Intestinal Permeability, Nutritional Status and MicroRNA Expression in Children Living in Slum Area of Bangladesh

THESIS FOR DOCTORAL DEGREE (Ph. D.)

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By

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Abstract

Background: Intestinal barrier dysfunction is a global problem especially in poor settings like Bangladesh where malnutrition and recurrent pathogenic infections are very common. The intestinal epithelial barrier maintenance is the most crucial role of intestinal epithelial cells. Inflammation and immune system instability are linked to intestinal epithelial barrier disruption. In vitro mild irritants, pathogens, toxins, pro-inflammatory cytokines, and other stimuli have been shown to open tight junctions and enhance paracellular permeability. Chronic exposure to toxins and fecal pathogens on a regular basis can cause intestinal inflammation, which opens the barrier to inflammatory molecules including undesirable toxins, colonic bacteria, and bacterial antigens, so enhancing the immune response. Children in Bangladesh who have ongoing infections from *Bacteroides fragilis, Campylobacter jejuni*, enterotoxigenic *Escherichia coli*, norovirus, rotavirus, adenovirus, or enteric protozoa (giardiasis, cryptosporidiosis, amebiasis,) are at risk for stunted growth and poor nutritional status. According to these findings, colonization of pathogens may cause persistent inflammation and alterations in the gut microbiota that result in intestinal barrier disruption.

MicroRNAs are non-coding, 18–23 nucleotide RNA molecules that are small, highly conserved, and bind to the UTRs of mRNAs to regulate gene expression post-transcriptionally. MicroRNAs have been demonstrated to play critical roles during infection with viruses such the enterovirus, adenovirus and rotavirus, which cause gastroenteritis and alter the bacterial and enterocyte microbiota. Pathogens can directly adhere to and infiltrate the intestinal epithelial barrier when there is intense contact with them during an infection, which results in inflammation.

Numerous intestinal disorders have been linked to changes in expression of miRNA, which have been seen in samples that are easily accessible such as faecal and tissue samples. It is thought that nutrition, toxins, and other environmental variables also affect the expression of miRNA. However, further research is needed to fully understand how human miRNAs are modulated. In this study, we looked at the expression levels of two human miRNAs (microRNA-21 and microRNA-122) in faecal samples taken from Bangladeshi children with increased intestinal permeability (IIP).

Hypothesis: In this study we hypothesized that dysregulation of microRNA is associated with increased intestinal permeability which could be used as a potential biomarker for Intestinal barrier dysfunction.

Aim: The aim of our study was to determine whether persistent contact with several fecal pathogens causes IIP and reduced barrier function, which result in aberrant expression of microRNA-122 and microRNA-21.

Methods: This study was a nested observational study which was performed within a longitudinal birth cohort study in the slum areas of Mirpur, Dhaka, Bangladesh. For this study, a total of 442, 2-year-old children were chosen. Mothers who agreed to sign an informed consent form and the absence of any birth defects or congenital abnormalities were the inclusion criteria for the study. Children under or over the age of 2, children without any major congenital defects, and parents who would object to having their child's blood drawn were excluded from this study. After the children (n = 442) ingested the lactulose and mannitol solution, samples of urine were taken from these children over the course of 2 hours. Children were chosen for the analysis of miRNA expression. We examined 120 mg of stool from eighty-five children who were chosen randomly. For gene expression analysis of miRNA-122, we chose 42 children from the NIP (L:M ≤ 0.09) group and 43 children from the IIP (L:M > 0.09) group. Identical fecal samples from thirty-six (36) children— sixteen with IIP and twenty with NIP—were chosen for miRNA-21 gene expression study which was performed by real time quantitative PCR. The remaining forty-nine children, were not included for the miRNA-21 gene expression analysis because their stool volumes were insufficient for qPCR.

The fecal biomarkers REG1B and Calprotectin were also assessed in the same stool samples used to determine miRNA-122 and miRNA-21 gene expression and was measured using ELISA kits. Human Bio-Plex Pro Assays were used to measure the levels of IFN-γ, IL-2, IL-1β, TNF-α, IL-10, IL-5, and IL-13 in serum samples. The TaqMan Array Card (TAC) system real-time polymerase chain reaction was utilized to detect gut enteropathogens from the total nucleic acid isolated from faecal samples.

Results: Children with IIP had higher expression levels of miRNA-21 (fold change 10; $p < 0.001$, 95% CI: 5.05-10.78) and miRNA-122 (fold change 11.6; $p < 0.001$, 95% CI: 6.14-11.01) in their fecal samples than those children with normal intestinal permeability (NIP). The inflammatory cytokines IFN-γ, IL-2, IL-1β and TNF-α were found to significantly correlate with miRNA-21 and miRNA-122 levels in faeces ($p<0.05$). Campylobacter jejuni, rotavirus, Bacteroides fragilis, astrovirus, norovirus, adenovirus and various Escherichia coli strains (ETEC_STp, ETEC_STh, EAEC_aatA EAEC_aaiC,) were

frequently found in children with IIP ($p < 0.001$). Calprotectin and REG1B, two fecal inflammatory markers, strongly correlated with miRNA-122 ($p = 0.030$, and $p = 0.015$, respectively), whereas miRNA-21 did not show any correlation with these fecal biomarkers.

Conclusion: We conclude by demonstrating that children from Bangladesh who have IIP have significantly changed miRNA expression levels in their feces. Two stool miRNAs were strongly associated with disease activity and readily available surrogate biomarkers such fecal Calprotectin, REG1B, and inflammatory cytokine concentrations in the blood. This work also shows that even after long-term preservation, the miRNA expression levels in feces are quite stable and may be measured in a consistent manner. Overall, our results show microRNAs merit further investigation as possible potential biomarkers of gut barrier diseases, which is consistent with earlier findings that the miRNA expression levels in feces correspond with disease activity.

Abbreviations

AhR	Aryl hydrocarbon receptor
B.fragilis	*Bacteroides fragilis*
C.jejuni	*Campylobacter jejuni*
CD14	Cluster of differentiation
cDNA	Complementary deoxyribo nucleic acid
CRC	Colorectal cancer
DC	Dendritic cells
E. coli	*Escherichia coli*
EED	Environmental enteric dysfunction
FIT	Fecal immunochemical test
FOBT	Fecal occult blood test
GCSF	Granulocyte-colony stimulation factor
GM-CSF	Granulocyte/macrophage-colony stimulation factor
IBD	Inflammatory bowel disease
IFN-γ	Interferon-γ
IgA	Immunoglobulins A
IGF-1	Insulin-Like growth factor
IL	Interleukin
IL-1β	Interleukin-1 β
IP	Intestinal permeability
JAM	Junctional adhesion molecule
kDa	Kilo Dalton
L:M	Lactulose: Mannitol
LBP	Lipopolysaccharide binding protein
LBW	Low birth weight
LMIC	Low- and middle-income countries
LNA	Locked nucleic acid
LPS	Lipopolysaccharide
MAMPs/PAMPs	Microbial/pathogen-associated molecular patterns
miRNA	Micro ribo nucleic acid
MLC	Myosin light chain
MLCK	Myosin II kinase
NIP	Normal Intestinal permeability
NK cells	Natural killer cells
PBMCs	Peripheral blood monocytes
PPRs	Pattern-recognition receptors
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA

qPCR	Quantitative real-time polymerase chain reaction
REG1A	Regenerating family member 1 alpha
REG1B	Regenerating family member 1 beta
RISC	RNA-Induced silencing complex
RNA	Ribo nucleic acid
SD	Standard deviation
TAMP	Tight junction-associated-MARVEL proteins
TER	Transepithelial resistance
TJ	Tight junction
TNF-α	Tumour necrosis factor-α
UTRs	Untranslated regions
WASH	Water, sanitation, and hygiene
WBCs	White blood cells
ZO	Zonula occludin

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1.1 Overview

The intestinal tract's fundamental function is to digest and absorb nutrients. The largest immune organ, the digestive tract is also the largest repository for bacteria and endotoxins. The intestinal epithelium performs the role of a barrier enclosing the body from the outside world. The intestinal epithelial barrier maintenance is the most crucial role of intestinal epithelial cells. Inflammation and immune system instability are linked to intestinal epithelial barrier disruption. In vitro mild irritants, pathogens, toxins, pro-inflammatory cytokines, and other stimuli have been shown to open tight junctions and enhance paracellular permeability (1). The epithelial barrier disruption is also significantly influenced by enteric infections. Interleukin- 1β (IL-1β), interferon -γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are examples of pro-inflammatory cytokines secreted by immune and intestinal epithelial cells that in turn are stimulated by pathogens. The intestinal barrier may become dysfunctional and intestinal permeability may rise as a result of these pro-inflammatory cytokines (2). It has been established that children from low- and middle-income countries (LMIC), such as Bangladesh, are susceptible to intestinal epithelial barrier disruption. Chronic exposure to toxins and fecal pathogens on a regular basis can cause intestinal inflammation, which opens the barrier to inflammatory molecules including undesirable toxins, colonic bacteria, and bacterial antigens, so enhancing the immune response. Children in Bangladesh who have ongoing infections from *Bacteroides fragilis, Campylobacter jejuni,* enterotoxigenic *Escherichia coli,* norovirus, rotavirus, adenovirus, or enteric protozoa (giardiasis, cryptosporidiosis, amebiasis,) are at risk for stunted growth and poor nutritional status (3-7). According to these findings, colonization of pathogens may cause persistent inflammation and alterations in the gut microbiota that result in intestinal barrier disruption.

MicroRNAs are non-coding, 18–23 nucleotide RNA molecules that are small, highly conserved, and bind to the UTRs of mRNAs to regulate gene expression post-transcriptionally. Specific mRNAs are translated repressed or degraded as a result of the miRNAs ability to bind to their complementary sequences and act as post-transcriptional regulators (8). Due to the fact that miRNAs regulate a wide range of important physiological processes in eukaryotic cells, changes in their expression have been connected to a number of diseases. This has the potential to significantly alter gene expression and cause inflammatory diseases (9). Pathogens can directly adhere to and infiltrate the intestinal epithelial barrier when there is intense contact with them during an infection, which results in inflammation. This disruption of the tight junctional protein complexes, which are made up of proteins like occludin and claudins, causes intestinal diseases to start and progress (10). MicroRNAs have

been demonstrated to play critical roles during infection with viruses such the enterovirus, adenovirus and rotavirus, which cause gastroenteritis and alter the bacterial and enterocyte microbiota (11-14).

The aim of our study was to determine whether persistent contact with several fecal pathogens causes IIP and reduced barrier function, which result in aberrant expression of microRNA-122 and microRNA-21. We also looked into whether aberrant levels of microRNA-21 and microRNA-122 could affect the likelihood of intestinal inflammation. Children who live in areas with frequently subpar sanitation and subpar hygiene practices were evaluated. It is generally agreed that a small percentage of people continue to experience IBS-like symptoms following a known episode of intestinal bacterial or viral illness. Additionally, a variety of pediatric non-neoplastic disorders have been linked to dysregulation of miRNAs (15). Therefore, based on the results of this research, we can say that diseases, such as those that primarily impact the gut, can be caused by aberrant microRNA expression in children with an IIP (16, 17).

