight junction-regulated paracellular pathway—which governs apical cell-cell adhesion—is of significant interest (Paradis et al. 2021). The most notable apical adhesive connection between adjacent cells of the epithelial and endothelial layers is the Tight Junction, also known as the zonula occludens (Furuse 2010; Bazzoni and Dejana 2004). TJ-integral membrane proteins are anchored to the cell cytoskeleton by cytoskeletal linker proteins like cingulin, whilst transmembrane and integral proteins like occludin, claudins, and JAM regulate gate function in the intercellular space (Heinemann and Schuetz 2019). Vulnerable people's immune systems can be overtaxed by an unchecked transmission of antigens via a breakdown of the intestinal barrier and digestive tract epithelial cells. These factors can also change the host-microbial harmony, which can trigger gut inflammation or increase the risk of mucosal infections (Meddings 2008), Yu et al. (2012). Examining how S1P signaling impacts the tight junctions of the gut epithelium is the aim of this work.

Pharmaceutical S1P analog, FTY720 is a FDA-approved drug used to treat multiple sclerosis (Sharma et al. 2011). FTY720 binds S1P receptors and sequesters making a blockage in S1P downstream signaling (Sykes et al. 2014). We studied the TJ protein expression and permeability associated with S1P signaling inhibition in intestinal epithelial cells as well as in the mouse model. We investigated the

siRNA-mediated silencing of SPHK-1, the cytosolic Sphingosine kinase-regulated tight junctional expression in HT-29 colon carcinoma cells. Likewise, mice were fed with FTY720 with continual dosing for 14 days to investigate gut permeability, bacterial translocation, and associated tight junction proteins' expression and their regulation.

This report investigates and reports that S1P signaling is critical for gut epithelial barrier maintenance. Also, as far as we are aware, this is the first study suggesting that the gut epithelial layer's vital barrier function may be compromised by the functional counterpart of FTY720, S1P.

5.1.1. Results

5.1.1.1. Intestinal epithelial cells' tight junction expression is impacted by siRNA-mediated suppression of Sphingosine kinase-1 (SPHK-1).

For twenty-four hours, siSPHK-1 RNA was transfected into HT-29 cells. Western blotting was used to determine the expression of tight junction proteins after cells were treated, isolated entire mRNA, then lysed to get a complete protein pool. A successful transfection with siSPHK-1 RNA significantly ($p<0.0001$) knocked down the SPHK-1 gene in HT-29 cells, was confirmed by both real-time PCR and western blotting (Figure- 5.1.1.1A). Lysates of transfected HT-29 cells were subjected to tight junctional protein analysis by western blotting. The data revealed a significant 2.2-fold and 1.97fold decrease in Occludin and JAM-A protein, respectively. In comparison, no significant change was observed in Claudin-4 and Claudin-5 proteins.

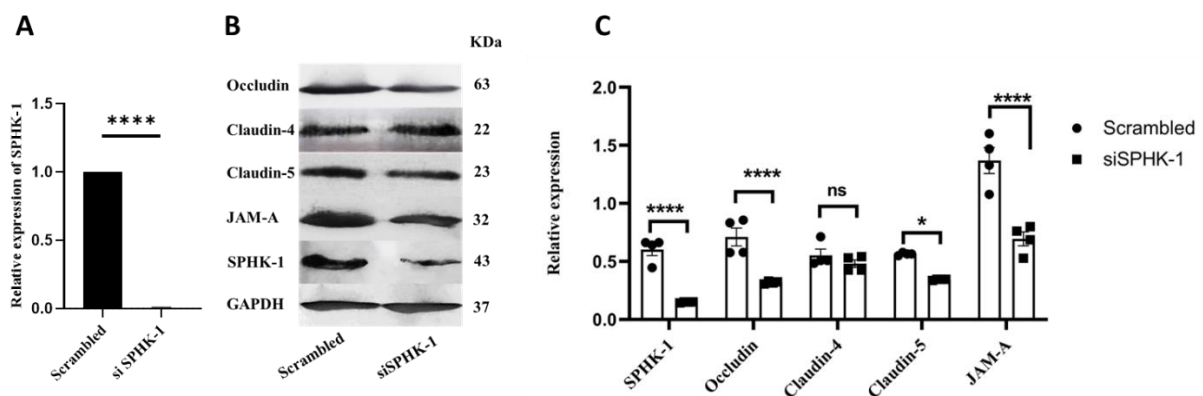


Figure 5.1.1.1A: HT-29 cells were transfected with siSPHK-1 RNA for 24 hours. **A.** Knockdown of the SPHK-1 gene was confirmed using the real-time PCR method. **2.** Upon transfection, SPHK-1 protein expression and expression of tight junctional protein analysis by western blotting. **3.** Densitometric analysis of the western blots. Results are expressed as mean $\pm$ SEM.

5.1.1.2. FTY720, an FDA-approved S1P signal analogue down-regulates tight junctional proteins in colon epithelial cells

FTY720 is a synthetic S1P receptor blocker used to treat autoimmune disorders like multiple sclerosis, atherosclerosis, etc. (Behara and Goudy 2023), (Keul et al. 2007), (Nofer et al. 2007). It binds to all five S1P receptors (S1PR1-5) and internalizes them, leading to the S1P downstream signaling blockage (Nofer et al. 2007). These experiments were carried out with HT-29 cells. For 48 hours, HT-29 cells were treated with FTY720 with the goal of blocking S1P signaling. Post-treatment, the viability of cells was determined by MTT assay (Figure- 5.1.1.2A). From the MTT assay, the dose of FTY720 was determined for the following experiments.

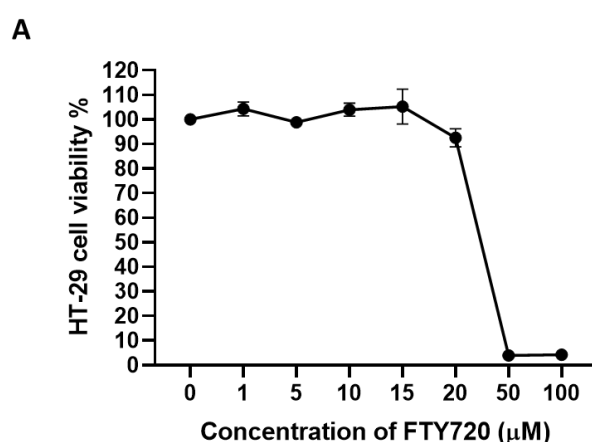


Figure 5.1.1.2A: HT-29 cells were treated with FTY720 of a series of concentrations. Cells were incubated for 48 hours. MTT was added for 4 hours and the purple formazan was measured at 595 nm. The viability of FTY720 treated cells was represented as a percentage against the viability of control cells.

Like any physical barrier, the gut wall has an electrical resistance across the epithelial layer (Powell 1981) primarily maintained by integration of the tight junctional proteins at the intercellular space of the adjacent epithelial cells (Gunzel and Yu 2013). In a Transwell insert HT-29 cells were grown as monolayers and upon treatment the Trans Epithelial Electrical Resistance (TEER) was measured by an Ohm meter and chopstick electrode. Changes in the TEER values of treated vs untreated monolayers were calculated as percentages. After FTY720 was added to HT-29 monolayers at escalating concentrations, the cells were incubated for 24 hours, with TEER measurements taken every two hours. An untreated control monolayer was also measured likewise. A decrease in TEER was observed in monolayers treated with FTY720 which continued to decrease up to 6 hours and remained decreased

even after 24 hours. The control monolayer remained constant for the first six hours. After 24 hours, the control monolayer's TEER doubled (Figure- 5.1.1.2B).

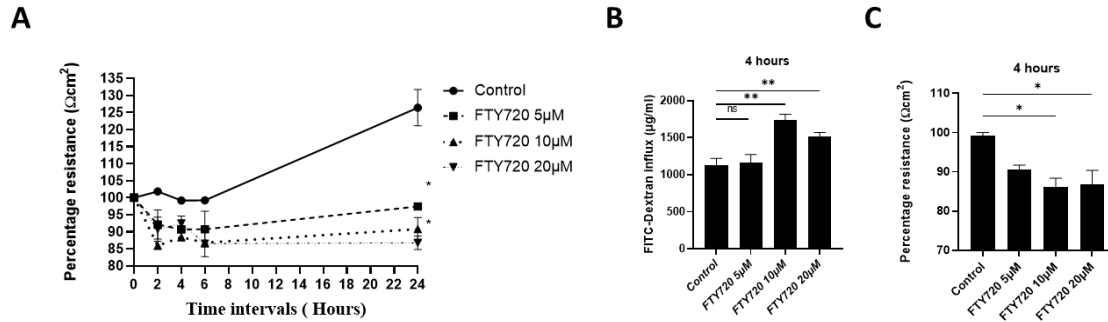


Figure 5.1.1.2B- HT-29 cells were treated with FTY720 for 24 hours in a transwell chamber. A. Time-dependent measurement of Trans Epithelial Electrical Resistance (TEER) of HT-29 cell monolayer upon FTY720 administration is represented as percentage resistance against the TEER of control monolayer. HT-29 monolayers were treated with FTY720 for 4 hours. B. FITC-Dextran influx across HT-29 monolayer in trans well chambers were. C. Percentage electrical resistance of control and treated monolayers. The mean± SEM is used to express all results. Three repeats of the experiments were carried out. The mean± SEM is used to express the results.

A decrease in TEER is an indication of the loosening of the junctional integrity of the epithelial monolayer (Ulluwishewa et al. 2011), (Shi et al. 2020), (Tatsuta et al. 2019). As it is directly associated with Tight junction (TJ) dysregulation, next we checked the tight junctional proteins from HT-29 cells treated with and without FTY720. Realtime-PCR and western blot were performed to investigate both the mRNA and protein levels of major TJ-associated proteins; Occludin, Claudin-4, and ZO-1 (Figure- 5.1.1.2C). We discovered a significant dose-dependent reduction in the expression of occludin and Claudin-4 from both analyses. Yet HT-29 cells showed no change in ZO-1 expression upon FTY720 (data not shown).

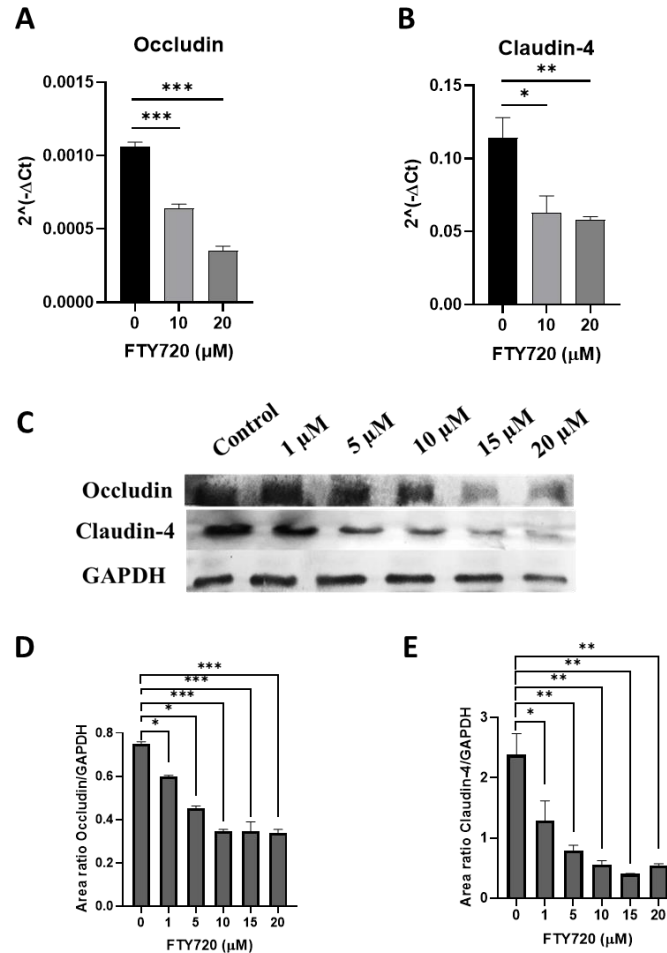


Figure 5.1.1.2C: A. Occludin and Claudin-4 gene expression changes relative to one another, as determined by real-time PCR. B. Western blot study of occludin and claudin-4 protein level expression. C. TJ protein blot densitometric analysis. The mean± SEM is used to express all results. Three repeats of the experiments were conducted.

5.1.1.3. Sphingosine-1-phosphate (S1P) analogue FTY720 impairs gut epithelial integrity in C57BL/6 mice model

To investigate the relation between the blockage of the S1P signal and tight junction proteins in the colonic tissues, we used S1P functional analog FTY720 in a murine model. C57BL/6 mice of 6 weeks old were fed with FTY720 (3mg/kg body weight) for 14 consecutive days by oral gavage. Control mice were given normal chow and drinking water. Gut permeability was assessed using FITC-Dextran influx. Compared to the control sera, the serum FITC-Dextran level in FTY720-mice was noticeably greater (Figure- 5.1.1.3A).

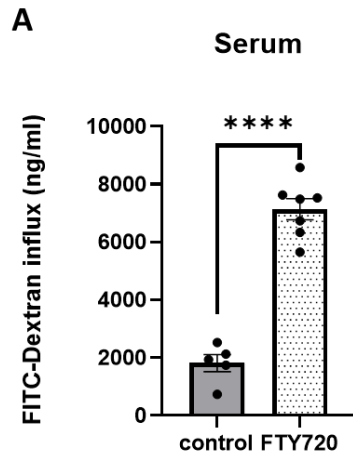


Figure 5.1.1.3A: C57BL/6 mice were orally fed with FTY720 (3mg/kg body weight) for 14 days. Control and FTY720 mice were given FITC Dextran (44mg/kg) orally. After 4 hours, FITC-Dextran concentration in the sera was measured by a fluorometer. Every result is shown as mean $\pm$ SEM. Three repetitions were used in the experiments. The results are shown as mean $\pm$ SEM.

We discovered leaky gut-associated gut commensal bacterial translocation to the liver in FTY720 animals, which is consistent with the FITC-Dextran permeability results (Figure- 5.1.1.3B).

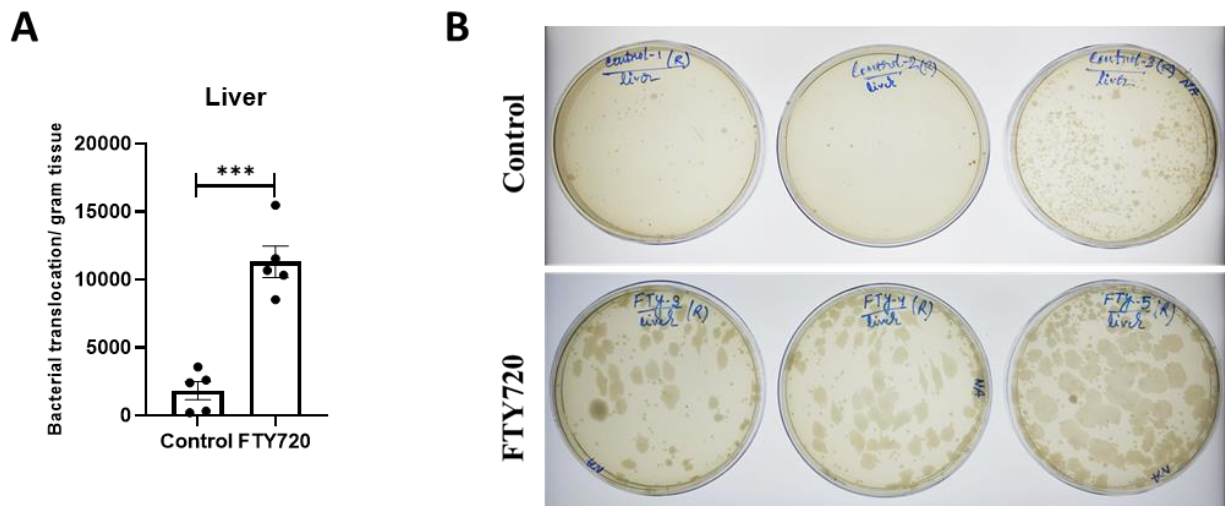


Figure 5.1.1.3B: A. The translocation of the gut commensal bacteria to the liver by counting the bacterial colonies that appeared on the nutrient agar plate. The data are presented as Mean $\pm$ SEM, and each group included a minimum of three animals. P-values were calculated alongside the control.

The expression of Tight junction-forming proteins, i.e., Occludin, Claudins (1,2,4), JAM-A was assessed by real-time PCR, western blot, and Immunofluorescence (Figure- 5.1.1.3C and Figure- 5.1.1.3D). The Real-time PCR data showing the gene level expression of Occludin and Claudin-4 greatly decreased in FTY720- mice whereas ZO-1, Claudin-1, and JAM-A remain unchanged. Conversely, there was a notable upregulation of Claudin-2, a tight junction regulating and pore-forming protein.

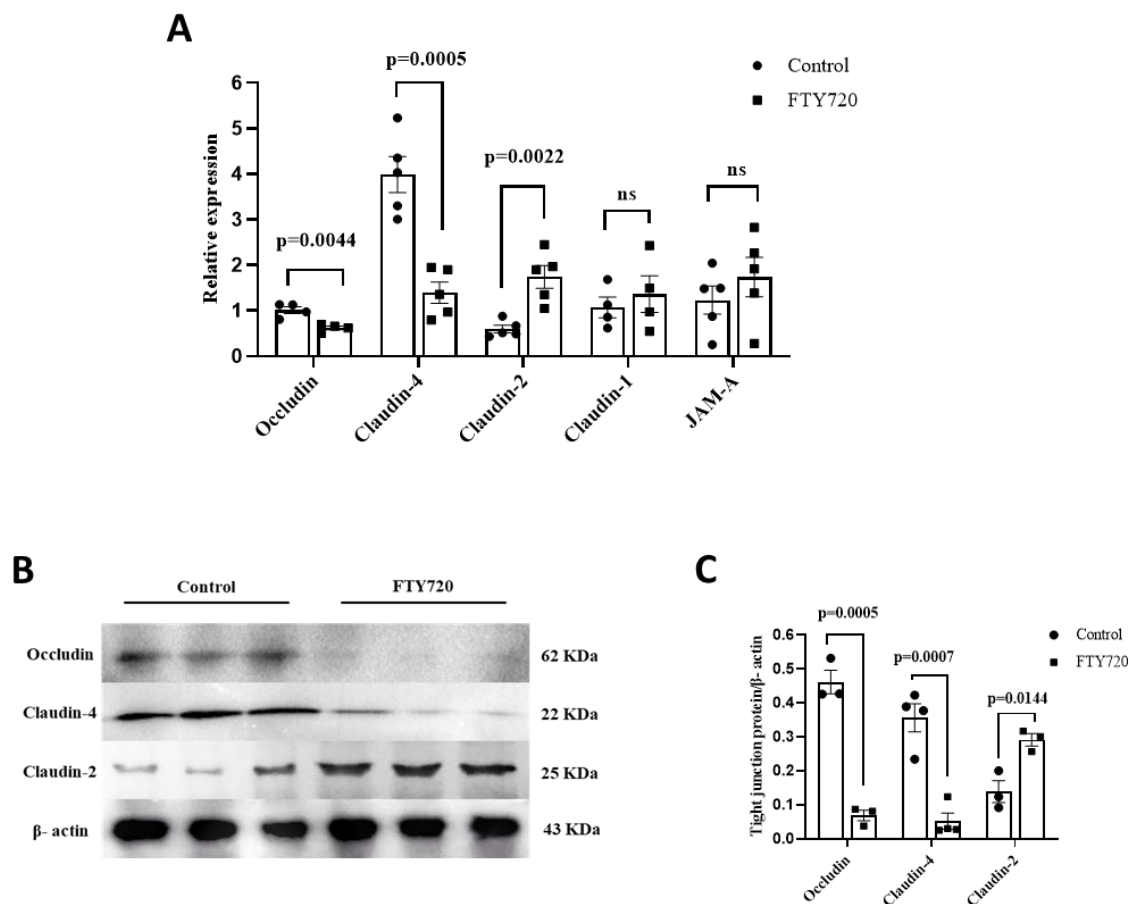


Figure 5.1.1.3C- A. Gene expression of Tight junction (TJ)-regulatory Occludin, Claudin-4, -2, and -1, as well as JAM, in real-time PCR analysis. **B.** For protein level expression analysis from FTY720 and control colon lysates for Occludin, Claudin-4, and Claudin-2 were done using Western blotting. β -actin was kept as an internal control. **C.** Densitometric evaluation of Occludin, Claudin-4, and Claudin-2 western blot images.

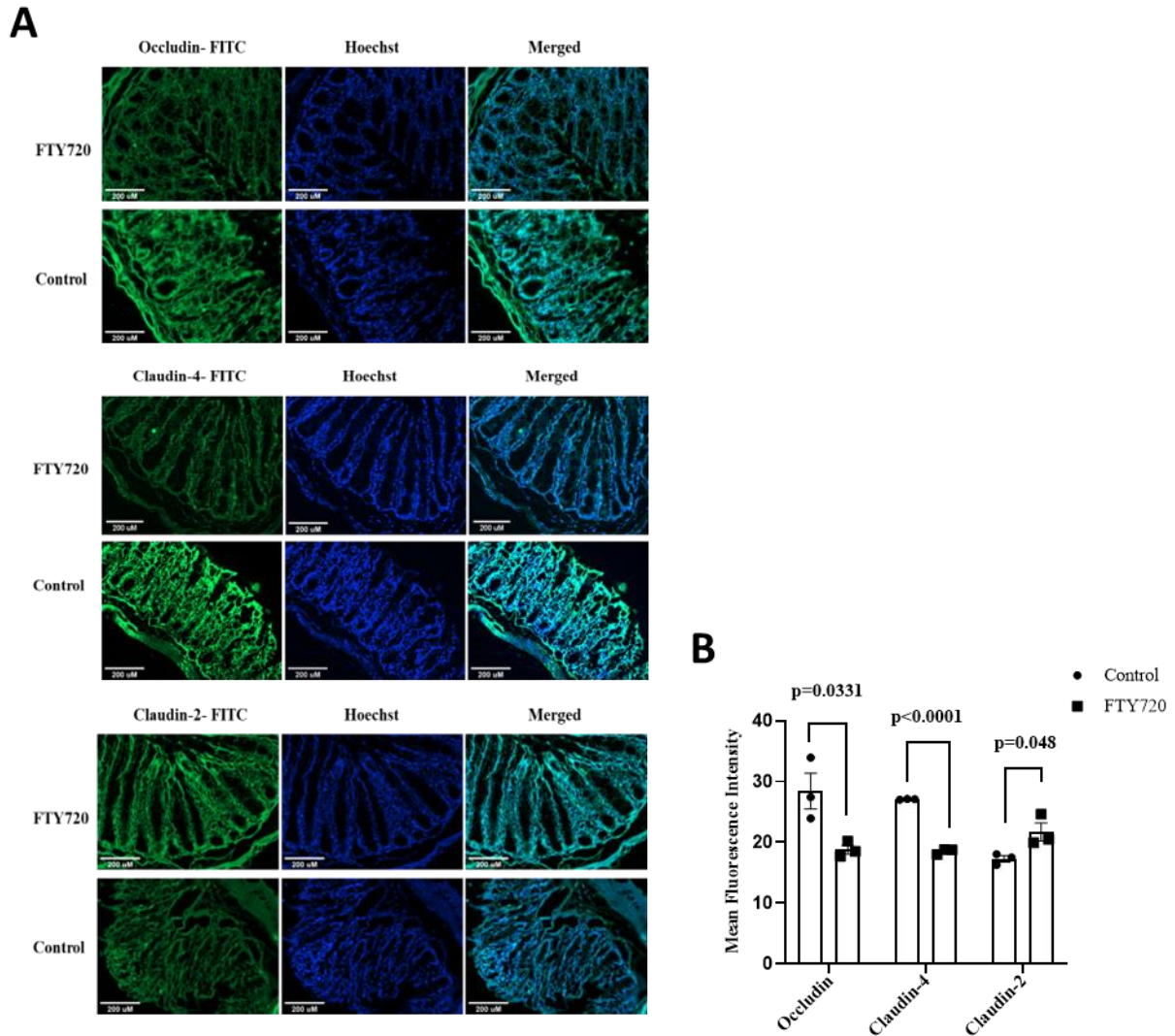


Figure 5.1.1.3D- A. Immunofluorescent images of FTY720 and control mice's colonic sections. Occludin, Claudin-4, and Claudin-2 antibodies labeled with green fluorophore FITC were used to stain colon sections. The nucleus was stained with Hoechst (blue). Photos that have been blended reveal the twin stains. **B.** the images' fluorescence intensity as assessed by the densitometric method. The data are presented as Mean $\pm$ SEM, and each group included a minimum of three animals. P-values were calculated alongside the control.

5.1.1.4. FTY720 targets the Akt- mTOR signaling axis to regulate tight junction proteins in HT-29 cells

mTORC1 is a major regulating factor of cells, which induces several mRNA translational pathways (Holz et al. 2005). Phosphorylation of Akt and mTOR activates transcription factor P70 ribosomal S6 kinase and eIF4E Binding Protein or 4EBP (Saxton and Sabatini 2017) to exert cellular physiology. Several previous studies have shown that mTOR signaling promotes occludin and claudin 4 tight

junction protein synthesis in epithelial cells (Wang et al. 2015), (Liu, Xie, et al. 2020), (Yan and Ajuwon 2017), (Liu, Xie, et al. 2020), (Yan and Ajuwon 2017), And Rapamycin, the mTORC1 inhibitor which suppresses the phosphorylation of PI3, Akt and mTOR proteins (Liu et al. 2021), also inhibits the tight junction protein synthesis (Mao et al. 2016). This study examined the effect of FTY720 on the Akt/mTOR/PS6K axis in HT-29 cells compared to rapamycin. We examined both the upstream Akt and downstream S6K of mTOR. FTY720 treatment significantly decreased the phosphorylation of Akt at Ser 473, mTOR at Ser 2448, and S6K at Thr 389/Thr 412 and decreased the expression of Occludin and Claudin-4 with respect to control and similar to rapamycin. In cells treated with FTY720, the ratios of P-Akt/Akt, P-mTOR/mTOR, and P-S6K/S6K were substantially ($p \leq 0.017$) reduced by 3.25, 1.79, and 1.99 fold in comparison to the control (Figure 5.1.1.4A).

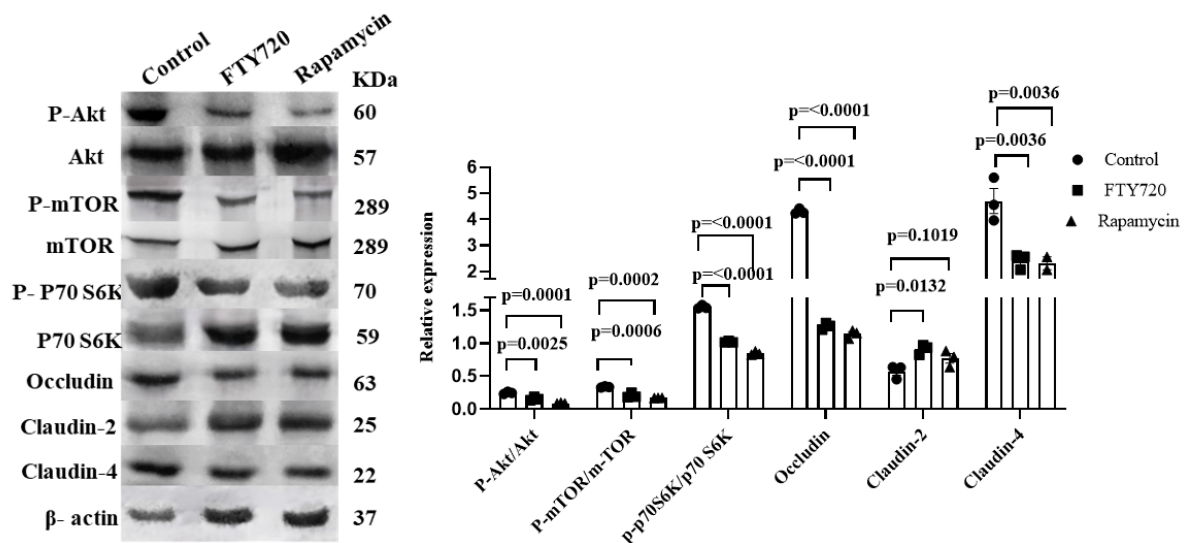


Figure 5.1.1.4A: FTY720 targets Akt-mTOR-P70 S6K signaling cascade to regulate the expression of occludin and claudin-4 and claudin- 2 in HT-29 cells. Rapamycin (500 nM) and FTY720 (20 μ M) were administered to cells, whereas a control group was not given any treatment for 48 hours. Western blots were employed to evaluate the cell lysates for p-AKT, AKT, p-mTOR, mTOR, p-P70 S6K, P70 S6K, Occludin, claudin-2, claudin-4, and α -actin. p-Akt/Akt, p-mTOR/mTOR, p-p70S6K/P70S6K, occludin/ α -actin, Claudin-2/ α -actin, and Claudin-4/ α -actin are all represented by densitometric analysis.

5.1.2. Discussion:

The gastrointestinal epithelium forms the body's largest interface with the external environment (Groschwitz and Hogan 2009). The "intestinal barrier" is made up of the intestinal mucosa and the underlying immune system and functions predominantly as a physical dam between the intestinal contents and the immune system underneath. By selective limitation of the permeation of the toxic

elements (luminal) and antigens through the mucosa, It effectively provides a barrier (Suzuki 2013). Thus, a breach in the mucosal barrier results in mucosal inflammation (Ahmad et al. 2017).

According to earlier research, TJ protein knockout animals exhibit inflammation of the gastrointestinal epithelium (Ahmad et al. 2017; Lu et al. 2013). More studies indicate the importance of permeability, especially in supporting the mucosal barrier's overall function in regulating mucosal immunological homeostasis (Castelli et al., 2015).

S1P is a signaling protein that triggers a number of signaling pathways within cells (Maceyka et al. 2012). S1P is generated by a sequence of events, including the hydrolysis of ceramide into sphingosine by ceramidase (Hanada 2003). Active S1P results from the phosphorylation of sphingosine by a pair of sphingosine kinase isoforms (SphK1 & 2) (Olivera et al. 1999). SphK2 has a nuclear location that enables the creation of nuclear S1P, a potent histone deacetylase inhibitor, whereas SphK1 generates cytosolic S1P (Kwong and Zhou 2019). Numerous investigations substantiate and validate S1P's ability to regulate the barrier in endothelial and epithelial tissue. The epithelial permeability is highly regulated by Tight junctions, Gap junctions, and Adherence junctions (Naser et al. 2022). There are reports of S1P-mediated barrier enhancement, wherein dose-dependent S1P-associated improvement of EC monolayer barrier properties and turnaround of the barrier disruptive effect of thrombin-stimulated actin stress fiber development, increased intracellular tension, and paracellular gap formation were observed in vitro using Trans Electrical or eTER (Garcia et al. 2001).

Consistent with this, our data also reports that SPHK-1 knockdown causes inhibition of Tight junctional (TJ) protein translation in HT-29 colon adenocarcinoma cells. A significant decrease in Occludin, Claudin-5, and JAM-A protein in HT-29 cells was associated with SPHK-1 gene knockdown. Occludin, a tetra-spanning membrane protein weighing near 60–65 kDa, interacts homophilically in dimers. It is composed of two extracellular loops separated by a short cytosolic loop, and the cytoplasm contains the amino- and carboxy-terminal domains (Feldman, Mullin, and Ryan 2005). A severely compromised occludin expression corresponds with intestinal inflammatory disease formation suggests occludin has a critical role in the maintenance of barrier integrity (Li et al. 2014), (Nighot et al. 2015), (Mennigen et al. 2009). Similarly, insufficient levels of Claudin-5 have been linked to lower mucosal electrical resistance in lymphocytic colitis (Barmeyer et al. 2017) and increased vascular permeability in mice models of diabetes, which causes diabetic retinopathy (Rudraraju, Narayanan, and Somanath 2020). Junctional adhesion molecule (JAM) proteins are TJ-associated type I transmembrane proteins with extracellular immunoglobulin-like (Ig) loops that form homotypic and heterotypic adhesive interactions (Luissint, Nusrat, and Parkos 2014). It has been demonstrated that JAM-A controls barrier function as well as epithelial cell proliferation (Laukoetter et al. 2007); (Vetrano et al. 2008); (Wang et al. 2018). Mucosal permeability and polymorphonuclear leukocyte infiltration were both elevated in JAM-A–/–

mice (Laukoetter et al. 2007). Our interest stems from the notion that S1P is directly linked to tight junctional proteins, thus in vivo evaluation of S1P-mediated gut permeability is necessary.

FTY720 or fingolimod was approved as a novel oral medication for multiple sclerosis due to its significant immunosuppressive properties. The purpose of this discussion is to clarify the impact of FTY720 on gut physiology. S1P is a crucial regulator of cellular barrier function in mammalian cells, according to a growing body of research (Lee et al. 2006; Gaengel et al. 2012; Cartier et al. 2020); this, in part, entails the manipulation of the proteins that govern cell-cell adhesion (Lee et al. 2006; Kato et al. 2014). Ozanimod, an FDA-approved selective S1P receptor antagonist, also has evidence demonstrating that it inhibits the reductions in HBMEC integrity caused by hypoxia plus glucose deprivation (HGD) via means of the tight junction proteins PECAM-1 and claudin-5 (Wendt and Gonzales 2023). However, the interaction between FTY720 and the gut epithelia agonizes or antagonizes is still in debate.

Consistent with this idea, we reviewed the literature on gut physiological function and discovered that FTY720 increased gut permeability and decreased tight junction proteins like occludin and claudin4, while maintaining other TJ proteins including Claudin 1, ZO-1, and JAM-A. Intricate signaling hubs in a dynamic environment, tight junctions serve as a permeability barrier to prevent solutes from readily flowing through the intercellular space (Weber, 2012). Together with the cytoplasmic scaffolds proteins, Zonula Occludens and claudins (1-15) form TJ strands and play important functions in the formation of the epithelial barrier (Furuse 2010). Together with claudins, additional essential membrane proteins found in TJs include proteins belonging to immunoglobulin superfamily, including junctional adhesion molecules or JAM-A, and occludin (Furuse 2010). Additionally, they are essential for controlling paracellular permeability. The structural and functional components of TJ are arranged by the occludin's coiled-coil domain (Nusrat et al. 2000). Tight junctions have a major role in controlling paracellular permeability; when their integrity, assembly, and expression are compromised, permeability increases (Liang and Weber 2014). A reduction in occludin expression or function results in an increase in permeability since it is a crucial component of tight junctions. According to Mu et al. (2017), the flow of luminal components from the intestinal lumen to the host may result in systemic as well as a localized inflammatory response due to increased intestinal permeability. We have shown that FTY720 alters the tight junctional integrity by increasing the pore-forming Claudin-2. The increased paracellular influx of FITC-Dextran into blood proves to be the direct consequence of epithelial integrity loss (Fischer et al. 2017), (Souza et al. 2016).

An increased transepithelial locomotion of gut bacteria to the Liver indicates the authenticity of our previous finding of gut barrier disruption. Numerous studies using chemotherapeutic agents, radiation, epigenetic modifications, alcohol, hyperglycemia, ischemia, and autonomic dysfunction following

stroke have shown increased bacterial translocation in distant organs due to damage in tight junctions in the gut epithelium (Nakao et al. 2012) (Viaud et al. 2013), (Meisel et al. 2018).

The most important new discovery was that FTY720 impairs the function of the intestinal barrier in human intestinal cells. When colon carcinoma cell line HT-29 was exposed to the pharmacological S1P inhibitor FTY720, they exhibited a reduction in both Trans Epithelial Electrical Resistance (TEER) and occludin and Claudin-4 expression.

Regulation of tight junctional protein synthesis is mainly associated with Akt-mTOR phosphorylation (Wang et al. 2015), (Yan and Ajuwon 2017). Both Akt and mTOR are serine/threonine kinases (Kandel and Hay 1999) that phosphorylate a cascade of proteins and contribute to ribosome biogenesis (Chan et al. 2011), protein translation (Showkat, Beigh, and Andrabi 2014), and autophagy (Zhang et al. 2020). Phosphorylated mTOR subsequently activates downstream P70 S6K and 4EBP transcription factors through phosphorylation (Saxton and Sabatini 2017). Interestingly FTY720-mediated suppression of tight junctional protein synthesis, followed the Akt-mTOR pathway as the data showed an inhibition of Akt, mTOR and P70 S6K phosphorylation followed by a decreased Claudin-4 and Occludin expression in HT29 cells exposed to FTY720. The mTORC1 inhibitor Rapamycin was kept as the positive control and showed similar results which further verifies and concludes the fact that FTY720 indeed follows the Akt/mTOR axis to regulate tight junction protein synthesis in HT29 cells.

Hence, we conclude that S1P synthesis as well as signaling is very much crucial for intestinal epithelial cells to maintain integrity by keeping the Tight junctional homeostasis within the cellular membrane. Also, the markedly increased permeability at the intestinal barrier associated with FTY720 oral consumption causes a deleterious effect in modulating gut integrity.

Objective Two:

***“Investigating the role of Sphingosine -1-phosphate (S1P) in
gut immunology”***

5.2. Introduction

The S1P needs to be extracellularly transported to release into the environment, where it attaches to cellular S1P receptors (S1PRs) and initiates downstream communication between S1P and S1PR (Nishi et al. 2014). S1P is said to control innate immune cells and their role in immune responses by acting on the cell surface through its five identified GPCR receptors (S1PR1–S1PR5) (Masana et al. 1995), (Goetzl, Kong, and Voice 2000), and (Hla et al. 2001). This is how S1P is said to play a significant role in tolerance.

S1P receptor expression and their functional modulation can significantly influence cellular functions in different conditions. As the S1P signal is linked with multiple physiological functions S1P is considered to be critical and a considerable amount of understanding is needed to unwind its dual nature sometimes. As in our first chapter we showed S1P is critical for barrier regulation in gut epithelial layer. But the deletion of the sphingosine kinase gene (Sphk), in the pregnancy model disrupted the sphingolipid pathway, causing the decidua to express neutrophil chemoattractants CXCL1 and CXCL2 at abnormally high levels, which further increased oxidative damage in the decidua and weaken the immune tolerance at feto-maternal interface eventually leads to the premature death of the allogenic fetus (Mizugishi et al. 2015). Decidual cells from the human first-trimester female secrete high levels of neutrophil chemoattractant CXCL-1 and IL-8 when treated with Dimethyl sulfoxide (DMS) a competitive inhibitor of SPHK-1 (Mizugishi et al. 2015). By altering S1P signaling, FTY720, also known as fingolimod, prevents lymphocytes from leaving the thymus, spleen, and lymph nodes (LNs) and entering the circulation and lymphatic system, stops the lymphocyte traffic from egressing from the LNs to target organs (Chiba 2005). According to Sykes et al. (2014), FTY720 enters the body, phosphorylates (FTY720-P), attaches itself to S1PR (1–5), and internalizes all receptors save S1PR3, preventing its downstream signaling. It is noteworthy that FTY720-P is limited to activating S1PR3, which is mostly present in monocytes, macrophages, B lymphocytes, and neutrophils (Bryan and Del Poeta 2018). This causes more downward signaling (Sensken et al. 2008). Furthermore, it affects dendritic cell (DC) migration (Han et al. 2015), regulates proinflammatory signaling mediated by DCs (Zeng et al. 2012), and effectively inhibits the proliferation of regulatory T lymphocytes (Wolf et al. 2009). It is currently unclear if FTY720 controls lymphocyte recirculation in vivo in an agonistic, functional antagonistic, or both ways (Blanc et al. 2015). The drug FTY720 (marketed under the brand Gilenya) has FDA approval to treat multiple sclerosis relapses (Sharma et al. 2011). Several studies demonstrated the protective effectiveness of FTY720 in a variety of preclinical models, including the IL-10 deficient mouse model (Mizushima et al. 2004), the oxazolone-induced murine model of colitis (Daniel, Sartory, Zahn, Schmidt, et al. 2007), the TNBS-induced murine model of colitis (Daniel, Sartory, Zahn, Geisslinger, et al. 2007), and DSS (Deguchi et al. 2006). FTY720 is in clinical trial for IBD patients (Danese, Furfaro, and Vetrano 2018). It was also effective in treating viral infection models (Miyamoto et al. 2001), rheumatoid arthritis (Tsunemi et al. 2010), graft-versus-host disease (Ryu et al.

2020), and (Gauthier et al. 2018) in mice. By modifying the bone marrow's colitogenic CD4+ T cells, FTY720 inhibits colitis (Fujii et al. 2008).

5.2.1. Results

5.2.1.1. S1P analog FTY720 consumption affects gut health in mice

Mice of 6 weeks old were fed with FTY720 (3mg/kg body weight) for 14 consecutive days. Daily body weight measurements were made during the trial and the change in body weight among mice groups was calculated. Compared to their control littermates, the FTY720-fed mice displayed a

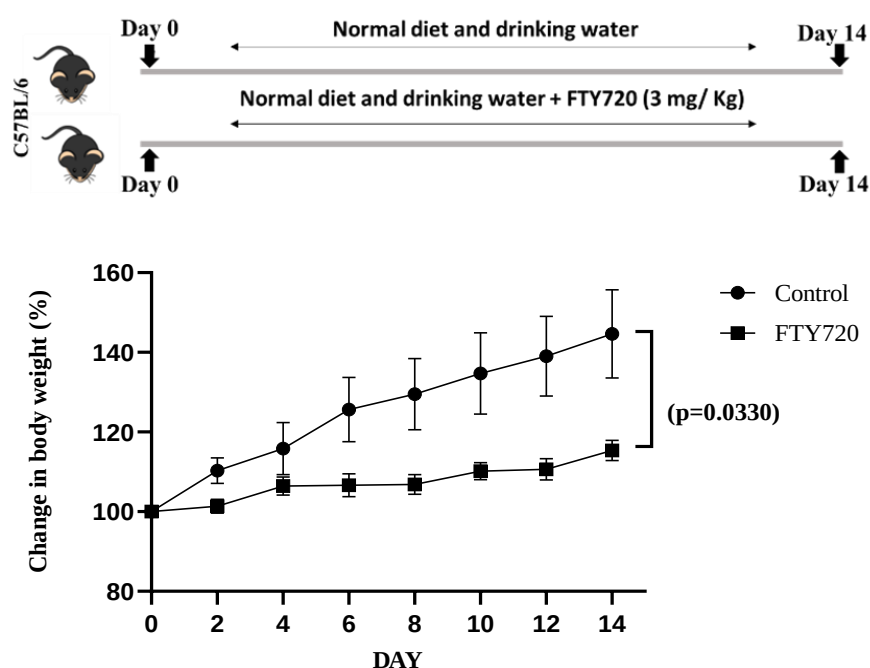


Figure- 5.2.1.1A: (a) Animal model with C57BL/6 mice, grouped as control and FTY720. The FTY720 group received FTY720 (3mg/kg body weight) for 14 consecutive days where the control mice were given standard chow. (b) Day-wise change of body weight percentage comparison between control and FTY720 fed mice. Experiments carried out in triplicates (n=3).

static development in body weight over time, while an increase in body weight was noticed in the control mice with healthy growth over time (Figure 5.2.1.1A). Data on the daily food and water intake of FTY720 and control mice were documented. Mice belonging to both groups did not show any noticeable change in their food consumption pattern, though FTY720 grouped mice showed comparatively lower thrust (Figure 5.2.1.1B). Changes in body weight of the control and FTY720 mice were regularly monitored and recorded as well.

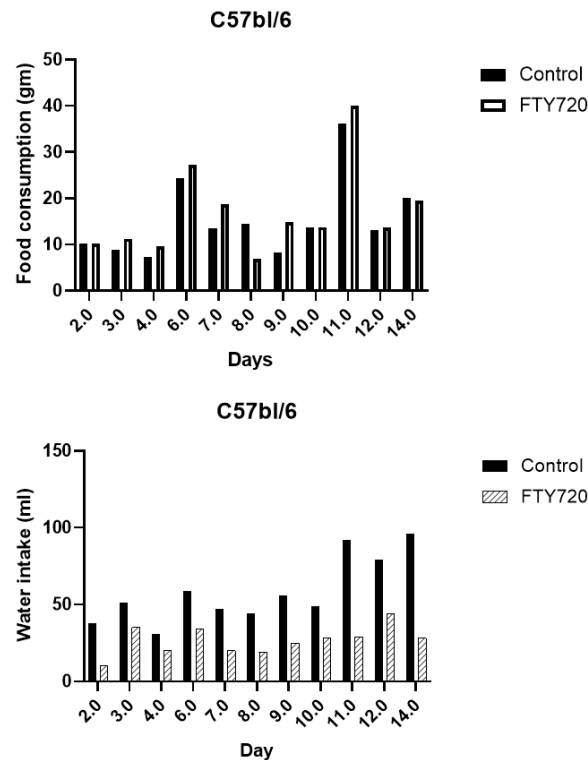


Figure-5.2.1.1B: 6-week-old Mice were grouped as control and FTY720. FTY720 mice were orally given 3mg/kg FTY720 for 14 days. A. Daily food consumption and B. daily water intake by the control and FTY720 mice.

5.2.1.2. Colon length change

Shortening of the colon was observed in the FTY720-fed mice when we compared that with the control colons (Figure 5.2.1.2A). Colon length constriction is one of the other symptoms such as fever, bloody stools, and loss of body weight which are associated with Inflammatory Bowel Disease (IBD) and can occasionally develop into colorectal cancer (Kim and Chang 2014). Despite the fact that the exact origins of IBD are unknown, it is believed that the intestinal mucosa's impaired immune response results from environmental factors leading to inflammatory conditions in the mucosa (Sartor 2008).

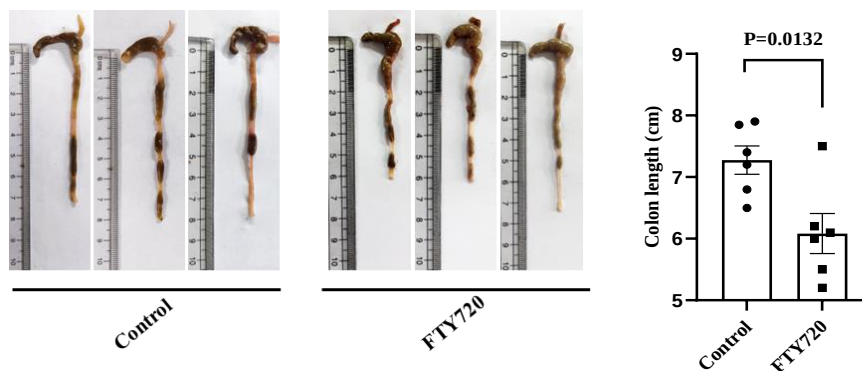


Figure- 5.2.1.2A: A. Images of the colons collected from control mice and FTY720-fed mice. B. Relative change in the colon length of control and FTY720 mice. All the experiments were conducted with $n \geq 5$ animals for each group. Data representing the Mean $\pm$ SEM values.

5.2.1.3. Tissue histology analysis

In search of the inflammatory responses in the gut, we examined the histological architecture of the FTY720- colon, and the control colon was compared. In comparison to the control, histological examination of the colon samples revealed that inflammatory cells had infiltrated the FTY720-mice. Colon histological sections from FTY720-mice revealed hyperplasia of mucous-secreting goblet cells and expansion of the colon's muscularis layer (Figure 5.2.1.3A).

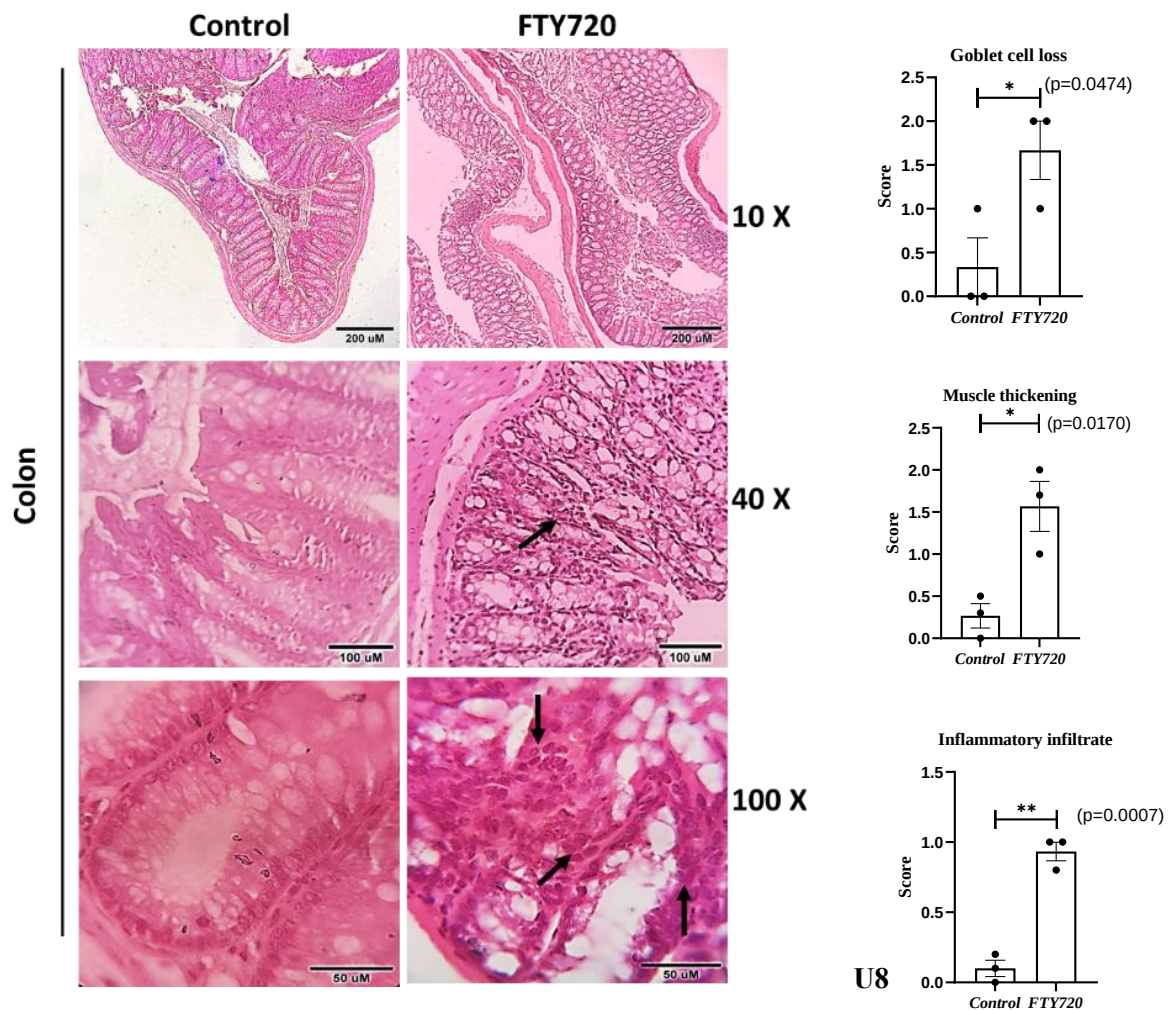


Figure- 5.2.1.3A: A. Histology of colon sections of control and FTY720 mice. Arrows were used to point the inflammatory infiltrates in the crypts. Images were taken at different magnifications (10X, 40X and 100X). B. Graphs denote histological scores for thickening of the outer muscularis layer, immune cell infiltration, and goblet cell loss. Experiments were conducted with $n \geq 5$ animals for each group. Data representing Mean $\pm$ SEM values.