1.2 Hypothesis

We therefore hypothesize that dysregulation of microRNA is associated with increased intestinal permeability which could be used as a potential biomarker for intestinal barrier

dysfunction

1.3 Objectives

1.3.1 Primary Objective

1. Determine whether dysregulated Expression of miRNA-122 and miRNA-21 in children is associated with Intestinal barrier dysfunction

1.3.2 Secondary Objectives

1. Determine whether an altered/increased Intestinal permeability and/or intestinal barrier dysfunction correlates with gut infection and the dysregulation of microRNAs.
2. Determine whether the additional biomarkers (Calprotectin, Reg1b) correlate with intestinal inflammation and gut barrier
3. Determine expression levels of miRNA-122 and miRNA-21 with inflammatory cytokines

1.4 Significance of the study

Persistent exposure to multiple fecal pathogens leads to an increase in intestinal permeability and decreased barrier function that can cause an aberrant expression of microRNA-122 and microRNA-21. Prevention of early gut functional changes and inflammation may preclude the later adverse vicious cycle of malnutrition and infection. The miRNAs dysregulated during any possible inflammation could be potentially involved in the pathogenesis of intestinal related diseases and their correlation with other inflammatory biomarkers offers new possibilities to use them as disease biomarkers. Aberrantly expressed miRNA-122 and miRNA-21 and their association with intestinal barrier functions have not been investigated in human feces till date in Bangladeshi children. microRNAs in feces could correlate with disease activity and maybe considered as a potential tool for further biomarker research in gut barrier diseases

1.1 What is Intestinal Barrier

The intestinal barrier serves as a barrier between the external and internal environments. A healthy intestinal barrier allows fluids and nutrients to be absorbed while also preventing substances that are harmful such as bacteria and toxins from entering past the epithelium and into the underlying tissue (18, 19). Physical defense mechanisms, such as junctional complexes between adjacent epithelial cells and the mucosal surface of the epithelial cell lining, maintain a precise balance to sustain a functional barrier **(Figure 2.1).**

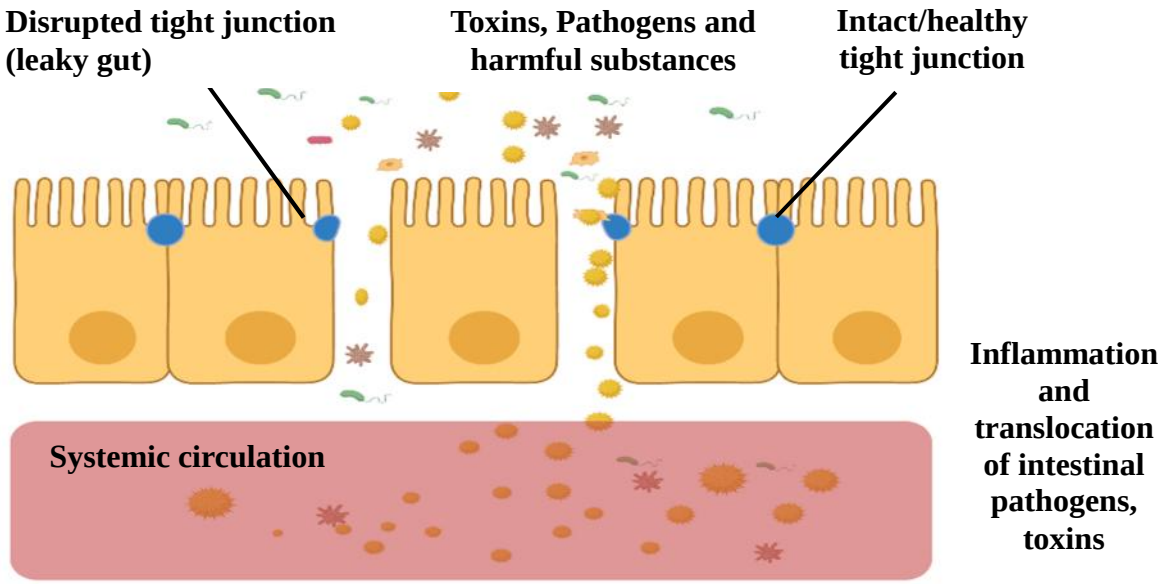


Figure Error! No text of specified style in document**..1** Tight junction disruption and the pathogenesis of intestinal diseases: In a healthy gut, epithelial tight junctions are present and function as a seal between nearby gut cells, allowing specific chemicals to enter and exit the intestinal epithelium and preserving homeostasis. Proinflammatory mediators, microbial gut dysbiosis, infections, certain foods, exposure to toxins or chemicals, stress, and epithelial tight junction disruption may increase intestinal permeability and damage the intestinal barrier by creating tissue lesions and punctures that may result in a leaky intestinal epithelium. This entire series of circumstances may result in the transfer of undesirable luminal gut content (microorganisms, poisons, undigested food particles), provoking an immune response in the host tissues.

A number of mechanical features of intestinal epithelial cells are required to maintain a working/functional barrier and form numerous defence mechanisms, the first of which is the lumen itself. Biliary fluids and stomach and pancreatic acids destroy antigens and bacteria here. Commensal luminal bacteria hinder pathogen colonization by producing bacteriocins, modifying the pH of the luminal content, and competing for resources essential for pathogen

growth. The commensal flora is constantly in contact with the intestinal epithelium, and it has been implicated in shaping the intestinal barrier structure by causing physiological paracellular permeability, which is necessary for food intake and mucus layer thickening (19-21). The microclimate, which contains the unstirred water layer, glycocalyx, and mucus layer, is the next line of defence (19, 22, 23). In close proximity to the epithelial cells, the mucus layer provides a protected environment for commensal bacteria. Mucus is made up of mucin, which is secreted and produced by goblet cells. In addition, IgA, one of the most prevalent antibodies in mucosal secretions, neutralizes pathogenic bacteria and promotes the maintenance of commensal flora through a variety of mechanisms (24).

The epithelium, a single cell layer that divides the body from the external luminal milieu, is the next component of the intestinal barrier. Paneth cells, enterocytes and goblet cells are among the cell types that make up the epithelium. The various roles that these cells play establish a tight barrier against the luminal milieu of the intestine (25). Antimicrobial peptides produced by Paneth cells aid in the removal of harmful bacteria, whereas enterocytes secrete chloride in response to unpleasant stimuli. T-regulatory cells, neutrophils, mast cells, and macrophages are a few examples of the adaptive and innate immune cells that make up the lamina propria, a layer of tissue that lies beneath the intestinal epithelium. In reaction to the invasion of foreign substances, these cells react quickly and work immediately to eliminate the inflammation. One of the earliest cells are neutrophils to reach inflamed sites and prevent microbe invasion via phagocytosis (26). Similarly, macrophages in the lamina propria are near to enterocytes and can phagocyte potentially harmful luminal substance that has made its way through the intestinal barrier and into the lamina propria (27). Due to their ability to reduce immune cell activation involved in intestinal inflammation and to promote immune tolerance to antigens obtained from food or commensal flora, T-regulatory cells play a significant role in immunological homeostasis (28). The endocrine and enteric nervous systems, which play a key role in gut barrier regulation, are also housed in the lamina propria. Mast cells, which are found near nerves can be triggered by neuronal mediators, have been linked to a variety of neuroinflammatory reactions as analysed by Keita et al (29).

The relationship between the intestinal epithelium and gut microbiota is crucial for maintaining gut barrier homeostasis. For gut homeostasis, the innate immune system, notably

pattern-recognition receptors (PPRs) produced in enterocytes (such as retinoic acid inducible gene I, and Toll-like receptors) is critical (30). Microbial signature molecules, also known as pathogen associated molecular patterns (PAMPs), are recognized by these receptors (31). The attachment of pathogen associated molecular patterns to pattern-recognition receptors triggers an inflammatory reaction to defend the host from foreign bacteria (32). Antigen presenting cells, such as macrophages and dendritic cells, can recognize foreign molecules due to this interaction. To defend the intestinal barrier, these cells move to the periphery, where they transmit antigens to T cells, causing the production of cytokines that are pro-inflammatory such chemokines, antimicrobial peptides and interferon gamma (IFN-γ) (33). The activation of tryptophan metabolism, which raises levels of indoleamine 2,3-dioxygenase enzyme and is essential for maintaining the intestinal epithelial barrier, is one of the important signaling cascades induced by IFN- γ. This enzyme converts tryptophan into kynurenine, an endogenous ligand for the transcription factor AhR, which, when activated, can reduce inflammatory reactions in the gut and protect against Inflammatory bowel disease (IBD) (34, 35).

The relationship between the host and gut bacteria is well balanced under normal conditions. However, in some disorders, such as inflammatory bowel disease, this balance is disrupted, resulting in intestinal inflammation (36). According to Luca et al., gut microbiota dysbiosis has been linked to a number of disorders associated with depression, including diabetes, leaky gut, and Alzheimer's disease (37, 38). These conditions have been linked to a decrease in the number of commensal flora strains that have been shown to have numerous positive benefits, such as Faecalibacterium. Fermentation of substrates that are non-digestible such as food fibers and endogenous intestinal mucus requires F. prauznitzii (38). Microbes that specialize in creating short chain fatty acids like butyrate thrive in the fermentation process. Human colonocytes use butyrate as their main energy source, which is necessary for maintaining the homeostasis of the intestinal epithelium (39). As demonstrated by research in cultured epithelial cells , butyrate has a role in improving intestinal barrier function, this could lead to an improvement in intestinal barrier function (40, 41). As a result, determining the composition of the microbial metabolites, gut microbiota, and inflammatory markers can be useful in determining the causes underlying a disrupted intestinal barrier.

1.2 Intestinal Permeability

The maintenance of a good barrier function, which allows nutrients, water, and ions to pass through but prevents pathogens and bacterial toxins from entering, is a critical function of the intestinal epithelium. Intestinal permeability is the increase intestinal transit of antigens and bacteria which results when the there is an alteration of the function of the intestine (42). As a result, increased intestinal permeability indicates a problem with the intestinal barrier function (43).

1.3 Epithelial Passage Routes

Solutes can travel between cells via the paracellular route or through cells via the transcellular route across the intestinal epithelium, as demonstrated in **Figure 2.2.** Depending on the solute's characteristics, transcellular passage can occur in a variety of ways. The pathophysiology of GI illnesses is thought to be heavily influenced by changes in how peptides move through the epithelium.

1.3.1 The Paracellular Route

The paracellular pathway refers to the path that connects cells via intercellular gaps and tight junctions (44). This pathway is employed by medium-sized hydrophilic, and protein sized molecules are generally impermeable, forming an efficient barrier against antigenic macromolecules. Junctional complexes, which include tight junctions, adherens junctions, desmosomes, and gap junctions, connect epithelial cells (45). Tight junctions, also known as zonula occludens, form a network of linking strands at the apical region of the lateral membrane. In addition to maintaining epithelial cell polarity, they participate in epithelial transport toward and away from the lumen (46). Tight junctions are multiprotein complexes embedded in the plasma membrane that communicate with the neighbouring cell. Occludin, tricellulin, claudin and Marvel D3 are transmembrane proteins (47). The claudin family has around 20 members, and their distribution in different tissues varies (48). Tricellulin, which is mostly found at three-cell contact points, permits big solutes (>10 kDa) to pass through. The amount of tricellulin expressed in cultivated epithelial cells controls macromolecular permeability. Positively charged molecules and ions can penetrate the tight junction permeability barrier more easily due to size and charge selectivity. ZO-1, ZO-2, and ZO-3, as

well as a number of other peripheral proteins including cingulin and symplekin, serve as scaffolding proteins that connect the tight junction with the cytoskeleton of the neighbouring cells (46, 49).