5.2.1.4. Detection of Neutrophil infiltration by anti-MPO antibody in the mice colon

To ensure the presence of neutrophils in the colonic crypts, we stained the colon sections with an anti-MPO (Myeloperoxidase) antibody. A secondary HRP-conjugated antibody was used. The peroxidase oxidizes DAB and generates a brown-colored precipitate. The presence of brown precipitates under the microscope indicates the presence of neutrophils. Neutrophils generate MPO in their granules and release it extracellularly. MPO oxidizes DAB by splitting hydrogen peroxide, resulting in the formation of a brown, insoluble precipitate (Bain, B. and Swirsky, D., 2012). In the FTY720 colon sections, a significantly ($p=0.0029$) high number of brown spots were detected under the microscope compared to that of the control sections (Figure 5.2.1.4A).

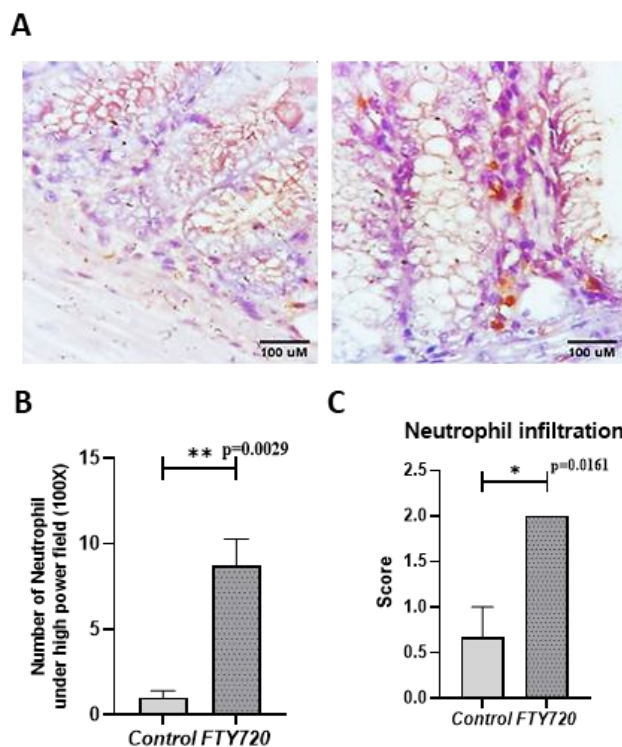


Figure-5.2.1.4A: A. Image showing the presence of MPO in the colon crypts of FTY720 and control mice. Images were taken under a light microscope with 100X magnification. B. The relative number of neutrophils was compared between FTY720 and control mice, under a high-power field. C. Neutrophil infiltration score of FTY720 colon and control colon. All the experiments were conducted with $n \geq 5$ animals for each group. The data presentation showed a Mean $\pm$ SEM value.

5.2.1.5. FTY720 affects the inflammatory markers in the gut

In order to confirm the gut's inflammatory response to FTY720 therapy, we assessed pro-inflammatory chemokines CXCL-1 and CXCL-2 and cytokines such as TNF- α , IL-6, and IFN- γ . Also, from anti-inflammatory cytokines, we checked IL-10 expression from the mouse colonic lysates. Interestingly, a

significant enhancement of CXCL-1 ($p= 0.0003$) and CXCL-2 ($p= 0.0180$), TNF- α ($p= 0.0206$), and IFN- γ ($p= 0.0103$) in FTY720 fed mice colon was seen when compared to the control; however, no change was observed for IL-6 and IL-10 expression. CXCL-2, as well as CXCL-1, had a substantial increase in FTY720 colons, which is a regulator of neutrophil migration, and inflammation (Sawant et al. 2021), further correlating the high MPO signal found in the FTY720 colons (Figure 5.2.1.5A).

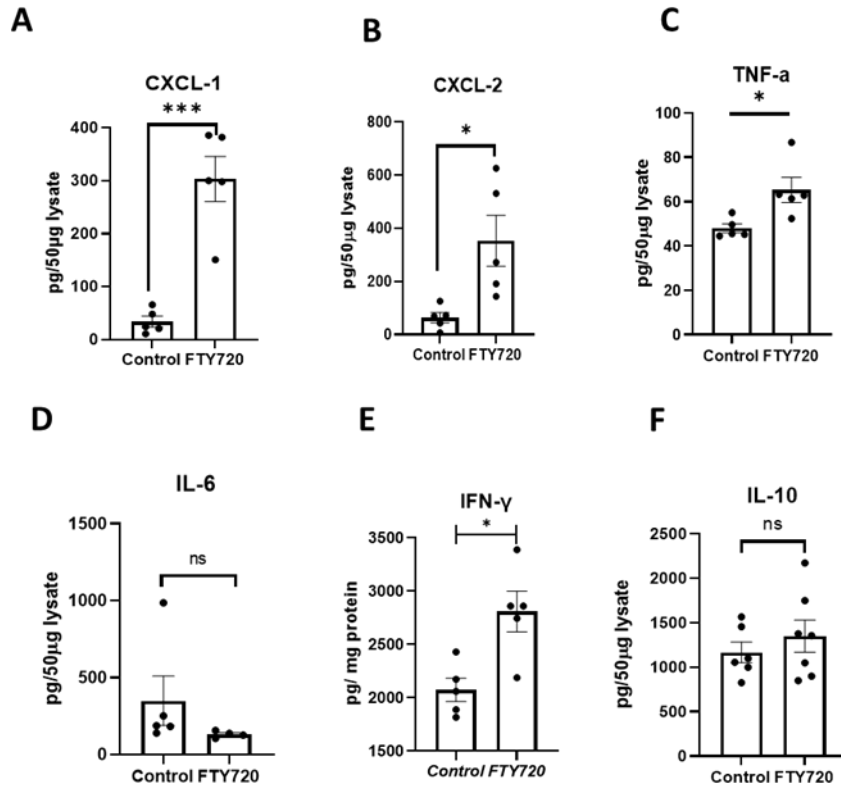


Figure-5.2.1.5A: ELISA analysis of the secretory cytokine CXCL-1 and CXCL-2 in the gastrointestinal tissue of FTY720 and control mice. FTY720 and control mice's gastrointestinal tissue TNF- α and IFN- γ production as determined by ELISA. All the experiments were conducted with $n \geq 5$ animals for each group. Graphs representing a Mean $\pm$ SEM value of data.

For the genomic expression of inflammatory cytokine genes from the colonic mRNA pool, we performed real-time quantitative PCR method for TNF- α , IFN- γ , TGF- β , and IL-10. For FTY720-fed mice colon, we found a significant increase in TNF- α ($p= <0.0001$) and IFN- γ ($p= 0.0088$). Surprisingly we observed a significantly increased expression of TGF- β ($P= 0.0079$) but IL-10 expression remained unchanged similar to the data acquired from the ELISA (Figure 5.2.1.5B).

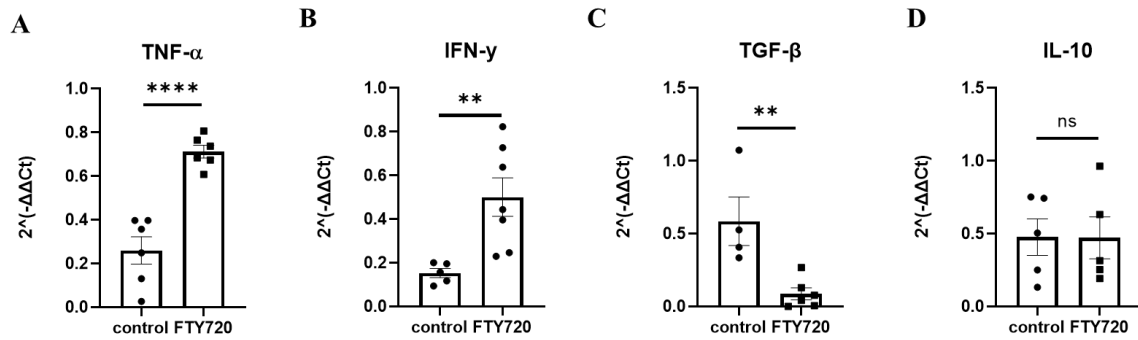


Figure-5.2.1.5B: Data represents the mRNA expression of TNF- α , IFN- γ , TGF- β , and IL-10 cytokines from the control and FTY720 tissue-derived RNA. All experiments were conducted with $n \geq 5$ animals for each group. Data representation as Mean $\pm$ SEM.

5.2.1.6. Immune infiltration in the colon

A dysregulated gut barrier often triggers mucosal immune response thus compromised gut permeability is closely related to immune cell infiltration (Lin et al. 2020). The increased cytokine and chemokine expression in the colon leads to the question of the involvement of the immune cells in the tissue. To address this question the study approaches the tissue immune cell population and the secondary lymphoid organ-specific population of different immune cells. Secondary lymphoid organs such as Payer's patch, mesenteric lymph nodes, and spleen were selected based on their proximity to the colon. The cell population which was curated from secondary lymphoid organs was examined by Flow-cytometry using fluorochrome-conjugated antibodies. And, the tissue-resident immune cell detection, Immune -Histochemistry analysis was done by using the same fluorochrome-conjugated antibodies.

An increased percentage of CD4 $^{+}$ T lymphocyte and CD8 $^{+}$ T lymphocyte cells were noticed in the mesenteric lymph nodes and the spleen. However, the Peyer's' patch population was unchanged for CD4 $^{+}$ and CD8 $^{+}$ T cell populations. The CD25 $^{+}$ FoxP3 $^{+}$ T-reg cell population and CD11b $^{+}$ macrophage population within MLN showed no change over time after FTY720 administration (Figure 5.2.1.6A).

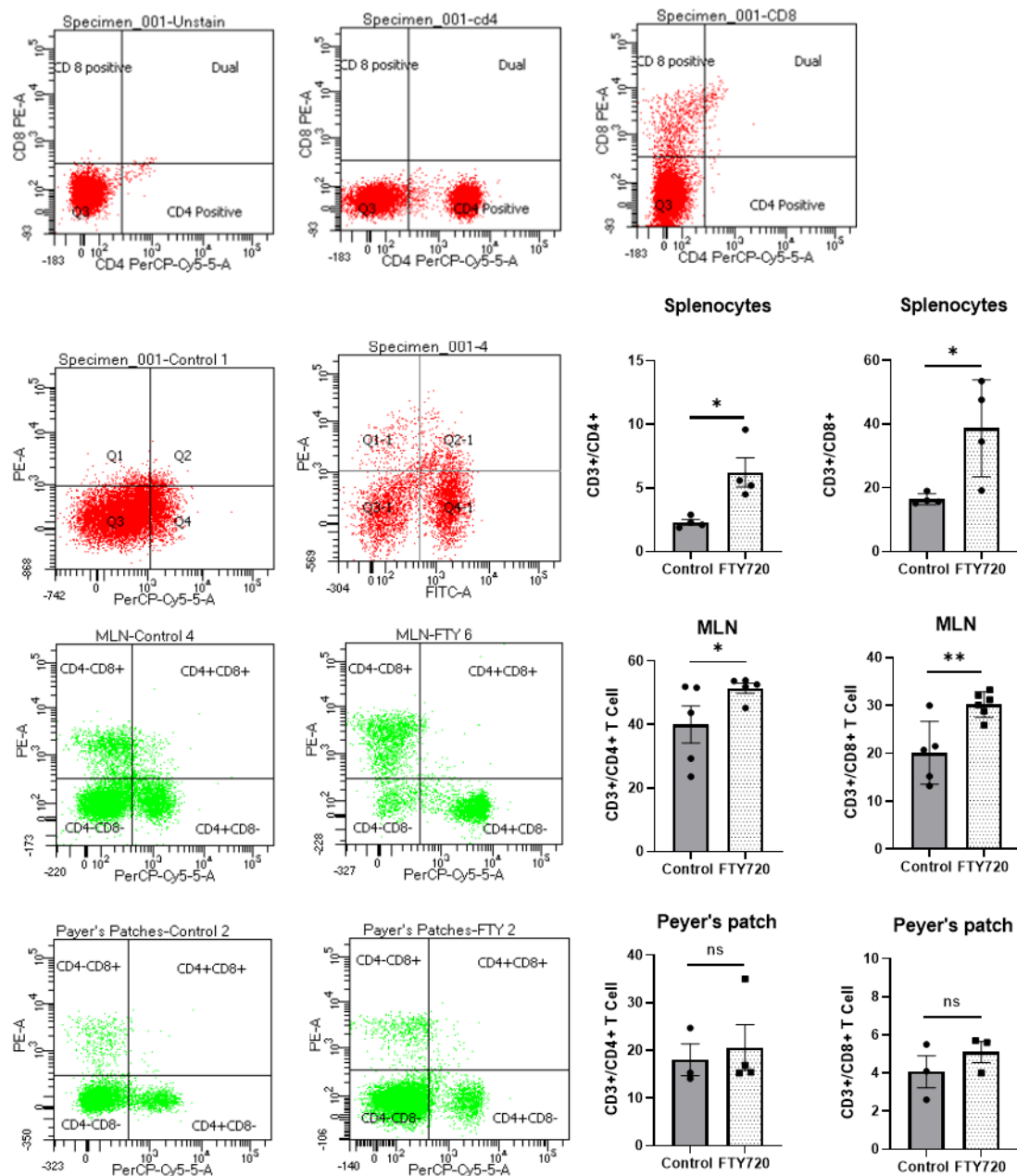


Figure-5.2.1.6A: The Flowcytometric analysis of CD4+ T cell and CD8+ T cell from Spleen, MLN (Mesenteric lymph node) and Peyer's patch. The CD3+ gated population from the singlet cells were gated for CD4+, CD8+, CD4-CD8-, CD4+CD8+. All the experiments were conducted with $n \geq 5$ animals and the data are presented as Mean $\pm$ SEM. P values were put, calculating with respect to control.

Surprisingly at the mucosa level, the immune fluorescence data showed a significant presence of B lymphocytes (represented by CD19+ cells), CD11b+ macrophage cells, Natural killer cells (NK1.1+cells), and Neutrophils (CD11b+Ly6G+ cells) in the colon tissue of FTY720 mice compared to the control. However, no change was observed in the case of T cells as the tissue signal for CD3+ cells was unaltered in both control and FTY720 tissue sections (Figure 5.2.1.6B- G).

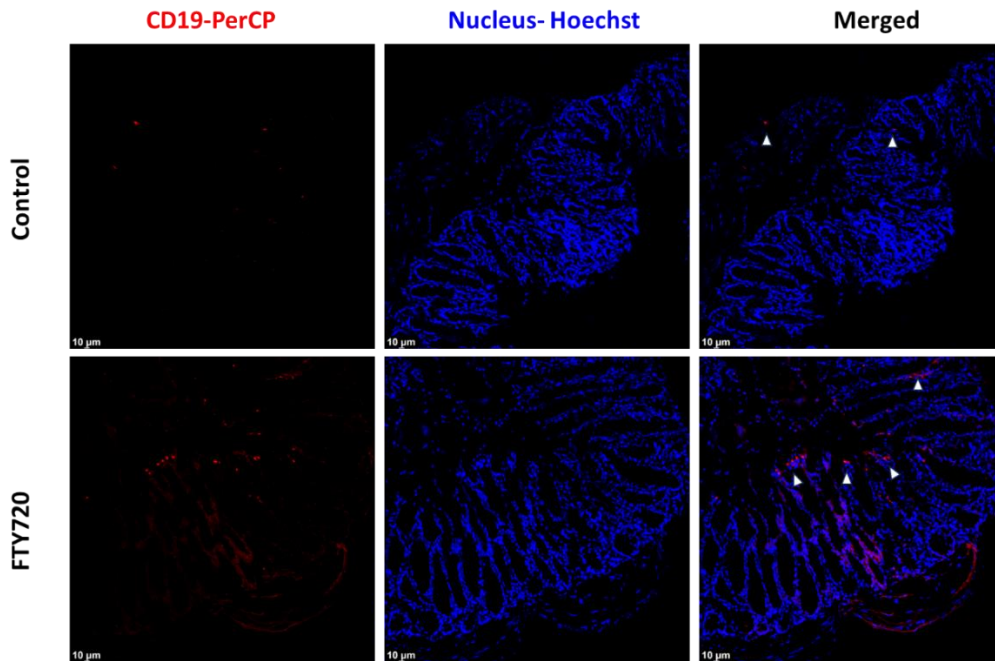


Figure-5.2.1.6B: Image indicates the presence of per CP conjugated CD19+ (red colour) B cells at the colonic crypts. Images were taken under the confocal microscope with 60X magnification. The small white triangles were used as indicators of the red dots. Images carrying a 10-micrometer scale bar at the left bottom.

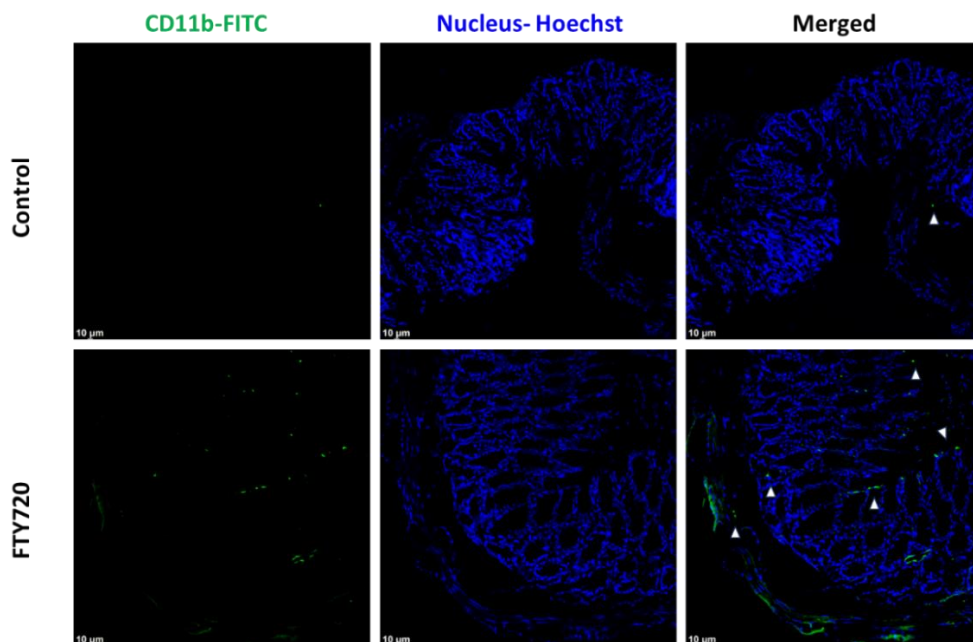


Figure-5.2.1.6C: Image showing the presence of FITC conjugated CD11b+ (green colour) myeloid cells at the colonic crypts. Images were taken under the confocal microscope with 60X magnification. The small white triangles were used as indicators of the red dots. Images carrying a 10-micrometer scale bar at the left bottom.

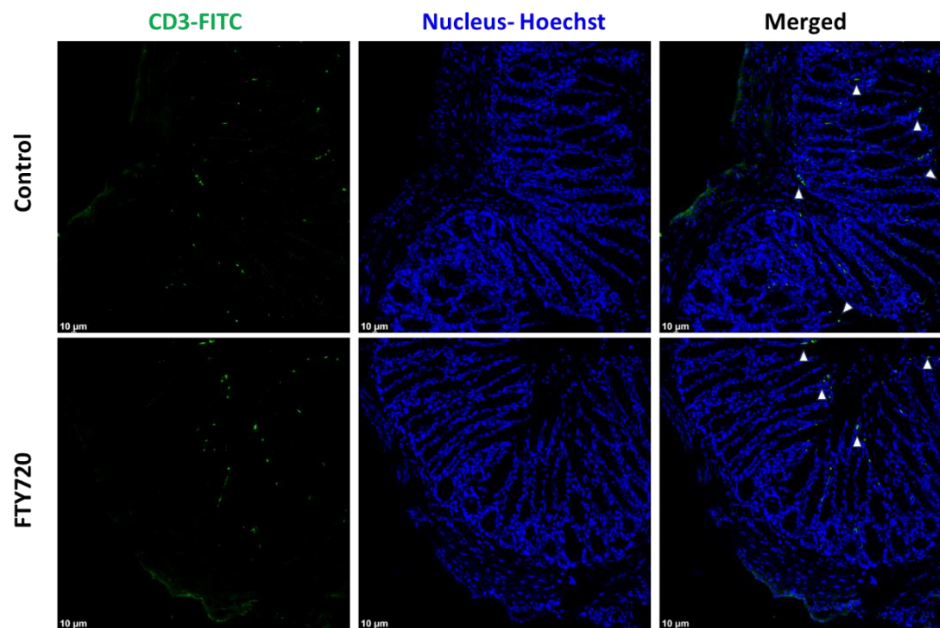


Figure-5.2.1.6D: B. Image showing the presence of FITC conjugated CD11b+ (green colour) macrophage cells and C. FITC conjugated CD3+ T- cells at the colonic crypts. Images were taken under the confocal microscope with 60X magnification. The small white triangles were used as indicators of the green dots. Images carrying a 10-micrometer scale bar at the left bottom.

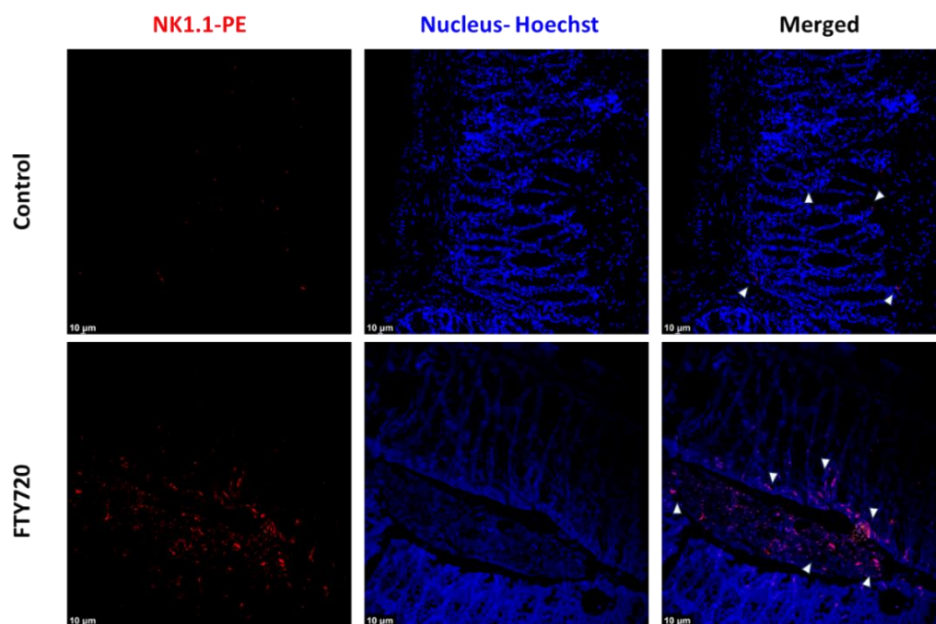


Figure-5.2.1.6E: Image showing the presence of PE conjugated NK1.1 (red colour) Natural killer cells at the colonic crypts. Images were taken under the confocal microscope with 60X magnification. The small white triangles were used as indicators of the red dots. Images carrying a 10-micrometer scale bar at the left bottom.

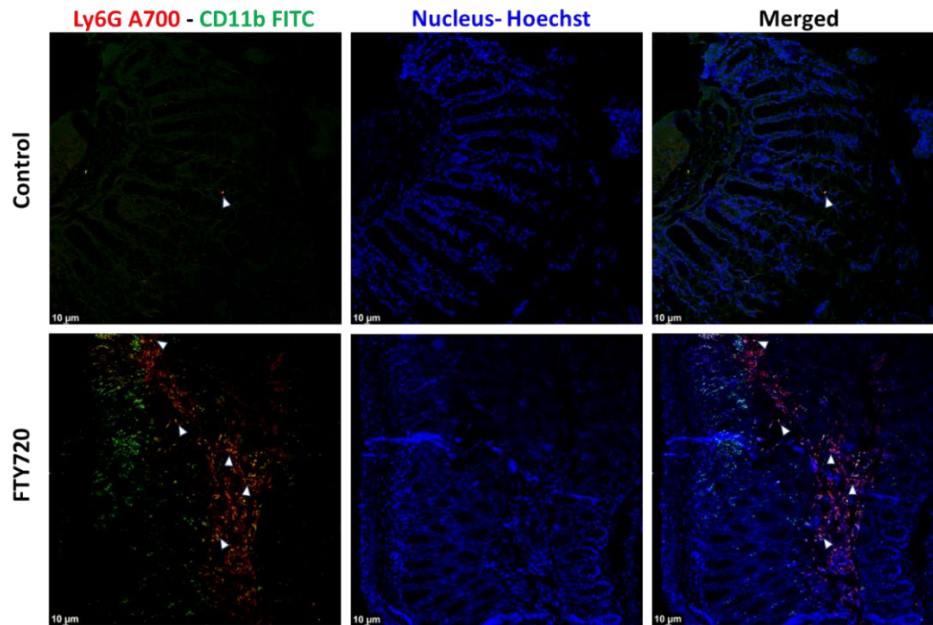


Figure-5.2.1.6F: Image showing the presence of PE-conjugated NK1.1+ (red colour) Natural Killer cells and E. A700 conjugated Ly6G+ (red) and FITC conjugated (green) CD11b+ cells (appeared as yellow/orange) at the colonic crypts. Images were taken under the confocal microscope with 60X magnification. The small white triangles were used as indicators of the green dots. Images carrying a 10-micrometer scale bar at the left bottom.

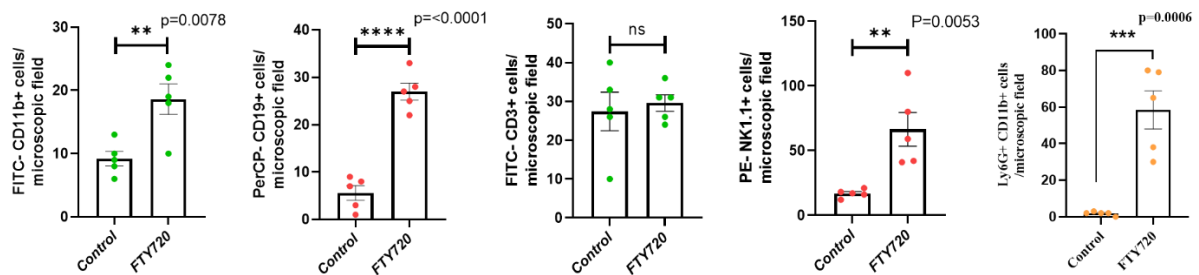


Figure-5.2.1.6G: Data represents the number of cells found at each microscopic field and compared between control and FTY720. This graph represents multiple replicative data. For statistical analysis, a simple T-test was done with $n \geq 5$ animals for each group. Data representation as Mean $\pm$ SEM.

5.2.1.7. Antimicrobial peptides

Antimicrobial peptides or AMPs are produced in the gut by the intestinal epithelial cells (Muniz, Knosp, and Yeretssian 2012). By rupturing the cell walls of harmful microbes, AMPs can eliminate them (Hartmann et al. 2010), (Krepuska et al. 2024). The data that we have got from the barrier protein expression of the FTY720 treated gut along with the gut bacterial translocation into the host tissue we

investigated the colonic expression of the AMPs. From the colonic mRNAs, we targeted Cathelicidin, Hepcidin and Mucin-2 gene as antimicrobial peptide regulating genes (Figure 5.2.1.7A). And,

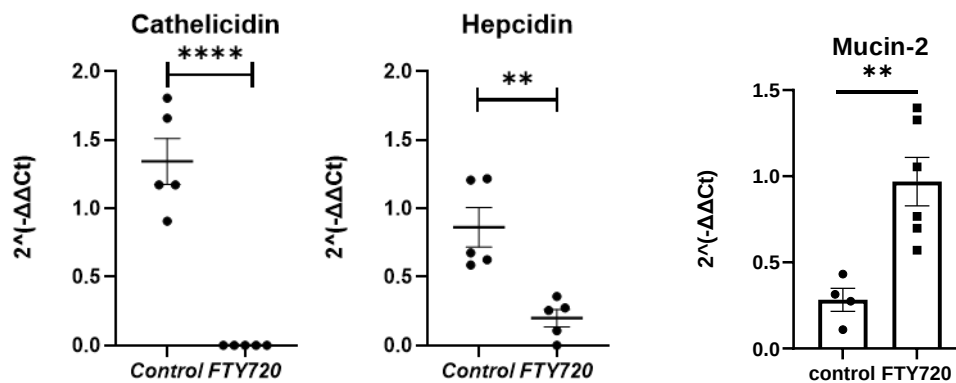


Figure- 5.2.1.7A: Data represents the expression of antimicrobial peptides Cathelicidin, Hepcidin, and Mucin-2. A simple T-test was done with $n \geq 5$ animals for statistical analysis and the data presentation showed Mean $\pm$ SEM.

surprisingly there we found a significantly decreased expression of cathelicidin and Hepcidin in the FTY720 colon samples. On the other hand, a significantly higher, almost 2-fold increased expression of the Mucin-2 gene was observed when we compared FTY720 with the control.

5.2.1.8. Metagenomic analysis of the gut microbiome of control and FTY720-treated mice

To determine whether FTY720-induced gut barrier disruption and inflammatory condition have any effect on gut microbial composition, we studied metagenomics of the gut commensal bacterial population from control and FTY720. We analyzed the relative abundance of bacterial phylum, Class, Order, Family and Genus (Figure 5.2.1.8A-D). We also did alpha and beta diversity analysis. From the relative abundance data of bacterial phylum (Figure 5.2.1.8M-O), we found FTY720-fed mice had a significant decrease in the Actinobacteria population and a slight decrease in the Bacteroidetes population compared to that of the control. No significant change in the Firmicutes and Proteobacteria population was found though. While analysing the relative abundance bacterial Genus, surprisingly we found a significant ($p=0.0051$) decrease in Bifidobacterium population and a decrease in Bacillus population, though it is not a significant difference. The lactobacillus population showed no change for both the groups. Though Clostridium population was significantly ($p=0.0006$) higher in FTY720 gut than in the control. The Bacteroides, Prevotella, Muribaculum and Campylobacter population remained unchanged in both groups (Figure 5.2.1.8E). To study the diversity of species or individuals within a particular sample at a particular time, we looked for alpha diversity analysis. We performed four methods, i.e., Chao1 (Figure 5.2.1.8I), Shannon (Figure 5.2.1.8J), Simpson (Figure 5.2.1.8K) and Fisher (Figure 5.2.1.8L), with the statistical method of T-test / ANOVA for Alpha diversity analysis. The

species richness and species evenness were analysed by using Chao 1 index and Shannon's index respectively. However, both the Chao 1 index and Shannon's index showed no comparable changes between FTY720 and control groups.

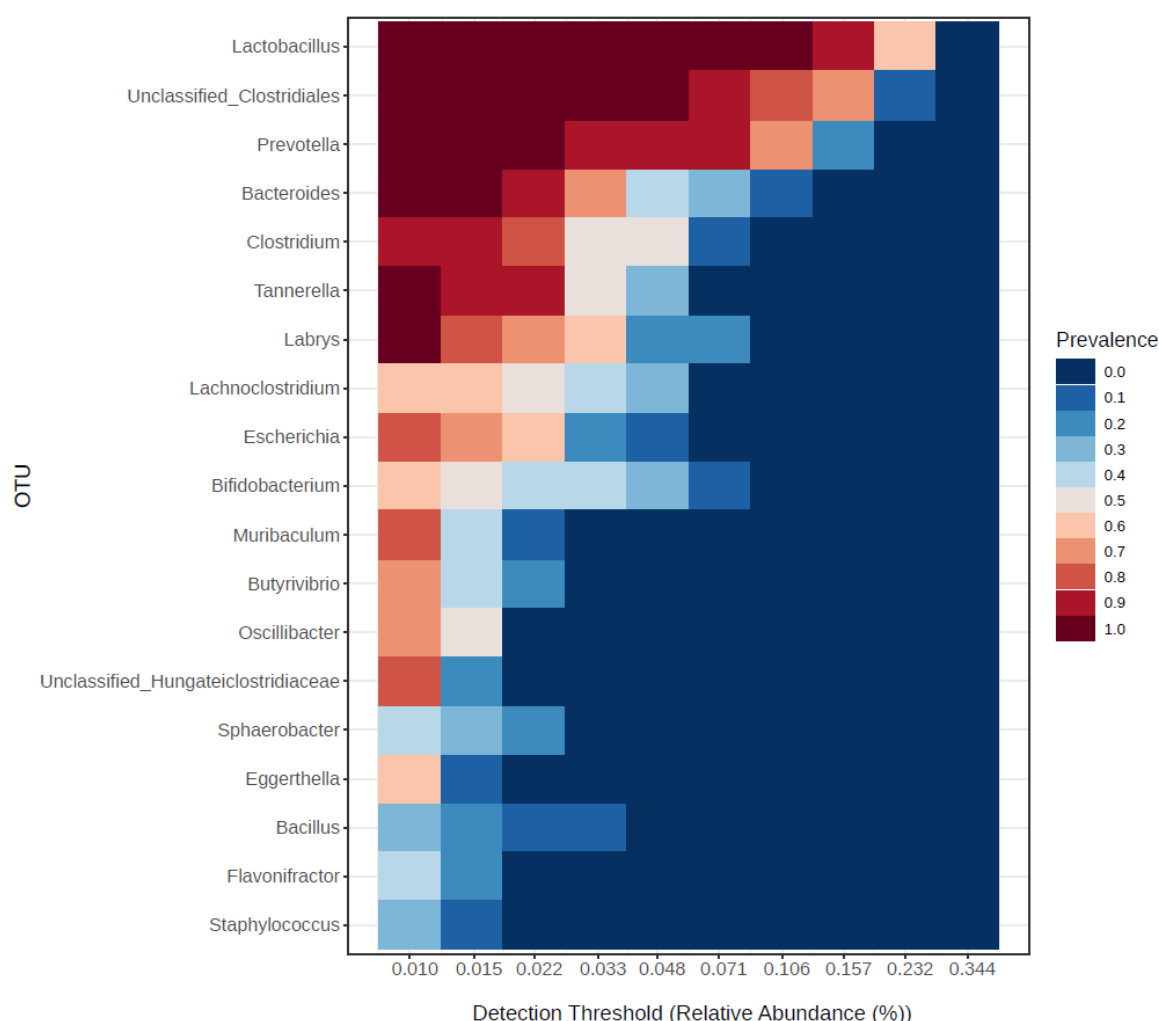


Figure- 5.2.1.8A: The data is representing the core microbiome that has been detected in a remarkable fraction of population above a given threshold. The data was visualized with the sample prevalence of 20% and relative abundance of 0.01 %. The detection threshold and the identifying core microbiota are represented on the X-axis and Y-axis respectively. Red coloured blocks represent the high prevalence of a taxa under the threshold and the blue boxes represents s taxa's low prevalence under the given threshold.

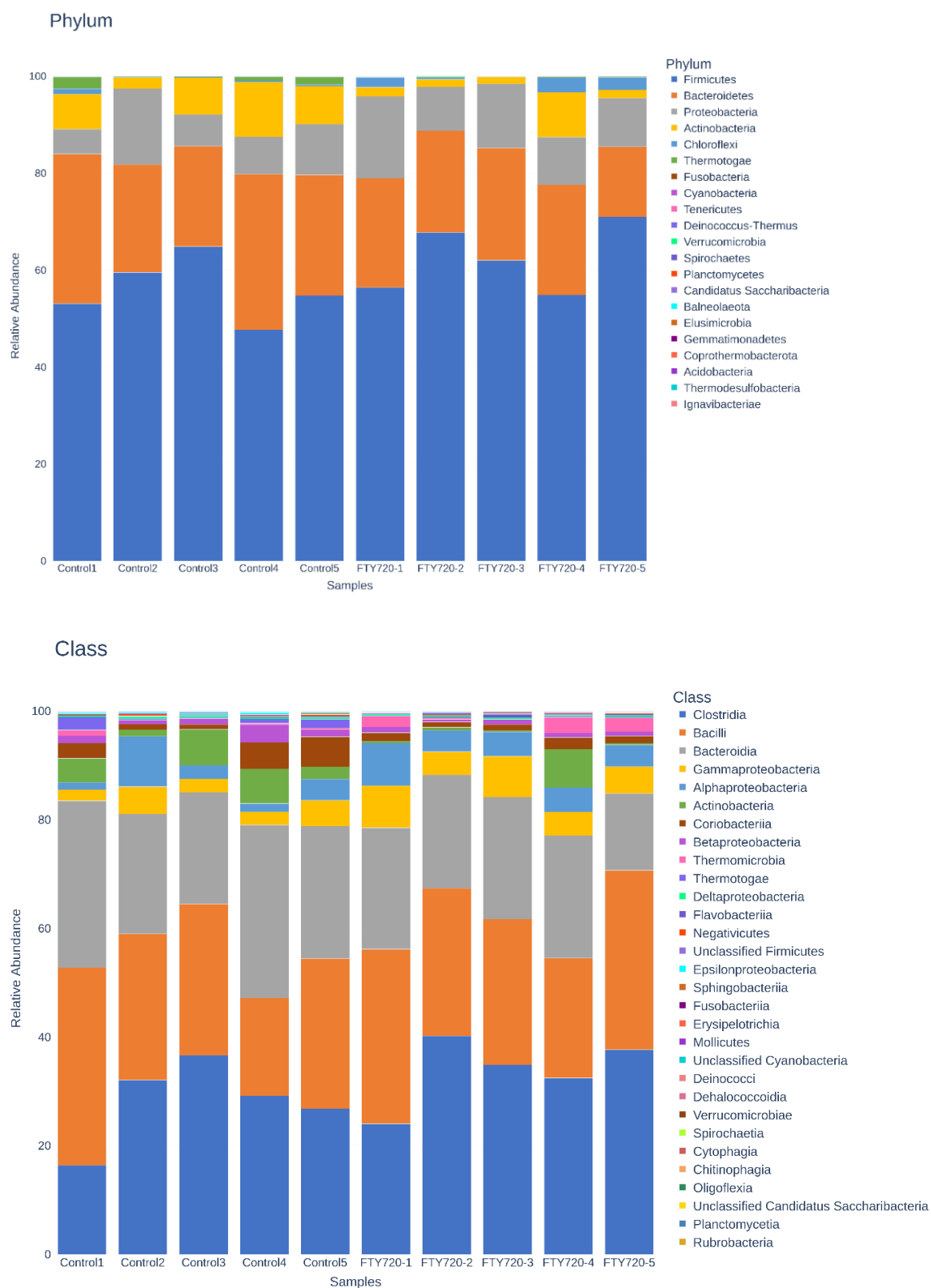


Figure-5.2.1.8B: The image represents the relative abundance, plotted against the OTU table matrix of A. bacterial Phylum and B. bacterial Class.

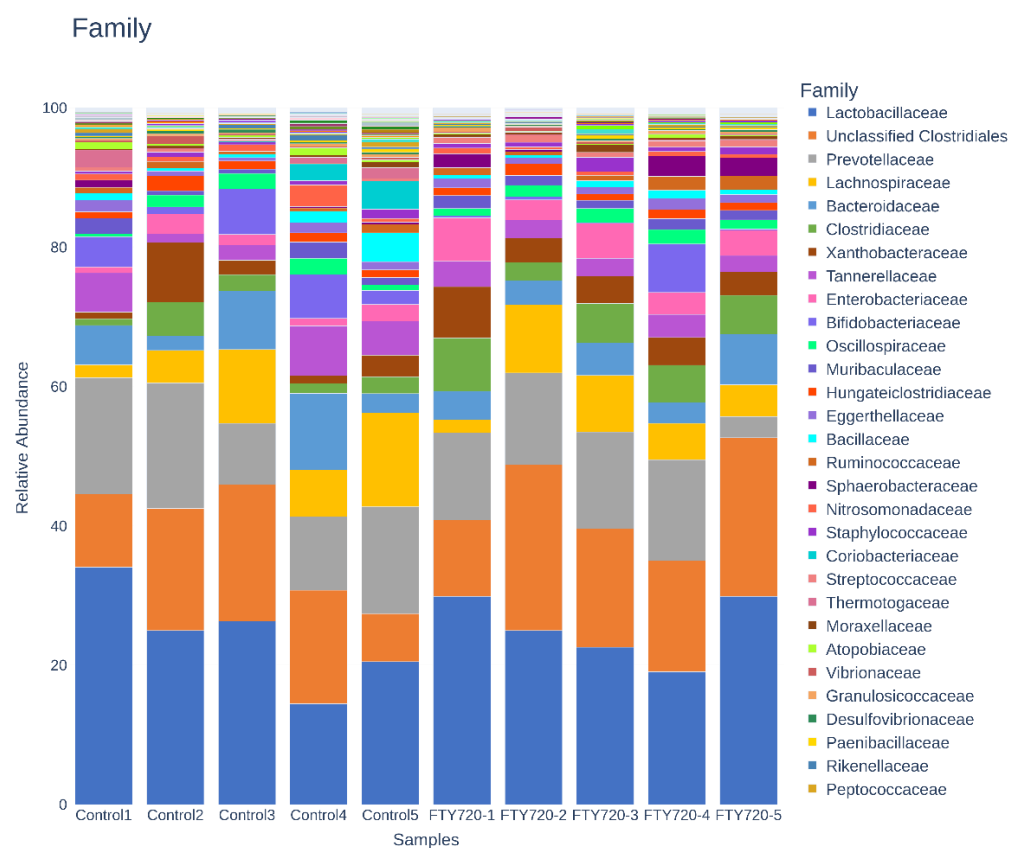
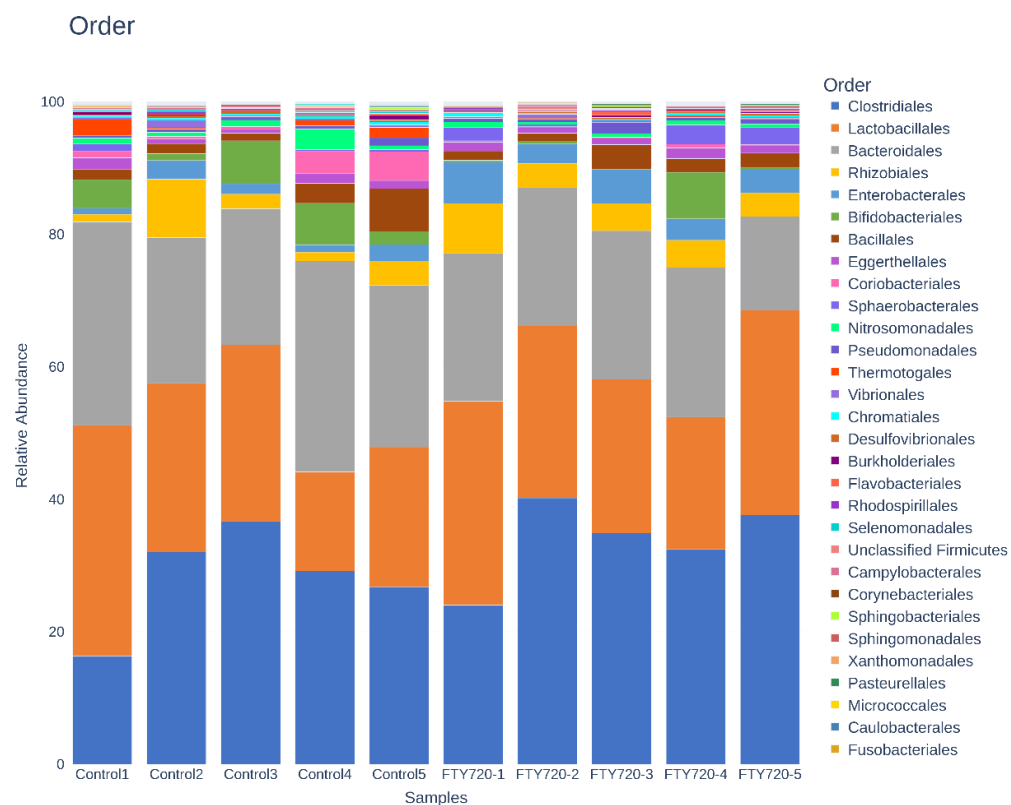


Figure-5.2.1.8C: The image represents the relative abundance, plotted against the OTU table matrix of A. bacterial Order and B. bacterial Family

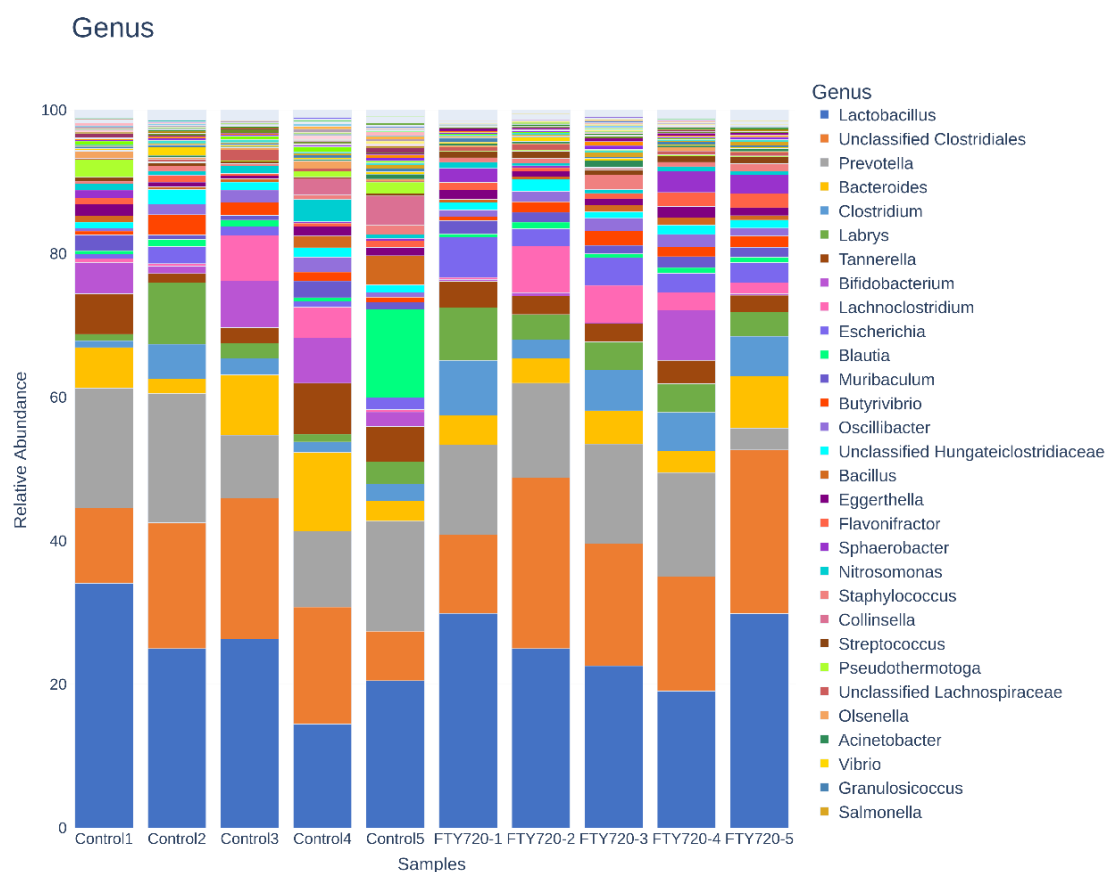


Figure-5.2.1.8D: The image represents the relative abundance, plotted against the OTU table matrix of bacterial Genus.

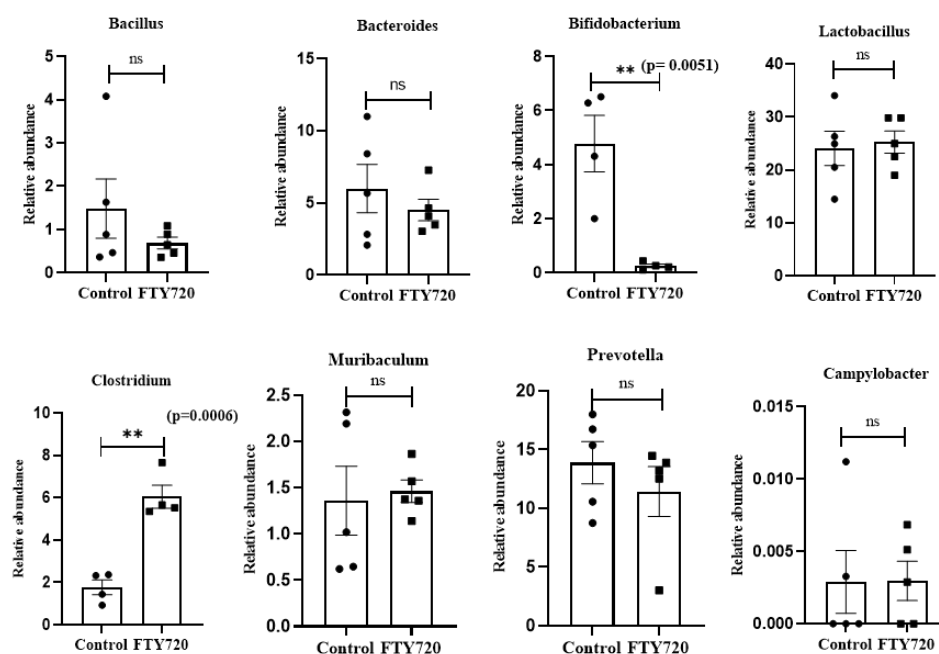
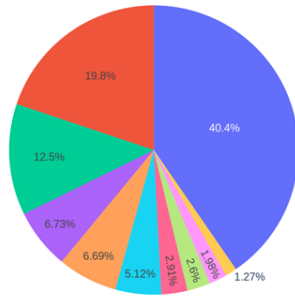
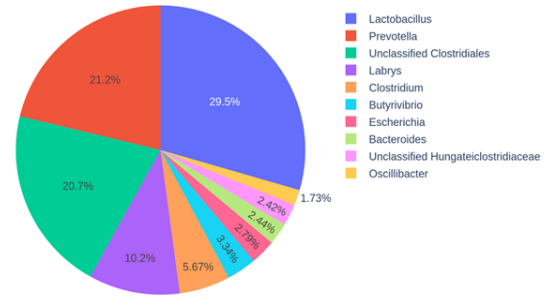


Figure 5.2.1.8E: Plot showing the relative abundance of specific bacterial genus from control and FTY720 and their comparative analysis using student's T test with GraphPad prism.