The adherens junctions, which are made up of molecules from the cadherin family, are located beneath the tight junctions. The apical junctional complex is made up of adhesion junctions and tight junctions that work together to form a single functional unit. The perijunctional F-actin ring connects this complex to the cytoskeleton (50). Desmosomes are thick adhesions that are found between epithelial cells, most typically below the adherens junctions. Desmosomes are made up of desmosomal cadherins such desmoglein and desmocollin, as well as connecting proteins including keratin and desmoplekin. Finally, there are gap junctions, which are intercellular channels that allow ions and tiny molecules to move between cells, therefore connecting the interiors of neighbouring cells. Paracellular permeability is a term used to describe TER measurements. The free flow of solutes and ions across the epithelium is correlated with TER, which can be known as the tissue's paracellular integrity (43). It's not unusual for TER to remain unchanged as paracellular permeability rises, or vice versa, during experiments. This most likely refers to different permeability pathway regulations, such as the pore and leak pathways, as defined by Shen et al. in 2011(51).

1.3.2 The Transcellular Route

The passive diffusion of lipid soluble and tiny hydrophilic substances through the cells is known as the transcellular pathway. Furthermore, the cell undergoes active and energy-dependent absorption. Endocytosis, which includes the cell invaginating the plasma membrane and creating vesicles, can be used to take up large particles and molecules that are not able to cross through the paracellular space or cell membrane, such as proteins and bacterial products. In studies that focus on intestinal barrier function, the transit of bacteria and bacterial products is examined in particular, together with paracellular indicators. Endocytosis is a process that allows the body to take in foreign antigens so that it can mount an appropriate immune response. Transcytosis is required for antigen surveillance in the Gastrointestinal tract after endocytosis because the substances that are engulfed are actively moved across the cytoplasm (52). Endocytosis and transcytosis are processes used by foreign

pathogens to gain access to the host, and a healthy barrier relies on the proper functioning of these pathways as well as the cell's ability to reject foreign substances. Depending on the nature of the substance taken up, endocytosis in epithelial cells can take place in a variety of ways.

The first pathway is through clathrin-mediated endocytosis, which is a highly selective receptor-mediated mechanism used mostly by viruses and immunoglobulins. Internalized molecules that have attached particularly to cells create clathrin-coated vesicles (53). The second method is phagocytosis, which involves molecule binding to cells via receptors and is employed by bacteria, viruses, and particles (54). Phagocytosis is involved in the uptake of antigens from the diet and bacteria, and it is initiated by invading bacteria's secreted solubles (55). Micropinocytosis, the third method, is a process that is non-specific allows extracellular fluid, as well as dissolved molecules, apoptotic cell fragments, bacteria, and viruses to be internalized. Micropinocytosis begins with the cell membrane invagination , which results in the creation of circular ruffles that are discharged in the cytoplasm as vesicles, or macropinosomes (56). The fourth path involves lipid rafts/caveolae, which are flask-shaped invaginations of cholesterol-rich microdomains within the plasma membrane that may contain caveolin, a coat protein (57). Certain enterotoxins and viruses have been demonstrated to be endocytosed into enterocytes via rafts/caveolae in studies (58).

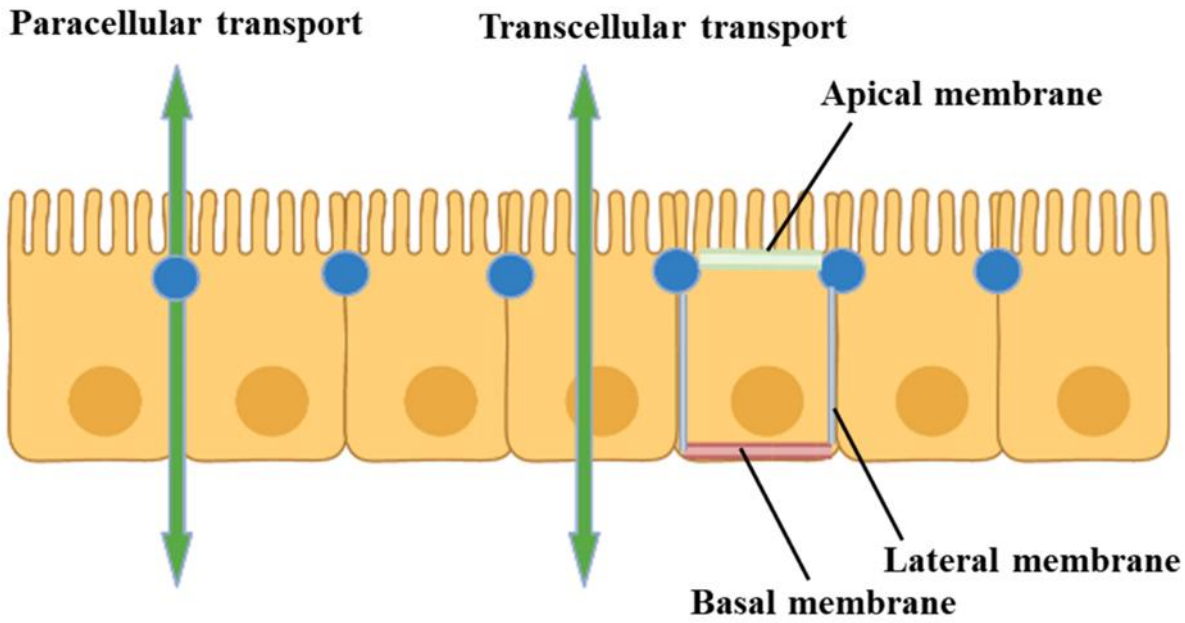


Figure Error! No text of specified style in document..**2** Transport processes across epithelium. There are 2 types of transport mechanisms of the epithelium**,** paracellular and transcellular transport. The intestinal lumen is in contact with the apical wall, which is characterized by villi. The tight junction complexes serve as the bonds between the epithelial cells. The term "paracellular transport" describes the movement of materials across the intercellular spaces between cells to cross an epithelium. It differs from transcellular transport, in which the substances move within the cell and pass through both

1.4 Causes of Increased Intestinal Permeability

1.4.1 Nonspecific feco-oral contamination:

Gut microorganisms play a role in the development or maintenance of Intestinal barrier dysfunction. Their relative importance varies depending on the geographical location, feeding practice and age. Children from cleaner Bangladeshi households had decreased risks of stunting and abnormal increased L:M ratio than children from less hygienic households (59, 60). An abnormal L:M test in Malawian children aged 3 to 5 was linked to insufficient access to latrines and low family water usage (61). However, a recent analysis of 14 water, sanitation, and hygiene (WASH) interventions trials on anthropometric outcomes for children aged 0 to 18 years found that WASH had only a minor impact on stunting and no effect on wasting or underweight (62). According to an observational research of young children and

infants in Zimbabwe, a major percentage of the oral and fecal contamination burden could occur not just from known sources (hand, water, and food), but also through direct consumption of animal feces and soil during exploration and play (63). A number of studies are now underway to determine the efficacy of various WASH strategies in the fight against Intestinal barrier dysfunction (64). Antibiotics have moderately favorable benefits in children growth in poor and middle income nations, according to a new systematic study (63). Intestinal barrier dysfunction has been incorporated in the results of several antibiotic investigations directed at specific pathogen species. During the transition period, complementary (weaning) meals put infants at risk for feco-oral infection, from exclusive nursing to complementary feeding, which often happens considerably sooner than the recommended 6 months (65). These can be made separately from family meals, stored for extended durations, and reheated insufficiently (66). The benefits of exclusive breastfeeding are lost during these crucial months of high energy need and development of immune system, and a confluence of poor nutrition and fecal and oral contamination occurs. During this time, the development of Intestinal barrier dysfunction is also noticed. For example, in Nepali children, switching from exclusive nursing to mixed feeding and then stopping breastfeeding completely are both related with deteriorating L:M ratios, while in Guatemalan infants, early breastfeeding termination has been associated with increased L:M ratios (67) . Although it's difficult to distinguish between the risk of nutritional deficit that comes with early breastfeeding termination, poor hygiene, which is commonly linked with complementary feeding, could be a probable reason of these observations.

1.4.2 Pathogenic infection

Increased paracellular permeability causes intestinal leakage, which is a prominent characteristic of the pathophysiology of many enteric pathogens and may ultimately increase the movement of intestinal bacteria into the body. Many pathogens interact with a specific component of the intestinal barrier. Several pathogens, for example, can disrupt epithelium tight junctions. Multiple intestinal parasites (Cryptosporidium, roundworm, and hookworm) have been associated to linear growth-faltering (68). In Brazilian and Ghanaian children with enteroaggregative *Escherichia coli* intestinal infection, elevated fecal lactoferrin was detected, as well as linear growth impairment in Brazilian children (69, 70). In children in underdeveloped nations, ETEC is a prevalent cause of acute watery diarrhea. Enterotoxigenic *Escherichia coli* is a multivalent enteric pathogen that produces heat-labile (LT) and/or heat-stable (ST) toxins, as well as more than 25 colonization factors (CFs). The heat stable phenotype of Enterotoxigenic *Escherichia coli* has been identified as the most common. In developing nations, *Escherichia coli* is the most known bacterial cause of infantile diarrhoea. *Escherichia coli* causes 41-46 percent of acute diarrhoea in Bangladesh. In Bangladeshi and Peruvian children, acute infection of rotavirus has been linked to increased L:M ratios (71, 72). Rotavirus is a serious public health issue and one of the leading causes of acute watery diarrhoea in children under the age of five. Electron microscopic study of duodenal samples from children with acute gastroenteritis identified this virus initially. Rotavirus is divided into seven categories, ranging from A to G. More than 90% of rotavirus gastroenteritis in babies and young children is caused by Group A. Rotavirus is spread by the faecal-oral route and has a low infective dosage. Rotaviral diarrhoea is a major cause of illness and mortality in children under the age of five. It is responsible for roughly 121,000 deaths per year in developing African and South Asian nations, as well as approximately 215,000 deaths per year in children under the age of five worldwide. Rotavirus is also the leading cause of diarrhoea and death in children under the age of five in Bangladesh. Rotaviral diarrhoea kills 1000-2700 children each year, according to the World Health Organization (WHO). Male gender, Low birth weight, 6-24 months age group (due to this age group's increased exposure to contaminated materials), poor food hygiene, children attending daycare, bottle-feeding, playing with toys, and low literacy status of mother are all risk factors for rotaviral infection in children.

Adenovirus, norovirus, and astrovirus infection may all have a role in microbiome dysbiosis

and alteration of intestinal homeostasis. *Campylobacter infection is a bacterial illness that is common in the gastrointestinal tract, especially in low- and middle-income nations. Children who live in poor living conditions are more likely to become infected, and their linear growth is generally stunted. Campylobacter was shown to be one of the most common enteric pathogens among the children who took part in the study, and it was linked to lower growth achievement at 24 months [2,12]. Campylobacter species are bacterial pathogens that are spread to humans by food, animal contact, water sources, and person-to-person transmission via the fecal oral route or fomites.*

The biological arsenal of enteropathogenic *Escherichia coli* (EPEC) is extensive. EPEC clings to epithelial cells surfaces and causes cytoskeletal protein buildup beneath the point of attachment, resulting in the typical attaching and effacing lesion. Effector molecules can be inserted into host cells by expressing a type III secretion machinery. These proteins cause a variety of cellular processes, including electrolyte secretion changes, TJ barrier breakdown, and inflammation. Enterotoxigenic *Bacteroides fragilis* (BFT) secretes an extracellular metalloprotease toxin that induces epithelial cells in the colon to expand without causing cell damage and changes the arrangement of actin filaments in intestinal epithelial cells. *Bacteroides fragilis* causes a "leaky epithelium" and stimulates active chloride secretion by cells, which may have a role in the pathophysiology of diarrhea in people (5).

1.4.3 Small intestinal bacterial overgrowth

The majority of nutrient absorption takes place in the small intestine, which is also where intestinal histological abnormalities can be found. When compared to the big intestine, the small intestine is comparatively sterile in good health. A condition called SIBO (small intestine bacterial overgrowth) can develop as a result of bowel stasis, as seen in muscular illnesses (73). SIBO diagnosis is done by sampling and fluid from the small intestinal lumen is cultured. Previous studies in 1980s it was discovered that fluid samples from small intestine in severely malnourished Gambian, Nigerian children and Indian adults, independent of the presence of diarrhea, were shown to be extensively contaminated with yeast and pathogenic bacteria (74, 75). SIBO was found in seemingly healthy newborns in Gambia, rising over the first few months when complementary foods were introduced, as exclusive breastfeeding ceased (76). Because the human body does not manufacture hydrogen unless through metabolically active intestinal bacteria, a reasonable method of identifying the bacterial

presence in the small intestine is by measuring the hydrogen concentration in exhaled air (77). After consuming a sugar substrate, there is a significant, late peak in the hydrogen concentration of the exhaled air, indicating that the sugar was digested by normally present bacteria in the large intestine. Abnormal bacterial presence in the small intestine is suggested by a smaller, earlier peak. This early peak has been linked to EED-like small intestine histologic alterations in slum-dwelling Brazilian newborns (78), urban Brazilian child poverty (79) , malabsorption and malnutrition in Burmese infants (80), and Chilean enteral vaccination failure. Antibiotics are an effective treatment for SIBO (81). Metronidazole, which is targeted at SIBO but also affects specific bacterial or parasite diseases like *Giardia*, helped Jamaican children recover from malnutrition (82), whereas the nonabsorbable oral antibiotic rifamixin, which is also targeted at SIBO, did not improve in the L:M ratios in children in Malawi (83). Study on probiotics are also available, however there is not much proof that they are useful in treating SIBO (73) , or that they can improve L:M ratios in children of Malawi (61).

1.5 Consequences of Increased Intestinal Permeability: Local and Systemic Inflammation

The intestinal barrier function is crucial for appropriate gut homeostasis, and its breakdown or dysfunction is linked to systemic and local effects, predominantly as a result of bacteria and bacterial products coming into direct contact with epithelial cells and spreading throughout the body. Through TLR4/MyD88-dependent signaling pathways, direct contact with bacteria or their products triggers the activation of immune cells in the lamina propria and causes the release of proinflammatory mediators which prolongs local inflammation. This activation is mediated by interactions with gut bacteria-derived LPS. Irritable bowel disease, ulcerative colitis, Crohn's disease, are a few of the gastrointestinal illnesses that are caused by local intestinal inflammation (84, 85).

The most detrimental effect of a disruption in the gut barrier is, however, the increased paracellular transport of LPS into systemic circulation. LPS interacts with immune cell surface receptors (such as TLR4) when it comes into contact with them and travels through the bloodstream coupled to either lipoproteins or LPS binding protein (LBP). This causes an inflammatory reaction. TLR4 needs CD14 as a cofactor because it is unable to bind LPS on its own. CD14 facilitates the transport of LPS to TLR4 and the modulation of LPS recognition by MD2 (86, 87). LPS binding protein shuttles LPS to CD14. The activation of

NF-B results in increased transcription of proinflammatory cytokines such as IL-1β ,TNF, and IL-6 as a result

of this cascade (88). Circulating LPS causes tissue inflammation by infiltrating activated macrophages or directly activating resident macrophages in peripheral tissues.

1.6 Impact of Increase Intestinal permeability in health and nutrition:

1.6.1 Impact on development and nutrition

Increase Intestinal permeability has been linked to stunting in children (low height-for-age) (89, 90). According to a 2008 comprehensive assessment of the effectiveness of existing supplemental feeding therapies for formal nutrition in 6- to 24-month-olds, they provided only average advantages, and intestinal barrier dysfunction may be a crucial reason limiting their effectiveness (91). Stunting in babies and children in low and middle income countries may not be entirely addressed by nutritional intervention alone (92). In addition to malabsorption, prolonged chronic intestinal inflammation can affect growth in a variety of ways. Appetite suppression is one potential mechanism, since it is often this, rather than food availability, that limits the food intake in children (93). Pro-inflammatory cytokines are known to affect the brain's appetite centers (94). Another hypothesized method is through the growth hormone insulin like growth factor 1 (IGF-1) axis, that has been well proven in Crohn's disease in children (95). A similar situation could arise in Intestinal barrier dysfunction, increased blood inflammatory markers were linked to decreased IGF-1 levels and stunting in Zimbabwean newborns (96). Stunting has a negative impact on a child’s health. It’s difficult to reverse once a child reaches the age of two, and it has long-term consequences for health and development (97). According to a meta-analysis published in 2007, 200 million children are stunted each year, preventing them from reaching their full developmental potential (98). Certain adult noncommunicable diseases are linked to childhood stunting, possibly due to epigenetic regulation or chronic inflammation (99).

1.6.2 Impact on immunity

The human gut is home to a sophisticated immunological surveillance system whose goal is to identify and destroy possible pathogens while avoiding unhelpful immune reactions to microorganisms that are nonpathogenic, food and other substances of the gut lumen that are harmless. Unusually large numbers of white blood cells (WBCs) in the gut walls of adults and children with Intestinal barrier dysfunction shows that this surveillance operation is under

strain. When the gut wall's barrier function is compromised (a feature of Intestinal barrier dysfunction), luminal contents cross the gut wall and immune system is activated. Chronic inflammation is induced, which might lead to growth failure and reduce antipathogen surveillance capacity. The exact nature of this persistent chronic inflammation is still unknown. This could be partly due to the fact that it is a challenging phenomenon to examine because of biopsy sample scarcity from the children who have an Intestinal barrier dysfunction. However, studies with mouse models of human disease has recently improved our understanding of mucosal immunity, and it's approaches based on animal model studies will enhance the understanding of intestinal barrier dysfunction and help guide public and clinical health management in the near future (100, 101).

1.7 Growth failure and stunting in altered Intestinal permeability

Children and infants whose lengths (or heights) are ≤ -2 SD (height-for age) as assessed by WHO growth guidelines are referred to as stunted. Chronic dietary deficits, recurring infection(s), or/and chronic inflammation are the most common causes of stunting in the developing countries. Children with familial or genetic forms of short stature, children with postnatal development failure due to metabolic or hormonal issues, and premature or small for gestational age infants are just a few examples of large cohorts of "stunted" participants. Indeed, development failure occurs in utero in 20% to 25% of "stunted" newborns and children: preterm and intrauterine growth restriction, especially when combined, raise the chance of postnatal stunting by two to seven fold (102, 103). This helps to explain why stunting is so widespread in developing nations, where typical length for age z scores at birth are about 0.5 and low birth weight (LBW) is 6 times higher than in developed countries. In the etiology of the growth of the infant post birth and LBW, maturity, the mother's health, and economic and social standing are all important factors. A history of severe or moderate maternal malnutrition, or pregnancy at an early age; stunting, maternal smoking; suboptimal pregnancy weight gain; and insufficient baby feeding practices are all risk factors for LBW and childhood stunting (104). The onset of Intestinal barrier dysfunction in early childhood is likely to amplify growth deficiencies that occurred throughout the perinatal and intrauterine periods, resulting in stunting. Because quickly growing infants and children are vulnerable to infectious, toxic and nutritional environmental exposures, stunted children's length for age z scores frequently fall from birth to between eighteen and twenty-four months of age. The imposition of Intestinal barrier dysfunction, in combination with deficits in nutrition

experienced during fetal, peri-natal, and early post-natal life, may limit nutrient delivery and utilization, impairing the proliferation and maturation of small intestinal epithelial cells, skeletal myocytes, renal nephrons, growth plate chondrocytes and pancreatic cells (105, 106).

1.8 Emerging approaches for the treatment and prevention of Increase Intestinal permeability

Intestinal barrier treatment is challenging. First, it can be difficult to identify Intestinal barrier dysfunction in an individual when reliable point-of-care biomarkers are not available. Furthermore, clinical trials have found no conclusive evidence that specific treatments can cure or alleviate the symptoms and signs of Intestinal barrier dysfunction. The best strategy to prevent intestinal barrier dysfunction is to "clean up" the environment because the condition is rooted in environmental factors. Implementing this idea will be difficult because 13% of people still defecate in the open and water scarcity still affects 40% of the world's population (United nation 2015). Additionally, just a quarter of the world's population has access to clean drinking water. Drinkable water, better hygiene practices and basic sanitation, break the chain of harmful bacteria colonizing the small intestine causing Intestinal barrier dysfunction. Infact, a research study conducted in Bangladesh discovered that providing a clean environment boosted population height for age by 0.54 standard deviations when compared to children living in poor conditions (60). Additionally, a 22% decrease in stunting prevalence was observed. More recently, Mali's sanitation program discovered that while better access to toilets did not reduce the prevalence of diarrhea, it did promote childhood development, especially in infants and children under the age of two (107). Lower chronic exposure to harmful microorganisms, resulting in reduced intestinal barrier dysfunction prevalence or severity, could be one explanation. Preventing intestinal barrier dysfunction requires ensuring hygiene at vital periods. According to a study in Bangladeshi villages and slums, 40 percent of supplemental foods were contaminated with *Escherichia coli*, resulting in increased rates of malnutrition and diarrhea (107, 108). Currently it is thought that deficiency in zinc, which is common in developing nations, coexists with intestinal barrier dysfunction and worsens the condition. Given that children in impoverished nations get extremely low zinc through diets, the need of supplementing, either long-term or at the very least as part of diarrhea treatment, cannot be overstated. In South African children, the idea that exclusive nursing helps lower gastrointestinal inflammation was recently validated.

In conclusion, new research suggests that a combination of the following factors can minimize

the prevalence, incidence, and severity of intestinal barrier dysfunction:

1. Improved hygienic practices and access to clean water in low-income nations;
2. Availability of sanitary restrooms and modifications to societal attitudes around their use;
3. Breastfeeding during the first six months and maintained breastfeeding beyond; and
4. Supplementation of zinc

Stunting is a defining feature of Intestinal barrier dysfunction, preventing and treating the disorder, as well as its repercussions, should be a top priority. Increased access to appropriate and enough meals incorporating foods from animal source, supplementation of zinc, and effective treatment of recurring diseases such as pneumonia and diarrhea are all therapeutic and preventive interventions that can be taken in the setting of poor socioeconomic conditions. Preventing intestinal barrier dysfunction at a population level will necessitate proper health and nutrition management for all girls and women of reproductive age prior to, during, and after pregnancy. It has been seen that supplementing children who are moderately or severely malnourished with nutritious ready-to-eat meals has been demonstrated to improve clinical recovery in situations of significant food insecurity (109, 110). Research is now being done on agents that can reduce or balance chronic inflammation in the gut mucosa. These include 5-aminosalicyclic acid, which was found to be ineffective in a recent research (110) budesonide, a nasal steroid, and oglufanide disodium, an immunomodulatory small molecule. Only well-designed, randomized controlled studies that analyze efficacy and side effects in areas where stunting/intestinal barrier dysfunction is common can lead to effective and safe treatments.

1.9 microRNA

1.9.1 The discovery of miRNAs

With the finding of the developmental regulator lin-4 in the nematode Caenorhabditis elegans over thirty years ago, the first miRNA was found. Originally thought to be a traditional protein coding gene, the Ambros and Ruvkun laboratories discovered lin-4 really codes for a 23 nucleotide regulatory RNA rather than a protein (111). They showed that the LIN-4 RNA may base pair with the LIN-14 mRNA, a different gene in the C. elegans developmental network, and influence the LIN-14 protein's production. This finding would not have had much of an impact outside of the C. elegans research community if a second miRNA, let-7, had not been found. Let-7 is also conserved throughout a wide range of organisms, including

humans, implying that this class of short regulatory RNAs has a broader function in biology (112). Another unexpected development at the same time was the identification of the RNAi pathway, more precisely the 21 nucleotide RNA triggers of the silencing mechanism. These two routes were later discovered to represent two arms of the same gene silencing pathway. Many thousands of miRNAs have now been found in many organisms, with the human genome now including 2588 annotated miRNAs. Given that each miRNA is projected to affect the expression of hundreds of target genes, the miRNA pathway as a whole represents an important method for controlling gene expression.