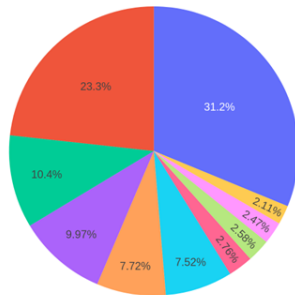
Control1



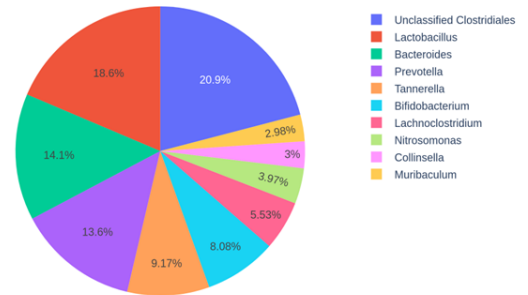
Control2



Control3



Control4



Control5

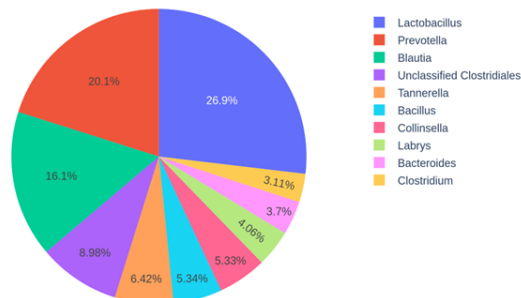


Figure 5.2.1.8F: The pie charts are representing the top 10 enriched Genus of each control sample.

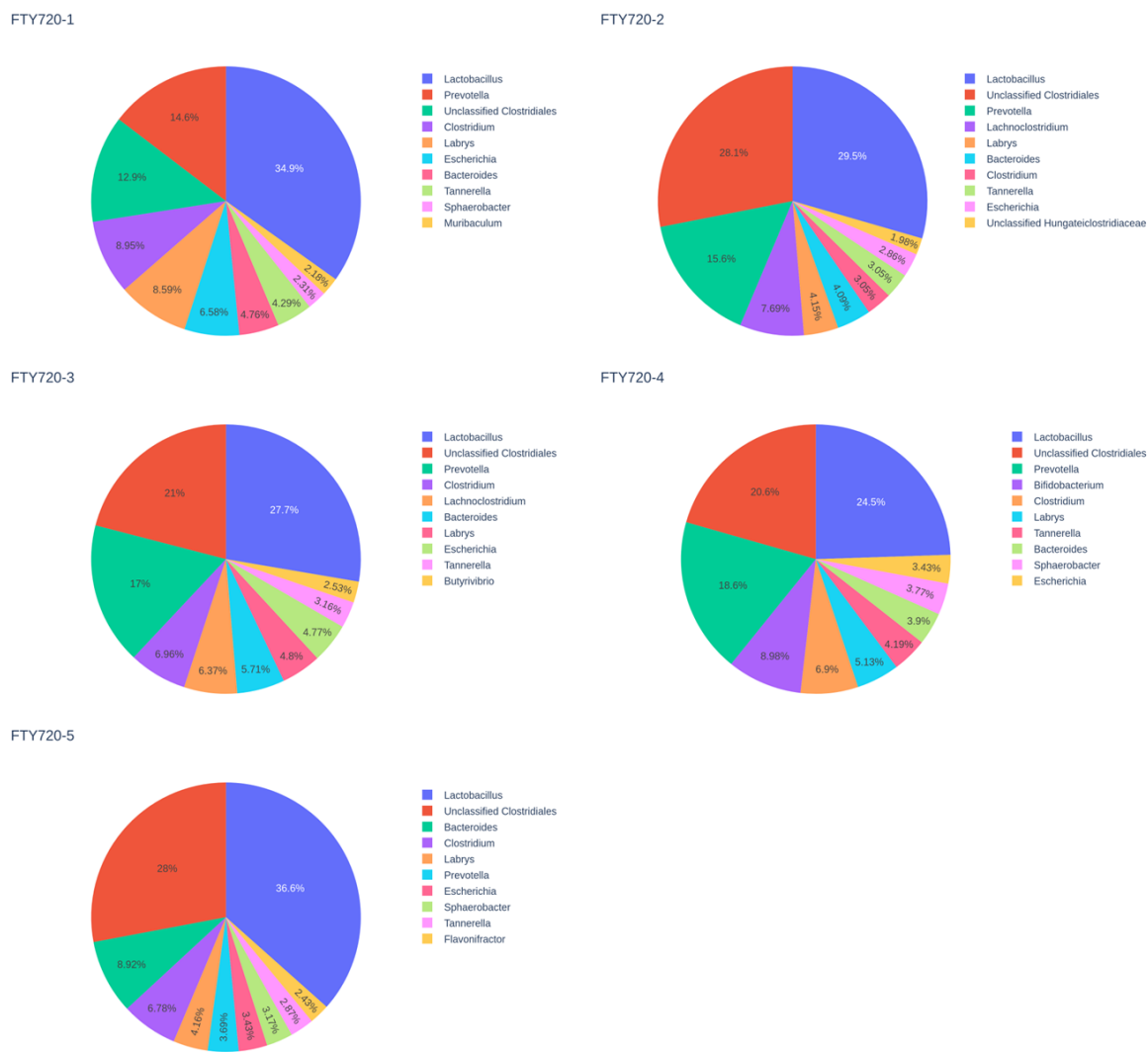


Figure 5.2.1.8G: The pie charts are representing the top 10 enriched Genus of each FTY720 sample.

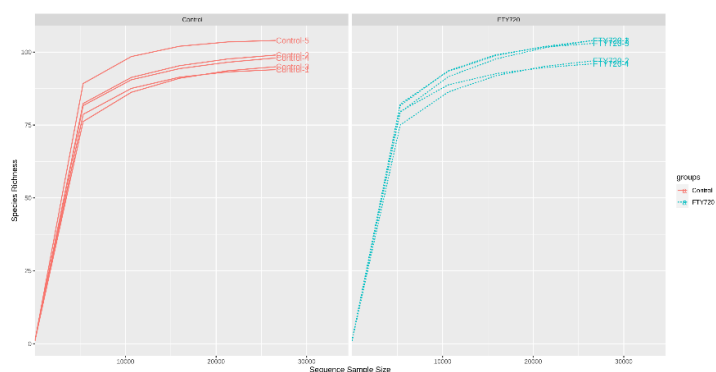
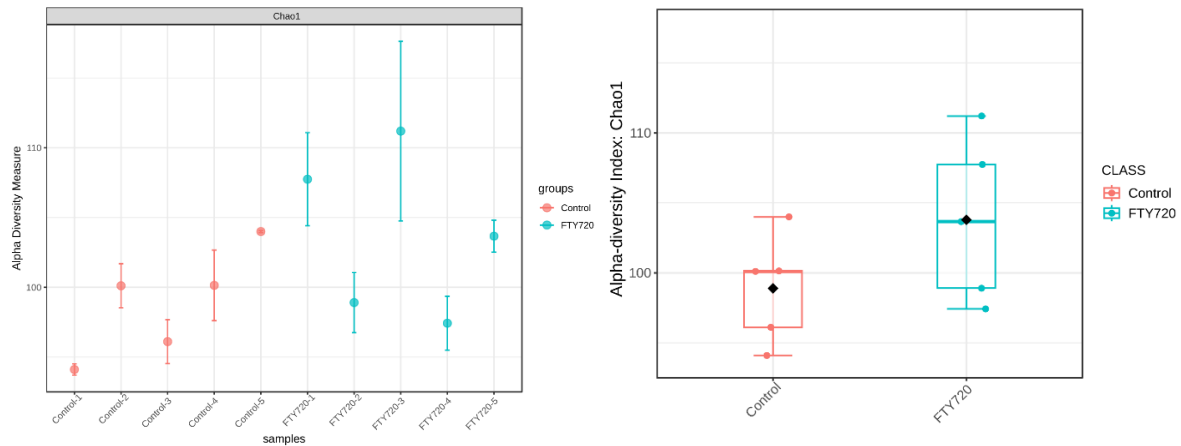
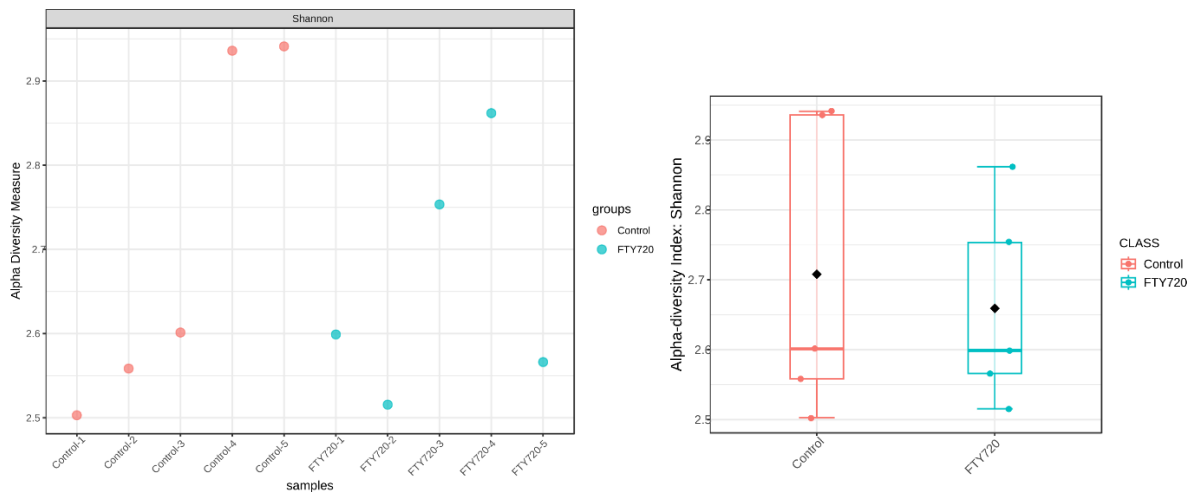


Figure 5.2.1.8H: Rarefaction curve



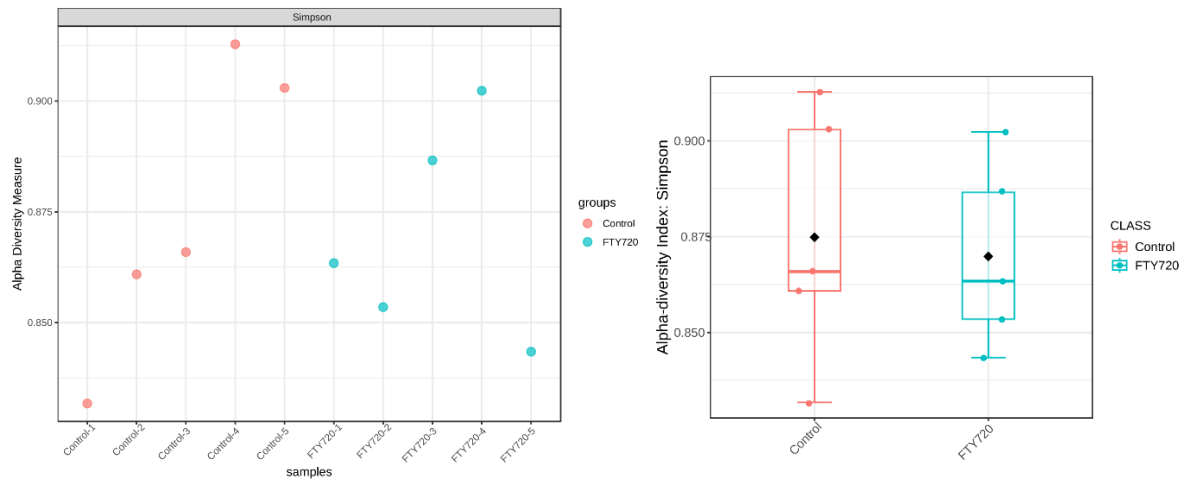
p-value: 0.16079; [T-test] statistic: -1.5694

Figure 5.2.1.8I: Data showing the species richness for control and FTY720 fecal microbiota by using Chao 1 index. Higher the Chao 1 value, higher the presence of rare the species in the sample. This experiment was carried out with n= 5 animals per group. For statistical analysis T- test was done.



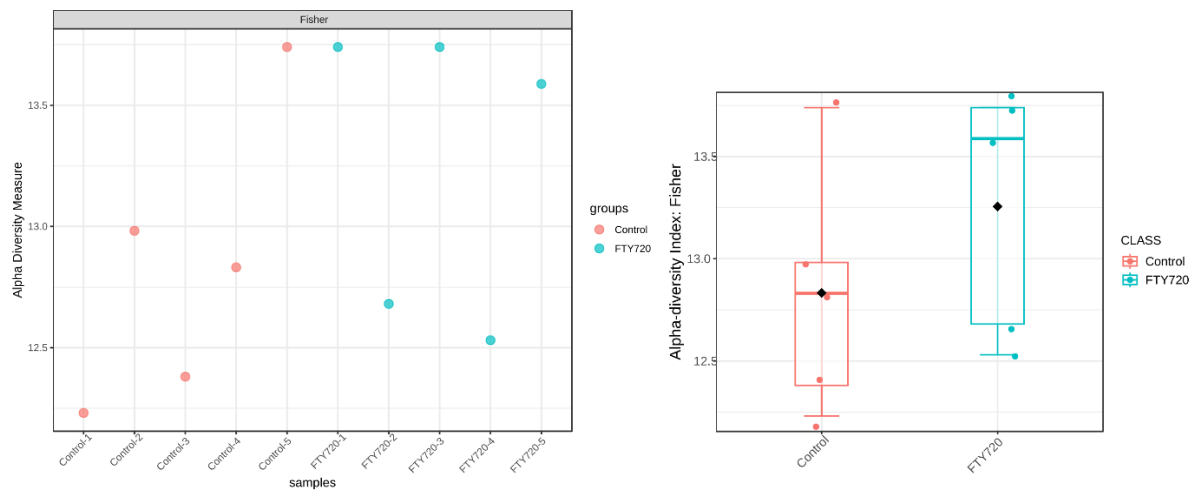
p-value: 0.6844; [T-test] statistic: 0.42381

Figure 5.2.1.8J: Data showing the Shannon's index of fecal microbiota of control and FTY720 mice. The range of this index is from 0 to $\ln(S)$. Total number of species which are present in the community is represented by S. If the value is 0 it represents that only one species dominant the community. Whereas, a value in $\ln(S)$ stands for equal representation of all species present here. This experiment was carried out with n= 5 animals per group. For statistical analysis T- test was done.



p-value: 0.79219; [T-test] statistic: 0.27326

Figure 5.2.1.8K: Data showing the Simpson's index of fecal microbiota of control and FTY720 mice to take the abundance and distribution within a particular community or ecosystem of each species into account. The values of Simpson's index ranges from 0 to 1. A greater diversity gives a value closer to 1. This experiment was carried out with n= 5 animals per group. For statistical analysis T- test was done.



p-value: 0.29496; [T-test] statistic: -1.1206

Figure 5.2.1.8L: Data showing the expected diversity of the community of control and FTY720 fecal microbiota using Fisher's Alpha. Higher values indicate more even distribution of individuals among species, indicating higher species evenness and lower value indicates lower species evenness by indicating more skewed or dominated distribution of individuals among species. This experiment was carried out with n= 5 animals per group. For statistical analysis T- test was done.

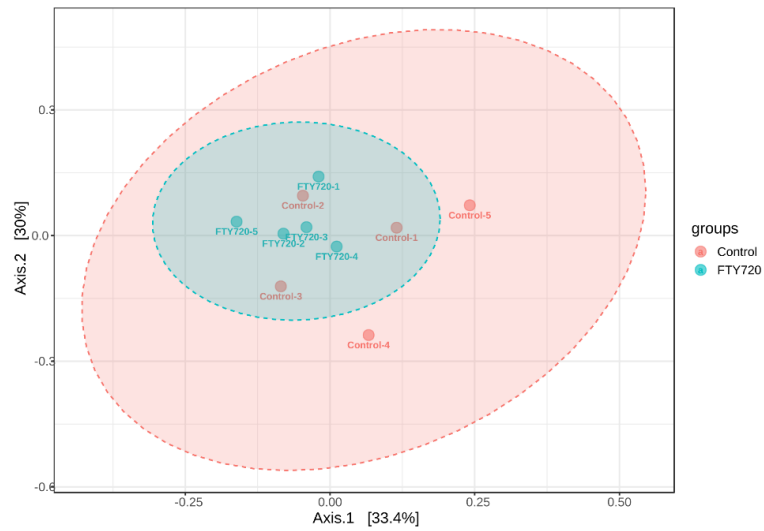


Figure 5.2.1.8M: Data showing the Beta diversity analysis at the taxonomical level of Genus with Bray-Curtis index method between control and FTY720 groups.

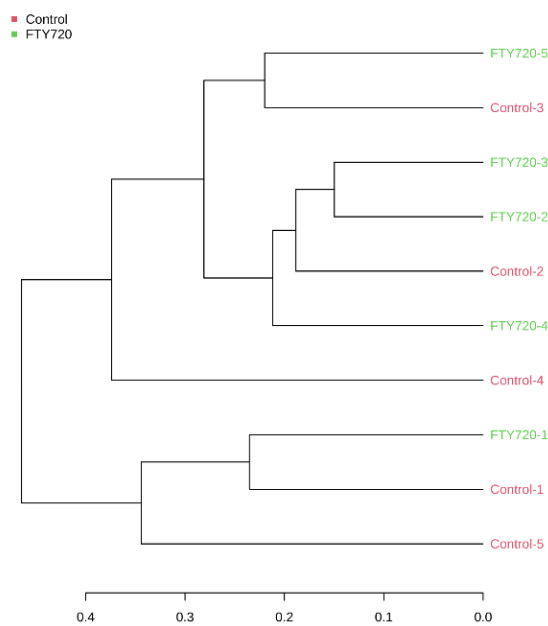


Figure 5.2.1.8N: Data showing the dendrogram of control and FTY720 samples based on the distance measured from the Bray-Curtis index with Ward clustering algorithm.

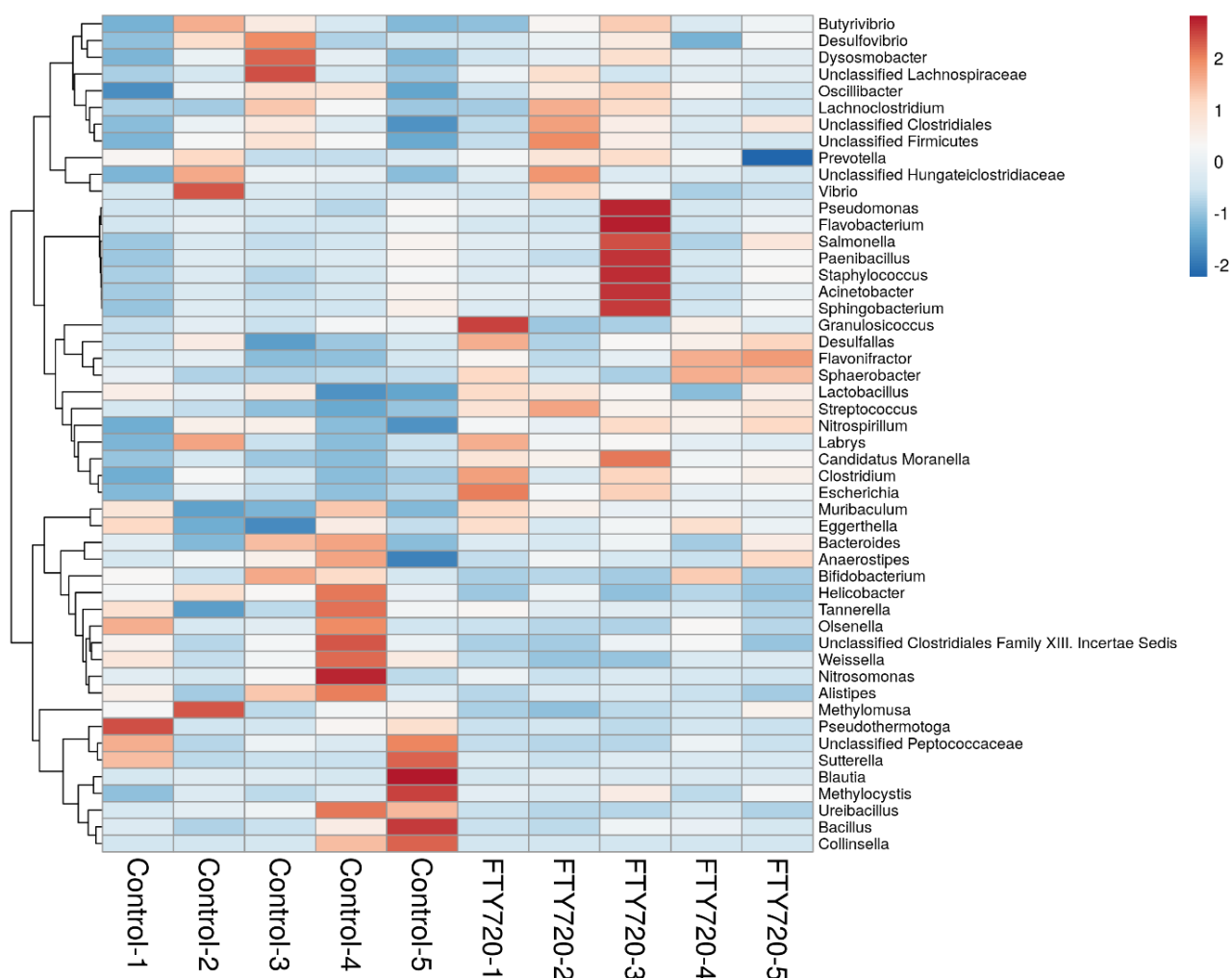


Figure 5.2.1.80: The data represents Heat-map clustering and visualization by applying the unit variance scaling to sequence abundance. Using correlation distance and average linkage Both taxon rows and samples columns were clustered. 50 Top organisms from the Genus level were taken to construct the Heatmap.

5.2.1.9. FTY720 treatment in mice lowers the Short Chain Fatty Acids (SCFAs).

From the fecal pellet from both control and FTY720-fed mice, a concentration profile of Butyrate and Propionate as SCFA was determined using GC-MS. Mice from FTY720 group showed a significantly ($p=0.0492$) low Butyrate and Propionate levels when compared to the control. A synbiotic (Probiotic + prebiotic) supplement was used as a compensatory mechanism for the enhancement of gut microbiome and SCFA as well.

6-8 weeks old C57BL/6 mice were grouped in- control, FTY720 (3mg/kg b.w.), and FTY720+ Synbiotic (*B. bifidum* 10^8 CFU/ml + Inulin 25g/Kg b.w.). FTY720 with Synbiotic supplementation showed a significant improvement in the Propionate production in mice. However, it showed a slight (not significant) increase in butyrate concentration (Figure 5.2.1.9A).

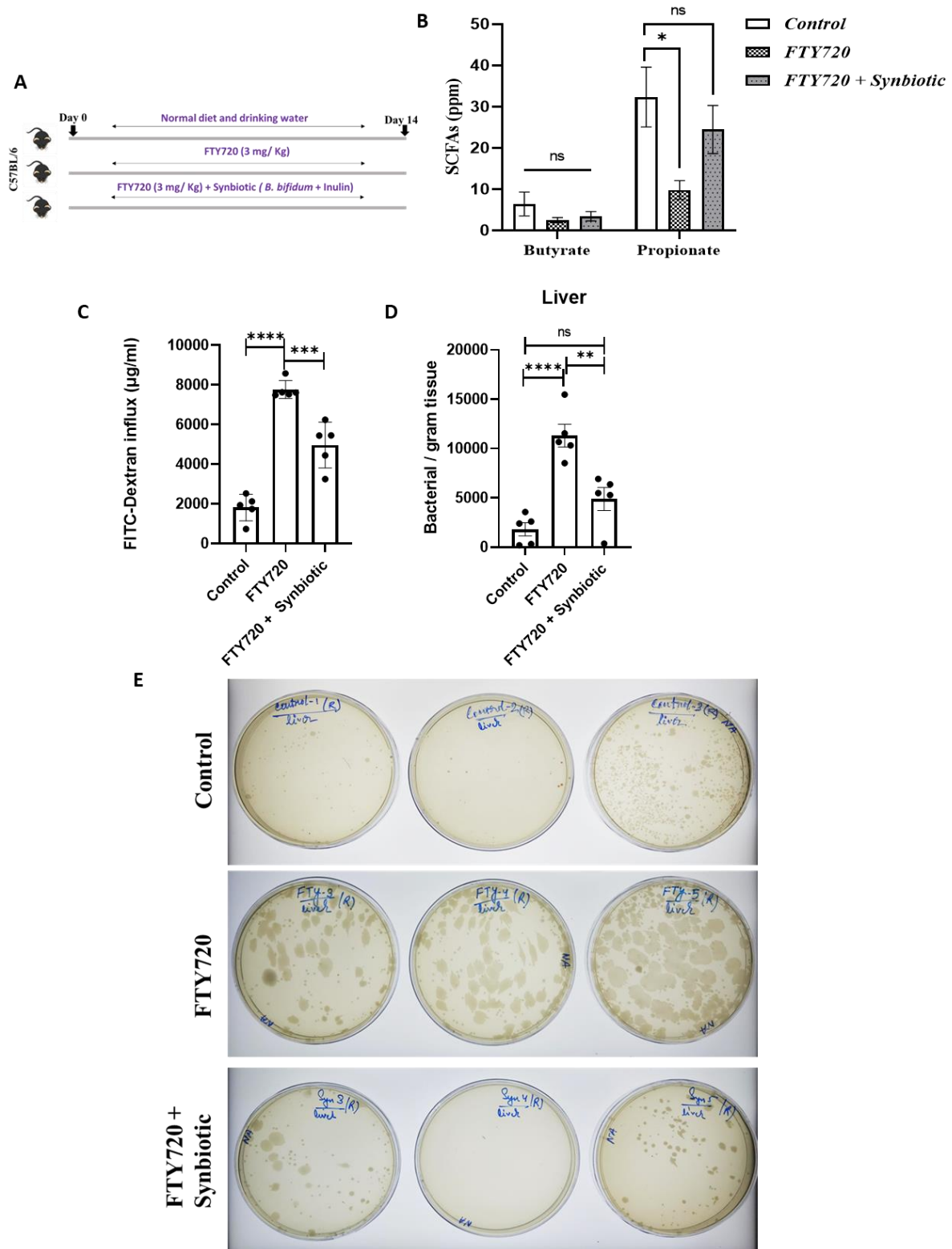


Figure 5.2.1.9A: (a) Animal with 6 weeks old C57BL/6 mice: control, FTY720 (3mg/kg b.w.), and FTY720 and synbiotic (*B. bifidum* +Inulin). (b) SCFA Butyrate and Propionate estimation using GC-MS technique control different experimental animal groups. (c) FITC influx assay for gut permeability. (d) Gut bacterial trans migration to liver. (e) Bacterial colonies from liver lysates of different experimental groups, on nutrient agar.

5.2.1.10. Synbiotic supplementation with FTY720 improves gut barrier function in mice.

An improved SCFA production in mice by the synbiotic supplementation with FTY720 lead us to question whether synbiotic supplement also improves the gut barrier function in mice or not. As a functional assay for intestinal permeability, FITC-Dextran influx from gut lumen to the blood sera was quantified using a fluorometer. Data showed the serum FITC-Dextran was significantly ($p<0.001$) higher (4.28fold) in FTY720 fed mice than in control sera, which we previously documented in the chapter one. Surprisingly, synbiotic supplemented animals showed a significant ($p<0.005$) decrease of 1.56fold of FITC influx compared to the FTY720 animals, suggesting a stronger integration in the gut epithelial cells. Also, a further experiment was conducted to observe the gut commensal's transmigration through the intestinal wall showed a decreased number of colonies in the liver of the control animals as well as in the FTY720+synbiotic animals. However, the FTY720-treated mice showed a greater load of bacteria in the liver (Figure 5.2.1.9A).

5.2.2. Discussion

FTY720 is the functional analog of S1P which upon phosphorylation by SPHK-1 binds to the S1P receptors and induces receptor internalization (Brinkmann et al. 2002). Continuous dosing of FTY720 induced intestinal inflammation which is evident by the immune infiltration in the colonic crypt and loss of crypt architectural integrity. Massive infiltration of the neutrophils at the site of colonic crypts was detected by the presence of MPO in the immune histochemistry and CD11b+Ly6G+ cells in the immune fluorescence study. Immune infiltration is the key factor in inducing inflammation in the colon, which can be seen in the case of Inflammatory bowel disease (IBD) (Lee, Kwon, and Cho 2018) and ulcerative colitis (UC) (Xue et al. 2021). Neutrophils migrate from peripheral circulation into inflammatory tissue as a result of IL-8, which primarily acts as a neutrophil chemoattractant. Studies showed that UC patients produce more IL-8 than normal controls, as is widely documented (Mahida et al. 1992). We demonstrated that FTY720 elevated colonic tissue CXCL-1 and CXCL-2 expression in the mouse model. A functioning mouse orthologue of the human chemokine IL-8 (often referred to as CXCL-8), CXCL1 is also called Gro/KC (Di Sapia et al. 2021). It has been thoroughly studied how chemokine (C-X-C motif) ligands 1 (CXCL1) and 2 (CXCL2) contribute to neutrophil recruitment. These two chemokines share the same receptor, CXCR2, and have 90% similar amino acid sequences (Jablonska et al. 2014), (De Filippo et al. 2013). A previous study demonstrated that CXCR2 is necessary for TNF-induced neutrophil emigration (Girbl et al. 2018). However, Neutrophils are the primary source of CXCL2. It encourages self-directed migration across EC junctions. Endothelial atypical chemokine receptor 1 can therefore, maintain extrinsic Chemokine (C-X-C motif) ligand 2 (CXCL2), generating the junctional chemokine "depot" required for continuous, effective transverse

luminal-to-abluminal neutrophil mobility. Surprisingly we looked and found that FTY720 treated colon had an enhanced concentration of TNF- α and IFN- γ cytokines. Sphingosine kinase phosphorylates FTY720 in vivo, transforming it into P-FTY720. Cell egress from lymphoid organs is prevented by P-FTY720, which binds to S1PRs on the surface of lymphocytes and captures all receptors-ligand complex except S1PR3 inside the cells. In contrast, phosphorylated FTY720 acts as a receptor stabilizer of S1P Receptor- 3, which is present on the surface of B cells, neutrophils, and myeloid cells and turns the downstream signaling on (Sensken et al. 2008). This phenomenon was reflected in our study, where continual dosing of FTY720 increases both CD4⁺ and CD8⁺-T lymphocyte populations in the secondary lymphoid organs such as Spleen and MLN, showing no significant change in the T cell population at the colon mucosal and submucosal layers. Nonetheless, FTY720 animals showed a substantial increase in CD19⁺ B cell, CD11b⁺ myeloid cell, and CD11b⁺Ly6G⁺ neutrophil infiltration when compared to control mice. It's unclear exactly how goblet cell hyperplasia happens, however, the FTY720 group of mice showed signs of colon architectural damage. According to earlier studies, Interleukin-13, the main controller of type-II immune response-induced inflammation, elevates inflammation and results in goblet cell hyperplasia (Huang et al. 2020). According to Zhao et al. 2018, IL-13 has been linked to S1PR2, which might lead to goblet cell hyperplasia.

FTY720 animals have higher levels of mucin-2 expression than control mice, indicating a rise in mucus production. According to earlier research, colonic inflammation may have a direct impact on colon mucin (Kang et al. 2022). FTY720-mice showed a considerably low existence of antimicrobial proteins, such as hepcidin and cathelicidin, than the control group. A decrease in cathelicidin and hepcidin that was evident in the FTY720-mice colon tissue was an indication of the likelihood of infectious disease (McHugh et al. 2019), (Scheenstra et al. 2020), and (Ganz 2018). These genes are believed to be essential for the defensive mechanisms of colonic macrophages against bacterial infections (Agier, Efenberger, and Brzezinska-Blaszczyk 2015).

Objective Three:

***“Investigating the role of Sphingosine-1-phosphate (S1P) in
ETEC induced infection model and its prevention”***

5.3. Introduction

One of the main causes of bacterial-associated diarrheal disease is the enteropathogen Enterotoxigenic *Escherichia coli* or shortly ETEC. The primary cause of traveler's diarrhoea, according to the Centers for Disease Control and Prevention or CDC, is ETEC infection. It is the leading cause of Diarrhoeal infection especially among the children of low-income countries. As per the World Health Organization, globally, childhood Diarrhoea is reported in 1.7 billion cases with an estimated 525000 deaths per year. According to UNICEF, most of the Diarrhoeal deaths among children are from South Asia and sub-Saharan. Though ETEC infection is not limited to any age group, it poses a significant threat, particularly among children who are yet to be immunized with prior infections (Qadri et al. 2007). There isn't an approved vaccination to prevent ETEC infection at the moment. The complex nature of ETEC and the lack of thorough understanding of the molecular mechanism of pathogenesis pose many challenges that restrict the development of a vaccine that is broadly protective, inexpensive and practical (Anderson et al. 2019).

Both heat-stable and heat-labile endotoxins—LT, a macromolecule that resembles cholera toxin, and ST, a low-immunogenic peptide—are used by ETEC to spread pathogenicity. ETEC strains exhibit different degrees of toxicity and either express LT, ST, or both (Bourgeois, Wierzbica, and Walker 2016), (Tribble 2017). Moreover, indiscriminate and irregular exposure to antimicrobial agents has also resulted in antimicrobial resistance in ETEC. Therefore, it is necessary to reduce the burden of the disease with an alternative approach that would reduce the rate of antimicrobial resistance and should have some health benefits. The primary limitation in vaccine development against ETEC was the heterogeneity of adhesins. The only oral vaccination that has been developed so far and is undergoing a phase 2b trial study is ETVAX (Kantele et al. 2023).

Our study focuses on probiotics and their protective effect on ETEC-induced diarrhoeal model in mice. Probiotics are live beneficial microorganisms that are often used as an alternative treatment for many gastrointestinal diseases. Probiotics have been observed to directly or indirectly reduce microbial toxicity in the intestine via bacteriocins, improving our gut functions and innate immunity (Brussow 2019). Regarding secondary outcomes, two trials (Gaon et al. 2003); (Basu et al. 2007) found that probiotic medication reduces the frequency of bowel movements on day 5. However, the World Health Organization (WHO) does not recommend probiotics for acute watery or persistent diarrhoea in children (<https://www.who.int/publications/i/item/9789240046702>). However, the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends probiotic strains like *L. rhamnosus* GG, *S. boulardii*, and *L. reuteri* (Szajewska et al. 2023). However, their recommendations are based on low to very low certainty evidence and are considered weak. Previous systematic reviews have remained inconclusive about probiotic efficacy (Collinson et al. 2020), (Ritchie and Romanuk 2012). A review by Szajewska et al. showed *L. rhamnosus* GG to reduce diarrhoea duration by

approximately 20 hours, but also noted high heterogeneity and methodological limitations (Szajewska et al. 2019).

Over the years, probiotics have shown several health benefits, proven by various disease models. It is known that strains of *Lactobacillus spp.*, *Lactococcus spp.*, and *Bifidobacterium spp.* considerably reduce pro-inflammatory cytokines like Interleukin-69 (IL-69) and raise anti-inflammatory cytokines like TGF- β and IL-10. The probiotic strain has been shown to increase tight junction proteins like ZO-1 and transmembrane proteins like occludin in duodenal samples creating a plausible approach to use probiotics to enhance gut epithelial barrier in humans (Brussow 2019).

In 1995 prebiotics were initially described as a "non-digestible food component by which the host is benefited by the selective stimulation of the growth or activity of one or a limited number of microbes residing in the colon" (Ianaro et al. 2018). Prebiotics require a colony of potentially beneficial microbes to ferment them specifically (Guarino et al. 2020). Fermentation of prebiotics gives rise to short-chain fatty acids (SCFAs) like lactic acid, butyrate, and propionic acid, which helps to decrease colon pH and positively influence the intestinal epithelial barrier. Fructo-oligosaccharides or FOS and galacto-oligosaccharides or GOS are common prebiotics which can be fermented by strains of *Bifidobacterium*, *Lactobacilli* and *Aspergillus* species (Stinson, Payne, and Keelan 2017). This study used Inulin, which is a plant-based fructo oligosaccharide with a low caloric content (1.5kcal/g or 6.3kJ/g) and non-digestibility. When intestinal bacteria are present, inulin changes into lactate, bacterial fuel, gasses, and short-chain fatty acids (such as acetate, propionate, and butyrate) (Nyman 2002). SCFAs therefore are consumed by Bacteria and partially used up by the host cells (Roberfroid 1999).

According to studies, probiotics and prebiotics work better together to shorten the duration of Diarrhoea and hospitalization episodes. When taken orally, synbiotics produce an inhibitory substance, lower the risk of infection, and improve the hosts' nutritional status. Different prebiotics are used in combination with a different probiotic to form synbiotics i.e. The first three are *Lactobacillus plantarum* 299 with oat fiber; the second is *Lactobacillus sporogens* combined with fructo-oligosaccharides; the third is Synbiotic 2000, which has inulin, betaglacans, pectin, and resistant amide along with each of *Pediococcus* , *Leuconostoc* , *Lactobacillus species*, fourth, *Pediococcus pentoseceus*, *Leuconostoc mesenteroides*, *Lactobacillus species*, and 2.5 g of inulin, oat fiber, pectin, and resistant starch; Fifth, inulin (SYN1) + Oligofructosa + *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12; vi) Golden Bifid: *Bifidobacterium bifidum*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* with FOS (Yang et al. 2019). Human intestinal probiotic bacteria *B. fragilis*, *S. parasanguinis* showed greater growth in Inulin in comparison with media supplemented with glucose only. On the contrary, *E. coli* can significantly grow faster in glucose medium but not in Inulin supplemented medium (Schropp et al. 2023) (Wang and Gibson 1993) (Buntin et al. 2017) (Crittenden, 2002) (Carlson et al. 2017). Synbiotic, is known to interfere with the adhesion and attachment of pathogens on gut mucosa, thereby preventing

disease and gut dysbiosis (Santosh Anand, 2018). Adhesion in the intestinal epithelial cells is the key step for colonization, which leads to the disruption of the gut barrier and integrity and causes intestinal infection (Collado, 2008).

Therefore, this study assessed the efficacy of different Synbiotics in relapsing the severity of ETEC infection in mice.

5.3.1. Results

5.3.1.1. Probiotics inhibit ETEC growth and survivability adhesion to the host cells

The growth of ETEC H10407 in Luria Bertani broth and probiotic strains in MPS broth showed an exponential time-dependent growth until they were co-cultured. A time dependent regression of growth of ETEC in the LB+ MRS growth medium co-cultured with probiotic strains was observed. After 2 hours and onward a significant ($p<0.05$) decline in ETEC population in the all three LB+MRS co-culture broth was noted. Although the proliferation of probiotic strains- *Lactobacillus. casei*, *Lactococcus. lactis* and *Bifidobacterium. bifidum* shows little to no inhibition of growth and proliferation patterns in the co-culture setup compared to their respective pure cultures (Figure-5.3.1.1A).

Int 407 small intestinal cells were co-cultured with all three pro-biotic strains separately. Cells received probiotics 4 hours prior to infection were for adhesion inhibition assay and cells received along with ETEC were for competitive inhibition assay. Adhered ETEC bacterial CFU data, acquired from the infection control groups was taken as 100 percent and the rest was calculated with respect to that. All three probiotics significantly ($p<0.0001$) inhibited ETEC adhesion to Int 407 cells comparing the infection control group (Figure-5.3.1.1B).

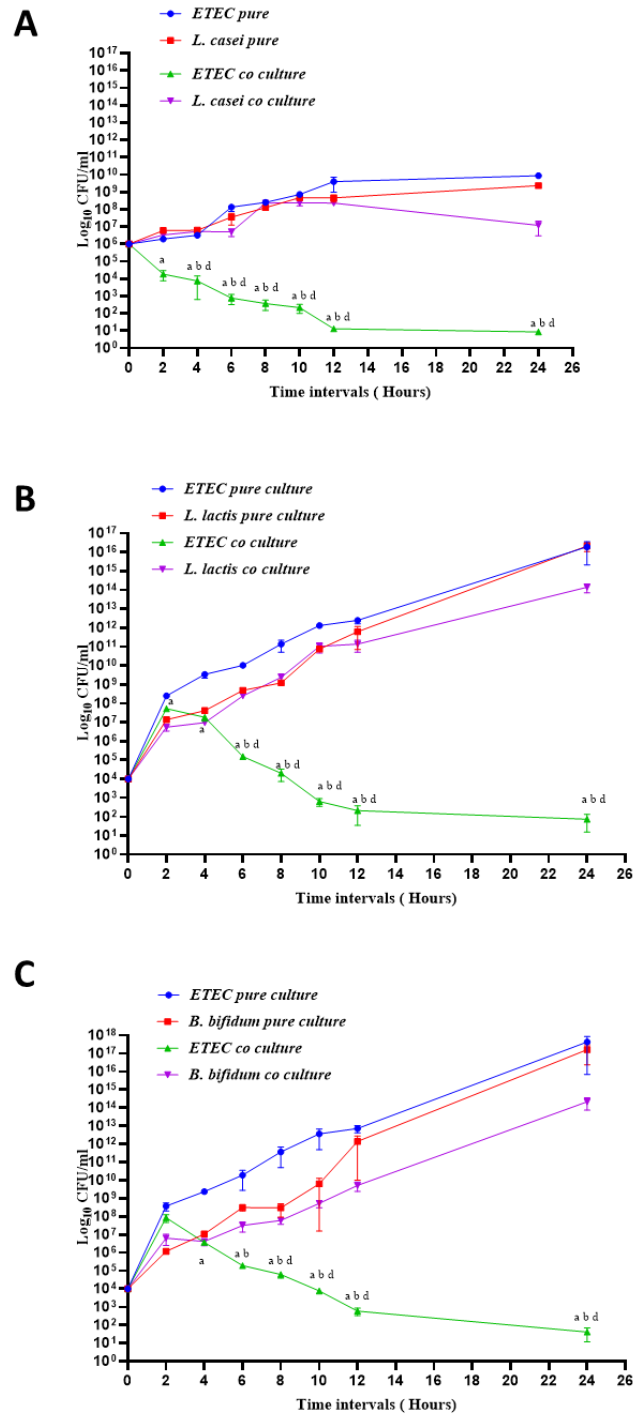


Figure-5.3.1.1A: Growth and survivability of ETEC and Probiotic strains *L. casei*, *L. lactis* and *B. bifidum* in pure culture vs co-culture system. Bacterial strains were cultured and examined for 24 hours. A time-dependent growth pattern of bacterial strains was plotted at Log₁₀ scale. Experiments were conducted thrice; the data is represented as Mean ± SEM.

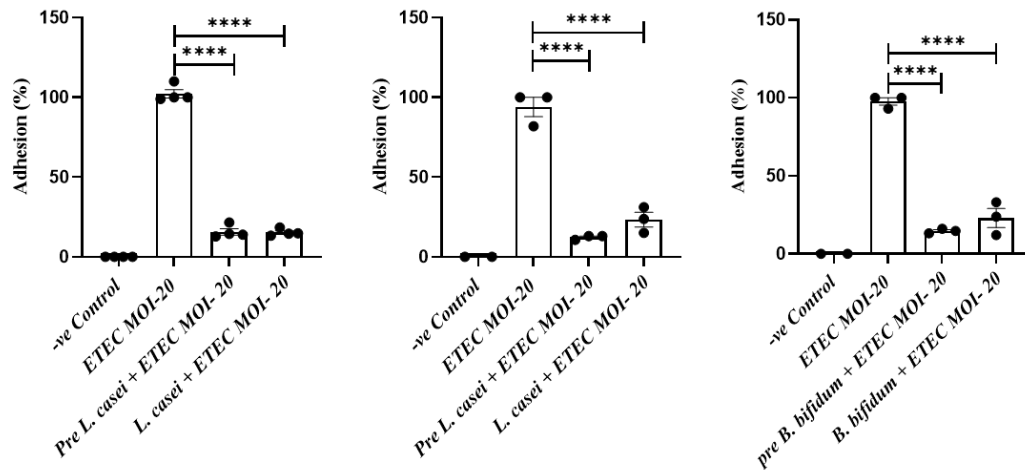
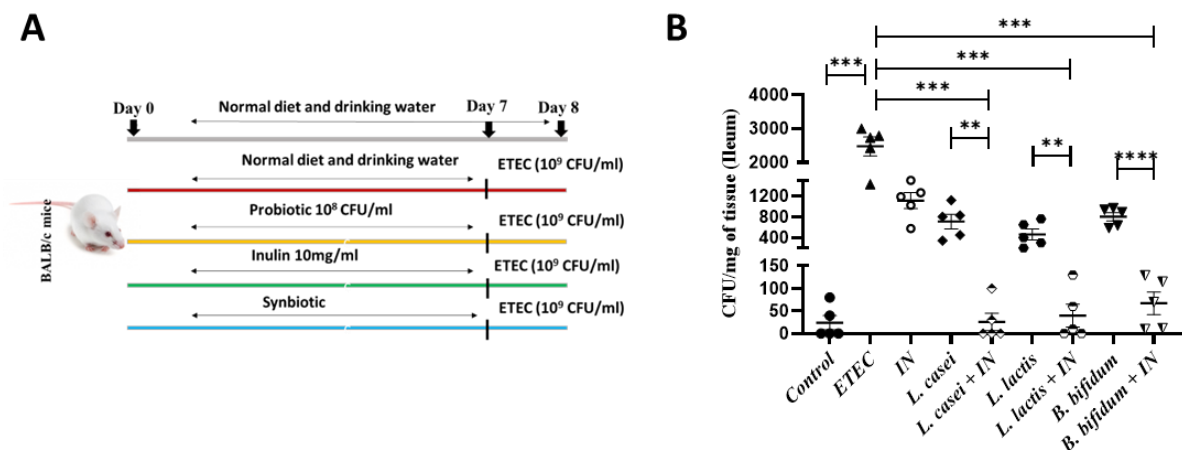


Figure-5.3.1.1B: ETEC H10407 adhesion to INT 407 small intestinal cells in the presence of *L. casei*, *L. lactis*, and *B. bifidum*, respectively. Results expressing the mean $\pm$ SEM.

5.3.1.2. Synbiotics ameliorate H10407 ETEC-induced Diarrhoea

In C57BL/6 mice, Diarrhoea was induced by ETEC H10407 strain with a concentration of 10^9 CFU/ml per animal. After 20 hours of infection, the number of bacterial colonies from the ETEC group detected on MacConkey agar plate was significantly ($p < 0.0001$) higher than the control. Colonies were further confirmed by RT-PCR and agarose gel electrophoresis. IN, *L. casei*, *L. lactis* and *B. bifidum* probiotic groups showed a significantly ($p < 0.0001$) less bacterial colonization with a percentage of 55.14433%, 71.37%, 81.34% and 67.60% when compared with ETEC group. Also, the Inulin supplemented probiotic groups showed a significantly ($p < 0.0001$) lesser number of colonies compared



C

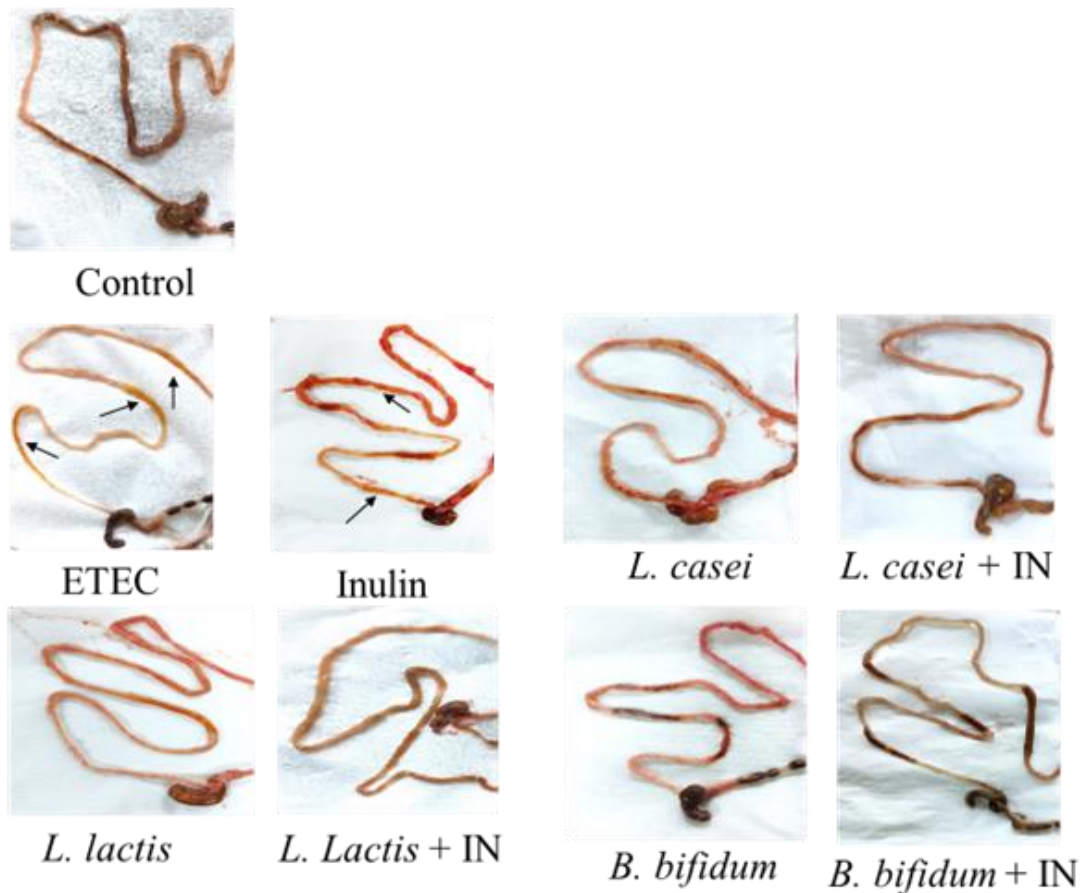


Figure- 5.3.1.2A: (A) Schematic diagram for the animal model. (B) 4-6 weeks-old mice were grouped into control, ETEC, IN, *L. casei*, *L. casei*+ IN, *L. latis*, *L. lactis* + IN, *B. bifidum* and *B. bifidum* + IN. Apart from the control, all groups were infected with ETEC H10407 at 109 CFU/ml concentration. To assess the colonization of ETEC H10407, small ileal sections were isolated from each group and homogenized in PBS and then the lysates were spread onto MacConkey agar plate. Infection loads in each and every group were compared with the ETEC group. And each synbiotic group was compared with the corresponding probiotic and IN group (C) The photographs of small intestinal contents of mice from different experimental groups. The consistency of intestinal content was visibly very liquid in the ETEC and IN-grouped mice. Experiments were conducted with $n \geq 5$ animals for each group. Data representing mean $\pm$ SEM. Experiments were done at $n=3$ repetitions.

to the ETEC positive group and even a further decrease ($p < 0.004$) in the bacterial colonization of 27.57%, 17.03% and 29.68% was noticed in the *L. casei* + IN, *L. lactis* + IN and *B. bifidum* + IN when compared to their corresponding probiotic group *L. casei*, *L. lactis* and *B. bifidum* respectively (Figure- 5.3.1.2A). ETEC-infected mice showed severe water loss in the S.I. lumen and loose stool. The

consistency of stool increased in the probiotic groups. Surprisingly the synbiotic groups showed no significant fluid accumulation in the small intestinal lumen (Figure- 5.3.1.2A)

5.3.1.3. Histological analysis of mice ileal tissue sections

Through histological investigations, the alteration in the cellular morphology of the small intestine's ileum was analyzed to look into tissue damage and inflammation. The results are shown in Figure-. (a) IN, (b) ETEC, and (c) Control In that order, the following are listed: (d) *L. casei*, (e) *L. lactis*, (f) *B. bifidum*, (g) *L. casei* + IN, (h) *L. lactis* + IN, and (i) *B. bifidum* + IN. The ETEC group showed severe loss of crypts and goblet cells, while the control group's animals showed intact mucosa, tubular crypts, and goblet cells with no discernible dispersed immune cell infiltration at the crypt and mucosal layers.