1.9.2 microRNA processing

MicroRNAs are short, non-coding Ribonucleic acids of 18–24 nucleotides length that control the expression of genes post transcription. The nucleus is the starting point of a complicated multi-step process called miRNA biogenesis **(Figure 2.3)**. In order to generate mature miRNAs, RNA polymerase II first produces a primary miRNA (pri-miRNA), which is then cleaved by enzyme Drosha which is a RNase III endonuclease, forming a precursor miRNA or pre-miRNA (113). The cytoplasmic endonuclease Dicer cleaves the pre-miRNA to generate a mature miRNA duplex(114). A mature miRNA's one strand is attached to the Argonaute (Ago 2) protein to form a RNA-induced silencing complex (RISC) (115, 116). The mature miRNA directs the Ago 2 protein and its associated components to specific target sites in the 3' untranslated region (UTR) of mRNA in order to carry out its activity. The degree of complementarity between a miRNA and its target mRNA dictates whether translation is suppressed or a target mRNA's stability is decreased. Translation initiation or translation elongation processes is inhibited ,promoting deadenylation (117). Thousands of target genes participating in various pathways are regulated by miRNAs, which also play a significant role in a wide range of developmental and cellular processes (118). Due to the fact that miRNAs control a wide range of crucial physiological processes in eukaryotic cells, changes in their expression have been connected to a number of diseases. This has the potential to significantly alter gene expression and cause inflammatory diseases.

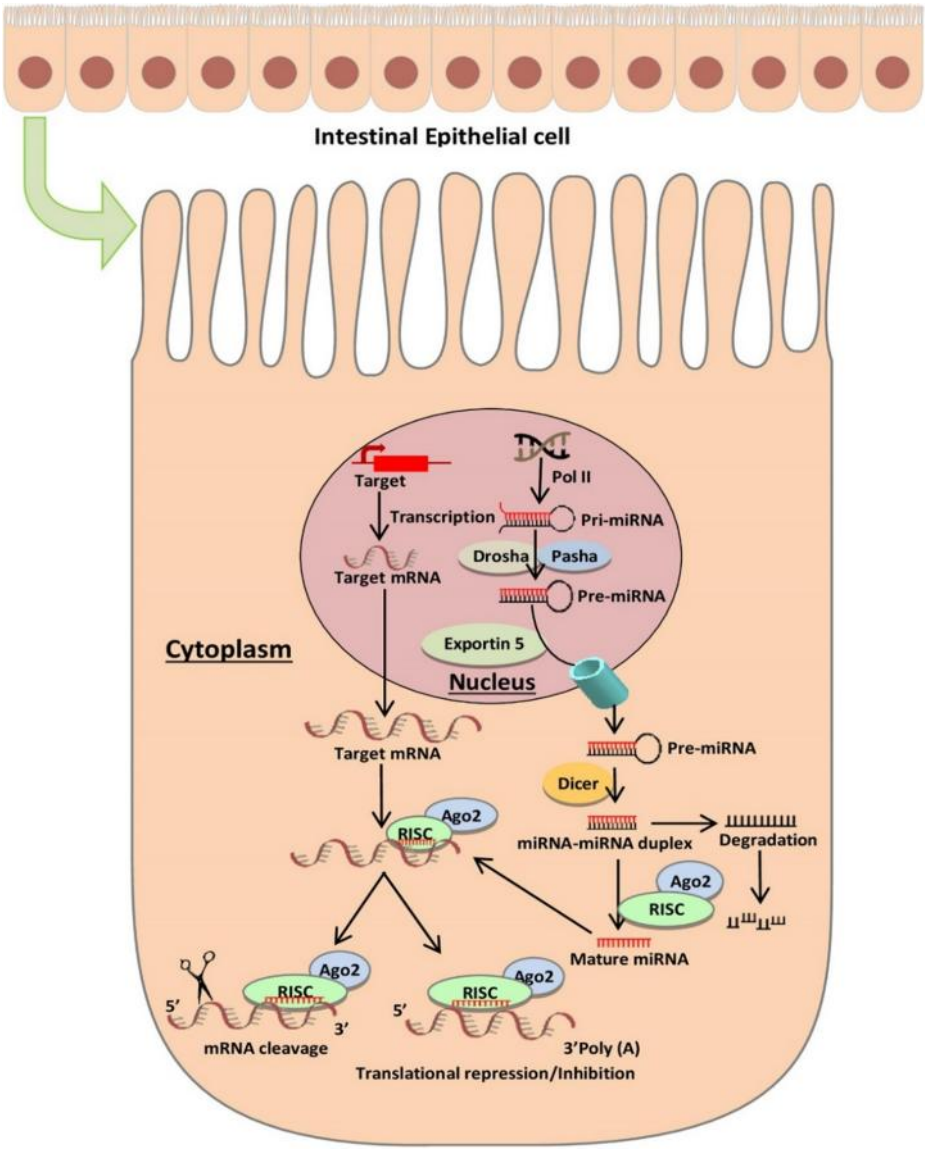


Figure Error! No text of specified style in document.**.3** Biogenesis and mechanism of action of miRNA: The primary miRNA (pri-miRNA) is generated when RNA polymerase II transcribes the miR genes in the nucleus. Drosha (RNase III endonuclease), in turn, cleaves the pri-miRNA to form a precursor miRNA (pre-miRNA). A duplex miRNA complex is formed when the cytoplasmic endonuclease Dicer cleaves the pre-miRNA. The mature miRNA binds with mRNA to cause translational repression or mRNA degradation by inducing the nuclease activity of RISC.

1.10 Fecal microRNAs as potential biomarkers for Intestinal Barrier Dysfunction

The complex anatomical and functional intestinal epithelium barrier protects the gut lumen from toxins and microbial invasions while allowing for the selective absorption of vital fluids and nutrients (119). Various intestinal disorders, marked by an increase in intestinal permeability, can be triggered by the disruption of this barrier (1). People living in low and middle income countries (LMICs), where there is a frequent inadequate sanitation and hygiene practices are subpar, are more likely to have intestinal epithelial barrier dysfunction (5, 120) . Different studies showed that Intestinal permeability is altered in children with enteric dysfunction, and this can lead to IBS and other inflammatory gut illnesses (121). To protect the integrity of the gut epithelia, it is vital to stop the invasion of harmful microorganisms. Repeated contact with irritants, toxins, fecal pathogens, and proinflammatory cytokines can compromise the tight junction's ability to function as a barrier, leading to inflammation and making it easier for pro-inflammatory molecules like bacterial antigens, unwanted toxins and colonial bacteria to pass through and aggravate the immune response (1, 122). Numerous studies have demonstrated an association between infections brought on by protozoa, enterovirus, adenovirus, astrovirus, rotavirus, and enterotoxigenic *Escherichia coli* and the dysregulated expression of host miRNAs, which has a significant effect on a child's development and growth (11, 123-127). miRNAs are essential for understanding infection-related intestinal problems because of their role in regulating inflammatory responses and, in turn, gastrointestinal ailments (128, 129).

According to recent research, the host's inflammatory response to dysbiosis may be mediated by fecal miRNA (130). An earlier investigation revealed the ideal balance between immunity and host metabolism and microbiome composition (131). Alterations or changes in the composition of the gut microbiota are linked to intestinal barrier failure, which can result in IBD and colorectal cancer (CRC) (132-134). Additionally, it has been demonstrated that miRNAs are crucial in controlling viral infections including enterovirus, rotavirus which mostly causes gastroenteritis, and changes in enterocytes and bacterial microflora (11, 135).

miRNAs have received a lot of attention in recent years as vital regulators of inflammation. Diseases, particularly those that primarily affect the gut, can be caused by aberrant miRNA expression **(Figure 2.4).** According to Cichon et al. (2014), a typical Dicer1-deficient mice model (i.e., the absence of mmu-miR-192) has reduced intestinal barrier function, which causes spontaneous intestinal inflammation. Notably, miRNAs like miR-122 and miR-21

target negative regulators of immune response to promote inflammation (127, 136, 137). Numerous studies have found that the miRNA expression in intestinal tissues, enterocytes, and cultured cells may be significantly increased by inflammatory cytokines such as TNF-α and IFN-γ (138). According to Lin et al., TNF-α increased miR-21 expression, which greatly improved barrier permeability. Increased intestinal permeability and barrier may result when proinflammatory cytokines are activated.

Intestinal barrier dysfunction is caused by a variety of pathophysiological processes that are still poorly understood. Despite the fact that miRNA has primarily been investigated in vitro using cell lines, there has been an increase in interest in how microRNAs can operate as a biological marker to identify intestinal barrier dysfunction in recent years. It is significant to note that stool miRNA may function as possible biomarkers for the diagnosis and detection of many intestinal illnesses in patients in underdeveloped countries because of its preservation, stability, and high abundance.

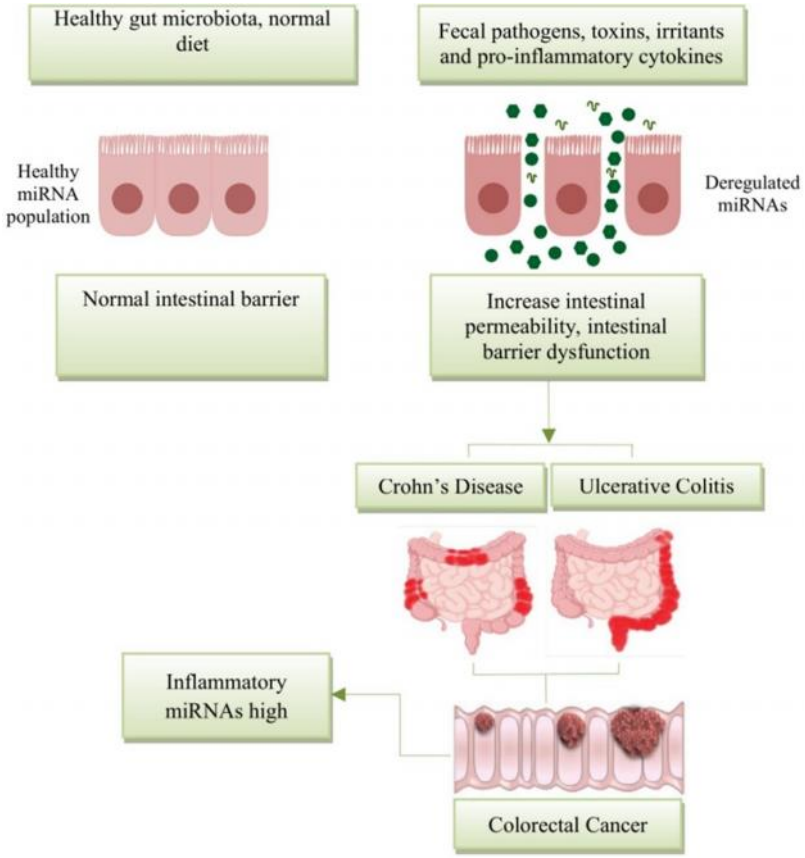


Figure Error! No text of specified style in document.**.4** The potential function of miRNA expression in intestinal disorders is depicted schematically. Controlling gene expression in both health and disease, the miRNA network is essential. miRNAs have a crucial role in controlling inflammation. Persistent inflammation following intestinal barrier disruption can alter the expression of microRNA, leading to the onset or development of IBD. When IBD prolongs in a patient, the chronic dysregulation of miRNAs targets transcripts that encode intestinal barrier components and tumor-suppressor miRNAs, can cause disease to progress from Inflammatory bowel disease to CRC.

1.11 Fecal microRNAs as Potential Biomarkers for different Intestinal Diseases

Numerous disorders, including hepatitis, gastrointestinal diseases, cancer , neurodegenerative, and cardiovascular disease are associated with miRNA deregulation (139, 140). According to studies, miRNAs can be secreted in exosome-encapsulated form in stool as well as in bodily fluids such urine, serum, saliva, and breast milk (141). miRNAs are protected from destruction by exosomes, which also serve as signaling molecules for mediating cell to cell communication (142, 143). miRNAs in fluids and feces are stabilized and protected from RNase destruction by exosomes (144). The identification of prognostic and therapeutic biomarkers for a variety of diseases has been aided by the specific and sensitive detection of microRNAs in the extracellular environment. MiRNAs have been

verified as possible biomarkers of non-neoplastic and neoplastic disorders in studies that have shown the relationship between miRNA dysregulation and disease progression.

Early disease detection is critical since it can reduce the risk of malignancies and enhance prognosis. Consequently, it is essential and crucial to create a technique that is safe, inexpensive, and has a high level of specificity and sensitivity. Colonoscopies, flexible sigmoidoscopies, faecal immunochemical tests (FIT) and faecal occult blood tests (FOBT), are methods for screening IBD and CRC in feces. Although sigmoidoscopies and colonoscopies are effective screening methods for identifying CRC and IBD (145, 146), their invasiveness and high cost make them less likely to be used. FOBT is a better method to screen for CRC and IBD since it is non-invasive and frequently identifies blood in stools(147); however, FIT, is which uses an antibody targeting human haemoglobin to measure the concentration of haemoglobin in feces, is preferred over FOBT as a non-invasive technique. These techniques either offer high specificity or sensitivity but not both (148, 149).

Disease-specific microRNAs can be discovered even after years of long-term preservation of fecal samples (150) , due to their remarkable stability and reproducibility. miRNAs are studied for their potential role as non-invasive clinical biomarkers due to aberrant expression of faecal miRNAs in CRC and IBD. RNA from human faeces has been extracted as shown in earlier studies, and miRNAs have been discovered as possible biomarkers for pancreatic and colon cancers (151, 152) . Another study found that miR-106a and miR-21 are substantially expressed in patients with CRC when total RNA from faeces is isolated, suggesting the viability of possible biomarkers for developing non-invasive screening tests for colorectal neoplasia. miRNA signatures may help predict cancer prognosis in addition to aiding in cancer diagnosis, which includes differentiating site or tissue origin , subtypes of cancer, and detection of cancer at an early stage, as recently suggested by different studies (153, 154). It is necessary to assess and validate possible prognostic biomarkers of CRC (155), such as the expression of faecal miR-21, in well-defined large cohorts of carefully chosen cases.

The expression of miRNAs in the intestine is dysregulated and is linked to a number of diseases, and this information can be used to create new miRNA biomarkers. As an illustration, faecal miR-1246 and miR-223 are observed to be increased in the feces of active Inflammatory bowel disease patients, indicating that active IBD patients have their expression profile of fecal miRNA composition altered when compared to controls (156).

Another study found that faeces had high levels of miRNA-155, miRNA-223, miRNA-21 expression. The intestinal epithelial barrier disruption is proved by miR-21 overexpression. The ROC curve analysis done on miR-223 in stool revealed the diagnostic precision for active Ulcerative Colitis (AUC: 0.93), with a sensitivity and specificity and of 90 and 86.7%, respectively (157-159). With a specificity and sensitivity of 81.08 and 86.05% (AUC: 0.829), respectively, Bastaminejad et al. (2017) demonstrated that expression of microRNA-21 in feces can be a viable biomarker for colorectal cancer detection.

Initially, expression of microRNA in the faeces of patients with CRC, Ahmed et al. (2009) found that 7 miRNAs, that include miRNA-106a, microRNA-21, microRNA-20a, microRNA-203, microRNA-96, microRNA-92, and microRNA-326, had elevated expression and 7 other microRNAs; microRNA-126, microRNA-320, microRNA-484-5p, microRNA-145, microRNA-143, microRNA-125b, and microRNA-16 had decreased expression, in the faeces of patients with CRC. Ahmad et al. also chose a panel of faecal microRNAs, of which twelve (microRNA-17, microRNA-7, microRNA-21, microRNA-20a, microRNA-96, microRNA-92a, microRNA-134, microRNA-106a, microRNA-196a, microRNA-183, microRNA-214 and microRNA-199a-3p,) had increased expression in patients with CRC and eight miRNAs (microRNA-29b, microRNA-9, microRNA-127, microRNA-143, microRNA-138, microRNA -146a, microRNA-938, and microRNA-222) had decreased expression in patients with CRC. With great specificity and sensitivity , these miRNAs could distinguish between CRC occurrences and healthy controls as well as between various TNM stages (160). The 2 microRNAs; microRNA-18a and microRNA-221 , which are expressed highly in the feces of patients with CRC and have a specificity and sensitivity of 75 and 66 %, respectively (AUC: 0.75), have the potential to be utilised as a non-invasive biomarker for screening CRC, according to a comparison study of faecal miRNA expression profile by microRNA microarray (161). The expression levels of microRNA-224, microRNA-29, and microRNA-223 have also been found to significantly decline in CRC patients, but miRNA-29 is overexpressed in patients with rectum cancer (162). According to the ROC curve analysis, the miR-29a AUC value was 0.777, with a specificity of 61% and sensitivity 85%; miR- 223 AUC value was 0.649, with a specificity of 71% and sensitivity 60%; and miR- 224 AUC value was 0.744, with a specificity of 63% and sensitivity of 75%. Being a strong biomarker for IBDs and CRCs, miRNAs in particular offer the potential to be utilised as a non-invasive

biomarker to identify a variety of intestinal disorders with an increased specificity and sensitivity.

1.12 Experimental methods for fecal miRNA isolation, detection and quantification

Being a good biomarker for IBDs and CRC, miRNAs in particular can be used as a non-invasive biomarker to diagnose a variety of intestinal disorders with a high level of specificity and sensitivity

Almost all bodily fluids, including faeces, have been found to contain microRNAs in a stable state (163, 164). miRNA stability is crucial in determining the miRNA expression profile and makes precise measurement and detection possible. To examine the changed miRNA expression profile and identify targets in various intestinal disorders, a number of methods have been established. The promise of miRNAs in diagnosis, prognosis, and therapy is revealed by the sensitive and precise methods for extraction and detection.

1.12.1 Isolation of miRNA from fecal samples

There are numerous techniques for miRNA isolation from biofluids, such as faecal samples, that are used in various studies (116, 165, 166). Numerous studies have concentrated on the technical optimization of miRNA isolation in order to generate high-quality miRNA. MiRNA extraction from stool samples can be done easily and effectively using guanidinium-phenol-based solutions, such as the TRIzol reagent. The cost-effective TRIzol approach produces high-quality miRNA, gets rid of the inhibitors, and doesn't require specific equipment (167). The most used technique for isolating total RNAs, including miRNAs, involves applying the TRIzol reagent after bead beating and alcohol precipitation. However, there is a chance that DNA, phenol, proteins or lipids will be cross-contaminated during miRNA extractions utilizing the TRIzol method. It has been discovered that combining TRIzol-based lysis and spin column-based isolation produces high, ultra-pure miRNA for delicate downstream operations. Intrinsic and exogenous inhibitors can be effectively removed using the miRNeasy extraction kit in conjunction with phenol/guanidine-based lysis, and RNA longer than 18 nucleotides can be purified for use in subsequent steps. Other commercially available kits, such as Norgen's Stool Total RNA Purification Kit, E.Z.N.A^{TM} stool RNA Kit (OMEGA, GA, United States), and the Macherey-Nagel's NucleoSpin RNAs stool kit, have been utilized in various research to extract ultra-pure, high-quality miRNAs. The immunomagnetic beads coupled with the target antibody are utilized for exosome isolation and exosomal RNA extraction. For use in later processes, the isolated exosomes are

homogenized and miRNAs are extracted using RNA isolation kits. High purity and extraction efficiency of miRNAs require additional technique optimization and validation (168). The patient's stool can then be utilized to extract miRNA, which can then be quantified and its miRNA profile examined.

1.12.2 Fecal miRNA quantification and detection by Quantitative real time PCR (qPCR)

Invasive endoscopic procedures are frequently used for the diagnosis and screening of several disorders, but they have a number of practical drawbacks, including the need for stool preparation and sedation. Therefore, a good screening method, preferably one that uses non-invasive diagnostic techniques, has a great chance of improving survival. miRNAs may be used as diagnostic biomarkers, and numerous research have reported on and explored this possibility. To profile miRNAs, an effective, reliable, and standardized approach for miRNA detection has been developed.

miRNA expression can be measured using the quantitative real-time polymerase chain reaction (qPCR), which is a popular technique. Traditional qPCR cannot be used to quantify mature miRNAs due to their small size. Therefore, it's crucial to increase the mature miRNA's length before running a qPCR to amplify and quantify it. The most popular technique for measuring miRNA is stem-loop reverse transcription PCR. This technique makes use of stem-loop primers designed specifically for the synthesis of mature miRNA's first-strand cDNA. Utilizing stem-loop reverse primer and forward primer specific to miRNA, the synthesized cDNA can be amplified. Fluorescence and expression of mature miRNAs are performed using the TaqMan probe (169). In a different technique, the 3′ ends of all mature miRNAs are polyadenylated with poly(A) polymerase, and a cDNA is produced using an oligodT primer with an adapter sequence at the 5′ end (170) . For Polymerase chain reaction amplification and SYBR Green based real time qPCR for quantification, a universal reverse primer complementary to adapter sequence and forward primers unique to miRNA are used (171). The conventional mature miRNA-qPCR, on the other hand, encounters difficulties when attempting to distinguish between closely related miRNAs that only differ in a few of the bases or when the RNA yield is extremely low. When compared to RNA and DNA and probes, Locked Nucleic Acid (LNA) primers are highly selective to targets and can distinguish single-nucleotide mismatches (172). The specificity for the identification of diagnostic biomarkers in difficult samples, such as faeces, is further increased by a novel

combination of 2 microRNA specific LNA primers. SYBR Green- and TaqMan qPCR-based expression assessments of stool miRNA were able to track changes at different phases of IBDs, CRC, and other intestinal disorders, enabling reliability in disease screening (151, 158). The miRNA quantitative real time PCR is a good screening technique with excellent detection sensitivity for biosignatures in clinical specimens.

1.13 Markers of Permeability and Absorption

The dual-sugar absorption test has some drawbacks while being the most extensively used biomarker for epithelium permeability and integrity. Villous atrophy decreases the amount of epithelial surface area and mannitol (or rhamnose) absorption, but intestinal inflammation creates tiny gaps between epithelial cells that permit lactulose to pass through the paracellular space. Despite the fact that the two sugars have been occasionally reported to disaggregate permeability and absorption, a higher or lactulose: rhamnose or lactulose: mannitol ratio has been employed as an indicator of Intestinal permeability.