The ETEC group showed a 55.55% reduction in crypt length, significantly less than that of the control group ($P < 0.0001$). Crypt hyperplasia was noticed in *L. lactis*, *B. bifidum*, compared to control which was 2 and 1.55-fold increase respectively. But in *L. lactis* + IN a 22%- or 1.22-fold increase and in *B. bifidum* + IN groups a 33.33%- or 1.33-fold increase in crypt length was observed compared to the control crypt length which was not a significant change. The ileal crypt structure was less altered in the *L. casei*, and *L. casei* + IN group; the mucosa and submucosa layer remained nearly intact, but the cells inside this layer were flaccid, and neutrophil infiltration was noted in these layers. When compared to the ETEC group, the immune infiltration was significantly ($p < 0.0001$) less in IN, *L. casei*, *L. casei* + IN, *L. lactis*, *L. lactis* + IN, *B. bifidum* and *B. bifidum* + IN group with a damage score of 5.26%, 15.23%, 55.39%, 55.38%, 74.17%, 75.53% and 87% less than ETEC. Which tells that the *L. casei* + IN showed a 40% improvement than the *L. casei*, *L. lactis* + IN had a 18.78% improvement than the *L. lactis* group, and *B. bifidum* + IN showed a 11.59% improvement than *B. bifidum*. Looking into the goblet cell loss in the ileal crypts, a significant ($p < 0.0001$) 95% reduction of goblet cells was observed in the ETEC ileal sections when compared to the control. However, the prebiotic and probiotic groups IN, *L. casei*, *L. lactis*, and *B. bifidum* exhibited a 55%, 61%, 48.6%, 83.33% reduction in disease score respectively. The disease score for goblet cell loss in the synbiotic groups *L. casei* + IN, *L. lactis* + IN, and *B. bifidum* + IN, showed a further reduction of 47.22%, 26.39% and 16.67% respectively (Figure 5.3.1.3A).

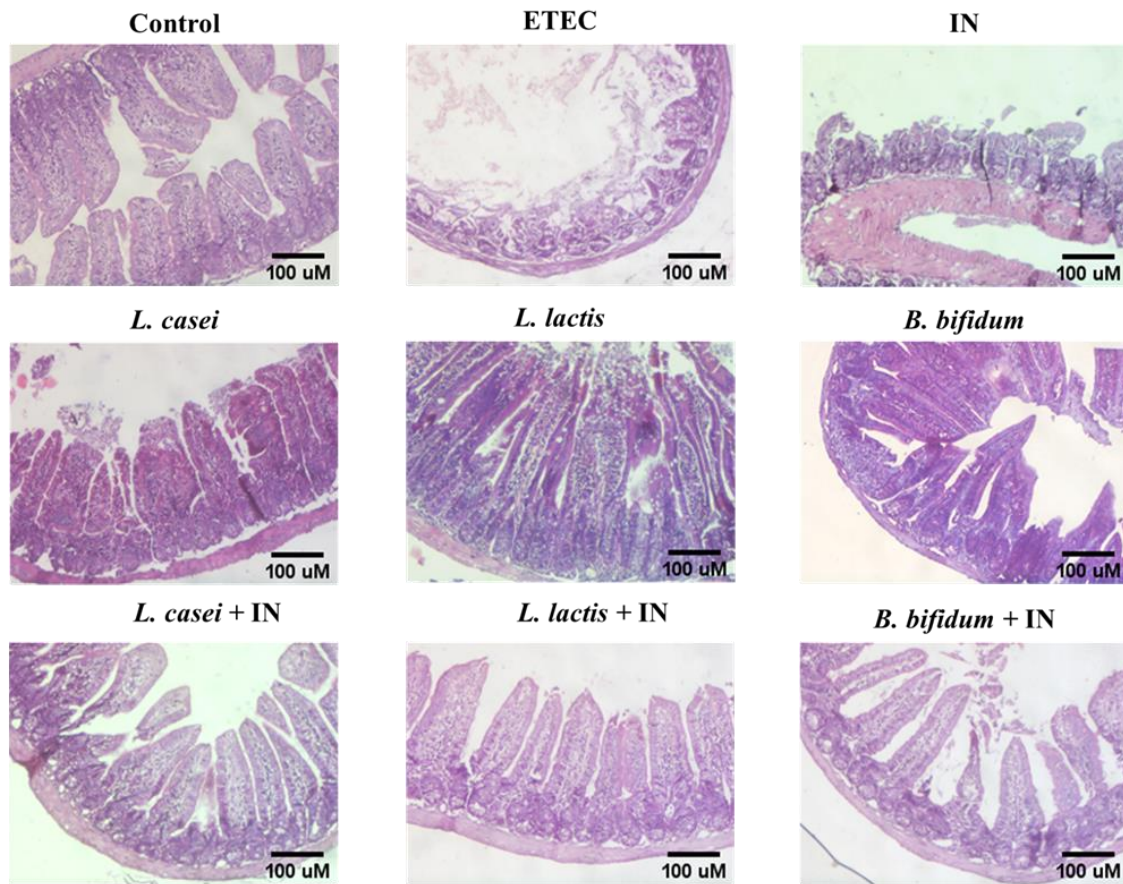
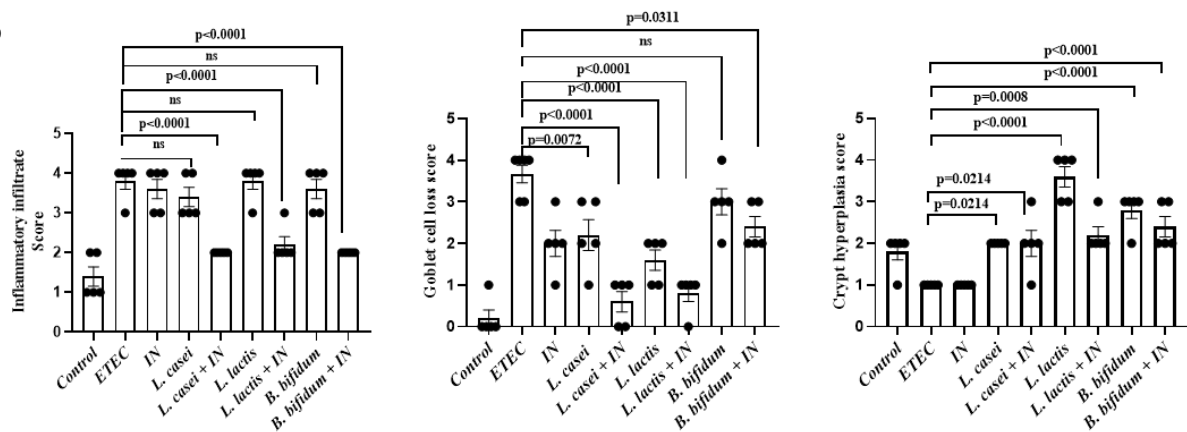
A**B**

Figure- 5.3.1.3A: (A) Cross-sections of ileum from different groups of mice were histologically examined using Haematoxylin and Eosin dyes under the light microscope with 40X magnification. (B) Graphs show small intestinal sections' histological scores for inflammatory infiltrates, Goblet cell loss, and crypt hyperplasia. $N \geq 5$ animals were taken per group. All the results are expressed as mean $\pm$ SEM. Experiments were done at $n=3$ repetitions.

5.3.1.4. S1P concentration in ETEC infected ileum tissue

The ileal tissue lysates of ETEC, a significantly ($p=0.0437$) high concentration of S1P was determined. On the other hand, the probiotic treated groups showed relatively low tissue S1P level in an ELISA dependent assay. ETEC infection caused a 1.21 fold increase in tissue S1P concentration. Interestingly, a significant decrease of 1.29 fold in the *L. casei*, 1.64 fold in *L. lactis* and 1.25 fold in *B. bifidum* pretreated infected group has showed (Figure-5.3.1.4A).

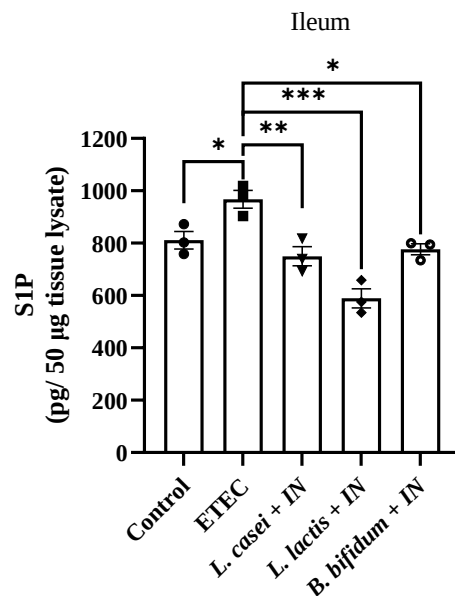


Figure-5.3.1.4A: The concentration on S1P in the ileal tissue from ETEC, *L. casei*, *L. lactis* and *B. bifidum* mice groups.

5.3.1.5. Synbiotics reduce CD3+/CD4+ Th1 cell population in ETEC-induced inflammation

In association with the cytokine profile, this study investigated each test animal's T- cell population of spleen and mesenteric lymph nodes (MLN) (Figure-5.3.1.5A) after 20 hours post-infection. In the spleen the CD3+/CD4+ T- cells increased 3.21fold in the ETEC group comparing the control with a p value of <0.0001 . CD3+/CD4+ T- cell showed significant population decrease in all the synbiotic groups which was 47.48% ($p= 0.0002$) in *L. casei* + IN group, 54.9% ($p= 0.0001$) in the *L. lactis* + IN group and 66.83% ($p <0.0001$) in the *B. bifidum* + IN group comparing to the ETEC group. Also, *L. casei* + IN, *L. lactis* + IN and *B. bifidum* + IN showed 40.95% ($p= 0.0033$), 44.51% ($p= 0.0026$) and 63.19% ($p= 0.0002$) decrease in CD3+/CD4+ T- cell population when compared with respective probiotic groups and a decrease of 56.11% ($p<0.0001$), 63.52% ($p<0.0001$) and 75.46% ($p<0.0001$) when compared with IN group. However, comparing ETEC group to the IN, *L. casei*, *L. lactis* and *B. bifidum* prebiotic and probiotic groups, no significant change was observed (Figure-5.3.1.5B).

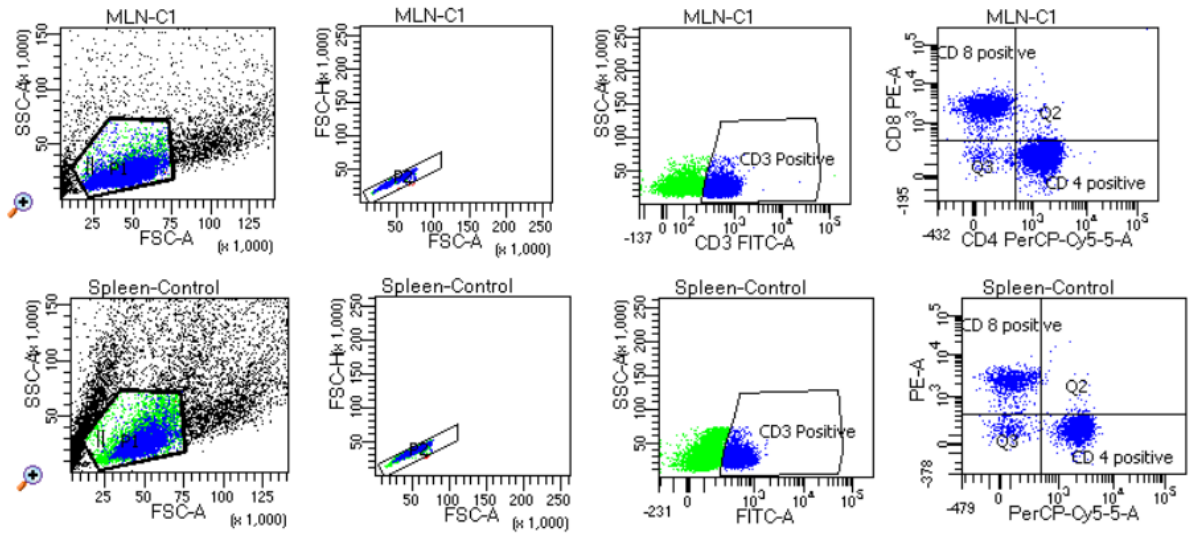


Figure 5.3.1.5A: Scattered plot for the gating strategy of CD3+CD4+ and CD3+CD8+ cell acquisition by flow cytometry from the cells of MLN and spleen of experimental mice groups. Singlet population of parent population were scattered according to their expression of CD4+CD8- (Q4), CD4-CD8+(Q1), CD4+CD8+(Q2) and CD4-CD8-(Q3).

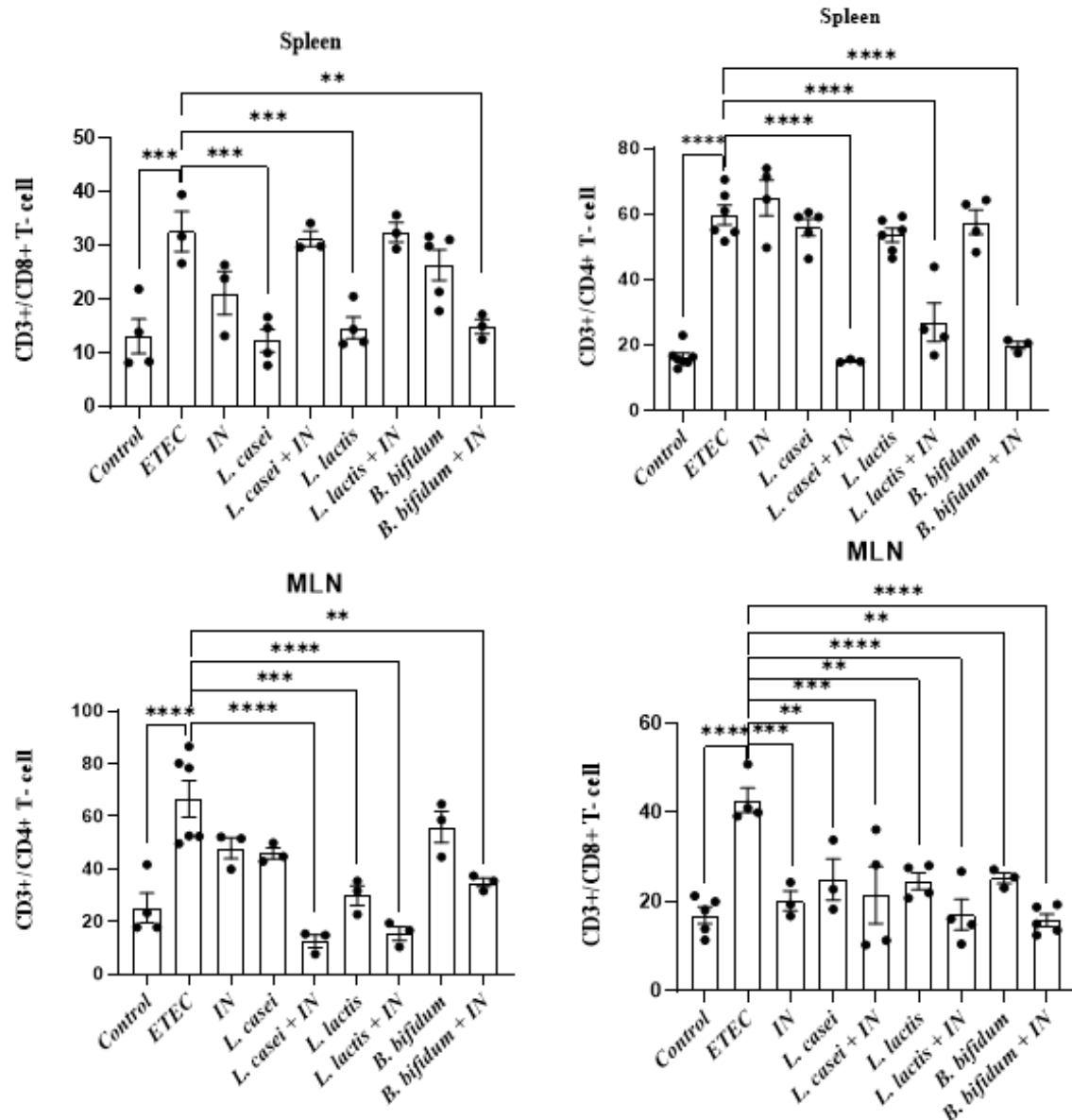


Figure- 5.3.1.5B: The population for CD3+CD4+ and CD3+CD8+ T lymphocyte cells in both MLN and spleen was determined by flow cytometry. Cells harvested from both MLN and spleen were tagged with anti-PerCP-CD3, anti-FITC-CD4, and anti-PE- CD8 antibodies. CD4+ and CD8+ cells were detected from the CD3+ parent population of T cells. N ≥ 5 animals were taken per group. All the results are expressed as mean± SEM.

5.3.1.6. Prebiotics, probiotics and synbiotics reduce inflammatory cytokines in the ileal milieu

In this study, we also investigated the state of inflammation upon ETEC colonization in the intestinal tissues. Where the major pro-inflammatory cytokines such as TNF- α , IL-6, IFN- γ , and anti-inflammatory IL-10 secretion were assessed by ELISA from the colonic tissue lysates of each sample.

In the ETEC group, the tissue TNF- α (pg/ml) 526.19 ± 173.31 , IL-6 (pg/ml) 482.97 ± 142.005 , and IFN- γ (pg/ml) 64.04 ± 13.18 got increased compared to the control group which was 99.816 ± 71.037 , 97.064 ± 70.63 and 23.12 ± 8.056 respectively. This data indicates a significant upregulation of TNF- α , IL-6, and IFN- γ in the ETEC group with a change in the fold of 5.27 ($p < 0.0001$), 4.976 ($p < 0.0001$), and 2.77 ($p < 0.0001$) respectively compared to the control cytokine levels. TNF- α expression in IN, *L. casei*, *L. lactis* and *B. bifidum* was 248 ± 61.07 , 296.53 ± 74.27 , 322.44 ± 99.49 , 68.67 ± 38.16 which indicates a reduction of 52.87% ($p = 0.0001$), 43.64% ($p = 0.0027$), 38% ($p = 0.0116$) and 86% ($p < 0.0001$) from the ETEC group. Surprisingly in the *L. casei* + IN, *L. Lactis* + IN and *B. bifidum* + IN group the TNF- α expression was 202.83 ± 92.45 , 141.54 ± 34.73 , and 49.6 ± 12.04 having further reduction of 17.8% ($p = 0.7808$), 34.38% ($p = 0.0013$) and 3.62% ($p = 0.9992$) from respective probiotic groups. Comparing to the ETEC group IL-6 expression got significantly decreased to 52.52% ($p = 0.0001$) in the IN, 69.24% ($p < 0.0001$) in the *L. casei*, 54.56% ($p < 0.0001$) in the *L. lactis* and 51.91% ($p = 0.0001$) in the *B. bifidum* group. Comparing to the respective probiotic groups we found that IL-6 production was further decreased with a percentage of 24.15% ($p < 0.0001$), 31.43% ($p = 0.0029$) and 22.95% ($p = 0.0471$) in *L. casei* + IN, *L. Lactis* + IN and *B. bifidum* + IN synbiotic groups respectively. Similarly, with a decrease of 25.24% ($p = 0.1663$), 47.18% ($p = 0.0002$), 22.9% ($p = 0.2709$) and 26.17% ($p = 0.1346$) respectively, IN, *L. casei*, *L. lactis* and *B. bifidum* groups showed significant decrease in IFN- γ production compared to the ETEC group. Having similarities with the previous data we found even more decrease in IFN- γ in the *L. casei* + IN, *L. Lactis* + IN and *B. bifidum* + IN synbiotic groups which were 21% ($p = 0.0125$), 48% ($p = 0.0005$) and 38% ($p = 0.0236$) compared to their respective probiotic groups. On the other hand, IL-10 as the anti-inflammatory cytokine which was 597.683 ± 148.219 in the control individuals, got significantly ($p < 0.0001$) decreased to 66.35 ± 43.67 in the ETEC group, showing a reduction by 88.89% or 9-folds decrease comparing the control. From this experiment we got a significant increase in IL-10 secretion in IN, *L. casei*, *L. lactis* and *B. bifidum* groups which is 74.95% ($p = 0.0092$), 83.58% ($p < 0.0001$), 45.39% ($p = 0.9455$) and 58.9% ($p = 0.6435$) respectively. Further production of IL-10 was seen in the *L. casei* + IN, *L. Lactis* + IN and *B. bifidum* + IN synbiotic groups which is 6.12% ($p = 0.0067$), 30.035% ($p = 0.0060$) and 23.05% ($p = 0.0020$) respectively compared to their respective probiotic groups. Besides all the inflammatory cytokines we investigated the expression of CXCL-1 production in the ileal tissues which is a major neutrophil chemo-attractant. In the ETEC group the CXCL-1 level was 332.183 ± 86.748 which is a 11.76 folds increase from the control 23.1 ± 8.53 . The prophylactic IN, *L. casei*, *L. lactis* and *B. bifidum* groups showed 49.75% ($p < 0.0001$), 48.02% ($p < 0.0001$), 44.41% ($p = 0.0001$) and 81.55% ($p < 0.0001$) decrease in CXCL-1 level compared to the ETEC group. Among all three synbiotic groups, only *L. lactis* + IN group inhibited 38.45% ($p = 0.0047$) more reduction in CXCL-1 expression when compared with its corresponding probiotic group (Figure- 5.3.1.6A).

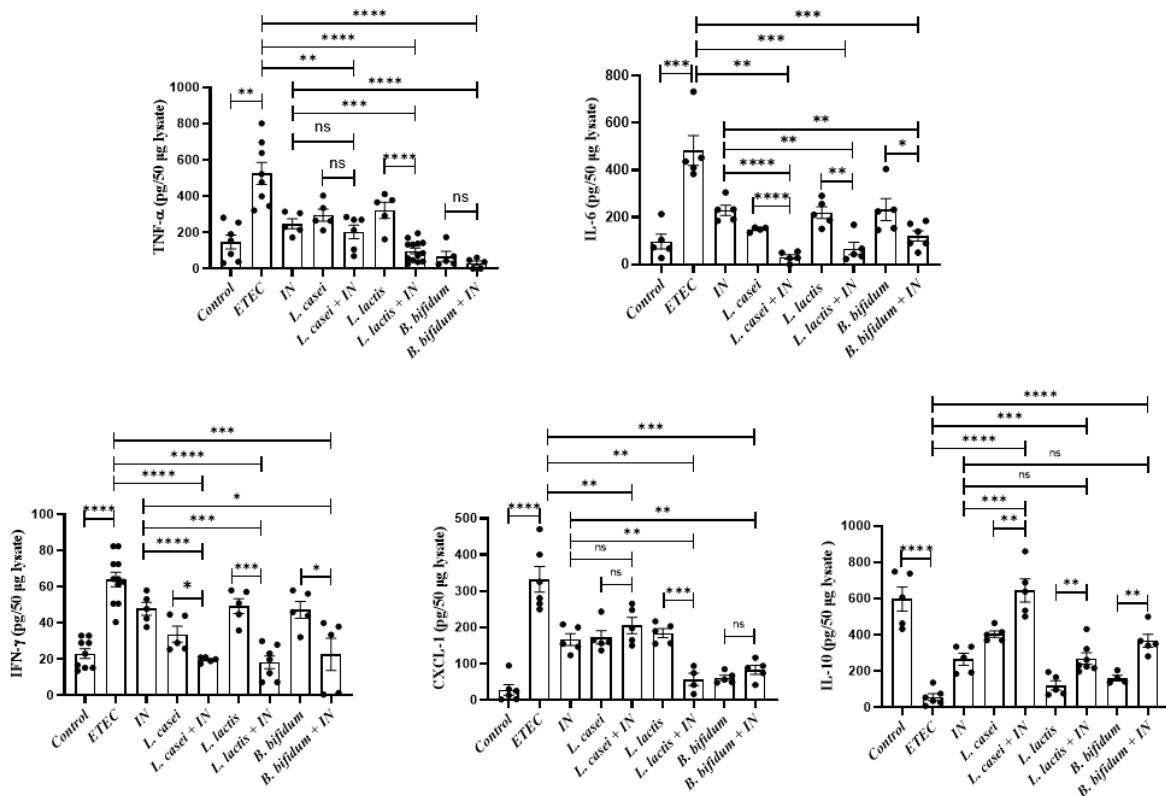


Figure- 5.3.1.6A: ELISA for the secretory TNF- α , IL-6, IFN- γ , CXCL-1, and IL-10 chemokine in ileal tissue lysates from each animal group. For each experiment, $N \geq 5$ animals were grouped. All the results are expressed as mean $\pm$ SEM.

5.3.1.7. Protective efficacy of prebiotic, probiotic, and synbiotics in tight junctional protein expression in the ileum

This study investigated major tight junction proteins associated with epithelial cells, including trans-membrane Occludin, claudins, and cytoplasmic protein ZO-1. A 2.34-fold reduction in the Occludin mRNA level was noticed in the ETEC H10407 infected ileal tissues compared to the control ileum. Among IN, *L. casei*, *L. lactis* and *B. bifidum* prebiotic and probiotic groups only *L. casei* group showed significant ($P < 0.05$) upregulation of Occludin with an increase of 1.8 folds. Occludin expression got significantly upregulated in all three synbiotic groups with an increase of 2.53, 2.35 and 3.18 folds or 60.43%, 57.4% and 68.63% respectively when compared to the ETEC group.

ZO-1 got decreased 2.53fold or 60.47% in ETEC group compared to the control group. *L. casei* probiotic group as well as *L. casei* + IN synbiotic group showed no significant improvement in the ZO-1 upregulation. Not the *L. lactis* probiotic group but *L. lactis* + IN synbiotic group exhibited a 3.1fold or 67.8% increase. Interestingly *B. bifidum* probiotic and *B. bifidum* + IN synbiotic group both showed significant upregulation of ZO-1 with an increase of 53.59% and 57.45% respectively. A 99% reduction

of JAM-A expression was observed in ETEC infected mice. We found no significant upregulation of JAM-A in all three probiotic groups along with the IN group. In contrary all the three synbiotic groups showed a significant increase of 98.63% for *L. casei* + IN, 97.54% for *L. lactis* + IN and 98.35% for *B. bifidum* + IN respectively. Claudin-2, the pore-forming claudin family TJ protein was significantly increased by 76.43% in ETEC-infected mice however, all synbiotic groups also showed significant upregulation (Figure 5.3.1.7A).

This data was further confirmed by the immune fluorescence staining of ileal tissue Occludin, ZO-1, Claudin-2, and JAM-A proteins (Figure 5.3.1.7B-C). The mean fluorescence for occludin, ZO-1, and JAM-A is significantly upregulated in the ileal tissues upon synbiotic treatment which decreased in the ETEC group. Likewise, Claudin-2, which was upregulated in the ETEC group, was significantly reduced by the synbiotic application.

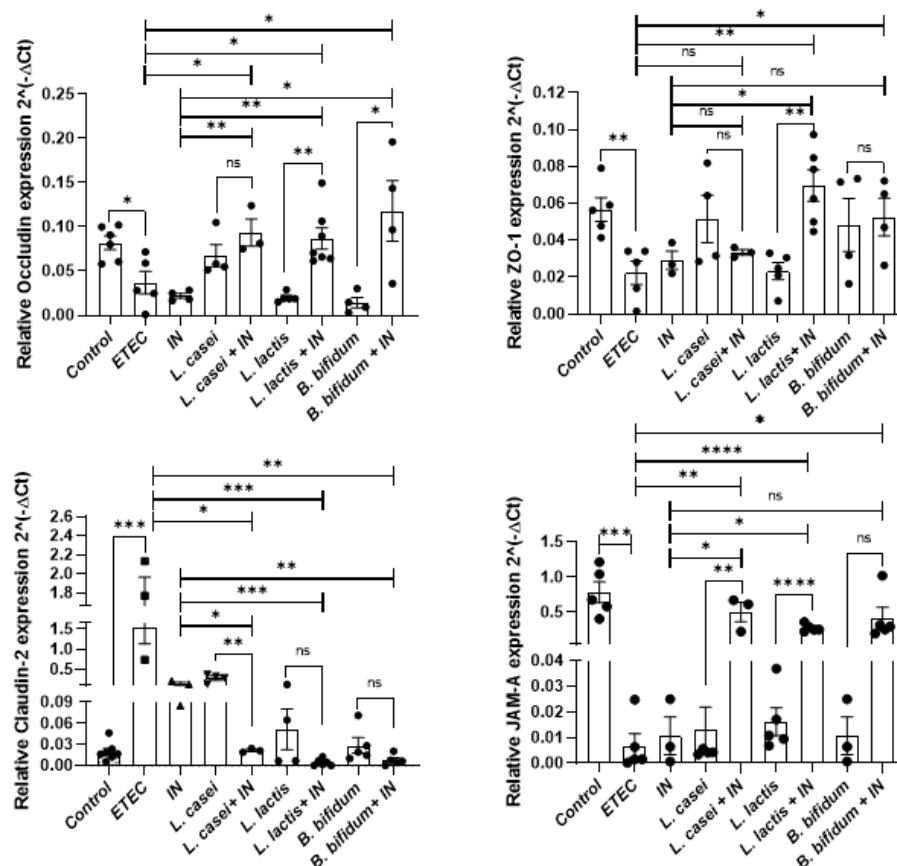


Figure-5.3.1.7A: The Epithelial tight junctional barrier integrity was analysed by determining the mRNA expression of Occludin, ZO-1 and JAM-A proteins by real-time PCR. mRNA expression of another tight junctional protein, Claudin-2, a pore-forming protein, was also detected by quantitative PCR. Housekeeping gene GAPDH was the internal control. Data was plotted using the relative gene expression values. Gene expressions of tight junction proteins of each synbiotic

group were compared with the corresponding probiotic group, prebiotic or IN group, and ETEC group. This study was conducted with $N \geq 5$ animals for each group, representing Mean $\pm$ SEM.

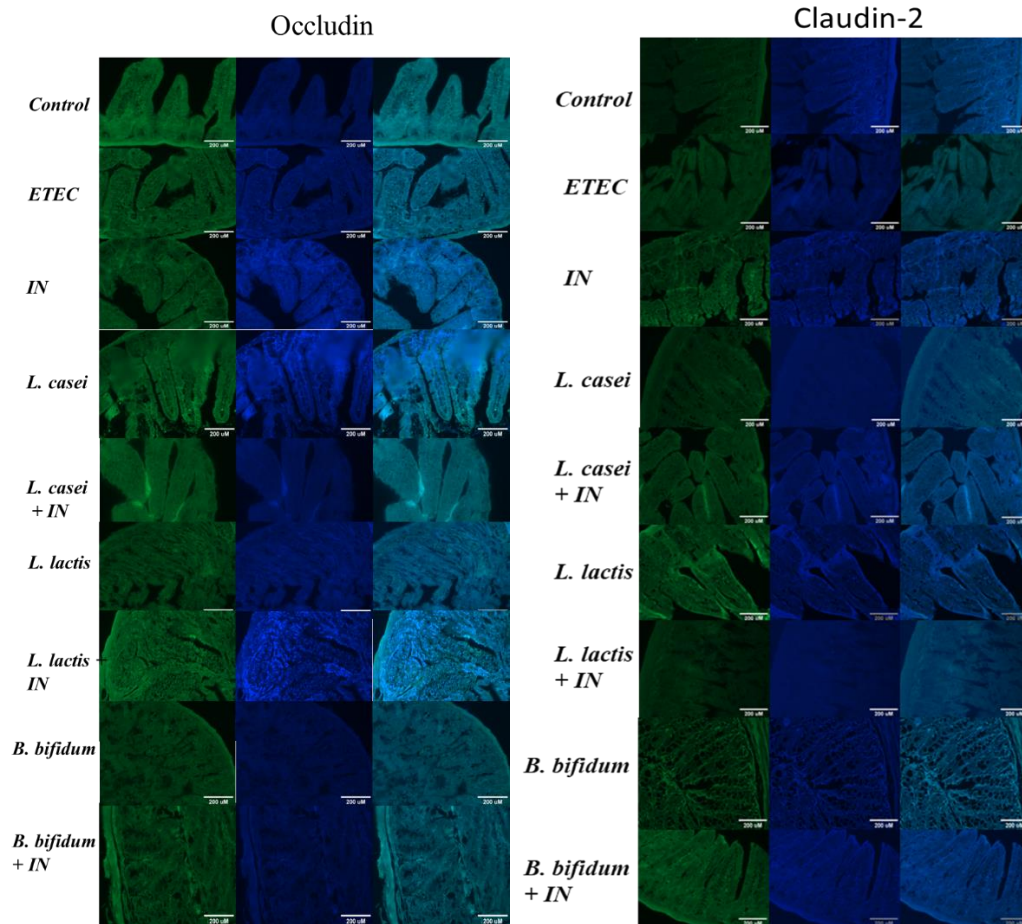


Figure 5.3.1.7B: (A) Immunofluorescence images of the small intestine. Tight junctional proteins were stained with FITC-tagged (green) anti-Occludin and Claudin-2 antibodies. To detect the Nucleus, the Hoechst stain (blue) was used. The fluorescence intensity of each image was plotted and compared with that of every other group. This study was conducted with $N \geq 5$ animals for each group, representing Mean $\pm$ SEM.

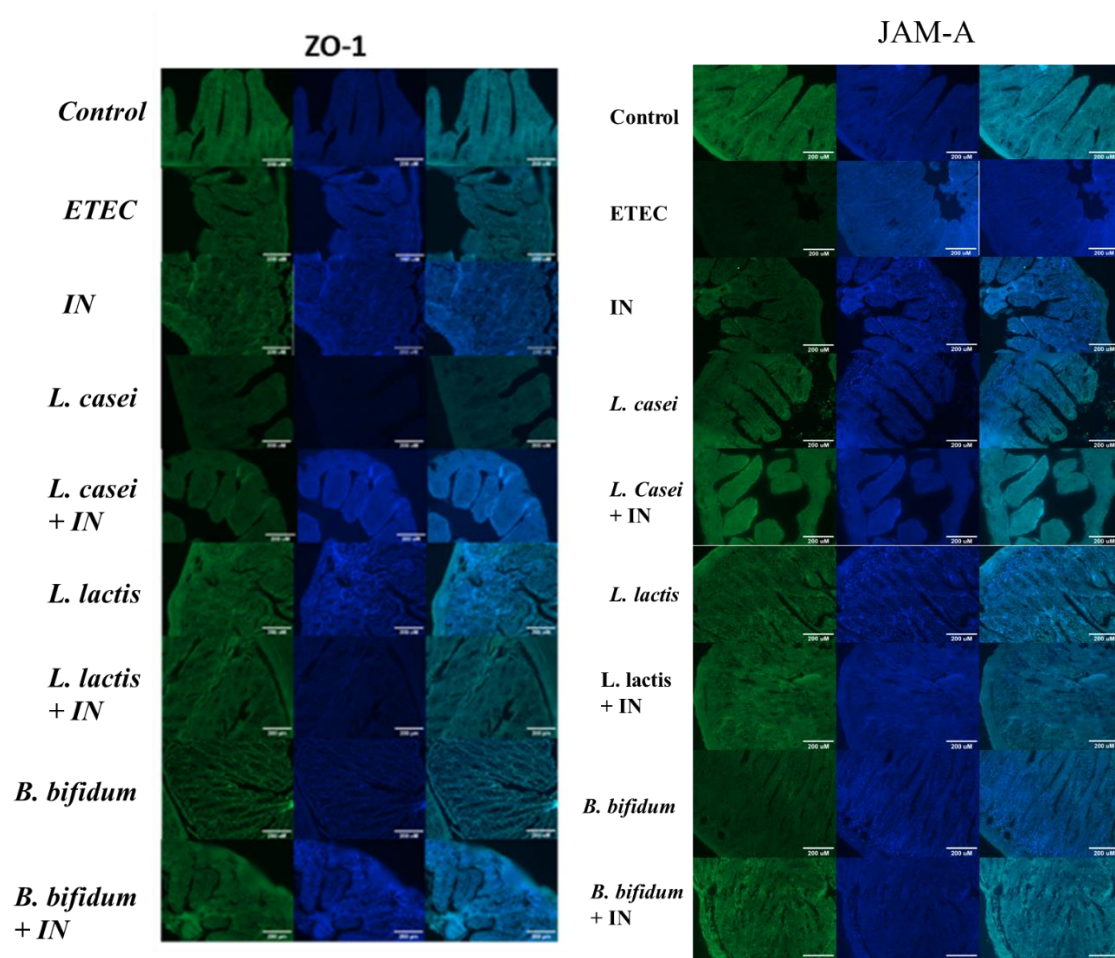


Figure 5.3.1.7C: Immunofluorescence images of small intestine. Tight junctional proteins were stained with FITC-tagged (green) anti-ZO-1 and JAM-A antibodies. To detect the Nucleus, the Hoechst stain (blue) was used. fluorescence intensity of each image was plotted and compared with that of every other group. This study was conducted with $N \geq 5$ animals for each group, representing Mean $\pm$ SEM.

5.3.1.8. Probiotic-induced gut microbiome alterations help ETEC clearance

In search for the beneficial effect of Probiotic consumption-mediated Gut microbial alteration, and its protection against ETEC colonization, the fecal pellets of probiotic-fed mice were collected and orally given to ETEC-infected mice. The kinetics of pathogen clearance among ETEC vs probiotic FMT groups was similar on day one. On day two, pathogen shedding was visibly increased in the probiotic-FMT-grouped mice compared to the ETEC group. A subsequent sharp decline in ETEC shedding was observed among the probiotic FMT groups from day 3 and onwards (Figure 5.3.1.8A) while in the ETEC group, declined ETEC load within fecal matter was observed on day 6 and onward.

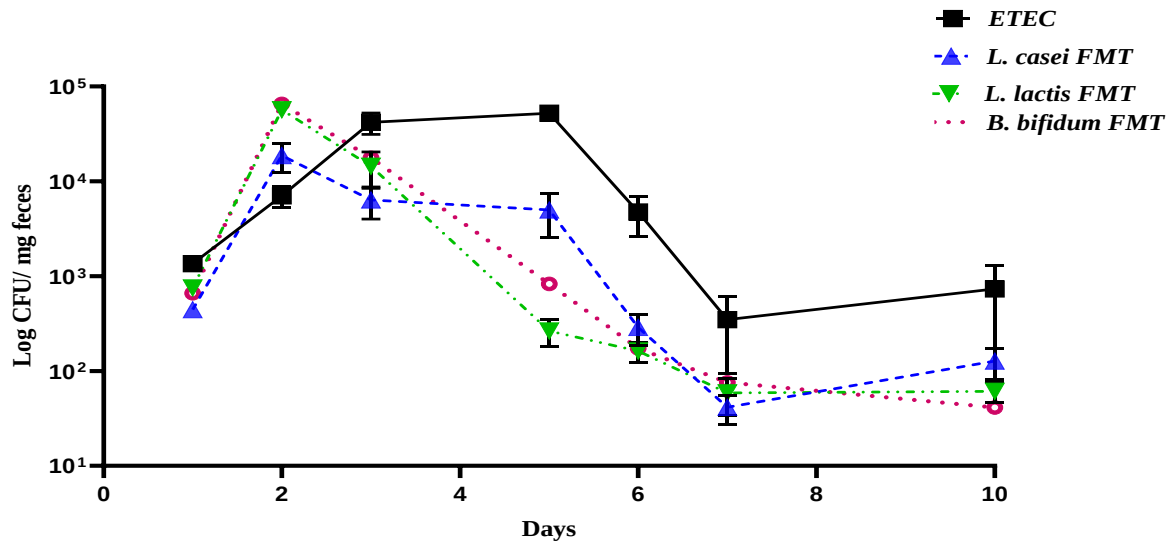


Figure 5.3.1.8A: Day-wise monitoring of the fecal ETEC CFU load. Pathogens (CFU) are excreted out with fecal pellets after infection. Data were collected at different time intervals. Error bars indicate sem.

5.3.2. Discussion

Over the years Diarrhoea caused by ETEC has been a threat to travelers visiting high-risk regions of developing countries. With the high rate of incidence and unavailability of vaccines with optimal efficacy it is necessary to treat ETEC infection with an alternative approach. Although antibiotics like fluoroquinolones are generally effective against ETEC, in healthy travelers, prophylactic usage is not advised due to the possibility of selecting resistant microbes (Riddle et al. 2017) (Diptyanusa, Ngamprasertchai, and Piyaphanee 2018), (Pamela Rendi-Wagner, 2002). Again, the risk of generating antibody response against ETEC toxin neutralizers keeps the problem unsolved (Duan et al. 2018). Because there is a chance of triggering an autoimmune disease-causing antibody response, administering a recombinant bacterium with an ETEC toxin-neutralizing compound on its surface is complex (Paton et al. 2005), (Wraith, Goldman, and Lambert 2003). A prophylactic intervention is much suited to ETEC-induced Diarrhoeal prevention. In this context, an effective and affordable alternative is critically needed. In this study, we approached a probiotic-prebiotic-based prophylactic therapy against the ETEC-induced Diarrhoeal onset in a mice model.

From the secret goat yogurt used by the Jewish Ottoman doctor in the 16th century to treat Francis I of France's severe gastrointestinal disease to Pasteur's 1857 discovery of lactic acid-producing bacteria; probiotics are now establishing as cutting-edge therapeutic and preventive measures (Ozen and Dinleyici 2015). The two genera *Lactobacillus* and *Bifidobacterium* species produce the majority of the probiotics that are most frequently utilized (Mariarosaria Matera, 2024), (Tham, 2012). *L. casei*, *L.*

lactis, and *B. bifidum* have proven to have probiotic efficacy against LT+ ST+ ETEC H10407 strain-induced infectious diarrhoea in mice models. This study also confirms a lesser ETEC colonization in the ileal part of the small intestine after *L. casei*+ IN, *L. lactis*+ IN, and *B. bifidum*+ IN prophylactic administration. The bile salts are broken down by the Lactobacillus species, which also aids in the creation of vitamins B and K. They contribute to the development of both host's innate and acquired immune responses. It also leads to suppression of pro-inflammatory mediators (Alok et al. 2017). In earlier research, probiotics were found to improve antibiotic-associated Diarrhoea (AAD) and dysbacteriosis of the gut microbiota by increasing the mRNA expression of Occludin, Zonula Occludens-1, and claudin-1 with an abundance of bacterial genera Lactobacillus, Bacteroides, and Muribaculaceae (Li et al. 2023).

Similar data was found, supporting our hypothesis where the intestinal multidrug-resistant *Acinetobacter baumannii* (MDRAB) mice infection model is used to investigate the prebiotic galactooligosaccharides (GOS) and the probiotic *Bifidobacterium breve* strain Yakult (BbY) having synbiotic combining effects (Asahara et al. 2016). Earlier in a study, a *Lactobacillus casei* ATCC 393 strain pretreated IPEC-J2 cells were protected against ETEC K88 induced Trans Epithelial Electrical Resistance loss due to restoration of tight junctional protein ZO-1 and Occludin. In PMMCs, ETEC K88 decreased levels of anti-inflammatory cytokines, but *L. casei* ATCC 393 decreased levels of IL-1 β , β -hexosaminidase, and tryptase, along with TNF- α , IFN- γ activity. Protein expression reductions produced by ETEC K88 were mitigated, and the rise of cytokines was reduced by intragastric pretreatment with *L. casei* ATCC 393. (Xu et al. 2020). In this segment, we found a similar protective role of *L. casei* ATCC 393 against ETEC H10407-induced gut barrier forming tight junctional protein dysregulation followed by neutrophil infiltration and induce pro-inflammatory cytokine release. On the other hand, the *Lactococcus lactis* oral vaccine showed protective efficacy against LT-producing ETEC and inhibited fluid loss in the rabbit model (Ahmadi Rouzbahani et al. 2021). We are the first to show *L. lactis* being a potential probiotic against ETEC-induced diarrhoea.

In humans, severe ETEC colonization correlates with high MPO in the stool as an outcome of systemic inflammation. This study also exhibited an increased concentration of IL-17A and IFN- γ in the sera of ETEC-infected volunteers indicating enhanced inflammatory molecular markers (Brubaker et al. 2021). An increased neutrophil in the ETEC-infected mice ileal villi showed a correlation to the increased MPO expression in ETEC infection in humans. Studies showed that the outer membrane vesicle (OMV) of ETEC has higher potency for pro-inflammatory response induction. WT ETEC H10407 triggered Interleukin-8 (IL-8) secretion from HT-29 human colon epithelial cells (Thapa et al. 2023), which shows similarity with the data we got from the mice ileal tissue expression of CXCL-1. The IL-8 cytokine in humans and CXCL-1 cytokine in mice play a similar role in neutrophil chemoattraction (Asfaha et al. 2013). In the previous chapter, we discussed how the proinflammatory cytokines, released by the

immune cells depend on the sphingosine-1-phosphate (S1P) concentration in tissue. A higher level of tissue S1P is essential to trap T cells in the mesenteric lymph nodes to promote a strong immune response when there is inflammation or infection (Sun et al. 2024) (Baeyens et al. 2021). In this chapter, we showed a prophylactic probiotic and synbiotic administration kept the tissue S1P concentration low and so did the T cell load in the MLN and spleen. The inability of the ETEC to colonize and generate toxins may be the reason it could not provoke a host immune response at the ileal portion of the small intestine in the presence of probiotic and synbiotic but an increased S1P level in the ileum with increased pro-inflammatory TNF- α , IFN- γ and CXCL-1 was visible in the ETEC infection group. Both in T-cell profiling experiment and cytokine parameters, the synbiotics showed a greater efficacy than that of the probiotics and prebiotic groups. The synergy between the probiotics and prebiotics in reducing the disease parameters was the most intriguing finding. Non-viable dietary ingredients called prebiotics alter the microbiota to improve health. They come from various carbohydrates, including breast milk, soybeans, inulin, raw oats, and non-digestible oligosaccharides (Pokusaeva, Fitzgerald, and van Sinderen 2011). Only bifidogenic, non-digestible oligosac carbohydrates, like inulin and pectin, meet prebiotic classification criteria. Prebiotics can reduce diarrhoea, alleviate inflammation, and prevent colon cancer (Pena 2007). Since the word "synbiotics" implies synergism, it should only be used for products in which the prebiotic substance or compounds preferentially affect the probiotic species or organisms. (Cencic and Chingwaru 2010). To help promote probiotics and to overcome potential survival challenges, the Synbiotics were created. Usage of synbiotics seems to be justified by data demonstrating that probiotic bacteria are more likely to survive when they transit through the upper gastrointestinal system (Pena 2007). In the previous chapter also, we found that synbiotic supplementation improved mice's short-chain fatty acid profile when it was given along with FTY720, a Shingosine-1-phosphate functional antagonist. Probiotics, when given separately, showed efficacy against ETEC a fewfold lesser than the efficacy it showed when given with Inulin as the synbiotics. From the colonization data, it is clear that the delivery of the probiotic strains was more successful when delivered with Inulin as well as their growth and survivability in the small intestine. We showed that a targeted enrichment of a probiotic strain with the help of a prebiotic, can make a big difference in pathogen survivability in the intestine and induce an early pathogen clearance. According to this study, synbiotics are a more effective way to prevent ETEC infections than standard probiotic dosage. Even, it can be established as an effective safeguard for travelers from having ETEC infection in advance. Because travelers' diarrhoea has a very high strike rate and makes it easy to identify those who are at risk before exposure, it is a perfect candidate for the prophylactic deployment of toxin-binding probiotics (Paton et al. 2005). As travel is generally a pre-planned event, a prophylactic administration of probiotics or synbiotics can be taken by travelers which not only offers protection against ETEC but can prevent many other pathogenic bacteria from colonizing in the gut and can help in coping with a foreign diet consumption.

Chapter 6:

Conclusion

The intestinal epithelium and the mucosal compartment are scientifically very complex yet fascinating areas to explore. Mucosal physiology, which includes physical and immunological barrier maintenance, nutrient absorption, antimicrobial peptide secretion, neuromuscular and neuroimmune response, and regulation makes a complicated network critical to unwind. Studies that we have encountered drew our attention to the sphingolipid signaling in gut physiology, targeting the barrier function. Through decades of research, we can now understand S1P as an essential immune regulator. But newly emerging studies disclose many other aspects of S1P signaling like, cell migration, angiogenesis, vascular permeability, and apoptosis. A concentration of S1P may influence the physiology in a deleterious or protective direction and how it depends upon the intracellular and intercellular signaling mechanism, is yet to be unveiled. S1P's protective function includes promoting wound healing and cellular functions including anti-apoptosis (cell survival). However, not much data is available regarding its effects on tight junction proteins and cell-to-cell adhesion, which are essential for tissue homeostasis and reducing inflammation. This thesis focuses on the epithelial tight junction of the gut barrier and the mucosal immune response underneath.

This study reveals that the S1P, generated from Sphingosine phosphorylation by Sphingosinekinase-1 (SPHK-1), is essential for the tight junctional protein expression by the colon epithelial cells. SPHK-1 knocked down HT-29 cells failed to express certain tight junctions forming “sealing proteins” i.e.; Occludin, Claudin-5, and Junctional Adhesion molecule-A. Even more interesting data was achieved when we used the SPHK-1 competitive inhibitor N, N-Dimethyl Sphingosine (DMS) in HT-29 cells and discovered a decreased transmembrane electrical resistance, followed by a reduced expression of Occludin. Occludin is a major transmembrane protein, associated with Zonula Occludens -1 (ZO-1), JAM-A, and claudins, it helps form the tight junctions in the epithelial membrane. Tight junctions are selectively permeable passages between epithelial cells in the gut wall that regulate the paracellular transport of cells and solutes. Upon synthesizing, S1P is exported to the extracellular scape where it binds to the S1P receptors (S1PRs) and transduces down stream signaling. We used FTY720, a functional antagonist and S1P receptor agonist, to treat HT-29 cells in order to examine whether tight junction regulation requires S1P signaling even when S1P is available. FTY720 treated HT-29 cells showed lower Trans Epithelial Electrical Resistance (TEER). A reduction in Occludin and Claudin-4 levels in FTY720 treated cells was spotted, further confirming that S1P signaling is essential to promote tight junction formation in the epithelial cells.

S1P receptors, expressed on the lymphocytes and other immune and nonimmune cells play a vital role in initiating innate and adaptive immune responses. Hence the emergence of S1P receptor analogs to treat several inflammatory conditions. FTY720 or Fingolimod, an FDA-approved drug to treat multiple sclerosis (MS), creates peripheral blood lymphopenia. Our curiosity lies in whether FTY720 makes the gut's epithelial barrier breach even more vulnerable to opportunistic infections by compromising local immunity. In the mice model, FTY720 created gut epithelial permeability by dysregulating Claudin-4,

Claudin-2, and Occludin. As we expected, the increased permeability induced the gut commensals to trans-migrate to the distal organs like the liver. This commensal exposure to the mucosal innate immune cells elevated the expression of pro-inflammatory cytokine synthesizing genes such as TNF- α and IFN- γ with an increased population of Neutrophils, macrophages, B- cells, and NK cells in the tissue. The inflammation was more evident in the colon histology where the colonic architectural loss was accompanied by massive neutrophil infiltration and goblet cell loss. Our observation became valid with the finding of an increased tissue level CXCL-1 and CXCL-2 of the FTY720 mice colon. As we assumed, continual dosing of FTY720, enhanced the population of the splenic and mesenteric CD4⁺/CD8⁺ -T lymphocyte cells as an outcome of the induced lymphocytopenia. Further investigation revealed that FTY720 decreased antimicrobial peptides (AMPs) Cathelicidin and Hepcidin (Figure-1). Taking a step further A metagenomic study for gut microbial composition, it was revealed that FTY720 mice showed a decreased Bifidobacterium abundance in the gut. This bacterial genus is one of the main contributors to Short-chain fatty acid (SCFA) synthesis bacteria. A brief reflection was the metagenomic data was unveiled in the CG-MS data for SCFAs profiling. FTY720 mice showed a decrease in Butyrate and propionate concentration in the gut. A probiotic and prebiotic supplement with FTY720 in mice showed a better SCFA profile and greater gut barrier function.