Furthermore, depending on their size and assimilation physiology, these sugars can be employed to investigate either combined function or individual processes or lesions. The L:M urine test is the most used intestinal permeability test in children to assess gastrointestinal function. This noninvasive test involves giving a dosage of both sugars (lactulose and mannitol) orally, followed by a urine collection at a specific time. Lactulose is a large sugar (a disaccharide) is only weakly absorbed from an intact small intestine, as previously stated. If an alteration in intestinal permeability occurs, this disaccharide passes through the gut's intracellular spaces, and is removed by glomerular filtration without renal tubular reabsorption, and is detectable in the urine. Mannitol a sugar alcohol is absorbed proportionally to the absorptive capacity (i.e., surface area) of the small bowel (through transcellular routes). Mannitol, like lactulose, is filtered and not reabsorbed, therefore shorter microvilli reduce absorption and consequent urine excretion.

1.14 Markers of Intestinal Inflammation

1.14.1 Fecal Calprotectin

Calprotectin a protein biomarker, is released by activated neutrophils, eosinophils, and monocytes, as well as some mucosal epithelial cells. Calprotectin is stable in the feces and is removed intact. The protein monomers S100A8 (10,835 Da) and S100A9 (13,242 Da) combine to form a 24 kDa dimer calprotectin, which accounts for up to 60% of the soluble

proteins found in the cytoplasm of human neutrophils (173, 174). Calprotectin concentrations in feces are a sensitive and easy-to-measure biomarker for inflammatory bowel disorders.

Calprotectin in the feces is a stable protein that can last for 4–7 days at room temperature. For a laboratory marker, this is a good advantage. Also, maintaining the specimen at a cold temperature (4 °C) appears to improve the stability of fecal calprotectin. At 20 °C, fecal calprotectin appears to be stable for up to a year. Calprotectin levels have been found to be increased in children and adults with inflammatory bowel illnesses such Crohn's disease and ulcerative colitis, and can be employed to determine how severe inflammation is in these patients. Calprotectin levels in the feces have been linked to atopic disease, cow-milk allergy, and gastrointestinal problems. Calprotectin's diagnostic value in infancy is also gaining popularity (175).

IIP, the establishment of intestinal microbiota, and/or a reaction to food antigens may all contribute to the higher concentration of fecal calprotectin in children. Increased faecal calprotectin concentrations have been linked to inflammatory bowel disease's severity of inflammatory processes (176, 177).

1.14.2 REG1B

C-type lectins that can be secreted by the intestinal epithelium are known as regenerating islet-derived (Reg) proteins. Colorectal cancer, and acute amebic colitis, diabetes and inflammatory bowel disease, are only a few of the gastrointestinal illnesses that upregulate Reg proteins. Reg proteins appear to be activated in response to injuries, suggesting that they might be involved in tissue healing. The regenerative gene family is divided into four subclasses (I, II, III, and IV), with REG1A and REG1B genes being substantially homologous (178). REG1 proteins may have a role in tissue healing (179-181), cell proliferation, and regeneration (179, 182). Early in life, greater regeneration gene 1 (REG1) protein concentrations in faeces could be a sign of persistent intestinal injury, which could predict future growth problems.

1.14.3 Inflammatory cytokines

Inflammation of the gut in IBD patients is characterized by the infiltration and activation of both the adaptive system such as T and B lymphocytes, innate systems, as well as macrophages and dendritic cells (DC), which produce large amounts of proinflammatory cytokines, contributing to the typical mucosal lesions.

1.14.3.1 Interleukin-1β

IL-1β is a proinflammatory cytokine that has both systemic and local effects. IL-1β, which is produced mostly by innate leukocytes, has the ability to influence the activity of both immune and non-immune cells. Dendritic cells, macrophages, and neutrophils are all activated and have effector activities when they are stimulated with IL-1β (183, 184).

Patients with IBD have also been found to have an aberrant increase in permeability in the small intestinal, as indicated by permeability markers such lactulose and mannitol absorption in the intestine.

The influence of circulating inflammatory markers such as TNF- α, IL-1β, IFN- **γ**, and lipopolysaccharide, which cause an elevation in intestinal permeability, is likely to account for increase in small intestine permeability. IL-1β has been found to promote an increase in intestinal epithelial tight junction permeability at physiologically and clinically significant doses, as seen in IBD (185-188). In an animal model of colitis, inhibiting the IL-1β induced increase in IP was observed to protect against the development of colitis (189), suggesting that IL-1β induced breakdown of the intestinal tight junction barrier may play a role in the intestinal inflammation process (190).

1.14.3.2 Interleukin-2

IL-2, along with interferon-gamma (IFN- **γ**), is produced by activated Th1 lymphocytes and promotes the cell-mediated immune response's natural killer (NK) cells, macrophages, and cytotoxic T cells. The presence of a high number of activated T cells in the mucosa of IBD patients shows that IL-2 is likely to be involved in inducing inflammation in some way (191-193). The measurement of IL-2 protein and in vivo, on the other hand, has been hampered by technical issues, and results have been inconsistent among investigations. Despite this, there is a growing consensus that IL-2 levels are elevated in intestinal barrier dysfunction implying that it is a Th1-driven disease

1.14.3.3 Interleukin-5

For appropriate gut homeostasis, the balance of anti- and proinflammatory cytokines is critical in the colonic mucosa. Disease conditions such as inflammatory bowel disorders (IBD) result from a disruption of the cytokine profile in favor of proinflammatory cytokine overproduction. Eosinophil differentiation factor, also known as IL-5, is a selective

eosinophil activating growth hormone that belongs to the chain dependent cytokine family (188, 194). Mononuclear cells, which release a large amount of this cytokine in inflammatory disorders, are the source of mucosal IL-5 (195, 196). IL-5, IL-13, and granulocyte/monocyte colony stimulating factor have all been found to activate eosinophil activity, which includes migration to the site of inflammation.

1.14.3.4 Interleukin-10

IL-10 suppresses the inflammatory process in the mucosa by inhibiting antigen presentation and the generation of proinflammatory cytokines. In CD, the anti-inflammatory cytokine IL-10 plays a vital role. In mice, inactivating IL-10 boosted the production of pro-inflammatory IFN- γ and IL-12 (197). Many cells in the adaptive and innate immune systems express it. The former causes IL-10 expression in both TLR-dependent and TLR-independent ways. dendritic cells and Macrophages are the primary generators of IL-10 (198). IL-10 is the most essential cytokine in the immune system for inhibiting pro-inflammatory responses. IL-10 works to safeguard the body by suppressing overactive immune responses and autoimmune diseases. The human IL-10 gene is 4.7 kb long and has 5 exons. It is found on chromosome 1q32. IL-10 inhibits not only cytokine production, but also chemokine (9) and co-stimulatory molecules (CD80, CD86, and MHC Class II) expression (10). IL-10 interacts to the proteins IL-10R and IL-10R, which are found on nearly all immune cells. As a result, IL-10 has the ability to regulate a variety of innate and adaptive immune cells in order to dodge the development of immunological diseases in a variety of ways.

1.14.3.5 Interleukin-13

Interleukin-13 (IL-13) often known as a multifunctional cytokine has a primary goal of reducing inflammatory reactions. T-helper 2 (Th-2) lymphocytes produce IL-13, which inhibits the synthesis of proinflammatory cytokines such as IL-1, interleukin-8, and TNF- α by monocytes and B lymphocytes. The granulocyte-colony stimulation factor (GCSF) are inhibited by IL-13. Furthermore, IL-13 suppresses the chemotaxis of both CD8+ and CD4+ T cells locally. IL-13 appears to play a key role in the downregulation of immunological responses via a variety of pathways. Inflammation of the intestine has been linked to dysregulation of IL-13 production in IBD patients. Previous research on IL-13 production in inflammatory bowel disease patients has found that mitogenically stimulated peripheral blood monocytes (PBMCs) have lower IL-13 activity. Circulating PBMCs, on the other hand, may

not adequately reflect lamina propria production. Vainer et al examined IL-13 concentrations directly from biopsies of the intestinal lamina propria, but found no significant changes between IBD and control patients.

1.14.3.6 Interferon-gamma (IFN-γ)

Intestinal barrier failure is exacerbated by IFN- **γ**. IFN- **γ** is an immunoregulatory cytokine that regulates T helper cell development, activating macrophages, and increasing MHC I and II expression. In chronic inflammatory illnesses such as IBD and after microbial infection, IFN- **γ** levels are frequently high, both locally and systemically. In addition to its significant role in immune modulation, multiple in vivo and fewer in vitro studies show that IFN- **γ** reduces the function of the intestinal epithelial barrier. Reduced expression of the tight junction (TJ) proteins occluding and zonula occludens-1 (ZO)-1 is associated with IFN- **γ** induced breakdown of epithelial barrier function, allowing greater material penetration between neighboring cells via the paracellular pathway. IFN- **γ** promotes bacterial transcytosis across epithelial monolayers.

1.14.3.7 Tumor Necrosis Factor (TNF-α)

TNF is a superfamily of 18 type 2 proteins that can be membrane bound or soluble (199). Type 1 transmembrane proteins serve as receptors for these ligands(200). TNF- α like ligands bind to receptors and activate intracellular pathways that are important in cell proliferation differentiation, and survival (201). The majority of TNF/ TNF- α receptor protein superfamilies are expressed on immune cells and are involved in numerous aspects of the immune response, including microbial defense, programmed cell death, inflammation, and immune system development.

TNF- α is a proinflammatory cytokine that plays a key role in the pathophysiology of inflammatory bowel disease (202, 203). Its pleiotropic effects are mediated through fibroblast proliferation, adhesion molecules, procoagulant factors, and the beginning of apoptotic, cytotoxic, and acute-phase responses, among other mechanisms (204).Tumor necrosis factor is a key modulator of gastrointestinal inflammation (205, 206). TNF- α induces an increase in intestinal TJ permeability in vivo and in vitro, allowing for greater luminal antigen penetration (207-209). Enhancement of the intestinal barrier has been proven in animal experiments to reduce cytokine-mediated intestinal inflammation and diarrhea (210, 211). Anti–TNF-α therapy causes the intestinal barrier to retighten, and normalizing intestinal permeability is linked to long-term clinical remission, according to clinical research.

1.1 Study Area and Human Subjects

This study is a nested observational study which was performed within a longitudinal birth cohort study in the slum areas of Mirpur, Dhaka, Bangladesh (212) . For this study, a total of 442, 2-year-old children were chosen. Mothers who agreed to sign an informed consent form and the absence of any birth defects or congenital abnormalities were the inclusion criteria for the current investigation. Children under or over the age of 2, children without significant congenital defects, and parents who would object to having their child's blood drawn were also excluded.

1.2 Sample and Data Collection

After the children (n = 442) ingested the lactulose and mannitol solution, samples of urine were taken from these children over the course of 2 hours. Children were chosen for the analysis of miRNA expression based on the availability of faecal samples from a bigger study that had already been completed **(Figure 3.1)**. We examined 120 mg of stool from eighty-five (85) children who were chosen randomly. For gene expression analysis of miRNA-122, we chose 42 children from the NIP group and 43 children from the IIP group. Identical fecal samples from thirty-six (36) children— sixteen (16) with IIP and twenty (20) with NIP— were chosen for miRNA-21 gene expression study (**Figure 3.2)**. The remaining forty-nine (49) children, however, miRNA-21 gene expression was not performed because their stool volumes were insufficient for qPCR. The same fecal samples used to identify microRNA-122 and microRNA-21 were also used to evaluate the faecal biomarkers REG1B and Calprotectin. We also examined 3 ml blood samples from the same children to measure inflammatory cytokines and correlate them with miRNA-122 and miRNA-21. Enteropathogens were looked for in diarrheal feces. A cold box was used to carry the blood, urine, and fecal samples from the field clinic to the Icddr,b Parasitology Lab, where they were kept at 70°C until analysis.