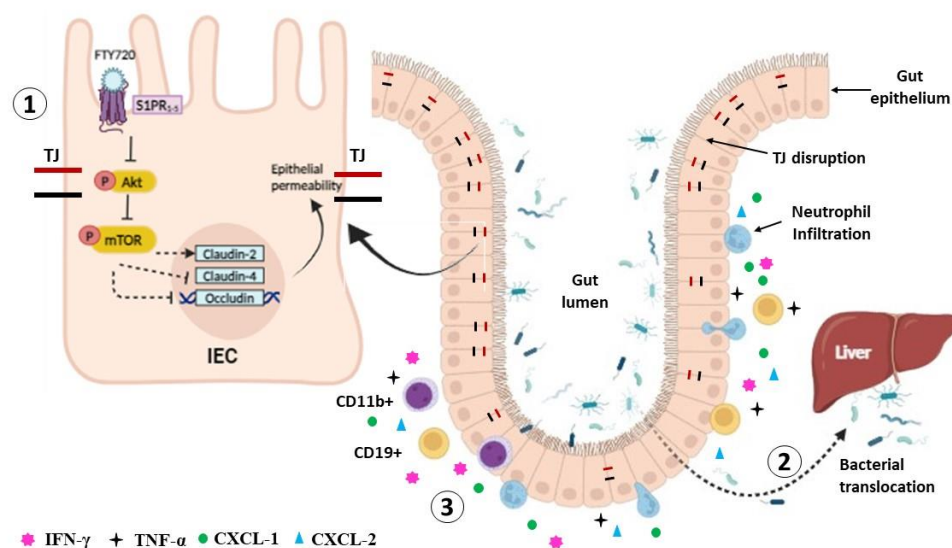


Figure 1: FTY720 decreases tight junctional protein Occludin, Claudin-4 and increases Claudin-2 via the Akt-mTOR signaling axis in epithelial cells of the colon and induces epithelial permeability. Which leads to 2. bacterial translocation to the liver. 3. Exposed microbiome to the host with enhanced immune cell infiltration increasing local inflammatory markers.

In another part of our research, we found an astonishing correlation between infection and S1P. Mice having an infectious diarrhoea with Enterotoxigenic *E. coli* (ETEC) H10407 clinical isolate, showed increased S1P concentration in small intestinal tissue. ETEC H10407 induced infection followed by

mucosal inflammation and gut barrier damage in mice. As Prebiotics and probiotics are coming into focus as potential therapeutic agents showing remarkable health benefits in several enteric diseases, we tried to establish a probiotic, prebiotic, and synbiotic (probiotic+prebiotic) based protective measure against ETEC. A prophylactic oral administration of probiotics, prebiotics, and synbiotics showed low bacterial load in ileum. Among these, synbiotics showed maximum protective efficacy over the other groups, showing decreased pro-inflammatory Tumor Necrosis Factor- α , IL-6, IFN- γ , and CXCL-1 expression and a lesser number of CD3⁺CD4⁺ and CD3⁺CD8⁺ -T lymphocyte cell populations in the spleen and mesenteric lymph nodes which increased upon ETEC infection. We also discovered that synbiotics have a profound protective efficacy in ETEC-induced gut barrier disruption by preserving the protein expression of the Zonula Occludens-1, JAM-A, Claudin-2, and Occludin.

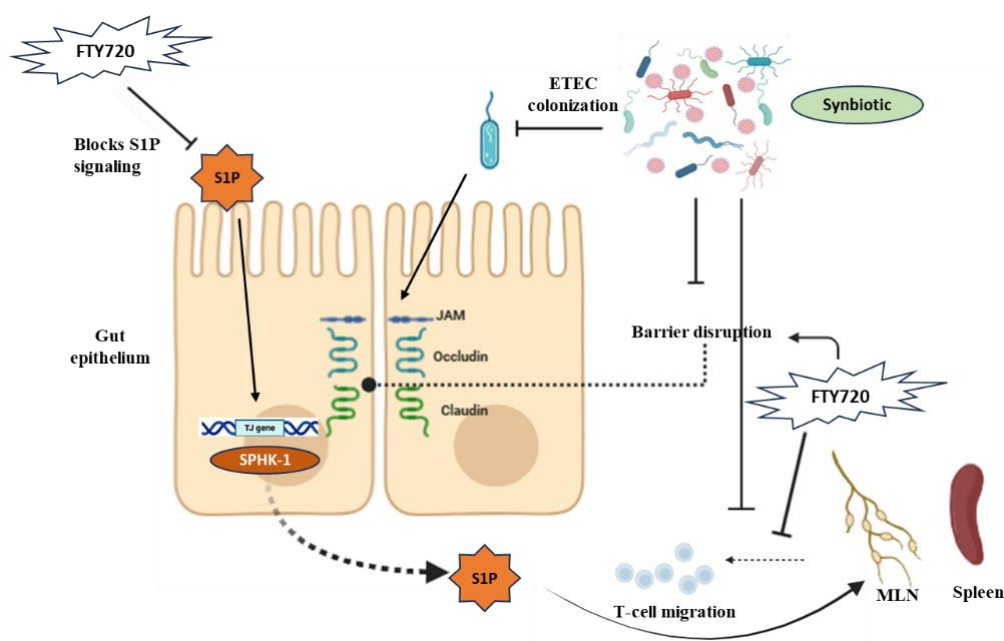


Figure 2: S1P has dual role. 1. S1P promotes Tight junction (TJ) synthesis. 2. S1P helps T-cell migration at the site of inflammation. 3. S1P functional analog reduces T-cell migration but, 4. disrupts the gut barrier. 5. Synbiotics reduce gut inflammation and, 6. Restore tight junctions. 7. Synbiotics inhibit ETEC colonization in the gut and, 8. Reverse the ETEC-induced TJ downregulation.

All these findings point to the mechanism of gut barrier disruption and immune response followed by S1P signaling alteration. The connection between S1P and infectious disease is relatively less inquired. This study may open a new perspective on Sphingolipid biology in infection-induced barrier disruption connecting S1P's role as a barrier regulator. However, forthcoming research work is needed to comprehend the complexity of immune-epithelial dynamics. The synergy between probiotics and prebiotics and becoming a more potent Synbiotic formula is a relatively new concept that can guide future studies and their intricate mechanism of action both in preventive and therapeutic modes of action.

Chapter 7:
Highlights of the
study

- Sphingosine-1-phosphate, a pleiotropic bioactive lipid of eukaryotic cells, regulates endothelial and epithelial barriers and the host's immune response.
- Sphingosine-1-phosphate (S1P) is crucial in forming tight junctions in the intestinal epithelial cell layer.
- In HT-29 colon carcinoma cells, the knockdown of the Sphingosine Kinase-1 (SPHK1), the key enzyme that produces S1P, leads to the disruption of the physical barrier property of the cells.
- SPHK-1 silencing in colon epithelial cells lowers the Trans Epithelial Electrical Resistance.
- S1P signaling inhibition affects Tight junctional protein synthesis of colon epithelial cells in mice.
- Continual dosing of S1P functional antagonist FTY720 in mice disrupts the gut barrier.
- FTY720 targets the Akt-mTOR signaling axis to dysregulate the tight junction forming Occludin, Claudin-4, and Claudin-2 proteins in the HT-29 cells.
- The absence of S1P-regulated tight junctional formation contributes to 'leaky gut' formation with massive neutrophil infiltration.
- FTY720 creates immune suppression by sequestering S1P receptors on T lymphocytes.
- S1PR antagonism increased gut epithelial permeability, leading luminal commensals to trans migrate to host tissues, creating low-grade tissue inflammation.
- Enteric infection caused by Enterotoxigenic *E. coli* or ETEC in mice increases tissue Sphingosine-1-phosphate concentration and triggers inflammation in the small intestine.
- Probiotic strains of *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* have protective efficacy against ETEC colonization in the small intestine of mice, protecting the epithelial barrier.
- These probiotic strains lower the tissue S1P level during ETEC infection and reduce inflammation by elevating anti-inflammatory IL-10 cytokine release in the epithelial tissue.
- *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*, conjugating with prebiotic, makes Synbiotics, which proved to have even greater protective efficacy against the Traveler's diarrhoea caused by ETEC.

Chapter 8:

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Chapter 9:

Publications



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EDITED BY

Jia-bo Wang,
Capital Medical University, China

REVIEWED BY

Zili Xie,
Icahn School of Medicine at Mount Sinai,
United States
Jiayu (Daisy) Ye, Stanford University,
United States

*CORRESPONDENCE

Moumita Bhaumik,
✉ drmmoumitabhaumik@gmail.com

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The functional antagonist of sphingosine-1-phosphate, FTY720, impairs gut barrier function

Sohini Sikdar¹, Debmalya Mitra², Oishika Das¹,
Moumita Bhaumik^{1*} and Shanta Dutta¹

¹Division of Immunology, ICMR-National Institute for Research in Bacterial Infections (NIRBI), Kolkata, India, ²Center of Radiological Research, Columbia University Irving Medical Center, New York, NY, United States

FTY720 or fingolimod is a known functional antagonist of sphingosine-1-phosphate (S1P), and it is effective in treating multiple sclerosis and preventing inflammatory bowel disease (IBD). Evidence shows that its use in mice can increase the susceptibility to mucosal infections. Despite the significant contribution of S1P to barrier function, the effect of the administration of FTY720 on the mucosal barrier has never been investigated. In this study, we looked into how FTY720 therapy affected the function of the gut barrier susceptibility. Administration of FTY720 to C57BL/6 mice enhances the claudin-2 expression and reduces the expression of claudin-4 and occludin, as studied by qPCR, Western blot, and immunofluorescence. FTY720 inhibits the Akt–mTOR pathway to decrease occludin and claudin-4 expression and increase claudin-2 expression. FTY720 treatment induced increased colonic inflammation, with notably greater immune cell infiltration, colon histopathology, and increased production of TNF- α , IFN- γ , CXCL-1, and CXCL-2 than that in control mice. Taking into account the close association of “the leaky gut” and gut dysbiosis among the major diseases, we therefore can infer that the vigilance of gut pathology should be maintained, where FTY720 is used as a treatment option.

KEYWORDS

FTY720, sphingosine-1-phosphate, occludin, claudin-4, gut permeability

Introduction

The mammalian gut is a multilayer system with an immunological barrier within and a physical barrier outside, thus enabling the gut microbiome to maintain equilibrium within the body. The gut barrier that maintains these luminal compounds needs to be considered. Bioactive phospholipid sphingosine-1-phosphate (S1P) has pleiotropic properties, which help in cell–cell integration (Santacreu et al., 2019), epithelial barrier regulation (Weigel et al., 2023), cell proliferation (Chen et al., 2017), migration (Chen et al. (2017) and Wang et al. (1999)), and survival (Van Brocklyn and Williams, 2012). S1P is ubiquitous in cells (Maceyka and Spiegel (2014), Le Stunff et al. (2004), and Schmidt et al. (2019)) and produced by key enzyme sphingosine kinase (SPHK) (Imamura et al. (2001) and Pulkoski-Gross et al. (2015)). SPHK catalyzes the ATP-dependent phosphorylation of sphingosine (Sph) and exists in two isoforms: SPHK1 and SPHK2 (Hatoum et al. (2017), Alemany et al.

(2007), and Liu et al. (2000)). S1P is reported to play an important role in tolerance by regulating innate immunity by acting through one or more than one of its five known receptors (S1PR1–S1PR5) (Chun et al., 2002), which are G-protein-coupled receptors (GPCRs) (Goetzl et al. (2000), and Hla et al. (2001)). FTY720 or fingolimod inhibits lymphocyte egress from the thymus, spleen, and lymph nodes (LNs) into the bloodstream and lymphatic system by modulating S1P signaling, thus restricting lymphocyte trafficking to target tissues (Chiba, 2005). When FTY720 enters the body, it is phosphorylated (FTY720-P) and binds to S1PR (Wang et al., 1999; Van Brocklyn and Williams, 2012; Chen et al., 2017; Santacreu et al., 2019; Weigel et al., 2023) and internalizes the receptors, except S1PR3, preventing its downstream signaling (Sykes et al., 2014). Interestingly, FTY720-P can only activate S1PR3, which is mostly present in neutrophils, monocytes, macrophages, and B cells (Bryan and Del Poeta, 2018), inducing further downward signaling (Sensken et al., 2008). Additionally, it influences the migration of dendritic cells (DCs) (Han et al., 2015), modulates DC-mediated pro-inflammatory signaling (Zeng et al., 2012), and is a potent suppressor of regulatory T-cell (Treg) proliferation (Wolf et al., 2009). Whether FTY720 regulates lymphocyte recirculation *in vivo* in an agonistic or a functional antagonistic manner, or in both, is still up for debate (Blanc et al., 2015). The FDA has authorized the medication FTY720 (trade name Gilenya) to treat multiple sclerosis relapse (Sharma et al., 2011). Several reports showed the protective efficacy of FTY720 in various preclinical models like oxazolone (Daniel et al., 2007a), TNBS (Daniel et al., 2007b), and DSS-induced murine models of colitis (Deguchi et al., 2006), as well as IL-10-deficient mouse model (Mizushima et al., 2004). FTY720 is in clinical trial for inflammatory bowel disease (IBD) patients (Danese et al., 2018). Furthermore, it showed therapeutic efficacy in the treatment of graft-versus-host disease (Ryu et al. (2020) and Gauthier et al. (2018)) and rheumatoid arthritis (Tsunemi et al. (2010) and Nakano et al. (2022)) in mouse and viral infection models (Miyamoto et al., 2001). FTY720 prevents colitis by modulating the colitogenic CD4⁺T cells from the bone marrow (Fujii et al., 2008). However, being a structural homolog of S1P, which helps in promoting barrier function, the effect of FTY720 on the mucosal barrier has never been elucidated.

The body's largest interaction with the outside world is the gastrointestinal epithelium, which effectively creates a barrier that limits the mucosa's ability to absorb luminal toxins and antigens while permitting nutrient and water absorption. Epithelial cells use both the paracellular and transcellular transport pathways to form this specific barrier (Laksitorini et al., 2014). The paracellular route, however, which is controlled by the tight junction (TJ), is in charge of the highest level of apical cell–cell adhesion and has drawn the most attention for its function in controlling mucosal permeability in both healthy and pathological settings (Paradis et al., 2021). The TJ (also referred to as zonula occludens) controls the most apical cell-to-cell adhesion between neighboring epithelial and endothelial cells (Furuse (2010) and Bazzoni and Dejana (2004)). These TJ proteins include integral and transmembrane proteins, including the claudins, occludin, and junctional adhesion molecules (JAMs), which extend into the intercellular space and regulate the gate function, and cytoskeletal linker proteins, such as cingulin, ZO-1, ZO-2, and

ZO-3, which anchor TJ-integral membrane proteins to the cell cytoskeleton (Heinemann and Schuetz (2019) and Heinemann and Schuetz (2019)). An uncontrolled flow of antigens across the intestinal epithelium and a malfunctioning intestinal barrier can tax the immune system of those who are vulnerable and alter the host–microbe balance, which can set off inflammatory processes in the gut or make mucosal infections more likely (Meddings (2008) and Yu et al. (2012)). A recent report showed that FTY720 therapy blunts the mucosal adaptive immune response, including the generation of Th1 cytokines, and impairs innate immunological responses, making mice more susceptible to *Citrobacter rodentium* infection (Murphy et al., 2012). However, the study did not assess the effect of FTY720 on the barrier function.

This study is designed and intended to investigate the effect of continuous dosing of FTY720 on the gut functional phenotype in a mouse model. We studied the TJ protein expression, gut permeability, and immune response in the gut of FTY720-treated mice compared with untreated control. Our results showed FTY720-treated mice exhibited downregulation of multiple TJ proteins, leading to an increase in gut permeability and inflammation, and these are associated with the inhibition of the AKT–mTOR signaling pathway. The mucosa of the mice shows indications of inflammation, including crypt hyperplasia, goblet cell loss, and immune cell infiltration at the crypt region. Our data clearly showed that continuous treatment with FTY720 increases the possibility of gut barrier disruption. To our knowledge, this is the first report indicating that FTY720 may jeopardize the critical barrier function and induce an immune response.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific). FTY720 (cat #SML0700) was purchased from Sigma-Aldrich. The cytokine detection ELISA kits were purchased from BD Biosciences (United States) and R&D Systems (United States). Primers were purchased from Integrated DNA Technologies. All the polyclonal antibodies for β -actin (sc-47778), occludin (ab216327), claudin-4 (ab53156), and claudin-2 (ab53032) and secondary anti-IgG (ab97080 and ab97023) antibodies were purchased from Santa Cruz Biotechnology and Abcam. For immunohistochemistry, an anti-MPO (PA5-16672, Invitrogen) antibody was used. Secondary IgG Alexa Fluor 488 (ab150081, Abcam) was used for TJ immunofluorescence. Rapamycin (cat #13346) was purchased from Cayman Chemical. The following antibodies were purchased and used for performing *in vitro* Western blot assay: p-Akt 1/2/3 (Ser 473) (cat #AF0016; Affinity), Akt (cat #GTX121937; GeneTex), P-mTOR (Ser 2,448) (D9C2) (cat #5536T; Cell Signaling Technology), and mTOR (7C10) (cat #2983S; Cell Signaling Technology). FACS antibodies used in immunofluorescence were as follows: FITC-CD3 (cat #100204; BioLegend), PerCP-CD19 (cat #115532; BioLegend), FITC-CD11b (cat #11-0112-85; eBioscience), and A700-Ly-6G (cat#127621; BioLegend).

Mice and animal ethics

C57BL/6 mice (6 weeks old) were procured from the ICMR-NICED Animal Facility of the institute. All the protocol for the study was approved by the Institutional Animal Ethics Committee of ICMR-NICED, Kolkata, India (PRO/151/July 2018–June 2021). Experiments were carried out in accordance with the guidelines laid down by the committee for the purpose of the control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India.

Treatment with FTY720

All mice were housed in cages containing straw bedding and held in pathogen-free facilities maintained at 24 °C with a 50% relative humidity and 12-h light:dark cycle. All mice had *ad libitum* access to standard rodent chow. The mice were divided into two groups (i.e., control and FTY720; $n = 5/\text{group}$). Mice in the FTY720 group were provided 3 mg/kg body weight (Choi et al., 2011) (Rau et al., 2011) of FTY720 orally for 14 days, whereas mice from the control group were provided normal saline. Food and water consumption was monitored throughout the experimental period. The body weights of the mice were also measured at regular time intervals. The mice were euthanized on day 15.

Gut permeability assay

On the day of euthanasia, mice from both the control and treated groups were force-fed 44 mg/100 g body weight FITC-labeled 4-kDa dextran (FD-4; MW, 4,000 Da; Sigma-Aldrich; Merck KGaA) via gavage following fasting overnight (Liu et al. (2020), Chakraborty et al. (2023), and Kang et al. (2017)). After 4 h of FD-4 administration, blood was collected and centrifuged at $2,500 \times g$ for 10 min at 4°C to collect the serum. The fluorescence intensity of FD-4 in the serum was determined using a multimode reader at an excitation and emission wavelength of 485 nm and 520 nm, respectively. Subsequently, a standard curve was generated using serial dilutions of FD-4 from 1,000 µg/mL to 0.

Bacterial translocation

A 40 mg portion of mice liver was collected under aseptic conditions after the mice were euthanized. Liver samples were washed in 200 µL PBS containing gentamicin (50 µg/mL) and then homogenized in ice-cold PBS. A measure of 100 µL of each tissue homogenate was spread onto the nutrient agar and incubated at 37°C for 24 h. The number of bacterial colonies was counted based on the dilution factor and expressed as colony-forming unit (CFU). CFU/g of tissue denoted the amount of bacterial translocation.

Real-time PCR

RNA was extracted using TRIzol Reagent (Invitrogen, Massachusetts, United States) from each colon sample and

quantified using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, United States). A measure of 0.5 µg of RNA was taken for reverse transcription with oligo dT primers, dNTPs, RNase inhibitor, and MuLV reverse transcriptase according to the kit manual (PrimeScript 1st strand cDNA Synthesis Kit, Takara Bio) to synthesize cDNA. Quantitative PCR was conducted using the SYBR green master mix (TB Green Premix Ex Taq-II) and ROX reagent in a real-time PCR system, and the ΔC_t values were calculated to determine relative changes in target genes. The primer sets used were as follows: GAPDH (>NM_001411843.1) (5'ACCCAGAAGACTGTGGATGG3') ($T_m = 59.01$); (5'CACATTGGGGTAGGAACAC3') ($T_m = 55.49$), product length = 170; occludin (>NM_001360538.1) (5'TCACTTTTCCTGCGGTGACT3') ($T_m = 59.53$); (5'GGGAACGTGGCCGATATAATG3') ($T_m = 58.93$), product length = 138; claudin-4 (>NM_009903.2) (5'TCGTGGGTGCTCTGGGGATGCTT3') ($T_m = 65.0$); (5'GCGGATGACGTTGTGAGCGGTC3') ($T_m = 62.8$), product length = 170; claudin-2 (>NM_001410421.1) (5'TATGTTGGTGCCAGCATTGT3') ($T_m = 58.08$); (5'TCATGCCACACAGAGATA3') ($T_m = 58.12$), product length = 205; claudin-1 (>NM_016674.4) (5'ATGCAAAGATGTTTTGCCACAG3') ($T_m = 58.60$); (5'TACAAATTCCTTGCAGCCC3') ($T_m = 59.17$), product length = 210; junctional adhesion molecule-A (JAM-A) (>NM_172647.2) (5'CTGATCTTTGACCCCGTGAC3') ($T_m = 58.27$); (5'ACCAGACGCCAAAAATCAAG3') ($T_m = 56.9$), product length = 187; mucin-2 (>NM_023566.4) (5'ATGTCCTGACCAAGAGCGAA3') ($T_m = 56.2$); (5'GATTTGAAGGCCACCACGTT3') ($T_m = 56.0$), product length = 142; cathelicidin (>NM_009921.2) (5'GGCAGCTACCTGAGCAATGT3') ($T_m = 59.6$); (5'CTGTGCACCAGGCTCGTTA3') ($T_m = 59.8$), product length = 122; and hepcidin (>NM_032541.2) (5'AGGGCAGACATTGCGATACC3') ($T_m = 57.5$); (5'GCAACAGATACCACACTGGGA3') ($T_m = 57$), product length = 111.

Cell culture

HT-29 cells, the human colorectal adenocarcinoma cells (kind gift from Dr. Sushmita Bhattacharya, ICMR-NICED), were cultured in DMEM with high glucose, supplemented with 10% FBS with 100 U/mL penicillin and 100 µg/mL streptomycin in humidified air/CO₂ (5% CO₂) at 37 °C within tissue culture flasks. Cell viability with FTY720 treatment at different doses was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. HT-29 cells were seeded with a density of 10^6 cells/well and grouped into control, FTY720 (20 µM), and rapamycin (500 nM) groups, and incubated for 48 h. Thereafter, the cells were lysed with RIPA lysis buffer (5M NaCl, 0.5M EDTA, 1M Tris-HCl (pH 8.0), 0.5% Tween-20, 10% SDS, PMSF, and dH₂O), followed by Western blotting, as described below.

SDS PAGE and Western blotting

Colonic sections were washed with PBS and homogenized in RIPA lysis buffer. The total protein concentration of the sample was determined using Thermo Scientific™ Pierce™ BCA Protein Assay Kit (23225). A measure of 30–50 µg of each lysate was loaded into

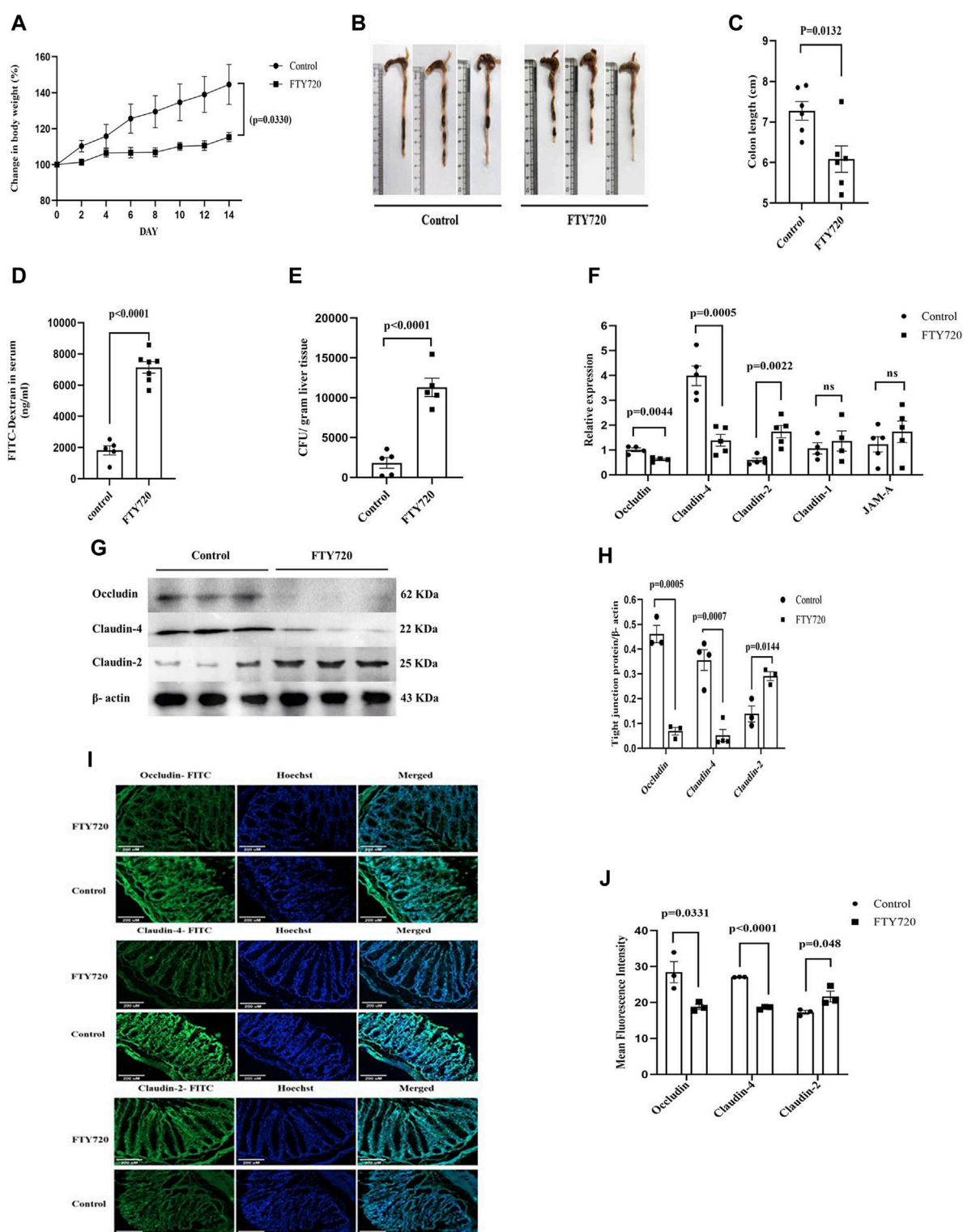


FIGURE 1 C57BL/6 mice were divided into control and FTY720 (3 mg/kg body weight) groups. After 14 days of continual dosing of FTY720, mice from both groups were euthanized. **(A)** The percentage change in body weight, **(B)** representative image of colon length, and **(C)** colon length of control mice and FTY720-treated mice were recorded. **(D)** After 4 h of oral administration of FITC dextran (44 mg/kg), there was a significant increase in FITC concentration in the sera among the FTY720 mice compared to that of the control mice. **(E)** The gut bacterial translocation in the liver was assessed by bacterial colony counting on a nutrient agar plate. **(F)** Real-time PCR data for tight junction-associated proteins: occludin, claudin-4, claudin-2, claudin-1, and JAM-A. **(G)** Western blot images of occludin, claudin-4, claudin-2, and as internal control, β -actin from the colonic lysates of both control and FTY720 groups (full blot images are provided in [Supplementary Figure S1](#)). **(H)** Densitometric analysis of the Western blot images of occludin, claudin-4, and claudin-2. **(I)** Immunofluorescent images of colonic sections from control and FTY720 mice. Colon sections were stained with FITC-tagged (green) and Hoechst (blue). Scale bars are provided for each image. **(J)** Bar graph showing mean fluorescence intensity for Occludin, Claudin-4, and Claudin-2. The FTY720 group shows significantly lower mean fluorescence intensity for all three proteins compared to the Control group. $p=0.0331$, $p<0.0001$, $p=0.048$. (Continued)

FIGURE 1 (Continued)

anti-occludin, anti-claudin-4, and anti-claudin-2 antibodies. Hoechst (blue) was used to stain the nucleus. Merged images represent the dual stains. (J) Densitometric analysis of the fluorescent intensity of the images. $n \geq 3$ animals were taken per group; these data are represented as the mean $\pm$ SEM. p values were calculated with respect to the control.

polyacrylamide gel (concentration of acrylamide was used according to the molecular weight of the target protein). After segregation, the proteins were transferred onto the PVDF membrane (IPVH00010, Millipore); 5% BSA in TBST was used as the blocking reagent. The membrane was incubated overnight with specific primary antibodies at 4°C. Then, the membrane was washed and incubated with AP/HRP-conjugated secondary antibody for 2 h at room temperature and detected using NBT BCIP substrate solution (Thermo Scientific)/chemiluminescent HRP substrate (Immobilon Western, Millipore). The HRP substrate-developed blots were visualized using the ChemiDoc Imaging System (Bio-Rad). Densitometric analysis of the protein bands was performed using ImageJ software.

Immunofluorescence staining and imaging

Colon samples from each group were collected and fixed in 4% paraformaldehyde. Paraffin-embedded 5- μ m-thick sections were generated. The sections were then de-paraffinized in xylene and rehydrated with graded ethanol, followed by distilled water. A measure of 1 mM EDTA buffer (pH 8.0) was used for antigen retrieval. The sections were then permeabilized with 0.1% sodium citrate and 0.5% Triton-X in TBST. The sections were incubated with blocking buffer (5% animal sera in TBST) at room temperature. The primary antibodies were added to the sections (1:200 for TJ antibodies and 1:50 for FACS antibodies) and incubated overnight at 4°C. The sections were then washed and incubated with the Alexa Fluor 488-conjugated secondary antibody for 2 h at room temperature, and counterstained with Hoechst 33342 (1 μ g/mL) for 10 min at RT and then washed. The sections were mounted and visualized using a Carl Zeiss microscope equipped with a CCD camera, and the images were processed using ZEN software (Gumber et al., 2014). The fluorescence intensity of the target proteins was measured using ImageJ software.

Histopathologic examination of the proximal colon

The proximal colon samples were fixed in 4% paraformaldehyde for 48 h at 4 °C. The fixed tissues were then dehydrated through graded alcohols and embedded in paraffin, and routine microtomy was then carried out to generate 5- μ m sections. The sections, in turn, were stained with hematoxylin and eosin for later microscopic examination (Chakraborty and Bhaumik, 2020).

Enzyme-linked immunosorbent assay for cytokine and chemokine profiling

To measure the inflammatory markers, that is, cytokines and chemokines, colon tissues were homogenized in PBS and

centrifuged at 10,000 g for 1 min to collect the supernatants devoid of any tissue debris. The protein concentration of the lysates was quantified using the BCA Protein Assay Kit (Thermo Scientific™ Pierce™, United states). A measure of 50 μ g lysate from each animal sample was used to perform ELISA for CXCL-1 (R&D Systems; DY453-05), CXCL-2 (R&D Systems; DY452-05), TNF- α (BD OptEIA; 555268), and IFN- γ (BD OptEIA; 555138) according to the manufacturer's instructions.

Statistical analysis

All experiments were conducted in triplicate, and data are expressed as the mean $\pm$ SEM. In Figures 1, 2, Student's t -test was used, and in Figure 3, one-way ANOVA was used using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, United States). Differences with $p < 0.05$ were considered significant.

Results and discussion

Having strong immunosuppressive potential, FTY720 was approved as the first oral immunomodulatory drug for multiple sclerosis (Cohen et al. (2010) and Kappos et al. (2010)). This discourse attempts to shed light on the understanding of the effect of FTY720 on gut physiology. A multitude of evidence showed that S1P critically regulates the barrier function of mammalian cells (Kono et al., 2008) and that S1P manipulates the proteins that are involved in cell-to-cell adhesion (Greenspon et al., 2011). Adult C57BL/6 mice were divided into two groups: one group of mice received 3 mg/kg body weight FTY720 daily through oral gavage (FTY720-treated mice), and the other group was fed saline (control mice). FTY720 used in our study is completely water soluble; therefore, a vehicle control other than water was not required. The dose of FTY720 administration was previously used to treat multiple sclerosis in mice (Choi et al., 2011). All the animals received food and water *ad libitum*. Interestingly, the FTY720-treated mice did not exhibit any significant increase in body weight (Figure 1A), indicating stagnancy in growth. The gut length was significantly reduced in FTY720-treated mice compared to the control mice (Figures 1B, C). The function of the intestinal barrier, as investigated by FITC dextran permeability (Figure 1D), revealed that the intestinal permeability of FTY720-treated mice was 4.7 times higher ($p < 0.0001$) than that of the control mice.

The increase in barrier permeability was further confirmed by studying the translocation of bacteria in the liver (Figure 1E). It was observed that a significant number of bacteria were translocated to the liver in FTY720-treated mice compared to the control mice. The translocation of bacteria

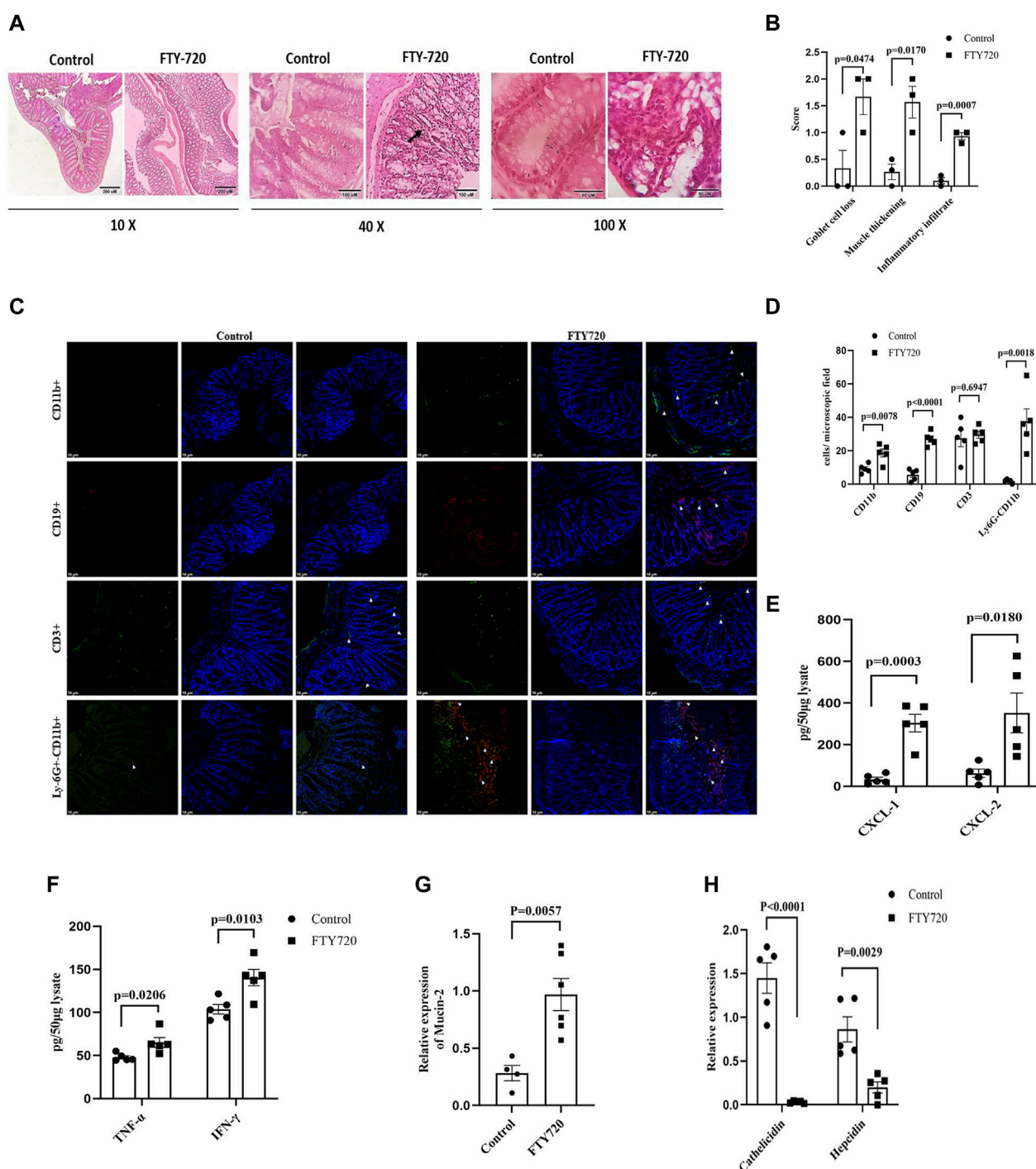


FIGURE 2

(A) Hematoxylin and eosin-stained colonic sections of mice from control and FTY720 groups (from left to right at 10 X, 40 X, and 100 X magnification, with the scale bar to the bottom right) for histological analysis. (B) Disease scores for goblet cell loss, muscle thickening, and infiltration of inflammatory cells. (C) Immunofluorescence of colon sections of FTY720 mice and control mice using FITC-conjugated anti-CD11b antibody, PerCP-conjugated anti-CD19 antibody, and FITC-conjugated anti-CD3 and A700-conjugated anti-Ly6G antibody. (D) The number of cells per microscopic field is represented. (E) CXCL-1 and CXCL-2 production in the gut tissue of FTY720 and control mice as measured by ELISA. (F) TNF- α and IFN- γ production in the gut tissue of FTY720 and control mice as measured by ELISA. (G) Real-time PCR analysis for mucus production regulating gene mucin-2. (H) Real-time PCR analysis for antimicrobial peptide cathelicidin and hepcidin in the colonic tissue of FTY720 and control mice. All the experiments were carried out with $n \geq 3$ animals, and the graphs are presented as the mean $\pm$ SEM. p -values were calculated with respect to the control.

to the liver is an indication that FTY720 may predispose the individual to major complications and play a role in chronic liver disease. We looked at the group of proteins

called occludin, classes of claudins (claudin-4, claudin-2, and claudin-1), and JAM-A that are associated with intestinal permeability (Figure 1F).

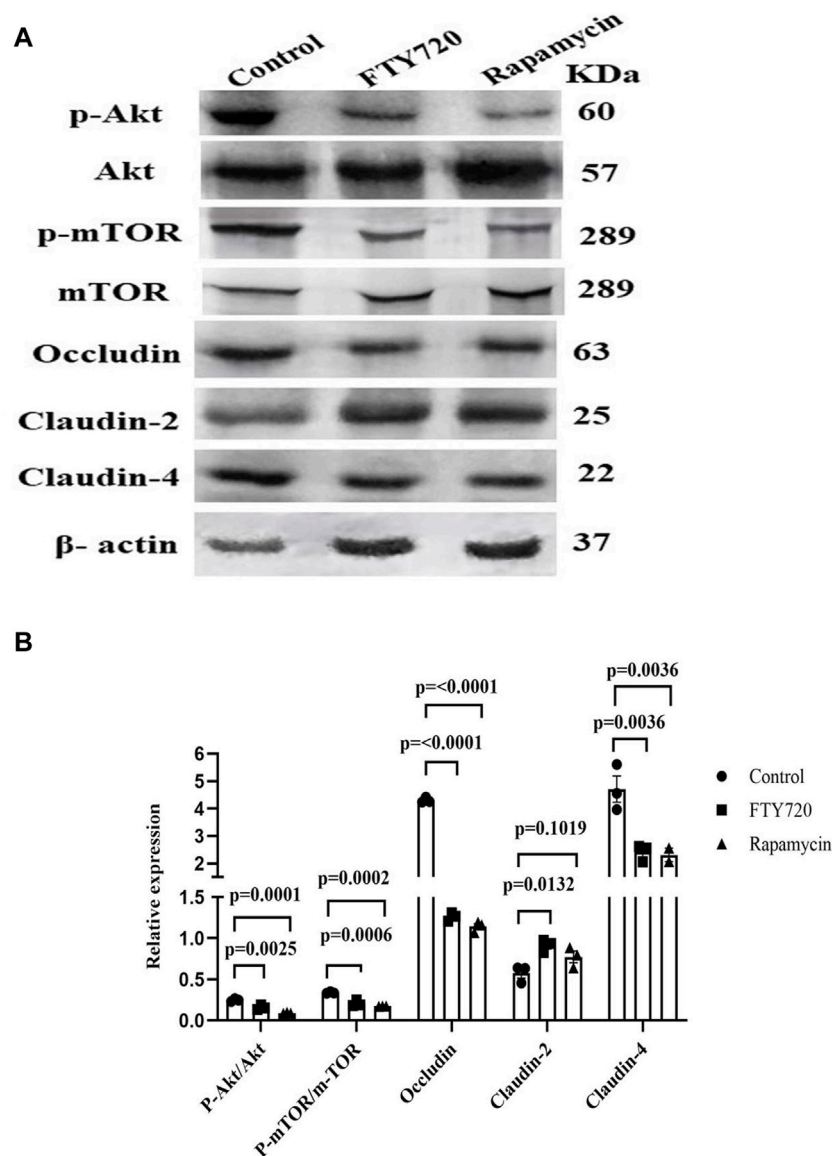


FIGURE 3

FTY720 inhibits Akt-mTOR to downregulate occludin and claudin-4 expression and upregulate claudin-2 expression in HT-29 cells. (A) HT-29 cells were treated with or without either FTY720 (20 μ M) or rapamycin (500 nM) for 48 h. Thereafter, the cells were lysed, and Western blotting was conducted for p-AKT, AKT, p-mTOR, mTOR, occludin, claudin-2, claudin-4, and β -actin. (B) Densitometric analysis is represented for p-Akt/Akt, p-mTOR/mTOR, occludin/ β -actin, claudin-2/ β -actin, and claudin-4/ β -actin (the full blot images are provided in [Supplementary Figure S3](#)).

qPCR analysis of the expression of these proteins in the colon revealed a 2-fold reduction in occludin expression and a 2.9-fold decrease in claudin-4 expression, whereas a significant 2.92-fold increase in claudin-2 expression was observed in FTY720-treated mice compared to control mice, and claudin-1 and JAM-A expression remained unaltered. The results were validated by western blot analysis (Figures 1G, H) (full blot images are provided in [Supplementary Figure S1](#)) and immunofluorescence (Figures 1I, J). Consistent to our findings in qPCR, there was a decrease in occludin and claudin-4 expressions but an increase in claudin-2 expressions in FTY720-treated mice compared to control mice, as found in the western blot and immunofluorescence analyses. Tight junctions, which are intricate signaling hubs in a constantly changing environment that serve as an impermeable

barrier to impede the free transit of solutes through the intercellular gap, make up the majority of proteins linked to gut permeability (Forster (2008) and Fasano (2012)). Paracellular permeability is largely regulated by tight junctional proteins; damage to their expression, assembly, or integrity causes an increase in epithelial permeability (Liang and Weber, 2014). Claudins form TJ strands with the cytoplasmic scaffold ZO and are essential for regulating the paracellular permeability (Furuse, 2010). In addition to claudins, TJs are the home of immunoglobulin superfamily proteins, including JAMs, and occludin, a tetra-spanning membrane protein (Furuse, 2010). The paracellular permeability is one of the main functions regulated by TJs. Occludin, with its coiled domain, functions to arrange the structural and functional components of TJs (Nusrat et al., 2000). As occludin is a crucial component of tight junctions,

permeability increases when its expression or function is reduced. Increased intestinal permeability can lead to both local and systemic inflammatory pathways when luminal components translocate into the host (Mu et al., 2017). The largest interface the body has with the outside world is the gastrointestinal epithelium (Groschwitz and Hogan, 2009). The passage of luminal toxins, as well as the antigens, through the mucosa is selectively limited by the gut epithelium (Suzuki, 2013). The concept that mucosal inflammation results from a breach in the mucosal barrier is maintained by the physical positioning of the intestinal epithelial layer, which wedges between the mucosal surface and the luminal contents (Ahmad et al., 2017). Studies using TJ protein knockout mice showed that the gastrointestinal epithelium became inflamed (Ahmad et al., 2017) (Lu et al., 2013). Additional research confirms the critical significance of permeability, particularly with regard to its ability to support the overall function of the mucosal barrier in controlling mucosal immunological homeostasis (Ahmad et al., 2017). Histological analysis of the colon samples (Figure 2A) showed the infiltration of inflammatory cells in FTY720-treated mice compared to the control. The muscularis layer of the colon showed enlargement, and mucous-secreting goblet cells also showed hyperplasia in FTY720-treated mouse colon histological sections. The breakdown of the gut barrier and immune cell infiltration are closely related events (Lin et al., 2020). We observed a significant presence of CD19⁺ B cells, CD11b⁺ cells, and CD11b⁺ Ly6G⁺ cells (neutrophils) in the colon tissue of FTY720-treated mice compared to the control. However, there was no change in CD3⁺ T cells in the gut of FTY720-treated mice compared to the control mice (Figures 2C, D). The infiltration of CD19⁺ B cells, CD11b⁺ cells, and CD11b⁺ Ly6G⁺ neutrophils increased significantly in FTY720-treated mice compared to the control mice. FTY720, after being phosphorylated *in vivo*, binds to S1PRs (Wang et al., 1999; Van Brocklyn and Williams, 2012; Chen et al., 2017; Santacreu et al., 2019; Weigel et al., 2023) that are present on the lymphocyte surface and sequesters all the receptors except S1PR3, hence preventing lymphocyte egress from lymphoid organs. Conversely, phosphorylated FTY720 binds to S1PR3, which is present on the surface of neutrophils, macrophages, and B cells, and causes downward signaling (Sensken et al., 2008). Therefore, it is likely to find increased neutrophils, macrophages, and B cells but not T cells in the gut of FTY720-treated mice compared to the control mice. To ensure the inflammatory response in the gut due to FTY720 treatment, we measured the expression of CXCL-1 and CXCL-2, TNF- α , and IFN- γ (Figures 2E, F). Interestingly, there was a significant increase in CXCL-1 and CXCL-2, TNF- α , and IFN- γ levels in the colon of FTY720-treated mice compared to control. With increased CXCL-1 and CXCL-2, the neutrophil chemoattractants (Sawant et al., 2021) in FTY720 mice are also indicative of inflammation. It is unknown exactly how goblet cell hyperplasia occurs. A previous study reported that goblet cell hyperplasia is induced by IL-13, the major regulator of type-2-mediated inflammation, which speeds up inflammation (Huang et al., 2020).

S1PR2 has been linked to IL-13 (Zhao et al., 2018), which could possibly lead to goblet cell hyperplasia. Increased mucin-2 expression in FTY720 mice compared to the control is indicative of increased mucous production (Figure 2G). Previous research

reported that colon mucin may be directly impacted by colonic inflammation (Kang et al., 2022). Subsequent investigation into the expression of antimicrobial genes, such as cathelicidin and hepcidin, revealed a notable decrease in FTY720-treated mice compared to the control (Figure 2H). It is thought to be crucial to colonic macrophage defense mechanisms against bacterial infection (Agier et al., 2015). Reduction in cathelicidin and hepcidin in the colon of FTY720-treated mice is indicative of the risk of opportunistic infection (McHugh et al. (2019), Scheenstra et al. (2020), and Ganz (2018)).

The expression of tight junction proteins is associated with the Akt-mTOR signaling pathway. In LS174T cells, human colon epithelial cells, it was shown that occludin expression is positively regulated by the PI3K-Akt-mTOR pathway (Mao et al., 2016). The Akt-mTOR pathway also activates autophagy, and the inhibition of autophagy contributes to the increase in claudin-2 expression (Zhang et al., 2017). Here, we show that FTY720 treatment to HT-29, a human colon carcinoma cell line, inhibits phosphorylation at Ser 473 of Akt and at Ser 2448 of mTOR, which is associated with a decrease in occludin and claudin-4 expression and an increase in claudin-2 expression compared to the control, as shown by Western blot analysis (Figures 3A, B) (full blot images are provided in Supplementary Figure S2). The dose of FTY720 treatment on HT-29 cells was determined previously by cell viability assay (Supplementary Figure S3). For Akt-mTOR phosphorylation, we used rapamycin, an inhibitor of mTOR, and found a similar decrease in the phosphorylation of Akt, mTOR, occludin, and claudin-4 and an increase in claudin-2 expression. The dose and time of rapamycin treatment were based on a previous report (Li et al., 2021). Therefore, it is tempting to speculate that FTY720 works through the inhibition of the mTOR signaling pathway to affect TJ protein expression. Further studies are warranted in this regard.

Among the latest findings, the most important one was that FTY720 impairs gut epithelial barrier regulation in mice. Moreover FTY720 caused a significantly increased intestinal barrier permeability and an elevated inflammatory response in the colon. These findings indicate that FTY720 causes deleterious effects in modulating the integrity of the intestinal barrier.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

All the protocol for the study was approved by the Institutional Animal Ethics Committee of ICMR-NICED, Kolkata, India (PRO/151/July 2018–June 2021). The experiments were carried out in accordance with the guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SS: data curation, investigation, methodology, software, writing–original draft, and writing–review and editing. DM: data curation, methodology, software, and writing–review and editing. OD: data curation, methodology, and writing–review and editing. MB: conceptualization, formal analysis, funding acquisition, investigation, project administration, resources, supervision, validation, visualization, writing–original draft, and writing–review and editing. SD: formal analysis, resources, supervision, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2024.1407228/full#supplementary-material>

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Gum Odina prebiotic prevents experimental colitis in C57BL/6 mice model and its role in shaping gut microbial diversity

Debmalya Mitra^a, Sohini Sikdar^a, Mainak Chakraborty^a, Oishika Das^a, Amalesh Samanta^b, Shanta Dutta^{a,*}

^a ICMR – National Institute of Cholera and Enteric Diseases, Kolkata, India

^b Division of Microbiology & Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

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ABSTRACT

Colitis is a multifaceted disease which is a global threat in the present times. It is hypothesised that novel prebiotics like Gum Odina (GO) are emerging as preventive therapy to combat chronic gut diseases due to their ease of incorporation into diet and established health benefits. The study illustrates the efficacy of GO in preventing dextran sodium sulfate (DSS) induced colitis in C57BL/6 mice and compares the results with Inulin (IN). Mice were grouped into Control, DSS, GO, and IN. Apart from the control, mice from other groups were administered with 2% DSS orally. GO and IN were also fed to the respective groups for 28 days. The reduction in body weight, colon length, mucosal dysplasia and disease activity index were found less in GO compared to DSS. CD8⁺ T cell population were reduced in GO ($10.28 \pm 0.09\%$) when compared to DSS ($30.03 \pm 0.23\%$) and IN (15.25 ± 0.25). GO increased the release of anti-inflammatory cytokine IL-10 and reduced TNF- α , IFN- γ , IL-1 β and IL-12 along with downregulation of iNOS expression in the colon. Next-generation sequencing data revealed the stool sample of colitis patient was dominated by *Prevotella* sp. along with other pathogenic bacteria. An increase in the diversity of gut bacteria and the abundance of commensal bacteria like *Lactobacillus* sp. and *Enterococcus* sp. was observed when the stool sample of a colitis patient was cultured in the gut simulator and GO was added into the feed. Thus, GO alleviate intestinal damage and slows colitis progression.

1. Introduction

Changes in dietary patterns and fast lifestyle are interconnected to a rise in colitis cases, impacting global healthcare costs with an estimate of more than 6.8 million active cases worldwide (Piovani et al., 2019). Humans are very vulnerable to colitis as the mammalian gastrointestinal tract is continuously exposed to food-derived toxins (Solanki et al., 2013), and houses numerous bacteria (Gibson & Roberfroid, 1995). Colitis is a multifaceted disease involving a disequilibrium of immune responses that occurs in the gastrointestinal tract (Xavier & Podolsky, 2007). One of the major causes of colitis is the colonization of pathogenic bacteria leading to the alteration of commensal microflora in the intestine which contributes to disease progression by causing epithelial damage (Yi et al., 2019) and up-regulating inflammatory genes (Randhawa et al., 2014). Dextran sodium sulfate (DSS) is a chemical colitogen having the properties of an anticoagulant and is most commonly used to induce colitis in animal models (Cader & Kaser, 2013) which closely resembles human colitis (Kaplan, 2015).

Prebiotics are carbohydrates that have the ability to alter the composition of the gastrointestinal microbiota and provide health benefits (Fuller, 1991). Prebiotics exerts their effect especially on the distal parts of the colon (Chassaing et al., 2014) making them a readymade therapeutic choice for the treatment of colitis. Prebiotics selectively stimulate beneficial bacterial species (Okayasu et al., 1990) and reduce the colonization of pathogenic bacteria (Gibson et al., 2004) which can slow the overall progression of colitis. Gum Odina (GO) is obtained from the plant *Odina wodier*, which is produced at around 60 tons per year with a processing cost of USD 1.50 per kilo (app) (De, Malpani, et al., 2020). GO is an established prebiotic (Mitra et al., 2016) with cancer-prevention properties (Mitra et al., 2017). GO contains arabinose and galactose as major constituent sugars and is a type I arabinogalactan (Bhattacharyya & Rao, 1964; De, Malpani, et al., 2020; De et al., 2020, 2020). GO is being used in various pharmaceutical formulations like tablet binders (Mukherjee et al., 2006), coacervates for colon-targeted delivery systems (Roy et al., 2013) and biodegradable spongy scaffolds for healing wounds of epidermal layer (Das et al., 2021). Considering the various application and prebiotic efficacy of GO, we

* Corresponding author. Division of Microbiology, ICMR NICED, P-33, CIT Rd, Subhas Sarobar Park, Kolkata, 700010, West Bengal, India.
E-mail address: shanta1232001@gmail.com (S. Dutta).

List of abbreviations

ANOVA	Analysis of variance
DSS	Dextran Sodium Sulfate
ELISA	Enzyme-Linked Immuno Sorbent Assay
DAI	Disease activity index
GO	Gum Odina
IN	Inulin
MGA	Meta Gene Annotator
ORFs	Open reading frames
PBS	Phosphate buffer saline
SEM	Scanning electron microscope
SCFAs	Short-chain fatty acids
SHIME	Simulator of Human intestinal Microbial Ecosystem
SEM	Standard error of the mean
wt	Weight

Table 1

Composition of diet fed to C57BL/6 mice to conduct DSS-induced colitis model.

Composition	Amount (g per kg)
Starch	385
Sucrose ^a	250
Casein	200
Refined oil	100
Mineral mixture ^b	10
Vitamin mixture ^b	50
Choline Chloride	2
Methionine	3

^a Sucrose was replaced with GO in mice groups fed GO and IN respectively gum odina and inulin respectively.

^b Vitamin and mineral mixtures were prepared and mixed according to (Pellizzon & Ricci, 2020).

hypothesised that GO can reduce the progression of experimental colitis induced by DSS of molecular wt. ~50 k Da in C57BL/6 mice, which mimics human colitis situation (Melgar et al., 2008) and has the ability to emerge as preventive therapy. The mechanism by which GO prevents colitis progression was investigated and the obtained results were compared alongside that of Inulin (IN) as a reference. IN is the most widely marketed prebiotic which is obtained from chicory roots, beet-roots, bananas, leeks etc and contains mainly fructose, glucose, xylose and galactosamine (Qiao et al., 2022). Subsequently, the gut simulator was used for culturing the microflora of a colitis patient by collecting stool sample and richness in terms of microbial diversity attained upon the incorporation of GO was profiled using Next-generation sequencing.

2. Methodology and materials

2.1. Collection & purification of prebiotic

GO was collected from the plant *Odina wodier* which is predominantly found in tropical forests of West Bengal, India and purified using acidified ethanol which induces stable conformational changes and brings out bioactivity. The raw polysaccharide was immersed in MiliQ water and left for 12 h to achieve uniform swelling, which was followed by 6 h of stirring using a laboratory stirrer (REMI, India) at 25 °C. The solution was filtered through clean and sterile muslin cloth and centrifuged (Thermo Scientific Heraeus Biofuge Stratos Centrifuge, Osterode, Germany) at 1500 rpm for 15 min. The collected supernatant was mixed with equal parts of acidified ethanol to generate a precipitate of white colour, the collected precipitate was further washed using ethanol and

dry ether (1:1) and lyophilized for 5 days to remove traces of organic solvents. The purified GO was powdered and used to conduct experiments (De, Malpani, et al., 2020).

2.2. Animals

Twenty-four C57BL/6 male mice of 6 weeks of age were collected from the animal house of ICMR – National Institute of Cholera & Enteric Diseases, Kolkata, India with a 12-12 h light/dark cycle (Mitra et al., 2017). A temperature and relative humidity of 25 °C and 50% respectively were maintained throughout the experimental period. The animals were fed with a standard rodent diet (Pellizzon, 2016) detailed in Table 1 along with ad libitum access to water. All the experiments were performed in accordance with the institutional animal ethics committee (No. PRO/162/March 2019–March 2022), abiding by institutional guidelines.

2.3. Induction of DSS colitis

C57BL/6 mice were divided into four groups as described below, n = 6 in each cage.

- Control:** Rodent diet
- DSS:** Rodent diet along with 2% DSS.
- GO:** Rodent diet, along with 2% DSS and GO.
- IN:** Rodent diet, along with 2% DSS and IN.

Each experimental mouse was weighed, labelled and fed a standard diet (ICMR – NIN, India). Additionally, mice from GO and IN groups were fed with GO and IN (2.5 g/kg body wt.) as prebiotics respectively (Zhu et al., 2021). The animals in DSS, GO and IN groups were administered with 2% DSS orally via gavages (Wirtz et al., 2007). The DSS was administered for Four days followed by intervening gaps of three days and the cycle was repeated for 28 days.

2.4. Examination of physical parameters i.e. body weight (wt.), colon length, spleen wt. and disease activity index (DAI)

At the end of the experiment, the animals were starved for 6 h after which the final body wt. were measured. The animals were euthanized following the permitted laws of the institutional animal ethics committee. The colons were separated from the small intestine at the ileocecal junction, collected, and washed in PBS and lengths were measured individually (Li et al., 2012). The colons were then fragmented to conduct further experiments. Similarly, the spleens of test animals were also collected, washed and weighed to draw differences among various groups. Splenocytes and colonic fluids were collected for further analysis (Qiao et al., 2022).

DAI was prepared by adding below mentioned scores (Yeom et al., 2016). The scoring standards followed are:

- body wt. loss (0: no loss; 1: 1–5% loss; 2: 5–10% loss; 3: 10–15% loss; 4: over 15% loss)
- stool consistency (0: normal; 1: loose but shaped; 2: very loose; 3: diarrhoea).

2.5. Histological analysis of colonic tissue sections

To investigate the severity of intestinal inflammation in the different groups, small distal colon sections were first washed with phosphate buffer saline (PBS) and fixed with 10% formaldehyde solution and kept for 48 h. The tissues were dehydrated in alcohol and embedded in paraffin. Ultra-microtome was used to cut sections of 5–6 µm thickness and stained with eosin and haematoxylin dyes, which were observed under the microscope (Breynaert et al., 2013). Tissue sections were graded with respect to the below-mentioned parameters and the values

of each parameter were added together to form the final score (Okayasu et al., 1990).

(i) degree of inflammation along with damage to colonic architecture:

0: normal morphology; 1: lost goblet cells, 2: lost goblet cells in large areas; 3: lost crypts; 4: lost crypts in large areas.

(ii) infiltration of leukocyte:

0: no infiltration, 1: infiltrating crypt basis, 2: infiltrating muscularis mucosa; 3: extensively infiltrating muscularis mucosa with thickened mucosa and obvious swelling; 4: infiltrating submucosa.

2.6. Examination of surface morphology by scanning electron microscope (SEM)

Colon tissues from the distal part of test animals were collected and washed with PBS and immersed in a 4% glutaraldehyde solution. After sufficient fixation, the colon tissues were cut longitudinally to expose the lumen. The tissues were subjected to dehydration by immersing in a series of alcohol (30%, 50%, 70% and 100%), dried and mounted to carbon tapes. The dried tissues were coated with gold and observed under SEM (SEM44 Zeiss SIGMA FE-SEMs, CARL ZEISS, Germany) (Mitra et al., 2017; Paulsen et al., 2005).

2.7. Analysis of splenic population of CD4⁺ and CD8⁺ T-lymphocyte by flow cytometry

Immediately after sacrificing the mice, the spleens were collected in PBS. The spleens were mashed with a sterile pestle and were strained with a cell strainer (BD Falcon™, NJ, USA) to remove any tissue debris. The splenocytes collected were mixed with ficoll plaque plus (GE Healthcare, Illinois, USA) in a graduated tube in a 3:1 ratio, and centrifuged at 2500 g for 30 min keeping acceleration and deceleration as 1 (Thermo Fischer). Splenocytes were collected and re-suspended in FACS buffer and stained with PE-conjugated anti-mouse CD 8⁺ T Cells and FITC-conjugated mouse anti-CD4⁺ T Cells (BD Bio Science, San Diego, USA). The population of cells were analysed using FACS (BD Aria II) (Chen et al., 2019).

2.8. Quantification of pro and anti-inflammatory cytokines in colonic tissue by Enzyme Linked Immuno Sorbent Assay (ELISA)

To conduct cytokine analysis, colonic sections of animals were washed and homogenized in PBS. Homogenized samples were centrifuged at 6000 rpm (Eppendorf, 5417 R, Hamburg, Germany) for 10 min to obtain the supernatant devoid of any tissue debris²⁹. The concentration of protein for each sample was estimated using BCA Protein Assay Kit (Thermo Scientific™ Pierce™, USA)³⁰. ELISA was conducted for TNF- α , IL-10, IL-17, IL-1 β , IL-12 and IFN- γ (BD Bio Science, San Diego, USA) as per the manufacturer's instruction taking 50 μ g of proteins for each sample (Chen et al., 2019).

2.9. Analysis of gene expression of *Tnf- α* , *Ifn- γ* and *Inos* by Real-Time PCR

RNA was extracted by TRIZOL reagent (Invitrogen, Massachusetts, USA) and quantified using nanodrop (NanoDrop 8000 Spectrophotometer, Thermo Fisher Scientific, USA). 5 μ g RNA was taken for reverse transcription with specific primers. dNTPs, MuLV reverse transcriptase, and other reagents were added according to the kit manual (Ambion INC, Austin, USA) to produce cDNA for *Tnf- α* , *Ifn- γ* , *Inos*. cDNA of *Gapdh* was also prepared and taken as loading control (Chen et al., 2019) qPCR was conducted using SYBR green mix (Qiagen, Maryland, USA) as per

Table 2

Composition of the nutritional medium used in SHIME for the cultivation of stool sample collected from the colitis patient.

Composition	Amount (g l ⁻¹)
Arabinogalactan	1
Pectin	2
Xylan	1
Starch	4.2
Glucose ^a	0.4
Yeast extract	3
Peptone	1
Mucin	4
Cysteine	0.5

^a The amount of glucose was completely replaced by GO during the prebiotic phase of SHIME run.

the provided protocol.

The detailed sequence of forward and reverse primers used was listed below.

Gapdh: 5'ACCCAGAAGACTGTGGATGG3'
3'CACATTGGGGTAGGAACAC5'
Tnf- α : 5'CATCTTCTCAAAATTCGAGTGACAA3'
3'TGGGAGTAGACAAGGTACAACCC5'
Ifn- γ : 5'TCAAGTGGCATAGATGTGGAAGAA3'
3'TGGCTCTGCAGGATTTTCATG5'
Inos: 5'CAGCTGGGCTGTACAAACCT3'
3'CATTGGAAGTGAAGCGTTTCG5'

2.10. Culturing of gut microflora using Simulator of Human intestinal Microbial Ecosystem (SHIME)

SHIME is an *in vitro* gut model mimicking the human gastrointestinal tract and physiological conditions. The nutritional medium used in SHIME is detailed in Table 2. The start-up and adaptation period was carried out for 2 weeks and the bacteria used for inoculation was from a faecal sample of a colitis male patient which was collected from ID & BG hospital as a specimen abiding Institutional Ethics Committee of ICMR NICED, Kolkata vide Ref no.: (A-1/2015-IEC). The test period was conducted for 2 weeks, during this time samples were collected from the 3rd, 4th and 5th compartments of SHIME (Mitra et al. 2017). Collected samples were used for microbial profiling. A similar SHIME run was done during the prebiotic phase with the same stool sample where sucrose was substituted with GO.

2.11. Analysis of Gut microbiota

DNA was extracted with Qiagen's power fecal pro kit using Qiacube from the fractions collected from the SHIME run during Colitis and treatment phase. The DNA yield was checked using Qiaexpert. The DNA yield for colitis and GO run was found to be 153.01 ng/ μ l and 174.76 ng/ μ l respectively. The samples were processed on Illumina Novaseq 6000 which gave a paired-end read of 150 $\times$ 2 bp and the samples were processed by downstream analysis. The de novo assembly of the adapter trimmed fastq files is carried out using MetaSPAdes (v 3.10.1). The contigs obtained from the assembly were used as input to MetaGeneAnnotator (MGA) for the prediction of open reading frames (ORFs). The predicted ORFs are searched against the non-redundant (NCBI-nr) database using DIAMOND (v0.7.9.58). The alignment file along with the filtered ORFs was used as input to the functional annotation using MEGAN6 (MetaGenome Analyzer) software. Further Fastq quality checks and base quality score distribution were carried out. Denovo metagenome assembly was carried out for the sample by assembling contigs from the reads using MetaSPAdes program. Further contigs were linked by the assembly algorithm to create scaffolds. Assembly was performed with default Kmer sizes 21, 33, and 55 using the de-bruijn

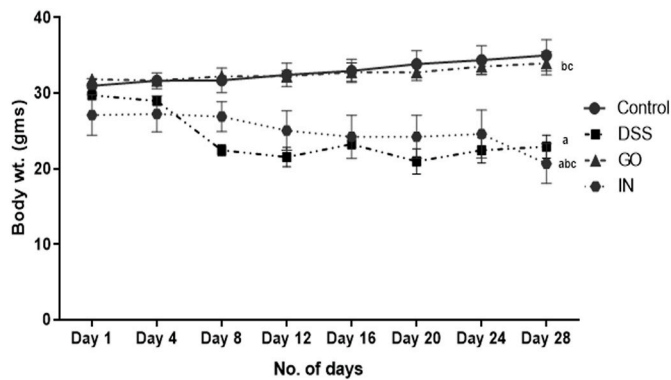


Fig. 1. GO administration halts DSS-induced reduction in body wt. of C57BL/6 mice. The variation in the body wt. of C57BL/6 mice during the development and experimental period of the colitis model. Results are expressed as mean $\pm$ standard error means ($n = 6$). Obtained values were analysed using one-way ANOVA followed by Tukey's multiple comparison test. $p \leq 0.05$ were considered statistically significant. ^a $p \leq 0.05$ compared to Control, ^b $p \leq 0.05$ compared to DSS, ^c $p \leq 0.05$ compared to GO. ANOVA, Analysis of variance; DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin; Wt., Weight.

graph method. PERL and Python code was used to parse the fastq files for the downstream analysis. As a result of de novo assembly, contigs in FASTA format were generated for all samples. The contigs obtained from the assembly are used as input to MetaGeneAnnotator (MGA) for the prediction of open reading frames (ORFs). The gene-finding program MGA was used to identify the coding regions and distinguish them from noncoding DNA. The ORF obtained from the samples was queried to DIAMOND BLASTX program with an optimum e-value of $1e-5$. The gene functions of all the ORF from DIAMOND BLASTX output were parsed using an in-house PERL script. The rarefaction curve was generated by comparing the species abundance between the samples based on the

number of leaves in the taxonomy and the number of sequences that occurred. The curve was made for all taxa including Bacteria, Archaea, Eukaryote, unclassified and other sequences (Sankarabramanian et al., 2020).

2.12. Statistical analysis

Experiments were conducted in triplicate and data are expressed as mean $\pm$ standard error of the mean (SEM), one-way analysis of variance (ANOVA) was conducted followed by Tukey's multiple comparison tests using Graph Pad Prism 6 software (Graph Pad Software Inc., San Diego, CA, USA) and difference with $P < 0.05$ was considered significant (Mitra et al., 2017).

3. Results

3.1. Examination of physical parameters i.e. body wt., colon length, spleen wt. and DAI

The body wt. of the test animals was measured for 28 days and represented in Fig. 1, the scores of stool consistency were represented in Supplementary Table 1. After the fourth day of DSS administration, a significant ($P \leq 0.0001$) fall in the body wt. by 6.38% was observed in DSS group (DAI: 2) compared to the control group, whereas no notable changes in the body wt. were observed in GO (DAI: 0) and IN (DAI: 0) groups in the first week. Further, there was a gradual fall in body wt. by 25.49% (DAI: 6) and 12.65% (DAI: 4) from day 8 which extended until the end of the experiment to 34.71% (DAI: 7) and 24.31% (DAI: 4) for DSS and IN groups respectively when compared to control group. A slight increase in the body wt. of GO group by 6.76% (DAI: 1) was observed at the end of the 28 days compared to the DSS group. The changes in colon length of experimental animals of test groups are represented in Fig. 2 (a) and pictures of the colon are represented in Fig. 2 (c) Control, (d) DSS, (e) GO, and (f) IN respectively. In the control

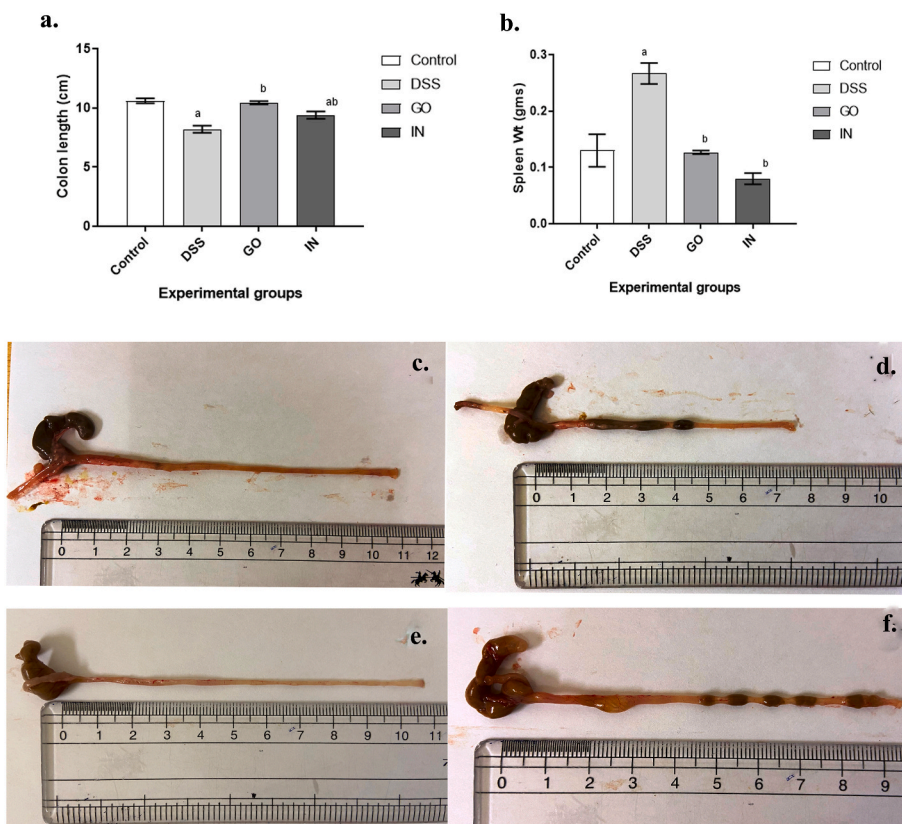


Fig. 2. GO inhibits DSS-induced shortening of colon length and splenomegaly in mice. The colon length and spleen wt. of C57BL/6 mice of different experimental groups (a) colon length (cm) (Control > DSS < IN < GO), (b) spleen wt. (gm) (DSS > Control > GO > IN). (c) picture of the colon from the control group, (d) picture of the colon from the DSS group, (e) picture of the colon from the GO group (f) picture of colon collected from the IN group. Results are expressed as mean $\pm$ standard error means ($n = 6$). Obtained values were analysed using one-way ANOVA followed by Tukey's multiple comparison test. $p \leq 0.05$ were considered statistically significant. ^a $p \leq 0.05$ compared to Control, ^b $p \leq 0.05$ compared to DSS, ^c $p \leq 0.05$ compared to GO. ANOVA, Analysis of variance; DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin; Wt., Weight.

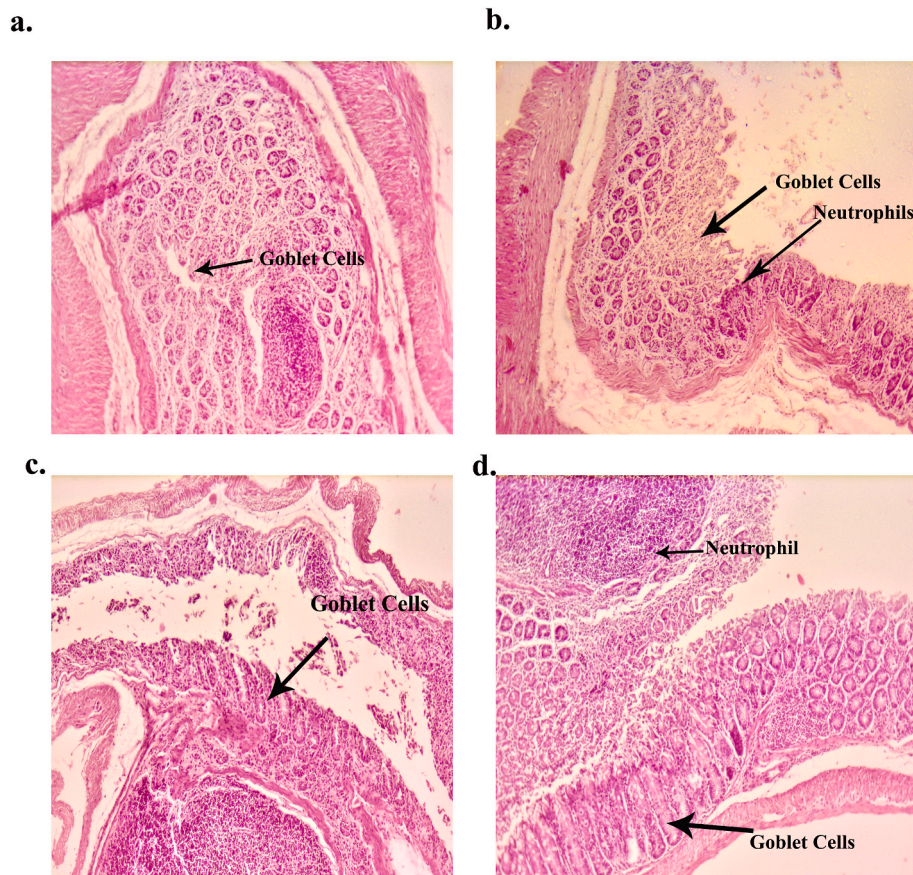


Fig. 3. GO reduces DSS-induced colonic damage at the cellular level of C57BL/6 mice. Haematoxylin and eosin stained cross-sections of colons at $20\times$ magnification (a) Control, (b) DSS, (c) GO, (d) IN. (Damage score: DSS $-7.75 \pm 0.5 > \text{IN} - 6.4 \pm 0.5 > \text{GO} - 6 \pm 0.81$). DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin.

group, the colon length was found to be 10.60 ± 0.36 cm which got significantly ($P \leq 0.0005$) reduced by 22.64% in DSS when compared to the control, the colon length of animals in the GO & IN group was found to be significantly ($P \leq 0.05$) larger by 21.22% (10.23 ± 0.29 cm) and 11.82% (09.33 ± 0.49 cm) respectively with respect to DSS group. The changes in the spleen weight have been represented in Fig. 2(b), the spleen wt. in the control group was found to be 0.13 ± 0.05 gms, which got significantly increased ($P \leq 0.005$) to 0.21 ± 0.03 gms in the DSS with respect to control group, whereas the spleen weight of GO and IN groups were found to be 0.13 ± 0.01 gms ($P \leq 0.0005$) and 0.08 ± 0.05 gms ($P \leq 0.0005$) respectively.

3.2. Histological analysis of colonic tissue sections

The change in cellular morphology of the colon to investigate colon injury and inflammation was done by histopathological studies and are detailed in Fig. 3 (a) Control, (b) DSS, (c) GO and (d) IN respectively. The animals from the control group had intact mucosa, tubular crypts of Lieberkuhn and goblet cells. On the contrary, severe mucosal dysplasia along with loss of integrity of crypts followed by bursting of cells and visible cell flaccidity near sub-mucosa layer in the DSS group was observed with a significant ($P \leq 0.0001$) damage score of 7.75 ± 0.5 to that of the control group. Alteration in sub-mucosa layers was also observed in the DSS group. In the GO group, the extent of dysplasia was significantly ($P \leq 0.001$) less by 22.58% with a damage score of 6 ± 0.81 compared to DSS. The crypts did not lose their orientation but a slight loss of the granular pattern of intestinal crypts was observed. Thus in IN group, we can see that the alteration in colonic structure was less, the mucosa and sub-mucosa layer were almost intact but the cells in this layer became flaccid and neutrophil infiltration in the mucosa and

submucosa layer was observed. The damage score in IN group was found to be 6.4 ± 0.5 which is significantly ($P \leq 0.0001$) less by 17.41% compared to DSS and 6.25% ($P \leq 0.01$) more than GO respectively.

3.3. Examination of surface morphology by SEM

Fig. 4 (a) depicts the colon surface of the control group which had intact intestinal crypts and goblet cells. The changes in the surface morphology when the mice groups were administered DSS and treated with prebiotics were visualized by SEM. In the DSS group, Fig. 4 (b) there was a complete loss of colonic structure and architecture with a massive loss of goblet cells, contrary to the control group. In GO, Fig. 4 (c) though we can visualize mucosal dysplasia, swollen crypts and the delineated boundary between the lesion area and the normal mucosa, the loss of goblet cells along with flat lesions was less when compared with that of the DSS group. Formation of the elevated lesion was also less in both GO and IN groups compared to DSS but the presence of flat lesions and swelling of goblet cells along with epithelial abnormalities was seen in IN group.

3.4. Analysis of splenic population of CD4^+ and CD8^+ T-lymphocyte by flow cytometry

From the flow cytometry data, we can observe that the population of CD8^+ and CD4^+ T cell in the control group was $8.9 \pm 0.13\%$ and $12.0 \pm 0.08\%$ respectively represented in Fig. 5 (a, e) which significantly ($P \leq 0.05$) increased to $30.03 \pm 0.23\%$ and $15.46 \pm 0.12\%$ respectively in DSS group as depicted in Fig. 5 (b, e). Further in GO and IN groups, it is seen that the population of CD8^+ T cells was significantly ($P \leq 0.05$) reduced to $10.28 \pm 0.09\%$ and $15.25 \pm 0.25\%$ respectively and a

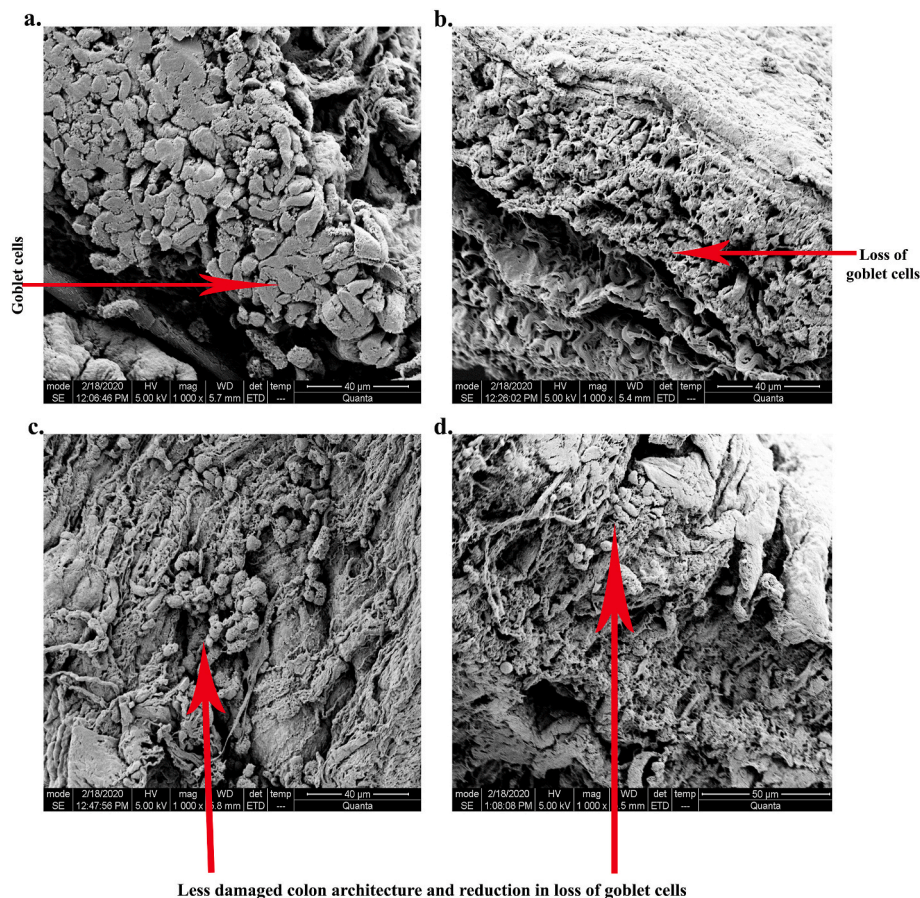


Fig. 4. Scanning electron microscopic images of colons of C57BL/6 mice depicting the changes in the surface morphology. GO reduces the loss of intestinal crypts and improves colonic integrity in C57BL/6 mice. (a) Control, (b) DSS, (c) GO, (d) IN. DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin.

significant ($P \leq 0.05$) increase in the number of $CD4^+$ T cells to $20.39 \pm 0.07\%$ and $25.53 \pm 0.14\%$ was seen with respect to DSS which is represented in Fig. 5 (c, e) and Fig. 5 (d, e) for GO and IN respectively.

3.5. Quantification of pro and anti-inflammatory cytokines in colonic tissue by ELISA

Cytokines were estimated and represented in Fig. 6. The levels of $TNF-\alpha$, $IFN-\gamma$, IL-12, IL-17, and IL-1 β in the colonic fluids of DSS significantly increased ($P \leq 0.005$) by 68.08%, 83.01%, 32.53%, 54.03% and 45.40% respectively with respect to that of the control group. Whereas the levels of the $TNF-\alpha$, $IFN-\gamma$, IL-1 β and IL-12 in the GO group got significantly reduced ($P \leq 0.005$) by 52.38%, 89.14%, 34.04% and 32.13% respectively with respect to DSS group. The levels of $TNF-\alpha$, $IFN-\gamma$, and IL-1 β in IN were found to be significantly ($P \leq 0.005$) reduced by 59.14%, 74.85% and 45.29% respectively to that of the DSS group. Minute reduction in the level of IL-17 was observed in GO and IN groups and no notable change in the level of IL-12 was seen in IN group. The level of IL-10 in the colonic fluids of the control group was found to be 27.50 ± 2.09 pg/mg and in the DSS group, the level was found to be 30.09 ± 9.17 pg/mg which got significantly ($P \leq 0.01$) increased by 64.70% in GO with respect to DSS group. However, the IL-10 level in the IN group was further significantly higher by 62.19% ($P \leq 0.0001$) than that of the GO group.

3.6. Analysis of gene expression of $Tnf-\alpha$, $Ifn-\gamma$ and $Inos$ by Real-Time PCR

Levels of $Tnf-\alpha$, $Ifn-\gamma$ and $Inos$ expression in the colonic tissues were represented in Fig. 7. The expression of $Tnf-\alpha$ and $Ifn-\gamma$ in the control

group were 8.11 ± 0.03 and 1.40 ± 0.38 respectively which got significantly elevated to 275.14 ± 8.09 ($P \leq 0.0005$) and 15.58 ± 2.55 ($P \leq 0.0004$) respectively in DSS group compared to control group. However a sharp fall in the expression of $Tnf-\alpha$ levels by 92.13% ($P \leq 0.0005$) and 81.46% ($P \leq 0.0005$) was seen in the GO and IN groups respectively when compared to the DSS group. The expression levels of $Ifn-\gamma$ in GO and IN groups were 11.16 ± 0.84 and 14.36 ± 2.34 which is less than that of the DSS group but significantly higher by 87.40% ($P \leq 0.004$) and 90.25% ($P \leq 0.0004$) respectively with respect to control group. The expression levels of $Inos$ in the control group was found to be 1.13 ± 0.70 which significantly increased by 93.10% ($P \leq 0.0001$) in the DSS i.e., 15.58 ± 2.55 when compared to the control group. In GO & IN groups the $Inos$ levels were significantly reduced by 53.56% ($P \leq 0.0001$) and 89.74% ($P \leq 0.0001$) with respect to the DSS group.

3.7. Analysis of Gut microbiota

The Next generation sequencing data in Fig. 8, revealed that *Prevotella* sp. was predominant at the genus levels accounting for 76.28% of gut flora of the colitis patient which got reduced to 1.85% after incorporating GO in SHIME feed for 14 days. Commensals like *Lactobacillus* sp., and *Enterococcus* sp. initially possessed 9.61% and 1.76% respectively which got increased to 11.53% and 23.07% respectively representing the maximum fraction of gut flora after GO administration. However, the percentage of *Bacillus* sp. was reduced from 3.68% to 2.56% after the addition of GO. The percentage of *Klebsiella* sp. in the gut flora of the colitis patient's stool sample was found to be 3.68% which accounted for 5.12% after the addition of GO in the SHIME feed. There was no change in the composition of *Enterobacter* sp. in the colitis patient's stool sample after the addition of GO. The microflora diversified

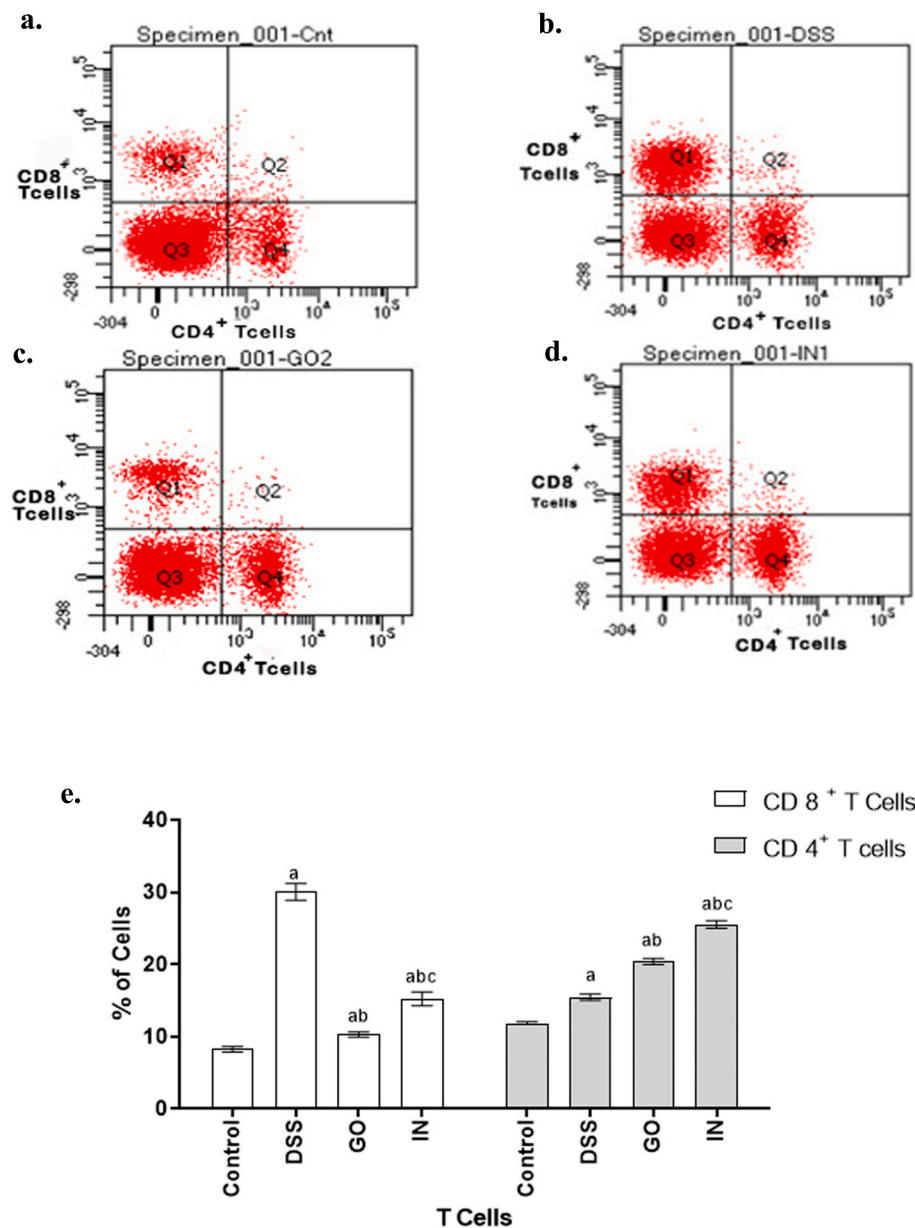


Fig. 5. GO reduces the population of CD8⁺ T cells and increases CD4⁺ T cells. Population of CD8⁺ T cells and CD4⁺ T cells in the spleen of C57BL/6 mice (a) Control, (b) DSS, (c) GO, (d) IN (e) graphical representation of CD8⁺ and CD4⁺ T cells. (CD8⁺ T cells - DSS > IN > GO > Control and CD4⁺ T cells - IN > GO > DSS > Control). Results are expressed as mean $\pm$ standard error means (n = 6). Obtained values were analysed using one-way ANOVA followed by Tuckey's multiple comparison test. ^a $p \leq 0.05$ compared to Control, ^b $p \leq 0.05$ compared to DSS, ^c $p \leq 0.05$ compared to GO. ANOVA, Analysis of variance; DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin.

with *Burkholderia* sp., *Achromobacter* sp., *Stenophomonas* sp., *Aeromonas* sp. along with *Delftia* sp., *Elizabethkingia* sp., *Merozyma* sp. and *Methylobacterium* sp. in the GO treated stool sample in SHIME which was previously absent in the stool of colitis patient.

4. Discussion

The present study illustrates the application of GO in the prevention of experimental colitis induced by DSS in C57BL/6 mice. 2% DSS was used in the study which is less than most previously reported studies, where the concentration used for developing colitis was 3%–5% (Naito et al., 2006; Winkler et al., 2007). DSS was administered directly via oral gavage and not mixed in drinking water like most of the studies minimising the need of constant monitoring of mice and reducing the wastage of DSS solution. Intervening gaps of three days after four days of DSS administration allowed us to run the experiments for 28 days mimicking a chronic colitis situation. Moreover, an increase in microbial diversity was also observed when sucrose was replaced by GO during the processing of the stool sample of the colitis patient in SHIME. The results

obtained from the preclinical study and human gut simulator also fabricate a platform for future clinical trials of GO on chronic non-communicable diseases.

The visible parameters of DSS-induced colitis in the animal model like reduction in body weight, shortening of colon length and increase in spleen wt. i.e. splenomegaly (Miles et al., 2017) were reversed upon prophylactic administration of GO and IN till the 7th day. From the 7th day, the body weight and colon length of animals from the GO group did not decrease and GO exhibited better results than IN. Histological changes observed in the DSS group were mainly due to damage in goblet cells, depletion of mucin (Laroui et al., 2012) and widening of the gap at the base of the crypt leading to epithelial erosion and formation of ulcer (Okayasu et al., 1990). Ulceration in the mucosa layer was accompanied by erythema and loss of granularity depicting the presence of active inflammation (Cader & Kaser, 2013). Splenomegaly was not seen in both of the prebiotic groups depicting their anti-inflammatory properties. GO is pseudoplastic (De, Malpani, et al., 2020) and fibrous in nature having the ability to absorb dietary precarious substances within the gut and limit the progression of gut damage by bulking feces (Mitra et al., 2016).

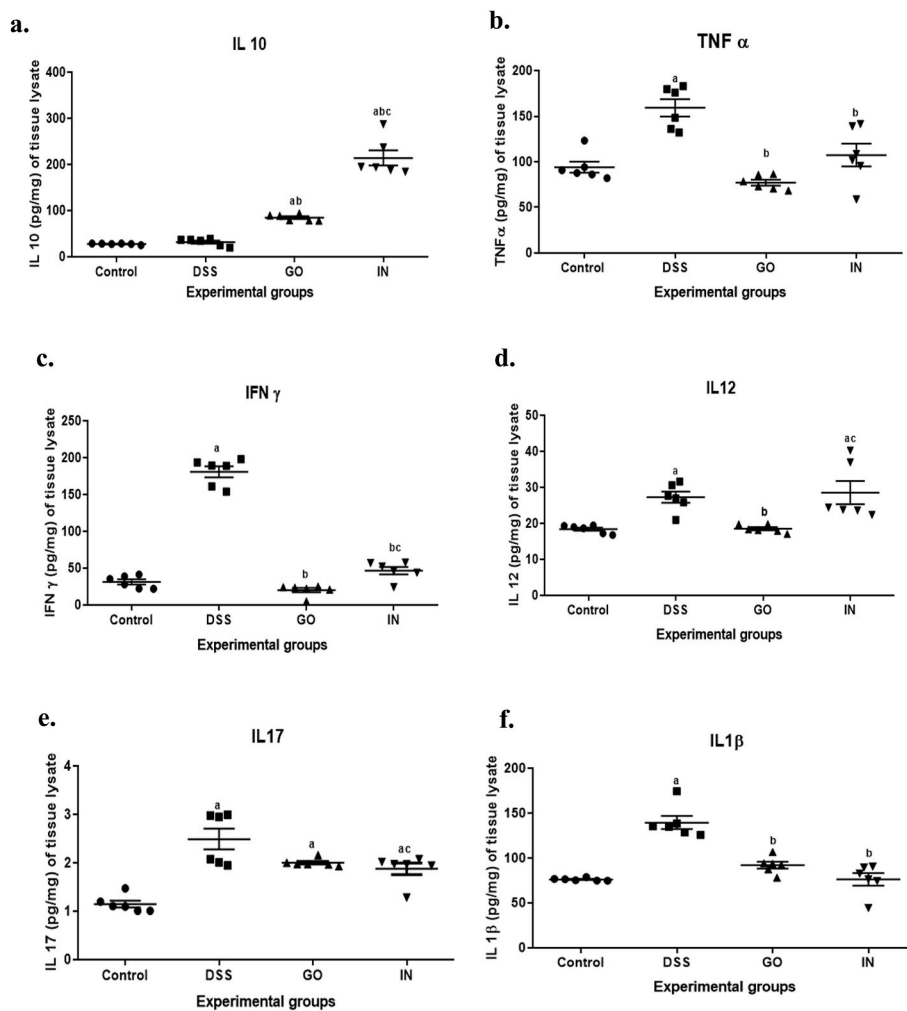


Fig. 6. Detection of proinflammatory and anti-inflammatory cytokine levels by ELISA in the colonic fluids of C57BL/6 mice of different experimental groups i.e. Control, DSS, GO, IN. GO reduced proinflammatory cytokines and increased IL-10 levels. (a) IL-10 (Control $\leq$ DSS $<$ GO $<$ IN), (b) TNF- α (Control $<$ DSS $>$ IN $>$ GO), (c) IFN- γ (Control $<$ DSS $>$ IN $>$ GO), (d) IL-12 (Control $<$ DSS $>$ IN $>$ GO), (e) IL-17 (Control $<$ DSS $>$ GO $\geq$ IN) and (f) IL-1 β (Control $<$ DSS $>$ GO $>$ IN). Results are expressed as mean $\pm$ standard error means ($n = 6$). Obtained values were analysed using one-way ANOVA followed by Tuckey's multiple comparison test. $p \leq 0.05$ were considered statistically significant. ^a $p \leq 0.05$ compared to Control, ^b $p \leq 0.05$ compared to DSS, ^c $p \leq 0.05$ compared to GO. ANOVA, Analysis of variance; DSS, Dextran sodium sulfate; GO, Gum Odina; IN, Inulin.

The dysplasia was confined to the mucosa and submucosa layers in the GO group, which extended to the muscularis externa in the DSS group. The damage in the colonic cells was also less in IN group however the cells remained swollen accompanied by leukocyte infiltration. SEM studies enlighten the loss of granular pattern of intestinal crypts (Naito et al., 2006) which were also less in both GO and IN groups whereas the DSS group had extensive damage in the surface of the colonic structure along with loss of integrity. Considering these attributes, GO had a better edge over IN to limit disease progression. GO contains serine in trace amounts possessing wound healing activity (De, Malpani, et al., 2020) and upon fermentation in the gut, it liberates short-chain fatty acids (SCFAs) mainly butyric acid and lactic acid followed by acetic acid and propionic acid (Mitra et al., 2017) which enhances the epithelial barrier by regulating tight junctional integrity (Andoh et al., 1999; Naito et al., 2006). Butyric acid is considered the major energy source for colonocytes and helps the colonic cells to regenerate their structure thus attenuating damage associated with colitis which is observed in our study (Venkatraman et al., 2000). Being a soluble dietary fiber, IN also liberates adequate SCFAs but its spherical and smooth surface (Qiao et al., 2022) makes it inefficient in absorbing toxins generated from food digestion and feces bulking.

Innate immunity plays a major role in the progression of colitis as it is a heterogeneous state of intestinal inflammation (Kalužna et al., 2022). Cytotoxic CD8⁺ T cells play an early role in the development of gut ulcers and lesions (Boschetti et al., 2016) and have the ability to deviate the regulation of CD4⁺ T cells (Chen et al., 2019). The imbalance between subpopulations of CD4⁺ T cells is the leading cause of abrupt

inflammatory responses (Hegazy et al., 2017) in colitis resulting in the increase in spleen weight in the DSS group due to the accumulation of splenocytes resulting from active inflammation. The cytokine levels of IL-12 were higher in the DSS group than in the control group. Increased levels of IL-12 deviated the differentiation of CD4⁺ T cells into Th1 cells (Vacaflores et al., 2016) as a result the levels of proinflammatory cytokines like TNF- α , IFN- γ , IL-1 β and IL-17 also increased in the DSS group. The increase in the population of CD4⁺ T cells in the DSS group might be due to colonic damage and associated inflammation. Though the population of CD8⁺ T cells subsided in GO and IN groups exhibiting a reduction in gut inflammation, the population of CD4⁺ T cells further increased by 24.17% and 39.44% respectively with that of the DSS group. As there was a reduction in the cytokine levels of TNF- α , IFN- γ , IL-1 β and an increase in IL-10 levels in both GO and IN groups it can be interpreted that prebiotics turned the CD4⁺ T cells differentiation into Th2 cells promoting anti-inflammatory functions. However, a detailed study was necessary to fully understand the fact. There was no drastic reduction in IL-17 levels with respect to the DSS group which could be also linked to neutrophilic migration to the inflammation site as seen in the histopathology analysis. The mRNA expression of *Tnf- α* , *Ifn- γ* became higher in the DSS group but suppressed in GO and IN groups and can be directly related to changes in cytokine levels of TNF- α , IFN- γ which also decreased in the respective group. Colonic damage incurred by DSS-induced colitis also disrupts the balance between oxidant and antioxidant status resulting in oxidative (Osman et al., 2006) stress causing an increase in mRNA expression of *Inos*. GO possesses antioxidant properties due to the presence of uronic acids and hydroxyl groups

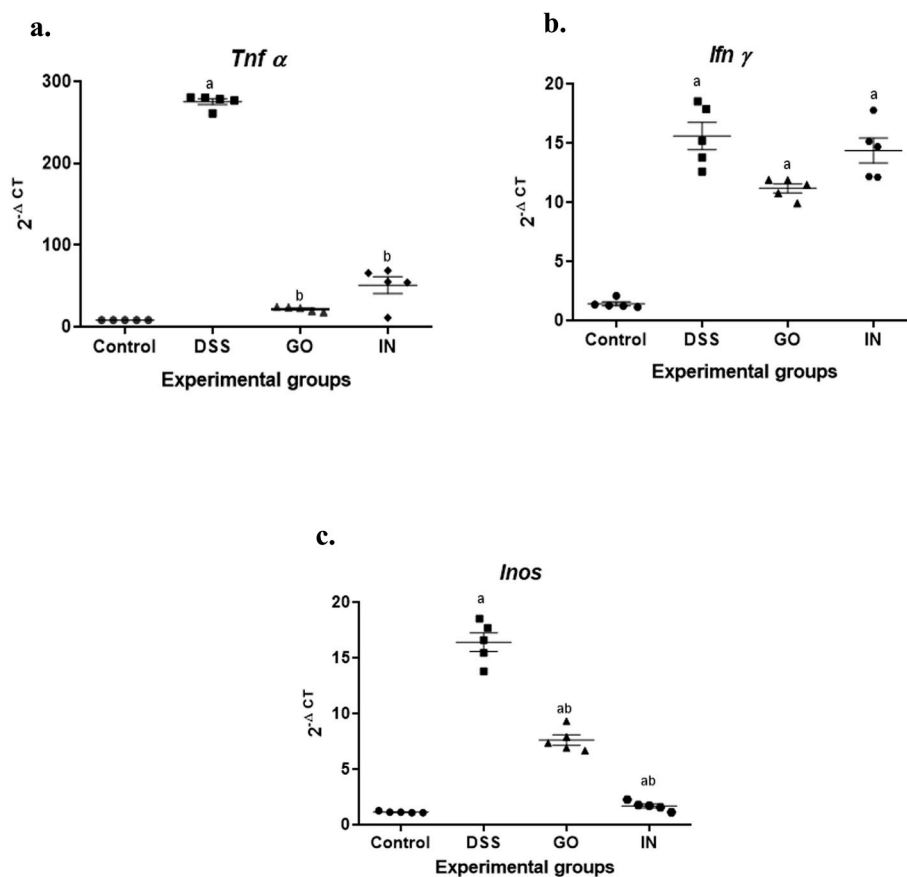


Fig. 7. GO reduces expression of *Tnf-α*, *Ifn-γ* and *Inos*. mRNA expression of (a) *Tnf-α* (Control<DSS>IN > GO) (b) *Ifn-γ* (Control<DSS>GO > IN) and (c) *Inos* (Control<DSS>GO > IN) by Real-Time PCR in colonic tissue of C57BL/6 mice of different experimental groups i.e. Control, DSS, GO, IN. Results are expressed as mean $\pm$ standard error means ($n = 6$). Obtained values were analysed using one-way ANOVA followed by Tuckey's multiple comparison test. $p \leq 0.05$ values were considered statistically significant. ^a $p \leq 0.05$ compared to Control, ^b $p \leq 0.05$ compared to DSS, ^c $p \leq 0.05$ compared to GO.

(De, Malpani, et al., 2020) in its structure, which reduces ROS and LPO levels and increases the production of antioxidants like GSH, GST, SOD, CAT (Mitra et al., 2017) in the gut resulting in low *Inos* expression (Fig. 7 c) diluting the extent of oxidative damage caused by DSS. The mRNA expression of *Inos* in the IN group was further low by 36.18% when compared to the GO group. The IL-10 levels and mRNA expression of *Inos* exhibited that IN had slightly better anti-inflammatory activity than GO. Though IN is an extensively used prebiotic and has been reported to have health benefits in several studies (Changchien et al., 2021; Pool-Zobel et al., 2002) it is mainly composed of fructose and glucose, which can be easily utilized by both commensal and pathogenic bacteria in the gut environment. Thus, GO becomes a better choice of prebiotic as a prophylactic treatment of colitis or related diseased state combining feces bulking, anti-inflammatory, antioxidant and gut-modulating factors (Mitra et al., 2017). GO on the other hand has less processing cost than IN and does not impart any taste (De, Malpani, et al., 2020).

Additionally, we also investigated the correlation between the gut microflora from feces of colitis patient before and after the incorporation of GO into the diet using SHIME. The gut microflora of colitis patient had low microbial diversity and was mostly dominated by *Prevotella* sp. followed by *Lactobacillus* sp., *Bacillus* sp. and pathogenic bacteria like *Enterococcus* sp. *Enterobacter* sp. and *Klebsiella* sp. The gut microbiome analysis revealed an increase in the diversity of bacterial species and a reduction in the percentage of pathogenic bacteria upon the addition of GO prebiotics in the experimental phase of the gut simulator. The change in the gut flora can be mainly due to SCFAs liberated from GO further limiting the growth of disease-causing pathogenic bacteria by acting as a selective antimicrobial agent enhancing the growth of commensal bacteria in the gut (Parada Venegas et al., 2019).

5. Conclusion

The study revealed that repeated cycles of DSS administration induce shortening of colon length and splenomegaly in C57BL/6 mice, disrupt the cellular integrity, increases the expression of pro-inflammatory cytokines and *Inos* in colon, which is prevented when prebiotic like GO is introduced in the diet of mice. GO also increases gut microbial diversity. Thus, the anti-inflammatory effects of GO to combat colitis were elucidated, allowing the availability of new prebiotic i.e. arabinogalactan and extending the choice of bioactive compounds in food and pharmaceutical industries which are presently dominated by fructooligosaccharides and galactooligosaccharides.

Authors contribution

Debmalya Mitra: Conceptualization, Methodology, Investigation, Writing-Original Draft, Funding acquisition, Formal Analysis.: **Sohini Sikdar:** Methodology, Investigation, Writing-Original Draft, Investigation, Writing-Review and Editing, Formal Analysis.: **Mainak Chakraborty:** Investigation, Formal Analysis, Writing-Review and Editing.: **Oishika Das:** Investigation.: **Amalesh Samanta:** Investigation, Writing-Review and Editing, Resources.: **Shanta Dutta:** Conceptualization, Resources, Methodology, Writing-Review and Editing, Supervision, Project administration.

Declaration of competing interest

The authors confirm that they have no conflict of interest to the work described in this manuscript.

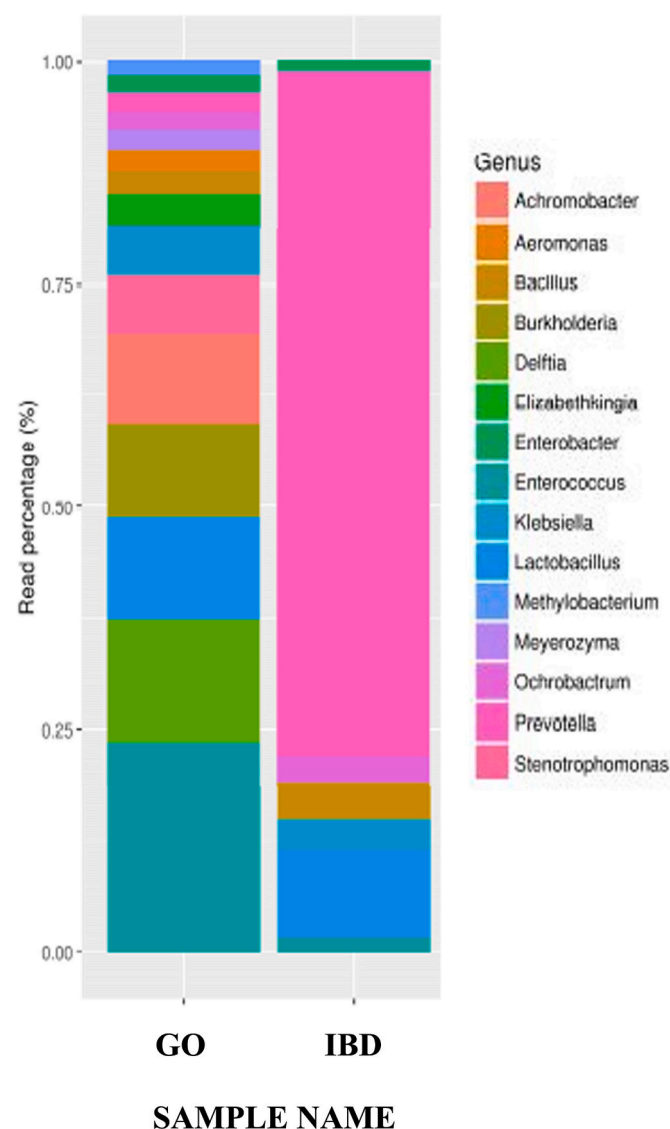


Fig. 8. Comparison of microbial communities at genus levels before and after the addition of GO in SHIME. GO increased microbial diversity in the stool sample of colitis patient. GO, Gum Odina; SHIME, Simulator of the human intestinal microbial ecosystem.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2023.102509>.

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Butyrate ameliorates inflammation in colon biopsy samples of IBD patients and experimental colitis in mice involving RNA binding protein, AUF1-IL-27 axis and accelerating B1a to B10 polarization

Aaheli Masid¹, Oishika Das¹, Diganta Roy^{1#}, Ankita Dutta^{1#}, Sohini Sikdar^{1#}, Atanu Ghosh², Arpan Banerjee², Ujjal Ghosh², Sutanu Acharya Chowdhury³, Sankhasubhro Majumder⁴, Mohammad Yahya⁵, Surajit Sinha², Moumita Bhaumik^{1*}

Affiliation:

¹ICMR-National Institute of Cholera and Enteric Diseases, Belegghata, Kolkata 700010, India

² Indian Associations for Cultivation of Science, Jadavpur, Kolkata, 700032, India

³Department of Medical Biotechnology, Ramakrishna Mission Vivekananda Educational and Research Institute, Kolkata, India.

⁴Department of Biochemistry and Biophysics, University of Kalyani, India.

⁵ Calcutta gastro & endoscopy clinic, Park Circus, Kolkata, 700017, India

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***Correspondence:**

Moumita Bhaumik, ICMR-National Institute of Cholera and Enteric Diseases, P-33 C.I. T Road, Belegghata, Kolkata 700010, West Bengal, India.

Email: drmoumitabhaumik@gmail.com

Telephone: 033-2363-2390.

Equal contribution

Disclosures

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Abstract

The pathophysiology of Inflammatory Bowel Disease (IBD) is significantly influenced by the decline in B regulatory (B10) cells, which produce IL-10. Therefore, it is important to identify the key genes and pathways that regulate the B10 cell generation in order to develop more effective therapies. Here, we have shown that one of the short chain fatty acid, butyrate regulates the expression of RNA binding protein, AUF1 which is responsible for increasing the half-life of p28 mRNA, coding for p28 protein which associates with overexpressed EBI3 and forms functional IL-27. This effect is mediated through AUF1 binding to 3'UTR of IL-27p28 mRNA. As a consequence, IL-27 signals splenic CD19⁺CD5⁺ (B1a) cells but not CD19⁺CD23⁺ (B2) cells to polarize to B10 cells. We proved the importance of AUF1 and the sequential downstream players in unique cell penetrating morpholino induced AUF1

knockdown (AUF1-KD) in mice, establishing the roster of events in splenic B1a cells: butyrate-AUF1-IL-27-IL-10. We showed that there was a significant decrease in AUF1, IL-27 and IL-10 expression in the colon biopsy of IBD patients compared to non-IBD control. We have used DSS induced colitis in mice as a surrogate of IBD in human and showed the reduction in AUF1 in spleen and colon could be correlated with the decrease in IL-27 and B10 cells in spleen and mesenteric lymph nodes which were reversed with butyrate treatment. We further established AUF1 as the role player by showing adoptive transfer of butyrate stimulated B1a cells from wild type mice conferring protection against colitis while adoptive transfer of butyrate stimulated B1a cells from AUF1 KD mice failed to suppress the disease. Finally, we propose that butyrate driven B1a cells as a glimmer of new hope of therapeutic possibility against colitis.

Keywords: butyrate, AUF1, IL-27, half-life, B1a cell, B10 cell

Introduction

Variety of pathobiological pathways epitomize complexities of colitis, but steady barrage of reports resonates that the altered immune homeostasis due to gut resident microbial dysbiosis posits short-chain fatty acids (SCFAs) like butyrate, propionate, and acetate to wrench local inflammatory response at bay (1). There are multiple narratives on human studies stating the beneficial role of butyrate in remission of variety of gut origin symptoms (2), (3). Albeit T-cells have created an endearing hinge, but the landscape of B-cells acceding attention in recent time to understand colitis pathogenesis. The murine model of colitis displays poorly regulated B-cells to cause exacerbated inflammation by blocking T_{reg} function (4, 5). During colitis, number of T_{reg} in gut associated lymphoid tissues in B-cell deficient mice reduced significantly which is restored upon B-cell transfer (6). Exploiting bone marrow chimera system, it was shown that in absence of IL-10 produced by B-cells (B10 cells), mice were unable to show cure from colitis (7). In human, B-cell depletion for unrelated diseases using anti-CD20 (rituximab) reported to either aggravate colitis or caused spontaneous colitis (8). The most studied and significant subpopulation of B regulatory cells that can produce IL-10 to inhibit the immune system are called B10 cells (9). B cells can be divided into several subclasses that differ in ontogeny, homeostasis, and functionality (10). The earliest B cells to form through development are B-1 cells mainly dwell in the peritoneal cavity or umbilical cord blood (11) and also found in the spleen (12). B-1a cells but not B-1b cells display cell-surface CD5 and functions by suppressing endogenous pathogens or microbiota (13), (14). B2 cells, which are characterized by presence of CD23 and the major constituent of B cells, are short-lived recirculating cells and collaborate with helper T cells to generate specific antibodies (15). Maintenance of mature B-cell homeostasis is reported to be dependent on AU-rich element RNA-binding factor 1 (AUF1). AUF1 encoded by *AUF1/hnrnpd* gene and have four isoforms AUF1^{p45}, AUF1^{p42}, AUF1^{p40} and AUF1^{p37} generated through

differential splicing (16). Based on the studies in AUF^{-/-} mice showing follicular-B cells reduced by two-fold whereas marginal zone-B-cells remained unaltered in as compared to wild type counterpart. Different B-cell subsets showed different levels of AUF-1 isoform expression. AUF^{-/-} mice showed unaltered cellular repertoire in lymph node, thymus, bone marrow coupled but two-fold reduction in splenocytes as compared to wild type mice. Interestingly, T- dependent antigen response is comparable whereas T-independent antigen response showed relatively compromised in AUF^{-/-} mice as compared to wild type counterpart (17).

AUF1 binds to AU-rich elements within the mRNA mostly at 3' untranslated region (3'UTR), leading to either stabilizing mRNAs such as encoding TWIST-1 (18), Jun-D (19), c-myc (20) or destabilising the mRNAs encoding such as TNF- α (21), GM-CSF (22), COX-1 (23), IL-2 (24). Overproduction of TNF- α , in AUF1 knockout mice accounts for higher susceptibility to septicemia (25). Previously we showed that AUF-1 is increased due to butyrate treatment (26) in mice liver and AUF-1 knock down using a cell penetrating morpholino in mice, inhibits butyrate mediated hypo-cholesterolemic effects in mice (26).

Butyrate activates RNA binding protein, AUF1 (26), is known to regulate cytokine mRNA stability (27), (28), (29). It is recently reported that B-1a cells (30) and Treg produce IL-27 (31). There is a report of IL-27 production from B1a (32) and IL-27 induces IL-10 production from Treg cells (33). The fact that IL-27 as a biologic to suppress auto immune processes (34) offers a valuable framework to address similar question in gut homeostasis. When IL-27 signalling is blocked during bacterial infection, enhanced pro-inflammatory response initiated through downregulation of IL-10 (35). Thus, one is left with a notion that AUF-1 and IL-27 may have a cross talk either directly or indirectly which may govern the colitis pathology. Interestingly there is a report that IL-27 can rescue the disease phenotype of acute severe colitis via IL-10 which is believed to be T-cell independent (34). IL-27 is encoded by two distinct genes, EBI3 and IL-27p28 and their heterodimer forms IL-27 and its receptor consists of heterodimer of IL-27R α (WSX-1) and gp130. IL-27p28 also called IL-27A or IL-30 can function independently without EBI3 (36).

We narrate here, the mechanism by which butyrate stabilizes IL-27 mRNA to polarize B-1a cells to B10 and suppresses DSS induced colitis in mice. We have shown that butyrate modulates AUF1 leading to increased half-life of IL-27p28 mRNA, resulting in the polarization of B-1a cells to B10. To provide further leverage in our understanding, we used morpholino to knock-down AUF1 in mice, and showed that butyrate indeed exploits AUF1 to accomplish its effect on B-1a cells. We have shown in IBD patients and DSS induced colitis in mice that AUF1 and IL-27 expression in colon tissue is significantly decreased. Remarkably, we found that transfer of butyrate stimulated B1a cells from wild type mice conferred protection against DSS induced colitis while adoptive transfer of butyrate stimulated B1a cells from AUF1 knock down mice could not suppress the disease. To our knowledge, we finessed here for the first time that butyrate orchestrates its effect through RNA binding protein AUF-1 to increase

IL-27p28 mRNA stability and resulting biologically active IL-27 which polarizes B1a cells to polarize into B10 cells as an effusive entity to control colitis.

Results

Butyrate but not propionate or acetate treatment in mouse splenocytes induced IL-10

We endeavoured to learn the ability of acetate, propionate and butyrate (SCFAs) to induce IL-10 production from mouse splenocytes. The splenocytes were exposed to 10 μ M and 100 μ M of each of the SCFAs in the culture condition and resulting IL-10 production was assayed by ELISA. Our results showed that there was hardly any induction of IL-10 in splenocytes in response to any one of the SCFAs at 24h (Fig 1A) but increased significantly with all three SCFAs at 10 μ M and 100 μ M at 48 h compared to control (Fig 1B). Interestingly at 72 h, treatment with 10 μ M acetate and propionate did not show enhancement in IL-10 production but at 100 μ M there was low but significant increase in IL-10 induction as compared to control (Fig 1B). Under identical scenario, butyrate treatment caused 1.3 and 1.8-fold higher at 10 and 100 μ M of butyrate respectively as compared to the corresponding doses of acetate and propionate (Fig 1C). Therefore, further experiments were conducted with 100 μ M at 72 h timepoint.

B1a-cell but not B2 cells as a source of IL-10:

We plan to navigate into the orbit of B-cell based on the fact that a unique phenotype of IL-10 producing B-cell posits as regulatory B-cells. To shed light on the process, two sub-lineages of B-cells: B1a cells as CD19⁺CD5⁺ and B2 cells as CD19⁺CD23⁺ (37) were considered. Splenocytes were cultured without and with 100 μ M butyrate for 72 h and B1a and B2 cells were gated and percentage of IL-10 producing cells was enumerated by intracellular cytokine assay. Our results showed that IL-10 producing B1a cells increased by 1.5-fold due to butyrate treatment whereas acetate but not propionate showed low but significant increase in frequencies (Fig 1D, E). Interestingly, B2 cells did not show any such increase in response to treatment with any one of the above SCFAs (Fig 1 F, G). Henceforth gated population of IL-10 producing B1a cells are defined as B1a cells for convenience. Gleaned from above studies accedes a quest to cultivate link between butyrate and B1a cells whether it is a direct effect or indirect.

rIL-27 increases the frequencies of IL-10 producing B-1a cells but not B2 cells

To address the link, if any, splenocytes were treated with 20 ng/ml rIL-27 as reported earlier (38), (39) and after 72h, frequencies of B1a cells but not B2 cells was enumerated. It was observed that IL-10 producing B1a cells but not B2 cells increased by 3-fold with respect to control (Fig S2 A, B). Based on this information we enquired if butyrate has any effect on IL-27 expression. Our results showed that there was hardly any change of EBI3 expression in splenocytes in response to 10 μ M and 100 μ M butyrate treatment but IL-27p28 expression showed dose dependent significant increase compared to control (Fig 2A). The increase in IL-27p28 were further verified by ELISA and western blot. The estimation in the increase of IL-27p28 protein with butyrate treatment by ELISA and western blot analysis gave similar results (Fig 2B, C, D).

With an objectivity to fit into the triptych of the following: butyrate, IL-27 and B1a cells, the following array of experiments were considered.

Both butyrate or IL-27 increased B10 cells in similar magnitude

To find the revelatory relation, if any, between butyrate and IL-27, the magnitude of B10 cells induction in splenocytes in response to either butyrate or rIL-27 treatment was evaluated as functional readout. It

was observed that butyrate treatment caused 2.4-fold increase in B10 cells which was reduced to basal level in the presence of anti-IL-27 mAb. Similarly, treatment of splenocytes with rIL-27 alone also rendered generation of B10 cells essentially similar magnitude as observed with butyrate (Fig 3A). This observation harnessed the interiority of butyrate effect on B1a cells was IL-27 dependent. The higher expression of CD1d in B-cells is considered as a marker of B_{reg} cells (40) and to assess the regulatory nature of B1a cells, expression of CD1d in IL-10 producing B1a cells were studied. It was observed that IL-10⁺CD1d⁺B1a cells frequency was increased significantly in the presence of rIL27. Furthermore, butyrate also increased the frequencies in similar magnitude as with rIL-27 which was reduced to the basal level in the presence of anti-IL27 Ab and corresponding flow cytometric analysis (Fig 3B, C).

Purified B1a cells produce IL-10 in response to butyrate or IL-27

To show that butyrate or rIL-27 activates B1a population directly to produce IL-10, B-1a cells (CD19⁺CD5⁺) were purified from whole splenocytes and cultured *in vitro* for 72 h either with rIL-27 or butyrate or butyrate + anti-IL-27 mAb and resulting IL-10 production was measured in culture supernatant by ELISA (Fig 3D). It was observed that there was a significant increase in IL-10 in culture supernatant due to rIL-27 or butyrate treatment as compared to untreated control. As expected, butyrate treatment in the presence of anti-IL-27 mAb, IL-10 production returned to basal value (Fig 3E). To establish credible evidence that B1a cells renders anti-inflammatory property upon butyrate treatment, functional assay was envisaged considering TNF- α as an inflammatory cytokine.

Butyrate or rIL-27 treated B1a cell supernatant inhibits TNF- α production

Functional analysis was done to study the ability of IL-10 produced by B-1a cells having anti-inflammatory property or not, the supernatant of cultured B1a cells treated either with butyrate or rIL-27 or butyrate + anti-IL-27 mAb and measured LPS stimulated RAW 246.7 cells derived TNF- α production. The LPS stimulation of RAW 246.7 cells caused 15-fold increase in TNF- α production compared to control. Addition of culture supernatant of either from rIL-27 or butyrate treated B1a cells caused significant reduction in TNF- α production but culture supernatant from butyrate treated B1a cells generated in the presence of anti-IL27 reversed the inhibitory effect (Fig 3F).

The above experiments deftly mirroring as follows: butyrate stimulates B1a cells to produce IL-27 which in paracrine manner producing IL-10 and this resulting IL-10 inhibited TNF- α production from LPS stimulated RAW264 cells. Now the tale unfolds with a notion of reciprocity between IL-27 and TNF- α and butyrate may deftly stroke such a reciprocity.

Butyrate reciprocally regulates IL-27p28 and TNF- α mRNA stability

We studied the decay of IL-27p28 and TNF- α mRNA to shed light on their reciprocity. The decay of mRNA was measured in terms of their half-life ($t_{1/2}$) in minute which was measured after blocking *de novo* RNA synthesis with Actinomycin D in splenocytes with and without butyrate treatment. It was observed that $t_{1/2}$ of IL-27p28 RNA with and without butyrate treatment were 53.75 ± 0.7 m and $118 \pm$

21.5 m respectively (Fig 4A) and corresponding $t_{1/2}$ for TNF- α mRNA was 52.11 ± 1.26 and 23.1 ± 2.23 respectively (Fig 4B). Thus, a reciprocity was noted as regard to $t_{1/2}$ of IL-27p28 mRNA and TNF- α mRNA in splenocytes in response to butyrate. This study showed that butyrate treatment caused dual events in tandem: increased and decreased stability of IL-27p28 RNA and TNF- α mRNA respectively. As a control, $t_{1/2}$ of housekeeping gene, GAPDH mRNA regardless of butyrate treatment remained unaltered (Fig 4C). Since, butyrate activates RNA binding protein, AUF1 (26), that exhibits characteristics of a trans-acting factor contributing to the stability and decay of many cellular mRNAs and therefore it was of automatic choice to study linking IL-27 with AUF-1.

Butyrate upregulates AUF1 and interaction of AUF-1 with TNF- α and IL-27p28 mRNA

To show the effect of butyrate, if any, on AUF-1, splenocytes treated with or without butyrate and the expression of AUF-1 isoforms (p45, p42, p40 and p37) were studied by western blot (Fig 4D). The western blot showed that the AUF1 isoforms: p45, p42, p40 and p37 were increased upon butyrate treatment (Fig4 D). It was clear from densitometry analysis that at 10 μ M butyrate treatment p42/p40 and p45 showed significant increase compared to control. However, 100 μ M butyrate treatment, all the AUF1 isoforms (p45, p42/p40 and p37) were increased 2-fold or more compared to control (Fig 4 E). Now to reveal if IL-27p28 mRNA remained associated with AUF1, we performed RNA immunoprecipitation (RNA-IP) with anti-AUF1 antibody in whole splenocytes without any treatment. The western blot showed the presence of AUF1 in anti-AUF1 pull down samples but not in control IgG pull down samples (Fig 4F, Inset) and qPCR analysis further showed that both IL-27p28 and TNF- α mRNA are present in anti-AUF1 pull down and not in IgG pulled down samples (Fig 4F). Since our study is to evaluate the mechanism of butyrate mediated IL-10 induction exploiting IL-27p28 mRNA stabilization we sought to understand where does AUF1 binds to IL-27p28 mRNA.

AUF-1 binds to 3'-UTR of IL-27p28 mRNA

As AUF1 mostly binds to the 3'UTR of its target mRNAs (41), (18) we seek to understand if it also binds to the 3'UTR of IL-27p28 mRNA. Therefore, we cloned the IL-27p28 3'UTR immediately upstream of EGFP gene and transfected it into RAW246.7 cells. To show that AUF1 stabilizes IL-27p28 mRNA by binding to the 3'-UTR, a co-transfection experiment was performed. The cells were transfected either with pEGFP-3'UTRp28 or pEGFP and the resulting EGFP fluorescence was analysed by fluorescence microscopy. Following the second round of transfection either with si-mock or siAUF1, the cells were treated with or without butyrate generating four experimental sets from left to right as follows: (i) si-mock, (ii) si-mock plus butyrate, (iii) siAUF1 and (iv) siAUF1 plus butyrate. The western blot showed the effective knock down of AUF1 expression which was not reversed even after butyrate treatment (Fig 4G). To bring further credence to our observation, transfection in RAW246.7 cells was done with pEGFP-3'UTRp28 (Fig 4H, upper panel) or pEGFP (Fig 4H, lower panel). In the upper first panel from left only si-mock showed basal level of fluorescence expression which was increased significantly with si-mock plus butyrate treatment (second panel). In the third and fourth panel either

siAUF-1 or siAUF-1 plus butyrate treatment showed significant decrease in fluorescence as compared to si-mock treatment. The fluorescence intensity was quantified and expressed as corrected total cell fluorescence (CTCF). The fluorescence intensity of 3' UTR IL27 EGFP and EGFP as per above specified conditions was expressed in bar diagram (Fig 4I).

Relevance of AUF-1 knock down on p28mRNA expression, stability, translational regulation and IL-10 producing B1a cells

To bring further strength to our notion on the importance of AUF-1 in the butyrate mediated effects, we opted to generate AUF1 knock down (AUF1-KD) in mice using novel cell penetrating morpholino oligomer of GMO-PMO (MO), to knock down AUF-1 in mice for further phenotypic analysis.

GMO-PMO driven knock down AUF1 isoforms in mice

To bring further strength to our notion on the importance of AUF-1 in the butyrate mediated effects, we opted to generate AUF1 knock down (AUF1-KD) in mice using novel cell penetrating morpholino oligomer of GMO-PMO. Here, anti-sense morpholino oligomer, GMO-PMO specific to AUF1 is denoted as AUF1-MO and corresponding scramble is denoted as scramble-MO (S-MO). The expression of AUF1 in the spleen cells from all the groups: control, S-MO, S-MO + butyrate, AUF1-MO and AUF1-MO+butyrate was analysed by western blot (Fig 5A, B). As expected, there was no difference in AUF-1 expression in the splenocytes of control and S-MO mice. Likewise, unsurprisingly S-MO + butyrate treatment caused significantly higher expression of all the isoforms of AUF-1 compared to S-MO. On the other hand, AUF-1 isoforms expression was hardly detectable in AUF-1-MO mice which was not restored upon butyrate treatment. (Fig 5B). Therefore, it was important to explore the impact of AUF1-Knock down (AUF-1KD) on IL-27p28 expression and its stability in in vivo system.

IL27p28 expression in AUF-1-KD mice

The IL27p28 expression in the splenocytes of mice was studied by qPCR after receiving the following treatment: (i) control (without any treatment), (ii) S-MO, (iii) S-MO + butyrate, (iv) AUF1-MO and (v) AUF1-MO+butyrate. The basal level of IL-27p28 expression remained essentially similar between untreated and S-MO treated groups. As expected, butyrate treatment caused 3-fold increase of IL27p28 expression compared to S-MO mice. Interestingly AUF-1 MO treatment caused significant decrease in IL-27p28 expression as compared to S-MO treated group which was not restored upon butyrate treatment (Fig 5C). The above observation calls for further enquiry of IL-27p28 mRNA half-life ($t_{1/2}$).

Measurement of $t_{1/2}$ IL-27p28 mRNA in S-MO and AUF-1KD mice

The process of mRNA degradation of IL-27p28mRNA was directly assessed by measuring the $t_{1/2}$ of IL-27p28mRNA following the addition of Actinomycin D in splenocytes culture isolated from S-MO and AUF1-MO mice. The $t_{1/2}$ of IL-27p28 in spleen cells of in S-MO and AUF1-MO mice was 53 ± 1.5 and 32 ± 3.6 respectively (Fig 5D). The $t_{1/2}$ of GAPDH mRNA remained essentially unaltered

regardless of AUF-1 knock down (Fig 5E). To understand if the decrease in $t_{1/2}$ of IL-27p28mRNA in AUF1-MO mice impacts its translation repression, we performed polysome analysis.

Polysome profiling and analysis of translational status of p28mRNA

The role of translational regulation of gene expression, polysome profiling has been exploited in this investigation to infer the translational status of IL-27p28mRNA. The absorbance at 254 nm for the fractions obtained from S-MO and AUF1-MO are showed the location of polysome, monosomes ((80S) or 40S and 60S) and free RNA (Fig 5 F, G). Our study showed that the distribution of IL-27p28 mRNA shifted towards upper fractions containing monosome (80S) or 40S and 60S in AUF1-MO mice whereas in S-MO mice its distribution restricted to polysome (Fig 5 H). As a control we showed that distribution of GAPDH mRNA remained in the polysome site regardless of any form of MO treatment (Fig 5I) showing that AUF-1 impacted translational regulation of IL-27p28mRNA.

AUF1-KD mice failed to produce butyrate driven B10 polarization of B1a cells:

Since AUF1-KD mice display translational repression of IL-27p28mRNA, we studied its impact in polarization of B1a cells to B10 in terms of IL-10 production on the gated Cd19⁺CD5⁺ population (B1a cells). The results were expressed as percentage of IL-10 producing B1a cells (Fig 5J) and also in absolute number per 10⁶ splenic cells (Fig 5K). As expected, in the S-MO mice receiving butyrate, there was a significant increase in the percentage and total number of splenic B10 cells. In AUF1-KD mice frequency of such cells became comparable to S-MO-group and remained unaltered even after butyrate treatment. The representative dot plots are provided in Figure 5L. It was important to note that the percentage of B1a cells (Cd19⁺CD5⁺) did not change upon butyrate treatment or AUF1 knock down compared to control (Fig supplementary S4). Thereafter we endeavoured to study the frequencies of IL-10 producing B1a cells in AUF-1-KD mice in response to butyrate or IL-27 treatment.

rIL-27 treatment but not butyrate rescue IL-10 production from B1a cells in AUF-1 KD mice

Splenic B-1a cells were sorted and purified from either S-MO or AUF1-KD mice. The purified population of CD19⁺CD5⁺ cells from S-MO mice and AUF1-KD mice having 93.7% and 91.1% purity respectively shown in the dot plots (Fig 6A, B). The cells from the above groups were treated with either butyrate (100 μ M) or rIL-27 (20 ng/ml) for 72h and thereafter IL-10 measured in the resulting supernatant. As expected, S-MO-mice showed basal level of IL-10 production which was enhanced significantly upon rIL-27 treatment or butyrate treatment. B-1a cells from AUF1-MO mice showed nearly 2-fold decrease in IL-10 production compared to the B-1a cells of S-MO mice. Further, treatment with butyrate to the B-1a cells from S-MO mice significantly increased IL-10 production but the cells from AUF1-MO mice with butyrate treatment essentially showed no change. Surprisingly that B1a cells from AUF-1-KD mice upon rIL-27 treatment caused copious IL-10 production which was comparable to the IL-10 produced by butyrate or rIL-27 treated B1a cells from S-MO. (Fig 6C). This dissonance in IL-10 production in AUF1-KD mice between butyrate and IL-27 treatment deftly captures a new revelation of AUF-1 independent effect of IL-27 in IL-10 induction in B1a cells.

Analysis of web-source of micro-array data base of AUF-1 knock down human cell line: molecular landscape of inflammatory genes

Evolving landscape from our preceding studies illuminated that AUF-1 KD using AUF-1-MO caused reduction in butyrate driven B10 polarization of B1a cells as compared S-MO treated mice. This observation impelled us to further explore general inflammatory response due to AUF-1 knock down. We searched in the web-source if such information is available in the public domain. The only available AUF-1 knock down cell line to our knowledge is of skin origin of human ([GSE138621](#)). Data curated linked with inflammatory processes were derived from the microarray datasets comparing AUF1 knockdown WS1 skin cells vs wild type control. KEGG pathway analysis revealed AUF1-affected networks involved multiple biological processes, including upregulation of IBD (underlined in red Fig 7A). It was observed that multiple mRNAs up/downregulated due to AUF1 knock down and many of them were associated cell adhesion, pathways, Th1/Th2 response, mucosal IgA production and the TGF- β signalling pathway garnering embodiments of gut inflammatory processes (Fig 7A, B).

Expression of AUF-1, IL-27, IL-10 and TNF- α in clinical human samples:

Early work from patients and IBD mouse models suggested that there is a blurring Th1/Th2 polarization coupled with altered cytokine repertoire. Enriched from the data derived from skin cell line showing signature of inflammatory processes. We analysed AUF1, IL-27, IL-10 and TNF- α expression by qPCR from colonic epithelial biopsies from 10 IBD patients suffering from ulcerative colitis. As controls, we utilized biopsies from 6 otherwise IBD-free individuals who had colonoscopy and whose diagnosis for intestinal diseases were ruled out. The details of the patients are presented in table 1. We observed that the median value of relative AUF1 expression in IBD cohort was nearly fivefold less than the median of control cohort (Fig 7B). Corroborating previous studies, our investigation broadly revealed a 1.89-fold decreased IL-27 expression in IBD patients as compared to the control cohort (Fig 7C). As expected, the IBD patients showed 2-fold decrease in IL-10 production with concurrent fourfold increase in TNF- α compared to control (Fig 7C).

To ensure that the observed results were not specific to just the human diseases and their matched mouse model of colitis induced by DSS can be used for validation of data generated from clinical samples. Mouse studies may provide further space to delve into mechanistic details like cytokine regulatory network and cell types involved in IBD pathogenesis which is not possible with clinical samples. Therefore, we undertook studies in DSS induced colitis in mice to validate some of the clinical results.

Disease phenotypes of DSS induced colitis in mouse and reversal with butyrate treatment

DSS colitis, despite shortcomings, has been of great use in understanding the pathophysiology of intestinal inflammation and, consequently, has informed the theoretical and clinical appreciation of active IBD (42). Here we induced colitis in mice with DSS and studied the pathogenesis. The following phenotype was noted in colitis mouse and subsequent butyrate treatment: (a) significant decrease in colon length and body weight (Fig 8A, B, C) (b) 13.36-fold reduction of faecal butyrate as compared

to control mice [Fig 8D] and (c) significant decrease in IL-10 and increase in TNF- α and IFN- γ as compared to control [Fig8E] and (d) decrease in AUF1^{p40} mRNA and IL-27p28mRNA as compared to control [Fig8F, G]. Butyrate treatment to colitis mouse impelled significant reversal of above changes.

Status of B1a cells in different secondary lymphoid organs in colitis mouse

Next, we went on to analyse percentage and number of IL-10 producing B1a cells not only in spleen but also in mesenteric lymph nodes (MLN) being in the proximity of the gut tissues. It was observed that frequency and absolute number of IL-10 producing B-1a cells were significantly decreased in colitis-mice regardless of origin and butyrate treatment increased their frequencies in low but significant measure [Fig 8H, I]. Notably the percentage of CD19⁺CD5⁺ cells (B1a cells) from spleen as well as in MLN remained unaltered irrespective of DSS and butyrate treatment (Fig S5).

Adoptive transfer of butyrate treated B-1a from wild type mice but not from AUF1 knockdown mice conferred protection against DSS induced colitis

Since AUF-1 was decreased in clinical samples of colitis coupled with reduced IL-10 production and AUF-1 knock down mice display decrease in IL-10 producing B1a cells, we asked the question concerning the ability of butyrate treated B1a cells to attenuate DSS-induced intestinal injury and to establish role of AUF1 in roistering B1a to B10 polarization and buoying disease phenotype. The functional assay involved adoptive transfer of B1a cells either derived from S-MO treated mice or AUF1-MO (AUF1-KD) and adoptively transfer to colitis-mice. The experimental design is schematically represented in Figure 9A. B1a cells were sorted from either S-MO (wild-type) and AUF1-MO (AUF1-KD) mice were stimulated with 100 μ M butyrate *in vitro* for 72 h. For convenience, B1a cells derived from S-MO or AUF1-MO defined as butyrate-B1a^{wild} and butyrate-B1a^{KD} cells respectively. The cells of butyrate-B1a^{wild} or butyrate-B1a^{KD} cells were injected into colitis-mice through tail vein. Adoptive transfer of butyrate-B1a^{wild} cells but not butyrate-B1a^{KD} cells to colitis-mice significantly reduced the severity of intestinal injury as observed by i) decrease in colon length (Fig 9 B, C), ii) significant decrease in TNF- α and IL-6 production in colon (Fig 9D) and iii) recovery from occult bleeding (Fig 9E).

Discussion

Composing together series of experimental results, we dodged here linking butyrate with IL-10 producing B1a cells and identified the intermediate players and their mechanistic sequel that dangle in gut pathology of colitis. We chose to study with whole splenocytes or its gated population as the experiment demands. The spleen was of choice based on the report that IL-10 producing B cells are numerically higher in the spleen than in peritoneal cavity, lymph node (LN), Payer's patch (PP) and gut associated lymphoid organs (37), (43). These numerically rare population of B cells producing IL-10 having unique functional program and suppresses inflammation are called "B10 cells" (37). Unlike acetate and propionate, butyrate has emerged as IL-10 producing short chain fatty acid from B1a cells but not in B2 cells, which has warranted a mechanistic look to understand how butyrate influences B10

polarization. The SCFA concentration used in the study was in tune with the report showing their physiological concentration in the peripheral blood in mice (44). The major regulatory pool of B cells come from B1a and B2 cells (45), therefore we have not considered B1b cells as it contributes mainly to adaptive responses (46).

Retroactively looking at the literature we found, IL-27, one of the IL-12 cytokine family members was shown to play an important role in IL-10 producing Treg cell development (33). Therefore, IL-27 induced B10 polarization was aptly looked into. We showed that B1a cells but not B2 cells produce IL-10 upon IL-27 stimulation. We studied the expression of the two subunits of IL-27: IL-27p28, and EB13 upon butyrate treatment and observed upregulation of IL-27p28 expression with butyrate compared to control. IL-27 is mostly produced from antigen presenting cells including macrophages, dendritic cells and also B1a cells but not from B2 cells (30). Therefore, in B1a cells IL-27 acts in autocrine as well as in paracrine manner. It is reported that B-1a cells express significantly higher IL-27 receptor (IL-27R α) mRNA compared to B-2 cells (30). The lesser number of IL-27 receptor expression in B2 cell may explain its failure to induce IL-10 production upon stimulation with butyrate or IL-27. Furthermore, regardless of B cell lineage development, CD5-expressing B cells have a higher propensity for IL-10 production and this property has been associated with CD5 expression (47). To substantiate our finding that butyrate may increase IL-10 production in B1a cells through IL-27 upregulation, we blocked IL-27 by using anti-IL-27 Ab in butyrate treated splenocytes. The significant reduction in the percentage of B10 cells from butyrate treated splenocytes upon IL-27 neutralization strengthens our hypothesis that butyrate increases the number of B10 cells by elevating IL-27 production. Earlier studies showed butyrate can increase IL-10 by increase in Blimp1 expression (48) and HDAC inhibition (49) whereas HDAC inhibitor inhibits Blimp-1-dependent gene repression (50). Since butyrate being an HDAC inhibitor (51), it may be speculated that it activates IL-10 by inhibiting Blimp-1 binding to its promoter. Interestingly, IL-27 is shown to activate IL-10 by c-Maf/ROR γ t/Blimp-1 signalling pathway (52). Therefore, earlier demonstration of butyrate mediated activation of IL-10 converges to the downstream of AUF1-IL-27 pathway.

IL-10 is regarded as a potent immune-suppressive cytokine with broad immunoregulatory functions (53). Thus, IL-10 suitably fulfils the profile of an effector molecule ‘downstream’ of IL-27 that exerts a general regulatory function of the B1a cells. Here, we showed that IL-27 not only increased expression of IL-10 but also promoted CD1d expression in B1a cells. Higher expression of CD1d is the hallmark of Breg phenotype (54). To further establish the anti-inflammatory function of butyrate stimulated B1a cells, we showed that the supernatant from B1a cells cultured in butyrate can reduce TNF- α production from LPS stimulated macrophage thereby endorsing the functional property of regulatory B cell in vitro. The cellular homeostasis of mRNA is not static and may vary in response to external stimuli through interacting with 3'-UTR. In addition to epigenetically regulating gene expression through its inhibition of HDAC activity, butyrate may also post-transcriptionally regulate the expression of various cytokine genes (27), (28), (29). To show the mechanism of regulation of IL-27 by butyrate, we undertook studies

on the determining the decay rate of IL-27p28 mRNA in splenocytes. We found butyrate stabilizes IL-27p28 mRNA increasing its half-life to 2.4-fold. TNF- α mRNA is reported to be destabilized by butyrate (27) therefore it was evident the half-life of TNF- α mRNA decreased upon butyrate treatment. Butyrate affects a number of other cellular functions which impact on gene expression, including RNA binding protein (RBP) mediated RNA stability (28), phosphorylation and ADP ribosylation of chromatin proteins and methylation of DNA (55). It is reported that butyrate regulates the expression of AU-rich element binding RBPs (56), (26) that can either accelerate or decelerate mRNA decay. The precise mechanism by which butyrate regulates these RBP expression is still not clear. AUF1 which is reported to have dual role in mRNA stabilization as well as in decay by binding to the AU rich elements of mRNA (57) is expressed in spleen > thymus > intestine (58). AUF1 having 4 isoforms: p45, p42, p40 and p37 are important for B cell development (17), survival and expresses in B cells (59), macrophages (60) and epithelial cells (58). Consistent with the biological function of AUF1, our data demonstrate that AUF1 stabilizes IL-27 production by targeting p28 mRNA via direct binding to the 3'UTR of p28 mRNA. Although AUF1 reported to predominantly bind to the motifs located in the 3'UTR of the mRNA and decides the fate of mRNA (24), (61), it does not rule out the fact that it may also bind to other parts of p28 mRNA. Further studies are under way to address the different regions of AUF1 binding to p28 mRNA and also to identify the isoform(s) necessary for p28 mRNA stability. Oddly enough, another AU rich element binding RBP, Tristetraprolin (TTP) which is also upregulated by butyrate (62) is reported to destabilize IL-27p28 mRNA (63). TTP is abundant in liver and reproductive tissues but negligible in spleen where AUF1 is copious (64). Therefore, it can be speculated that AUF1 outpaces TTP on IL-27p28 mRNA regulation in the lymphoid tissues. AUF1 has pleiotropic targets, one of which is stabilizing IL-10 mRNA, by binding of p40 isoform of AUF1 (60). Refutably, we observed that IL-27 inhibition completely shuts down IL-10 production from B1a cells thereby contradicting the fact that AUF1 directly can increase IL-10 by stabilizing its mRNA. This may be explained by IL-27 mediated regulation of AUF1 in a positive feedback loop as evident from the western blot of AUF1 by inhibiting IL-27 expression using IL-27 morpholino (IL-27 MO) (Figure S6). To our surprise it was observed that blocking IL-27 by IL-27MO decreases the expression of AUF1 isoforms which is restored upon rIL-27 treatment. Further investigations are required to understand the phenomena of IL-27 mediated AUF1 regulation. Further investigations are required to understand the phenomena of IL-27 mediated AUF1 regulation.

To further establish the link between AUF-1 and IL-10 production in B1a cells, we knocked down AUF1 by deploying morpholino oligomer. Morpholino oligomers (MO) which are short single-stranded DNA analogues that are built upon a backbone of morpholine rings and are resistant to host enzymes present, a characteristic that makes them highly suitable for application in primary cells and *in vivo* (65), (26). Earlier we have used the AUF1 knock down mice model using MO to establish the role of AUF1 in cholesterol synthesis (26). Unlike a knockout, AUF1-MO mediated knock down causes partial knock down of AUF1 in organs like in kidney, heart (26), but nearly 100 % knock down in liver (26),

colon and spleen. Therefore, warranting morpholino administration in mice a unique knockdown (KD) model to explore the role of AUF1 in B10 generation. The AUF1 KD mice showed low levels of IL-27 with decrease in the half-life of IL-27p28 mRNA compared to S-MO. Fundamentally, the process of mRNA decay is tightly linked to translation output (66) and we showed that absence of AUF1 represses the translation of IL27p28 mRNA. Reports showing the involvement of other trans factor(s) that are associated with translational repression with RBP mediated mRNA stability (67) provide fodder for further investigation of the trans factor (s) associated with AUF1 if any. The decrease in splenic B10 cells along with decrease in IL-27 in the spleen of AUF1 KD mice which butyrate failed to reverse, indicate that AUF1 is the master regulator of butyrate induced B10 polarization. Oddly enough, rIL-27 but not butyrate was perfectly capable of producing IL-10 from B1a cells in AUF1-KD mice bringing credence to the notion that butyrate mediates its effect through IL-27. By aligning the observations, we can formulate the roster of molecular events as follows: butyrate-AUF1-IL-27p28-IL-10 in B1a cells. Since AUF1 regulates many cytokines gene expression the AUF1 knock out is reported to be susceptible to septicaemia and skeletal muscle wasting with impaired regeneration following injury (25), (68). Dialectic to the defects in post transcriptional regulation of the cytokines may be involved in human inflammatory diseases, we harnessed the pathways of IBD associated with AUF1 knockout cells by exploiting publicly-available microarray dataset by KEGG pathway analysis. Based on the alliance of IBD with AUF1 knockout we investigated the association of AUF1 and IL-27 in colon biopsy samples from IBD patients and non-IBD control. Significant decrease in AUF1 and the half-life of p28 mRNA in the colon biopsy of IBD-patients compared to control indicates an indispensable role of AUF1 in inflammation. Absence of AUF1 can also act as a colitogenic trigger as evident from the destructive colon architecture and increased inflammatory cytokines (TNF- α and IL-6) in AUF1 knock down mice compared to wild type control (Fig S7).

The reduction in AUF1 can be linked to IBD which is characterized by severe diminish in the IL-10 producing T and B regulatory cells (69, 70) and gut microbial dysbiosis resulting to decrease in butyrate (71), (72). As a surrogate of IBD patients we showed in DSS induced experimental colitis in mice reduction in butyrate and IL-10 producing B cells in MLN and spleen which was reversed upon butyrate treatment. Multiple studies have shown butyrate and rIL-27 administration ameliorates colitis in mice model (73), (74), (34). As there are very low frequency of B10 cells in gut associated lymphoid tissue (37) in wild type mice we preferred to analyse B10 cells from MLN considering its proximity to gut and also due to its sufficient availability in MLN (37). The decrease in B10 cells in colitis was aligned with decrease in AUF1 and IL-27 expression. Earlier it was shown that adoptive transfer with regulatory B1a cells decelerates colitis (75). To examine the importance of AUF1 in butyrate mediated B10 polarization in murine colitis, we isolated B1a cells from wild type mice and AUF1-KD mice to perform adoptive transfer in colitis-mice. We showed that butyrate treated B1a cells from wild type mice but not from AUF1KD mice exhibited therapeutic effects on colitis indicating B1a cells from wild type mice but not from AUF1 KD mice is polarized B10 upon butyrate treatment which attenuated

inflammation. Therefore, we present quantifiable new data with the unique function of butyrate-driven B1a cells, offering an intriguing prospect for a colitis treatment.

In summary, this study unveils a novel regulatory pathway for IL-27 expression and B10 polarization mediated by AUF1, which contributes to the regulation of anti-inflammatory activity. It also indicates that butyrate induced AUF1 can dampen inflammation in DSS induced colitis through exploiting B10 polarization. Finally, we propose that butyrate driven B1a cells as a glimmer of new hope of therapeutic possibility against colitis.

Materials and Methods

Collection of colon biopsies and patient features Eight active Ulcerative colitis (UC) patients as defined by Ungaro et al. (76) and seven patients who had colonoscopy and whose diagnosis for intestinal diseases were ruled out were enrolled for the study (Table 1). This study was approved by the ethics committee of National Institute of Cholera and Enteric Diseases (ICMR-NICED/IEC-BMHR/003/2023), and all methods were performed in accordance with the respective approval. Patients were informed in advance and gave written consent to sampling additional biopsies to ones that were taken for diagnostics during the routine endoscopic examination. For these, patients allowed usage for analysis of RNA and protein expression. From the Mayo endoscopic subscore is the part within the full Mayo score to assess disease presentation during colonoscopy (77). Based on the aim of this study, patients with mild and moderate disease activity were unified as active (Table 1).

Mice and animal ethics:

C57BL/6 mice (4 weeks) were obtained from the animal facility of ICMR-NICED, Kolkata, India. All of the protocols for the study were approved by the Institutional Animal Ethics Committee of ICMR-NICED, Kolkata, India, (PRO/2001/-Nov 2023-26). Experiments have been carried out in accordance with the guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India.

The daily consumption of food in mice was recorded every day by weighing the food given (food placed in the food receptacle at time zero) and food remaining (food left in the food receptacle 24 hours later). The cumulative food intake by each group (5 mice / group) per day was determined.

Induction and Evaluation of DSS-Induced Intestinal Injury and calculation of Disease Activity Index (DAI)

2.5 % (w/v) of Dextran sodium sulphate (DSS) (molecular mass, 36 to 50 kDa) was dissolved in purified water and administered to mice for 14 days (78). In some experiments, mice were fed with 5% butyrate (w/w) mixed with food pellet from the day of experiment initiation till it ended (26). The DAI is the combined score of weight loss compared to initial weight, stool consistency, and bleeding. Scores are defined as follows: weight loss: 0 (no loss), 1 (1-5%), 2 (5-10%), 3 (10-20%), and 4 (>20%); stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhoea); and bleeding: 0 (no blood), 1 (Hemoccult

positive), 2 (Hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus) (78).

Histological Analysis

The mice were sacrificed 5 days after the induction of intestinal injury. Colon samples were removed and segments were fixed in 10% buffered formalin. After paraffin embedding, 5- μ m-thick sections were cut and stained with H&E. Histological scoring was based on a previously described method (79). Briefly, H&E-stained cross sections of the descending colon tissue were scored microscopically in a blinded fashion on a scale from 0 to 4, based on the following histological criteria: 0, no change from normal tissue; 1, low level of inflammation with scattered infiltrating mononuclear cells (foci, 1 to 2); 2, moderate inflammation with multiple foci; 3, high level of inflammation with increased vascular density and marked wall thickening; and 4, maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells. An average of four fields of view per colon was evaluated for each mouse. These scores were averaged for each group and recorded as the histopathological score.

Intracellular staining

Single-cell suspension of splenocytes from mice was prepared as described previously (80). The cells were treated as indicated in the experiments and then cultured in the presence of 10 μ g/ml Monensin for 8 h. Cells were stained by monoclonal fluorescent tagged antibodies either against CD3, CD4, or CD19, CD5 or CD 23 or CD1d fixed and permeated using Cytotfix/Cytoperm, followed by intracellular staining with anti-IL-10 antibody. Details of the antibodies are given in the supplement.

Cell Sorting and Adoptive Transfers

Spleen cells from scramble-MO and AUF1-MO mice were isolated and treated with 100 μ M butyrate for 72 h at 37°C in humidified CO₂ incubator. After incubation, B1a cells (CD19⁺CD5⁺ B cells) from butyrate treated spleen cells of scramble-MO and AUF1-MO mice were purified using a flow cytometer (FACSaria Fusion; Becton Dickinson), with purities of approximately 85% to 95%. The 2×10^6 cells in 200 μ l was transferred by intravenous injection into 7 days post DSS fed mice. On day 7th of post injection, the mice were sacrificed and colon were collected, length of the colon was measured and further processed for histology and cytokine analysis.

GMO-PMO treatment to knock down AUF1

The GMO-PMO synthesis and its administration in mice was performed as described earlier (26). Briefly, The GMO-PMO was dissolved in saline to make a concentration of 1 mM which was equivalent to 8.2 mg/ml. In vivo knock down of AUF1 was carried out in mice by injecting AUF1 GMO-PMO (AUF1-MO) through tail vein on day 1 and day 7 at a dose of 3 mg/kg body weight. Scramble GMO-PMO treated animals are called as S-MO controls. Animals were sacrificed after day 14 and tissue were collected for further analysis.

Sequence of AUF1-MO = 5'-TCCGAAGTCTCCTCCGACATAGTG-3'

Sequence of IL-27-MO = 5' CAAGGTCTCCTGTACCTGGCCCAT3'

Sequence of S-MO = 5'-TCGCAAGTCTCCTCCAGCATAGTG-3'

Cloning of IL-27p28 3'UTR

Mouse IL-27 p28 3'UTR plasmid was cloned by inserting p28 3'UTR into MCS of the pEGFP-C1 vector (Clontech, California US) between XhoI and KpnI sites. Primers used for amplification of p28 3'UTR from genomic DNA of splenocytes were CCGCTCGAGTTCTAGACACCTAGCTTCAAGCCCTATGG (sense); and CGGGGTACCGGCCCGCCCGGGCTGGATGGCTTTATTA (anti-sense). All plasmid DNA were prepared with QIAGEN Endo-free Mini-Prep kits.

Transfection

RAW264.7 cells were plated in 6-well culture plates at the density of 1×10^6 cells/well and cultured in 2 ml serum-free medium for 24 h to 80% confluence. Transfection was performed with Lipofectamine 2000 according to the protocol recommended by the manufacturer. Briefly, 25 μ l Opti-MEM medium was used to dilute 1.0 μ l lipofectamine and 0.5 μ g plasmid or 0.27 μ g siRNA, and equal volume of the plasmid or siRNA and lipofectamine was mixed at room temperature for 15 min. Cells were transfected by adding 100 μ l of the Opti-MEM medium containing lipofectamine and plasmid or siRNA at 37° C for 6 h, and then cells were grown at DMEM containing 10% FCS.

Fluorescence microscopy

RAW264.7 cells grown on glass cover slips were transfected with either pEGFP-IL-27p28-3'UTR or p-EGFP for 24 h. After washing with PBS, the cells were treated with 1 μ g/ml Hoechst 33342 for 5 min at room temperature and washed again with PBS three times. Fluorescence images were captured with Carl Zeiss microscope equipped with a CCD camera controlled with ZEN software (Carl Zeiss, Gottingen, Germany). The fluorescence intensity was quantified and expressed as corrected total cell fluorescence (CTCF). $CTCF = \text{integrated density} - (\text{area of selected cells} \times \text{mean fluorescence of background readings})$ (81).

Determination of mRNA half-life

Splenocytes were challenged with Actinomycin D (5 μ g/ml) for increasing periods of time. Total RNA was then extracted and subjected to qPCR. The one-phase exponential decay curve analysis (GraphPad Prism 9) was used to assess the mRNA decay kinetics, considering the values at time 0 as 1. The time corresponding to 50% remaining mRNA was considered as mRNA half-life ($t_{1/2}$) (63).

RNA immunoprecipitation

Proteins extracted from whole splenocytes were incubated with beads pre-coated with anti-AUF1 antibody and control IgG. After washing three times, RNA was extracted from Beads, and reverse-transcribed into cDNA, followed by detecting p28 and TNF α mRNA by qPCR (63).

Quantitative reverse transcription-PCR

Total RNA was extracted from B cells or colon tissue using Trizol reagent and RNA quantification was performed on Nanodrop 2000. RNA (0.5–1 μ g) was reverse transcribed using the cDNA Synthesis Kit according to the manufacturer's instructions. The qPCR reaction was performed using the SYBR Green

Kit and on QuantStudio 5/7 (Applied Biosystems, Massachusetts, USA). Primers were designed and synthesized with the sequences listed below. All primers were purchased from IDT (Lowa, USA) (Supplementary Table).

Polysome analysis

Ribosomal fractions were obtained as described earlier (75). In brief, spleen cells were homogenized in polysome lysis buffer containing cycloheximide (0.1 mg/ml) and cytosolic extract was obtained by centrifugation at 10000g for 20 min. The extract was overlayed on a 10–50% (w/v) sucrose gradient and centrifuged at 100000g for 4 h in ultracentrifuge (Thermo, Massechusetts, USA). Fractions were collected manually and absorbance at 254 nm was measured. RNA was isolated from the fractions by phenol-chloroform extraction and ethanol precipitation.

Western blot

Tissue protein or cell lysate were extracted in RIPA Lysis buffer. Protein concentration was measured using Pierce BCA Protein Assay Kit. Proteins (50 µg/lane) were separated by using SDS-PAGE on 10% gel under reducing condition and electro transferred to PVDF membrane in a transferred buffer (25mM Tris-HCl, 150mM Glycine, 20% Methanol). Membranes were blocked at room temperature with 5% non fat skim milk in TBS for 2 hours, and then incubated with primary antibody against specific protein. The membranes were incubated either with the horseradish peroxidase (HRP)-conjugated secondary antibodies or Alkaline phosphatase (AP)-conjugated antibodies at 37° C for 1 h. For HRP-conjugated antibody treatment, SuperSignal West Pico chemiluminescent substrate kit (Thermo) was used to visualize the blotting results. The blots were imaged with Fluor Chem R system (ProteinSimple, San Jose, CA, USA) (76). For AP-conjugated antibody treatment, BCIP-NBT substrate kit (Thermo) was used to visualize the blotting results (77).

KEGG pathway analysis

The data (GSE138621) FastQ files were obtained from NCBI-SRA, specifically processing samples belonging to the specified groups (AUF1-knockdown WS1 cell line vs WS1 wild type cell control). Initially, the samples underwent quality filtering using trim_galore. Next, quality-controlled samples were aligned to the human reference genome using the STAR aligner (78). Subsequently, featureCounts (79) was employed on the generated BAM files to produce a count table. This count table underwent further analysis using the DESeq2 (80) package in R. Additionally, KEGG pathway over-representation analysis was conducted separately for upregulated and downregulated genes, based on specific criteria ($\log_2$ fold change ≤ -0.5 or ≥ 0.5 and a p-value less than 0.05), utilizing the clusterProfiler (81) package.

Measurement of butyrate in faeces by LC-MS

Faecal content of each mouse (10-50 mg wet weight) was dissolved in distilled water at 1:10 (w/v) and homogenized in a Dounce homogenizer. Then, the content was centrifuged at 10,000g for 10 m and the supernatant was filtered through 0.45µm syringe filter. The supernatant was further diluted in LC-MS grade distilled water up to a final volume of 1 ml and were subjected to LC-MS analysis for SCFA as described by Cheng et al (82). In brief, a calibration curve was generated by using varying calibration of internal standards of butyrate from 2 µmoles to 50 µmoles. The LCMS separation was performed on

the Agilent 1290 Infinity LC system which was coupled to Agilent 6545 Accurate-Mass Quadrupole Time-of Flight (QTOF) with Agilent Jet Stream Thermal Gradient Technology with electrospray ionization (ESI) source. The suitable MS parameters were optimised and the high-resolution mass spectra were obtained by performing the analysis in negative ionisation mode. The Chromatographic separation was achieved on Agilent ZORBAX SB- C18 column (2.1 × 100 mm, 1.8 µm) as stationary phase. The mobile phase consisted of a linear gradient of 0.1% (v/v) aqueous formic acid (A) and Methanol (B): 0–1.0 min, 0 % B (v/v); 1.0–3.0 min, 0 30% B (v/v); 3.0–6.0 min, 30-100% B (v/v); 6.0–12.0 min, 100% B (v/v); 12.50–15.0 min, 0% B. The column was reconditioned for 5 min before next injection. 0.2 mL/min flow rate and a varying injection volume was used for analysis. The UPLC system assembled with a Diode array detector (DAD) and an auto sampler. The peak area of each SCFA was used to calculate the amount of SCFA present which was further normalized by the injection volume. The data was further represented as the amount of SCFA present per gram of faeces.

Statistics.

All graphs and statistical significance were calculated using GraphPad Prism 9.00 (GraphPad, San Diego). For mouse studies, we chose a sample size of five mice per group. Unpaired two-tailed Student's t test was performed for comparison between two groups. One-way ANOVA (Nonparametric) with Tukey (Compare all pairs of columns) or Kruskal–Walls test with Dunn's Multiple Comparison was used for comparison among multiple groups. Data shown are mean+SE. of at least three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 between indicated groups. Curve fitting, the mathematical modelling, was done with Origin Lab data analysis.

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Author contributions

AM performed majority of the experiments and analysed data. OD showed that AUF1 knockdown mice develops colitis and performed experiments on clinical samples. DR and SAC performed the cloning. AD, SS, SA performed experiments. RS and MY provided clinical samples and helped in diagnosis of the disease by colonoscopy. SS synthesized the morpholino. MB conceptualized, designed, supervised, arranged funding and wrote the manuscript.

Data availability statement

All data generated in this study are included in this article and supplementary information or are available from the corresponding author on reasonable request.

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Table1: Details of the patients from whom the colon biopsy samples were collected

Patient ID	Age (y)	Sex	UCEIS score	Colonoscopy finding	Samples taken as control or IBD
7	65	Male	NA	Normal mucosa, Ileal ulcer	Control
10	59	Male	NA	Normal mucosa	Control
5	44	Female	5	Both rectum and sigmoid colon is found with complete loss of vascular pattern with spots and tears.	Active IBD (UC)
6	50	Female	5	Complete loss of vascular pattern, large superficial ulcer of 75mm seen	Active IBD (UC)
8	24	Male	--	Complete loss of vascular pattern seen with multiple superficial ulcers of varying size> 5mm in size.	Active IBD (UC)

12	33	Male	7	Mucosa is involved in form of continuous loss of vascular pattern with spots and steaks of blood ahead of scope which can be washed away with jet of water. Multiple superficial and deep ulcer.	Active IBD (UC)
11	49	Female	NA	Normal mucosa	Control
13			NA	Normal mucosa, illeal ulcer	Control
15	45	Female	5	Complete loss of vascular pattern, expanded lamina propria with inflammatory cells.	Active IBD (UC)
1	52	Male	NA	Normal mucosa	Control
2	36	Female	7	ulceration of epithelium, Cryptitis, crypt abscess, mucodepletion. Focal crypt distortion, crypt branching and crypt loss. Lamina propria is expanded with dense amount of inflammatory cells.	Active IBD (UC)
3	30	Male	NA	Chronic sigmoiditis	Control
4	58	Male	7	Ulceration in the lining of epithelium, presence of inflammatory cells in lamina propria; no granuloma was found.	Active IBD (UC)
5	32	Male	NA	Normal mucosa	Control

Figures:

Figure1

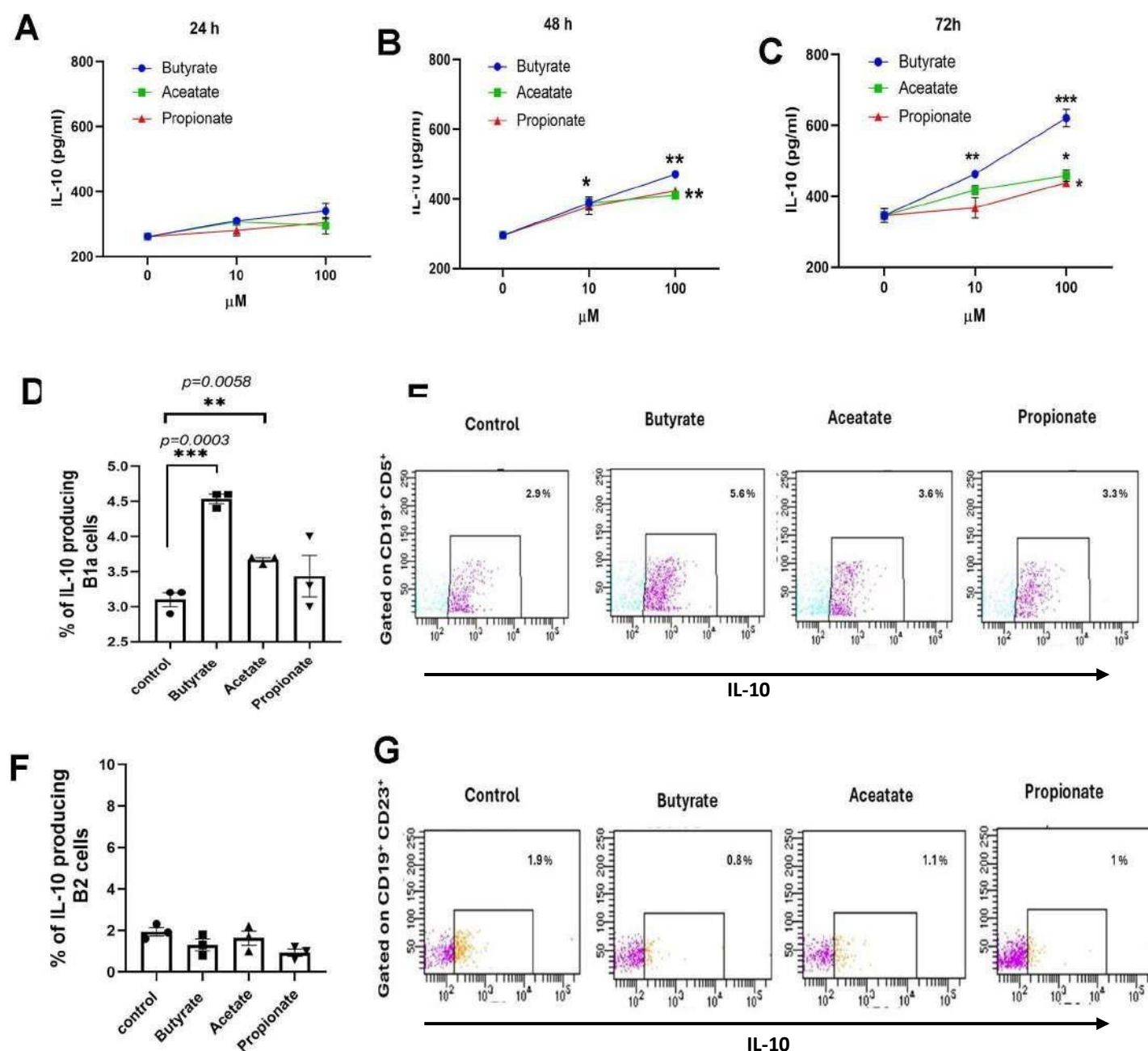


Fig. 1: Effect of butyrate or propionate or acetate (SCFA) on mouse splenocyte on IL-10 production and phenotypic characterization of IL-10 producing cells.

Splenocytes were treated with either butyrate or propionate or acetate at a concentration of 10 μ M and 100 μ M, where without SCFAs were used as control and resulting IL-10 production in the culture supernatant was measured by ELISA at 24h (A), 48h (B) and 72h (C). Having established that 100 μ M of SCFAs caused significantly higher IL-10 induction in splenocytes compared to without SCFAs, rest

of the studies were carried out with 100 μ M. For phenotypic characterization of IL-10 producing cells, splenocytes were treated with 100 μ M of each SCFAs for 72 h where monensin treatment was added during the last 8 h of culture. B1a cells and B2-cells in the splenocytes were characterized as CD19⁺CD5⁺ and CD19⁺CD23⁺ respectively and on the gated population intracellular IL-10 production was assayed staining with anti-IL-10 mAb and results were expressed % IL-10 producing cells in B1a (D and E) and B2 cells (F and G). Gating strategy is shown in supplementary Fig S1. All experimented were repeated thrice (n=3) and represented as Mean $\pm$ SE. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to control calculated by student's t-test.

Figure 2

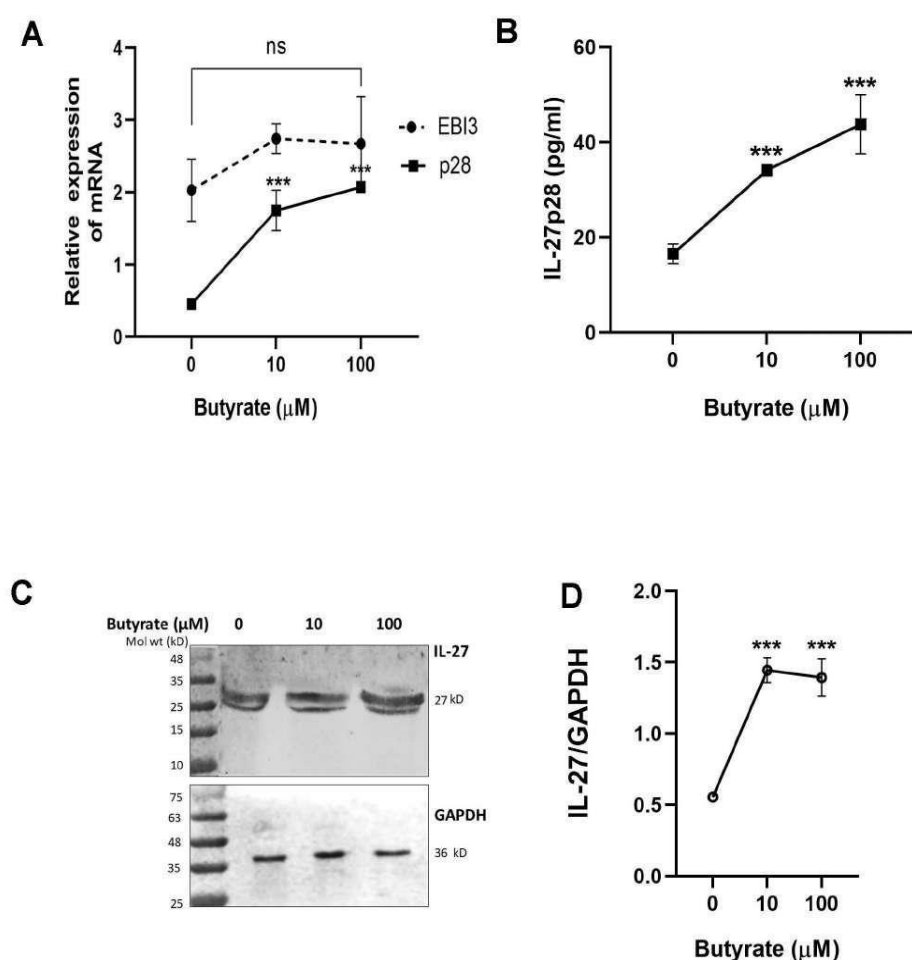


Figure 2: Effect of butyrate on IL-27 production by normal mouse splenocytes

Splenocytes were treated with butyrate at a concentration of 10 μ M and 100 μ M for 72 h. The expression of p28 and EBI3, the two subunits of IL-27 was determined by qPCR (A) and corresponding IL-27p28

was determined in the culture supernatant (B), expression of IL-27 by western blot (C) and corresponding densitometry of the western blot using ImageJ showing relative expression of IL-27 with respect to GAPDH (D). All experimented were repeated thrice (n=3) and represented as Mean±SE. p values calculated with respect to control by student's t-test.

Figure 3

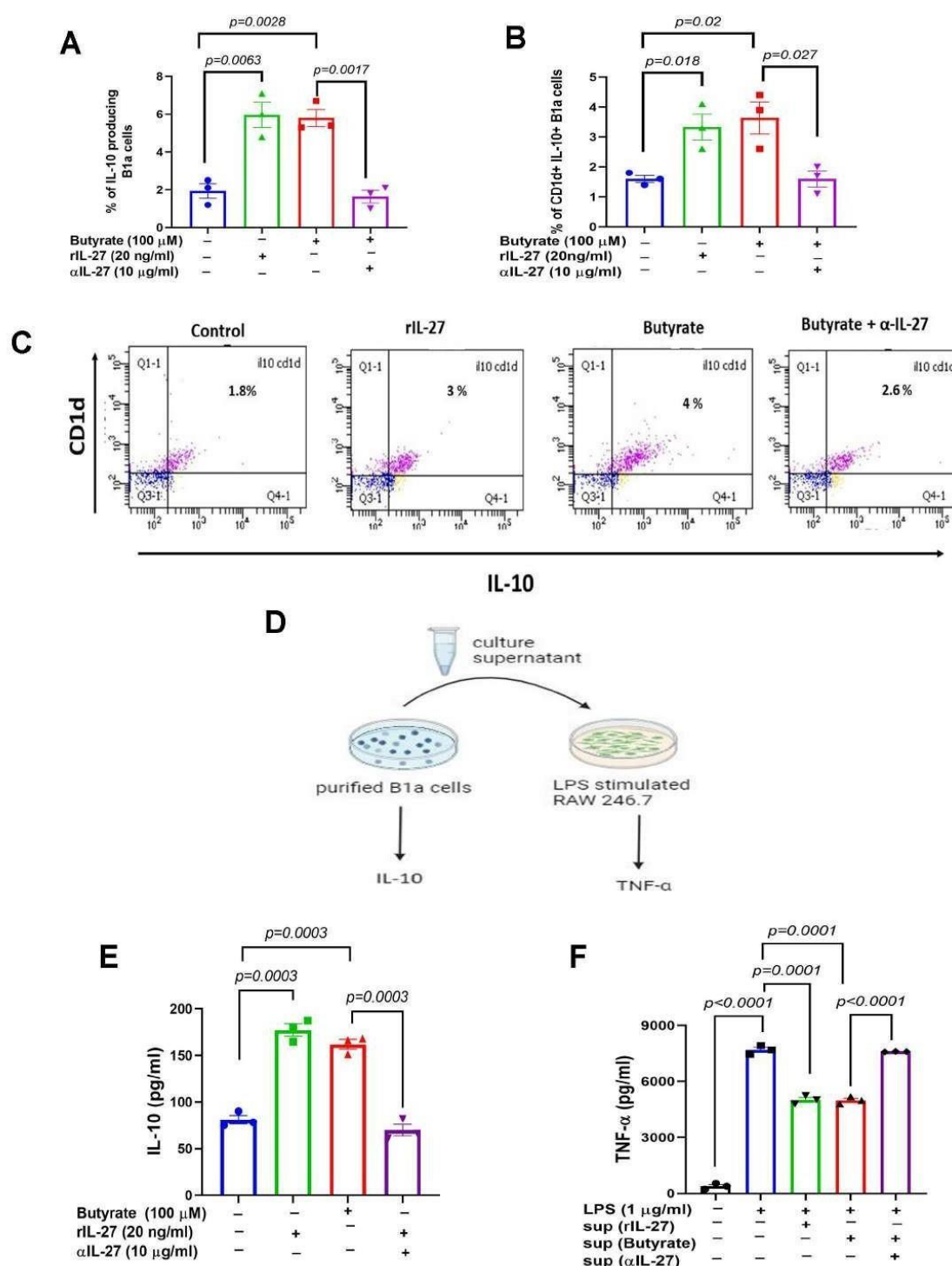


Figure 3: Butyrate or rIL-27 induced IL-10 production from splenocytes and B1a cells and functional assay of supernatant of B1a cells as inhibitor of TNF- α production

Splenocytes treated either without (□) or with butyrate (100 μ M) (◻) or rIL-27 (20 ng/ml) (◻) or butyrate (100 μ M) plus α -IL-27 mAb (10 μ g/ml) (◻) for 72 h and monensin was added to last 8 h of treatment. Thereafter cells were stained with antibodies against CD19, CD5 and CD1d. The percentage of IL-10 positive B1a cells in the splenocytes were detected after staining with anti-IL-10 Ab and gated on CD19⁺CD5⁺ cells (A). The percentage of CD1d⁺IL-10⁺ B1a cells was detected by gating CD19⁺CD5⁺ cells (B). The representative dot plots showing CD1d⁺IL-10⁺ B1a cells gated on CD19⁺CD5⁺ cells (C). The CD19⁺CD5⁺ cells were purified from untreated splenocytes and were cultured *in vitro* without (□) with either butyrate (100 μ M) (◻) or rIL-27 (20 ng/ml) (◻) or butyrate (100 μ M) plus α -IL-27 mAb (10 μ g/ml) (◻) for 72 h. A part of the supernatant was collected to measure IL-10 production by ELISA and the other part was added to 24 h LPS (10 ng/ml) stimulated RAW264.7 cells which were further cultured for 24 h. The TNF- α was measured by ELISA from the resulting supernatant from LPS stimulated RAW264.7 after 24 h. The diagram represents experimental design (D). The ELISA results of IL-10 (E) and TNF- α (F) are presented. The pre and post sorting is represented in supplementary figure S3. All experimented were repeated thrice (n=3) and represented as Mean $\pm$ SE. p values calculated with respect to control by student's t-test.

Figure 4

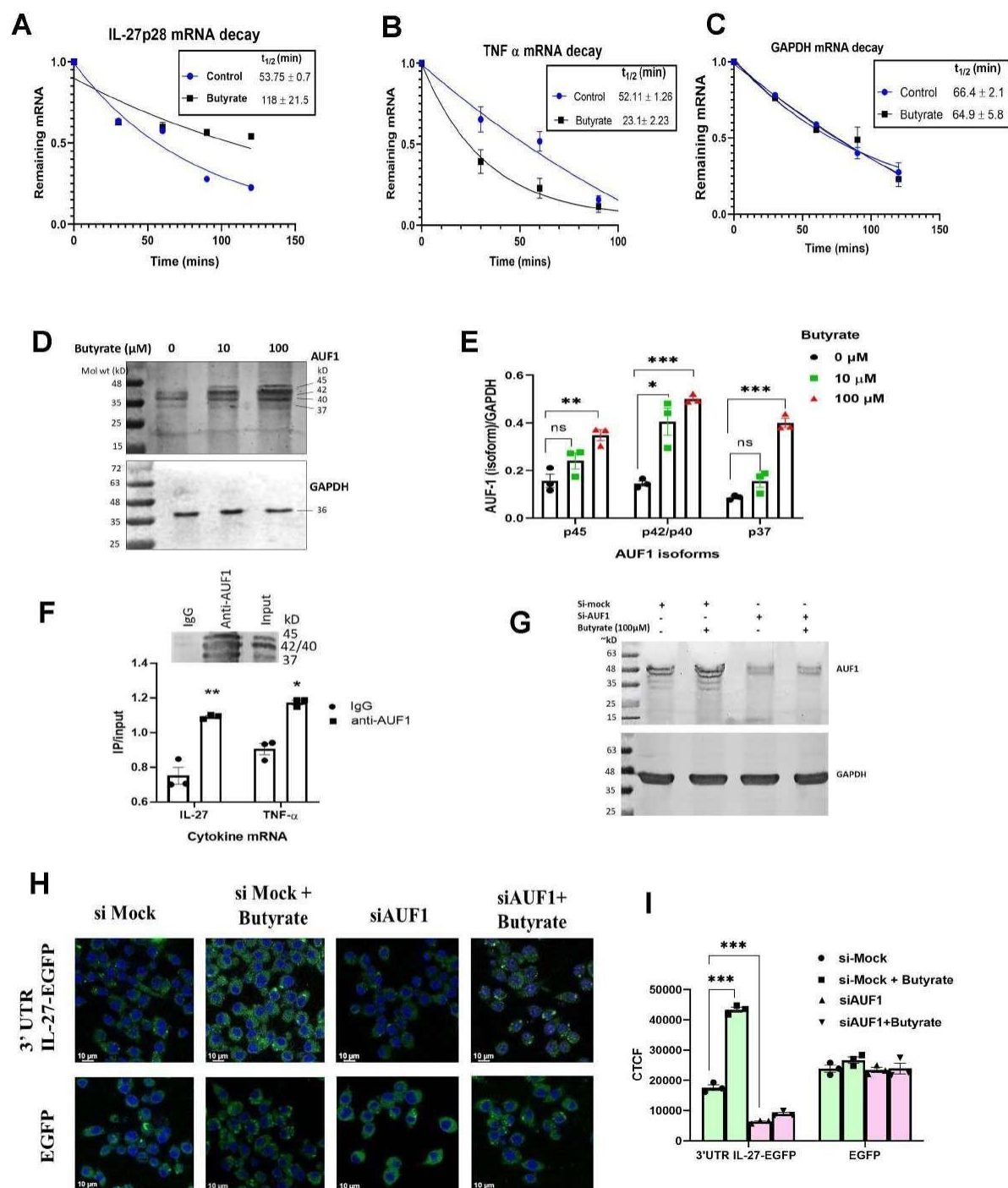


Fig 4: Butyrate regulates p28 mRNA stability by binding to 3'UTR of IL-27p28 mRNA

Splenocytes treated with or without 100 μ M butyrate for 72 h. Then Actinomycin D (10 μ g/ml) was added to the splenocytes and RNA was extracted at indicated timepoints to measure mRNAs by qPCR. The remaining IL-27p28 mRNA levels (A), TNF- α mRNA (B) and GAPDH mRNA (C) were measured by qRT-PCR and normalized to the results at 0 min and represented as a function of time. AUF1 isoform

expression in the splenocytes after 72 h of treatment with 10 μ M and 100 μ M butyrate by western blot (D). The corresponding densitometry showing relative AUF-1 isoform expression with respect to GAPDH (E). Whole cell lysates extracted from splenocytes were used for RNA-IP with anti-AUF1 Ab (inset, F). Both p28 and TNF- α mRNA were detected by qPCR in RNA extracted from the RNA-IP complex (F). RAW246.7 cells were transfected with either si-AUF1 or mock RNA and further treated with or without butyrate for 72 h. The AUF1 expression was measured by western blot (G). EGFP constructs carrying the 3'UTR of gene encoding p28 (3'UTRp28) or empty vector (pEGFP) was transfected into siRNA transfected cells and EGFP fluorescence was measured by fluorescence microscopy in si-mock, si-mock+ butyrate, si-AUF-1 and si-AUF1+butyrate (H). The relative fluorescence intensity was determined (I). All experimented were repeated thrice (n=3) and represented as Mean $\pm$ SE. p values calculated with respect to control by student's t-test.

Figure 5

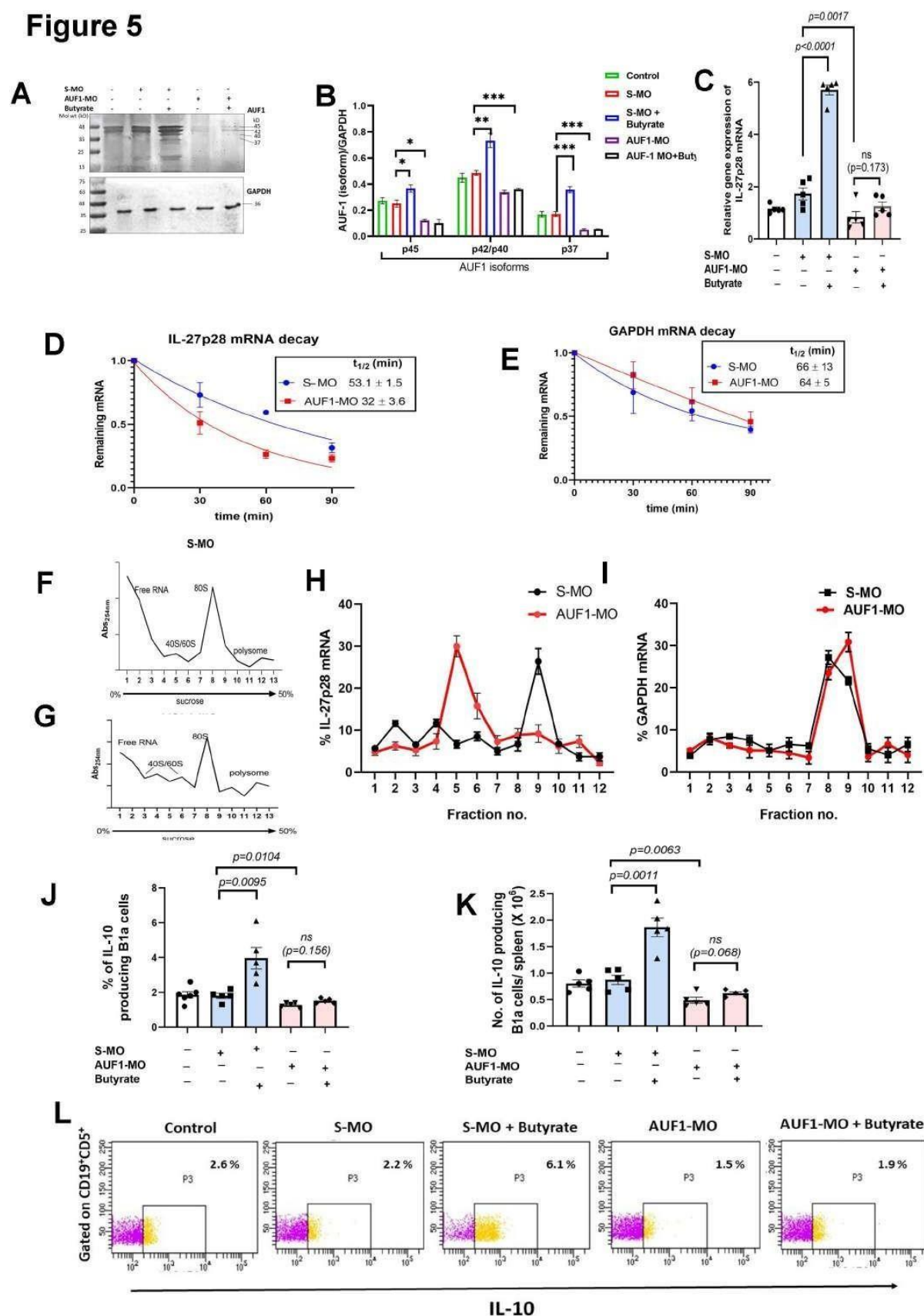


Figure 5: Effect of AUF1 knockdown (AUF1-KD) by Morpholino oligomer (GMO-PMO) on the half-life of IL-27p28 and B10 polarization

Mice were divided randomly into five groups having n=5mice/group: (i) mice without any treatment (ii) mice receiving scramble-MO (S-MO-mice), (iii) mice receiving S-MO fed with butyrate supplement (butyrate-S-MO-mice), (iv) mice receiving AUF-1-MO (AUF-1-MO-mice) and (v) receiving AUF-1-MO fed with butyrate supplement (butyrate-AUF-1-MO-mice). Western blot analysis of AUF1 in spleen in groups i-v (A), corresponding densitometric analysis of AUF1 isoforms expression normalized to GAPDH (Data represent >3 independent experiments. (B). Expression of IL-27p28 mRNA in spleen determined by qPCR in groups i-v (C). Splenocytes isolated from AUF1-MO mice and S-MO mice were treated with or without 100 μ M butyrate for 72 h and Actinomycin D (10 μ g/ml) was added. Thereafter RNA was extracted at indicated timepoints to study mRNA expression. The remaining p28 mRNA levels and GAPDH mRNA were measured by qPCR and normalized to the results at 0 min after Actinomycin D treatment (D, E) (n=3, t-test). To quantify the mRNA associated with ribosomal fractions from splenocytes were analysed by 0–50% continuous sucrose density gradient fractionation. Ribosomal RNA content, measured at 254nm, from S-MO mice (F) and AUF1-MO mice (G) were plotted against fraction numbers. RNA isolated from selected fractions were analysed by qPCR using appropriate primer sets for IL-27p28 (H) and GAPDH (I) (n=3). The splenocytes isolated from each mouse were treated with monensin for 8 h. Thereafter the cells were stained with antibodies against CD19, CD5 and IL-10. The intracellular IL-10 positive cells were detected with their respective antibodies, gated on CD19⁺CD5⁺ cells(L). Gating is shown in supplementary figure S4. The frequency (J) and absolute number (K) of IL-10 producing CD19⁺CD5⁺ cells are represented in the groups i-v. All experimented were repeated thrice with n=5 mice/group. (Data from J and K representation of 3 independent experiments having n=5mice/group, p value one-way ANOVA with post hoc Tukey's test). *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA with post hoc Tukey's test.)

The cells were then treated either with rIL-27 (20 ng/ml) or butyrate (100 μ M) for 72 h. Thereafter the resulting supernatant was collected and IL-10 was measured by ELISA (C). Experiment was repeated thrice each time with the cells from 3 independent mice from each group and represented as Mean $\pm$ SE. p value one-way ANOVA with post hoc Tukey's test.

Figure 7

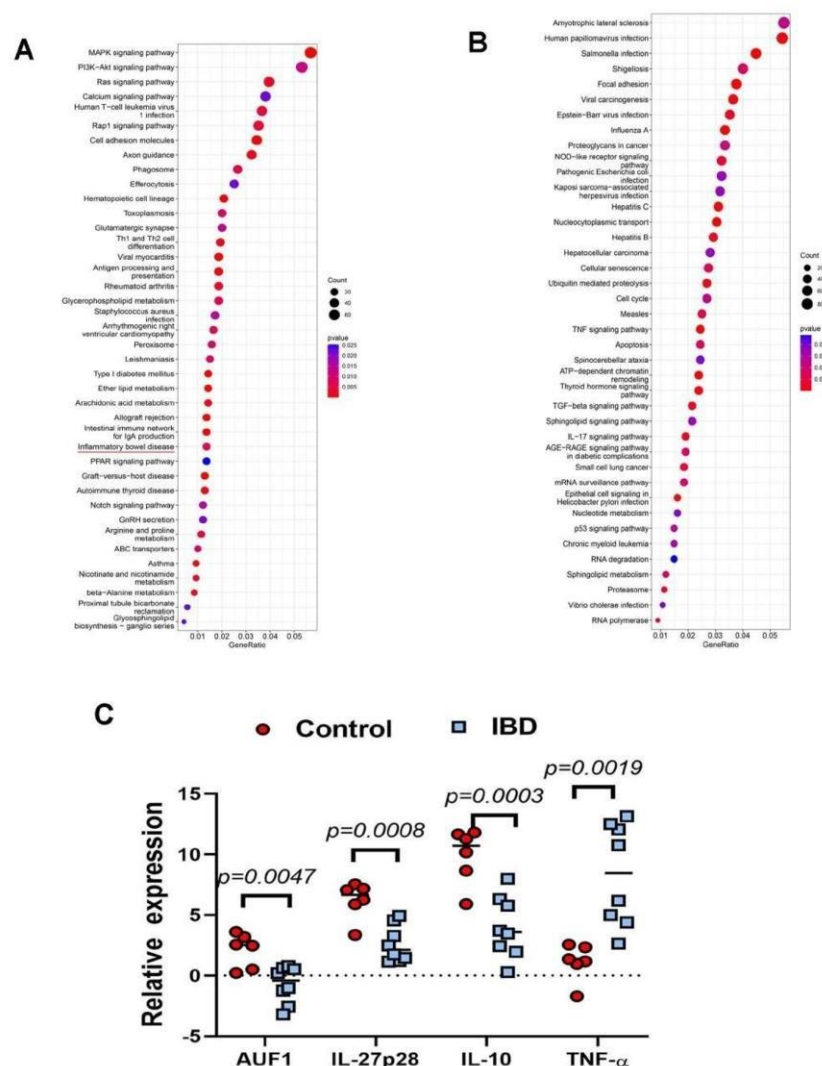


Fig 7. Analysis of web-source of micro-array data of AUF-1 knock out human cell line and AUF-1 status in Inflammatory Bowel Disease (IBD) patients

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of in AUF1 knock out skin cell line, WS1 vs control ([GSE138621](#)) was performed showing the upregulated (A) and downregulated pathways (B). The range of colour from blue to red represents the p value as indicated. To gain more insight in real clinical scenario, status of AUF-1 and inflammatory and anti-inflammatory cytokines were studied in the biopsy samples from 6 IBD patients (6 UC, 0 CD) and 6 non-IBD patients were collected from IBD clinic of SSKM hospital. The mRNA expression of AUF1, IL-27p28, IL-10 and TNF-a were determined by qPCR (C). The details of the patients are provided in Table1. Results are representative of triplicate measurements. The data is represented with Median. p values calculated with respect to control by student's t-test.

Figure 8

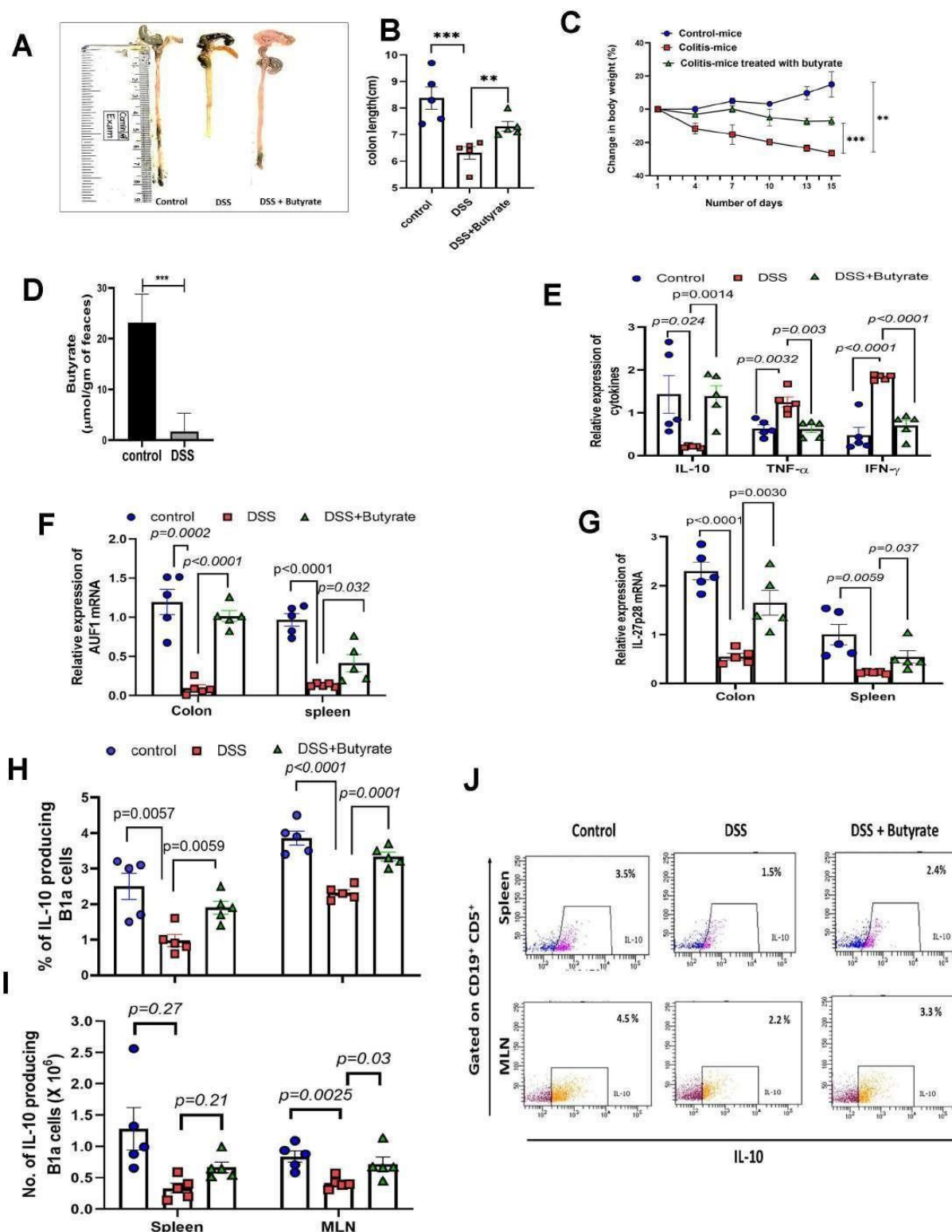


Fig 8 Butyrate protects DSS induced colitis in mice by increasing B10 polarization in spleen and mesenteric lymph nodes.

Mice were randomly selected and divided into 3 groups (n=5 mice/group). First group was fed with 2.5% DSS for 14 days (colitis-mice), second group was fed with 2.5% DSS plus 5% butyrate with normal chow diet for 14 days (butyrate-colitis-mice). Third group received normal drinking water and food served as a control (control-mice). On day 15, mice were sacrificed and following parameters were analysed from all the mice from each group: colon length (A, B), % change in body weight (C), faecal butyrate measured by LC-MS and expressed in $\mu\text{mol/gm}$ of faeces (D), expression of IL-10, TNF- α , and IFN- γ in the colon tissue of control-mice, colitis-mice and butyrate-colitis-mice was measured by qPCR (E). Expression of AUF1 (F) and IL-27p28 (G) in the spleen and colon tissue of control-mice, colitis-mice and butyrate-colitis-mice was measured by qPCR. The cells from spleen and MLN isolated from each mouse were treated with monensin for 8 h. Thereafter the cells were stained with antibodies against CD19, CD5 and IL-10. The intracellular IL-10 positive cells were detected with their respective antibodies, gated on CD19⁺CD5⁺ cells. The percentage (H) and absolute number (I) of IL-10 producing CD19⁺CD5⁺ cells are represented. The representative dot plots showing IL-10 producing CD19⁺CD5⁺ cells (J). The gating and % CD19⁺CD5⁺ cells of spleen and MLN were shown in supplementary figure S5. Data represented of 3 independent experiments with n=5 mice/group. p value calculated by one-way ANOVA with post hoc Tukey's test).

Figure 9

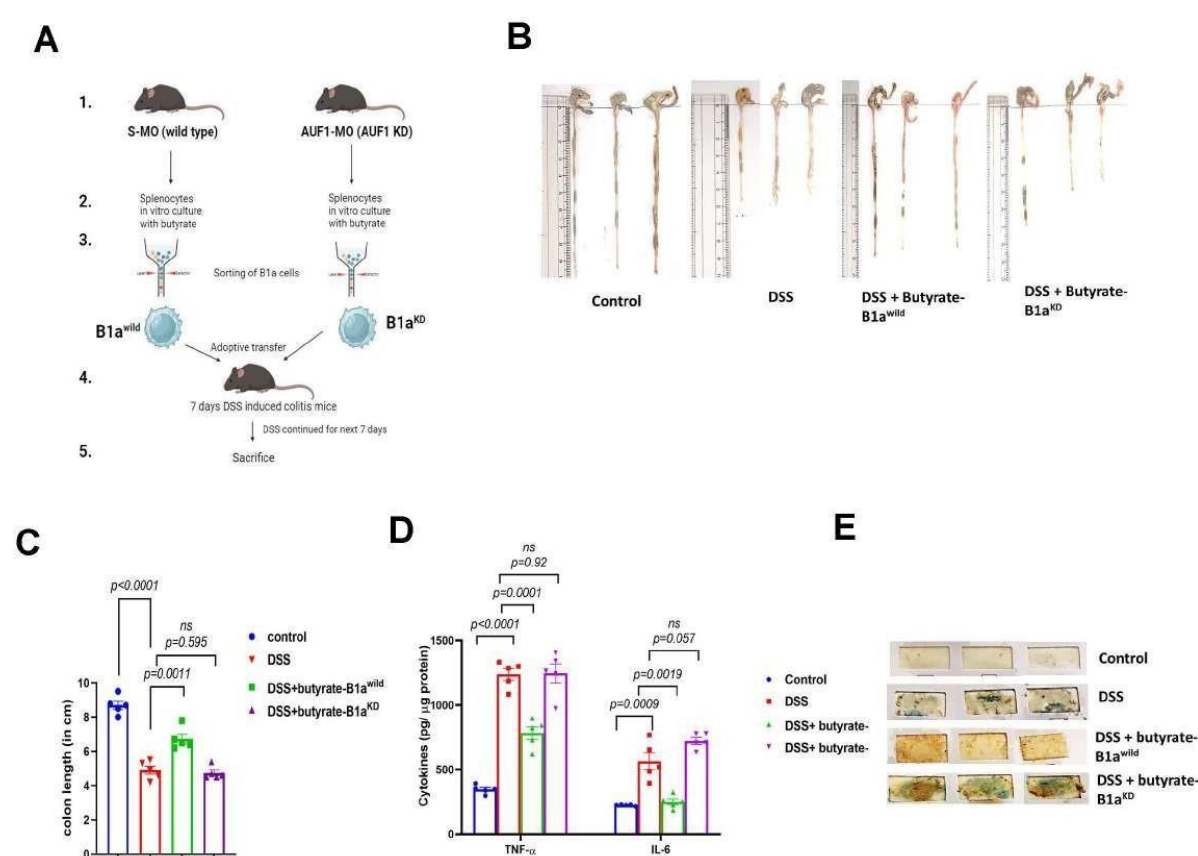


Fig 9: Butyrate treated B-1a from wild type mice but not the B-1a cells from AUF1 knockdown confer protection from DSS induced colitis

The experimental design is represented in five steps. A. Mice were injected with either S-MO or AUF1-MO as described in methodology. Splenocytes isolated from S-MO and AUF1-MO mice were then treated with butyrate (100 μ M) for 72 h in vitro. Thereafter cells were stained with antibodies against CD19 and CD5 and gated and sorted by flowcytometry. The pre and post sort analysis is represented in Figure 6A and B. Butyrate treated CD19⁺CD5⁺ cells from S-MO mice and AUF1-MO mice were represented as butyrate-B1a^{wild} and butyrate-B1a^{KD} cells respectively. Mice were fed with 2.5% DSS for 7 days and then injected with either 2X10⁶ cells butyrate-B1a^{wild} cells or 2 X 10⁶ cells butyrate-B1a^{KD} cells. The DSS administration was continued next 7 days. On day 15, the mice were sacrificed and following parameters were analysed: colon length (B) (C), expression of TNF- α and IL-6 in colon tissue as measured by ELISA (D) and occult bleeding (E). All experimented were repeated thrice with n=5 mice/group and represented as Mean $\pm$ SE. p value calculated by one-way ANOVA with post hoc Tukey's test).

Chapter 10:

***Abstract presentation at
various conferences***

➤ ***“Studies on synbiotic therapy diminish colonization and inflammation in ETEC induced murine diarrheal model”***

Sohini Sikdar*, Debmalya Mitra, Bidisha Pal, Paulami Dutta, Prolay Halder, Soumalya Banerjee, Hemanta Koley*, Shanta Dutta*

ASCODD 2022 16th Asian Conference on Diarrhoeal Disease and Nutrition November 11-13, 2022 | Kolkata, India.

➤ ***“The antagonist of Sphingosine 1 phosphate receptor, FTY720 impairs gut barrier function and increases the risk of inflammation”***

Sohini Sikdar*, Shanta Dutta**, Moumita Bhaumik**.

91st Annual Meeting of the Society of Biological Chemists (India), during 8th to 11th December, 2022, at the Biswa Bangla Convention Centre, Kolkata.