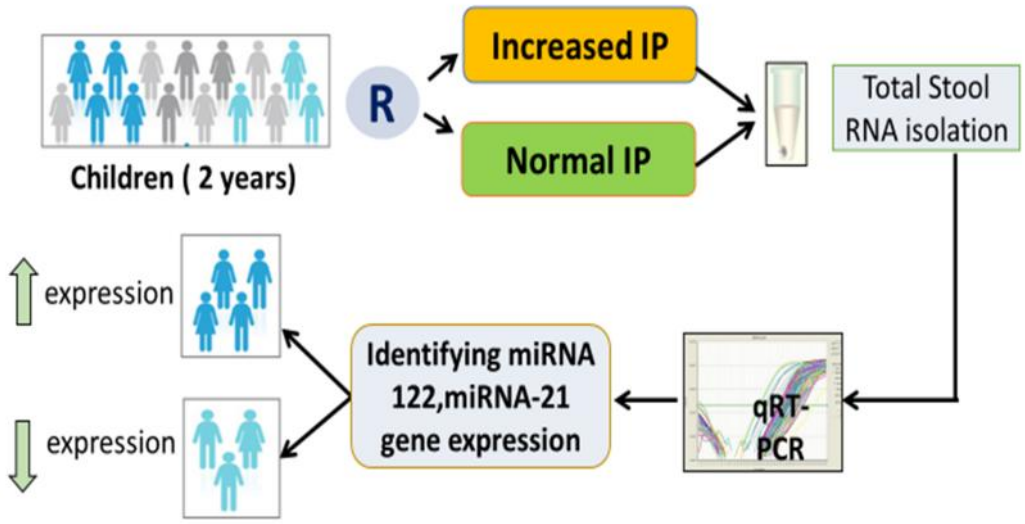


Figure Error! No text of specified style in document.**.1** Study design for microRNA gene expression. RNA extraction from fecal samples was performed and these samples were run for SYBT green real time quantitative PCR to see miRNA gene expression

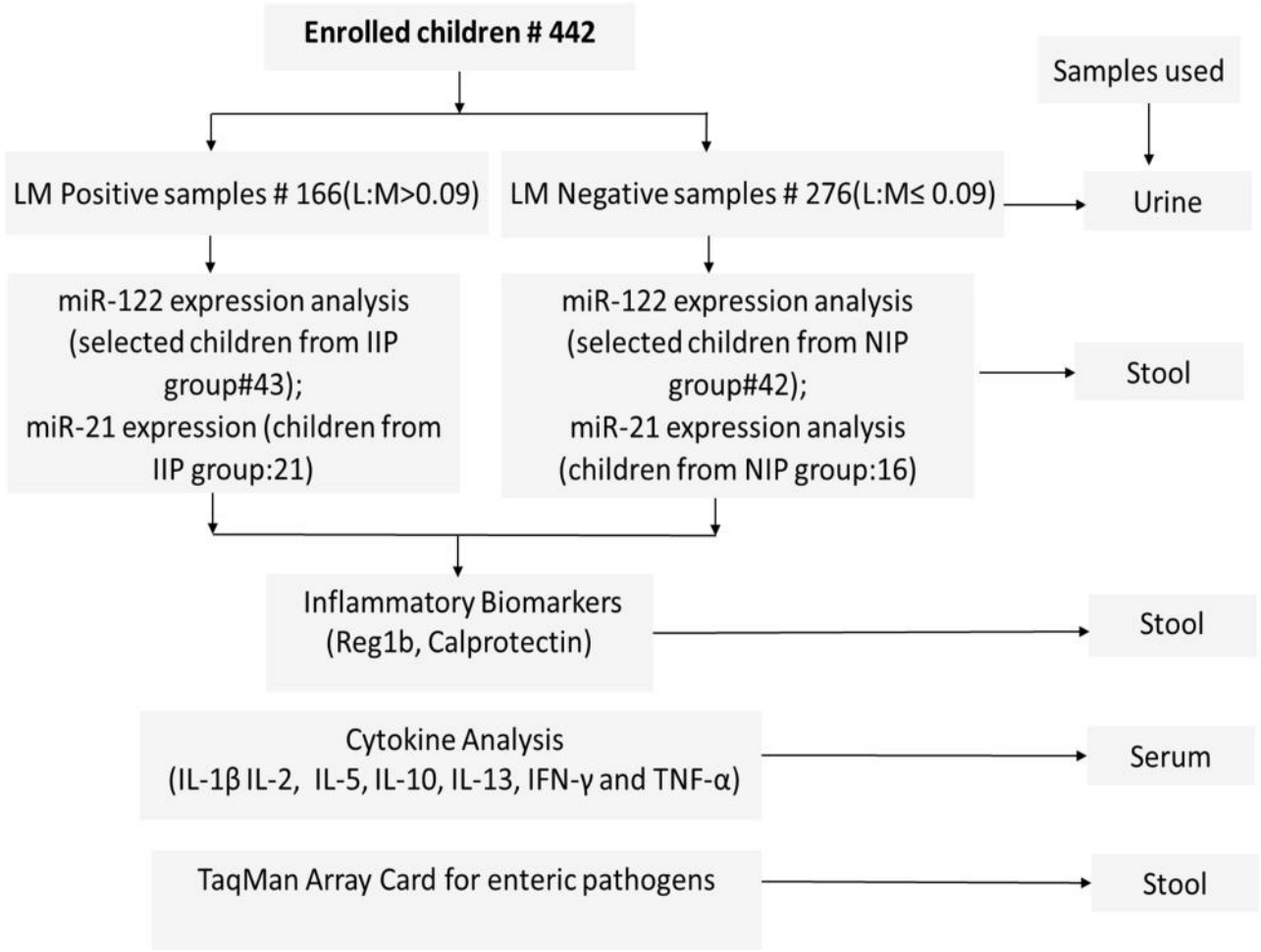


Figure Error! No text of specified style in document**..2** Flow Diagram showing the number of children enrolled for L:M ratio and miRNA expression analysis and the type of samples used for each assay.

1.3 Anthropometry

At the time of enrolment, anthropometric measurements were taken using a calibrated digital baby scale (UC-321; A & D Co., Tokyo, Japan) and standardized supine length measurement apparatus (TALC, St. Albans, Herts, UK) to calculate the measures of underweight and stunting and which are the weight-for-age (WAZ) z-score and height-for-age (HAZ) z-score, respectively (World Health Organization, 1995). Stunting is defined as a HAZ less than −2 SD and underweight as a WAZ less than −2 SD.

1.4 Measures of Intestinal Permeability (High performance anion exchange chromatography-pulse amperometric detection)

A measurement of intestinal permeability is the L/M ratio. Due to IIP and decreased intestinal surface area, individuals with intestinal barrier dysfunction absorb more lactulose and less mannitol, respectively (71) . The children were administered 2 ml/kg of body weight of lactulose and mannitol. 250 mg/ml of lactulose and 50 mg/ml of mannitol were included in the water-based solution. After consuming mannitol and lactulose, urine was collected for up to 2 hours. High-performance anion exchange chromatography was used for the test (HPAEC-PAD). Children were categorized as having elevated IIP if their L:M ratio was > 0.09, and NIP if L:M ratio was ≤ 0.09 (213).

1.4.1 Assay Procedure

- Urine samples was taken out from the freezer and thawed
- Urine samples was vortexed and centrifuged for 10 minutes @ 10000 rpm
- After centrifuge,100µl of supernatant urine and 50 µl of a solution containing melibiose (3.0 mM) diluted in 2.85 ml of distilled deionized water was taken in a 50 ml falcon tube
- Internal/calibration standards Lactulose (50 µM), Mannitol and Melibiose of (10µM, 20µM, 30µM, 40µM, 50µM)was prepared in separate falcon tubes
- Both standard and urine samples was then vortexed
- 700 µL of the above samples and standards were transferred to the Poly Vial with 20 µm Filter cap and placed in the auto-sampler and read in the detector. Each sample can be automatically filtered during loading by an optional 20 µm filter in

the vial cap, so that particulates can be removed during sampling.

Values were electronically transferred into the software program and results calculated **(Figure 3.3 and 3.4)**

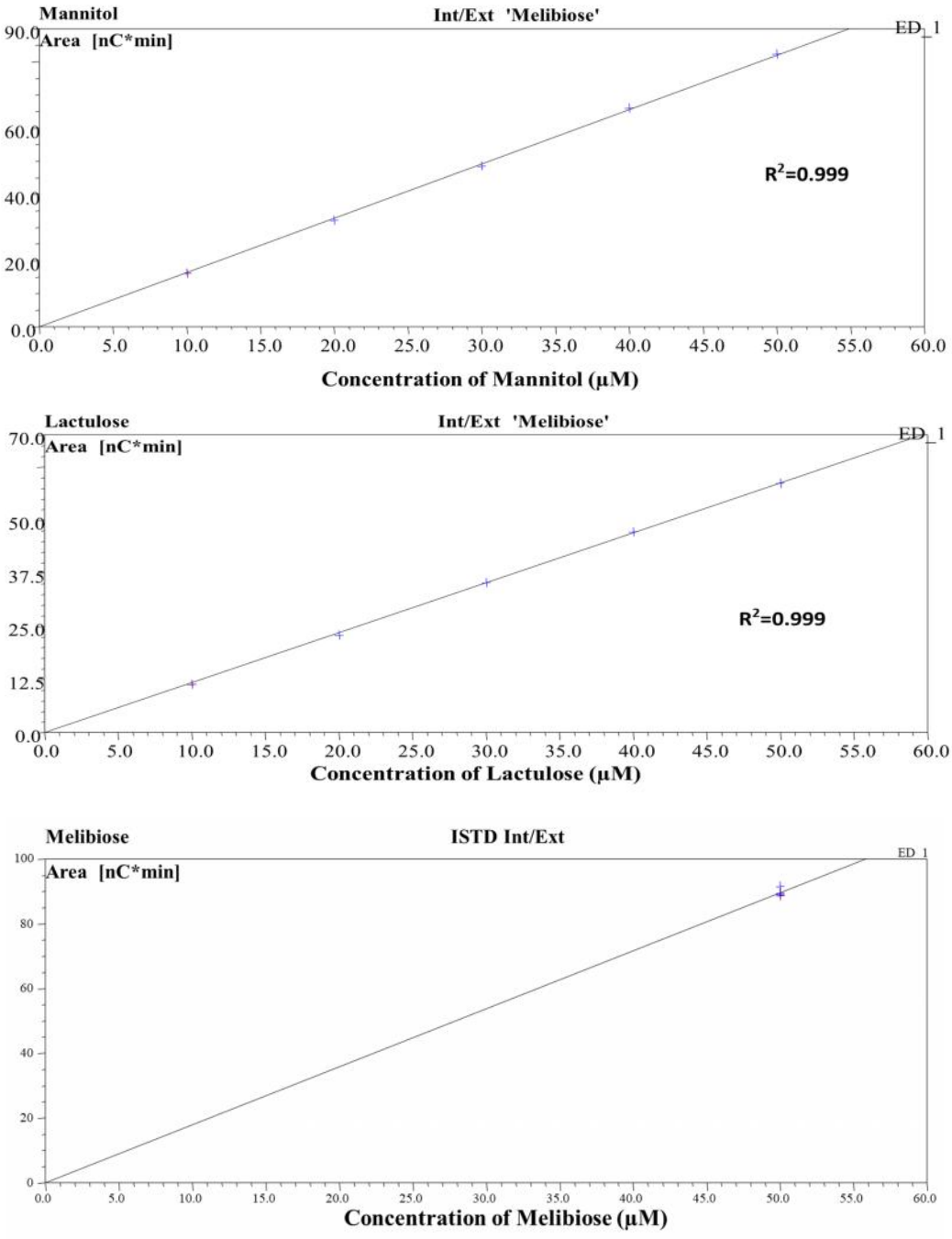
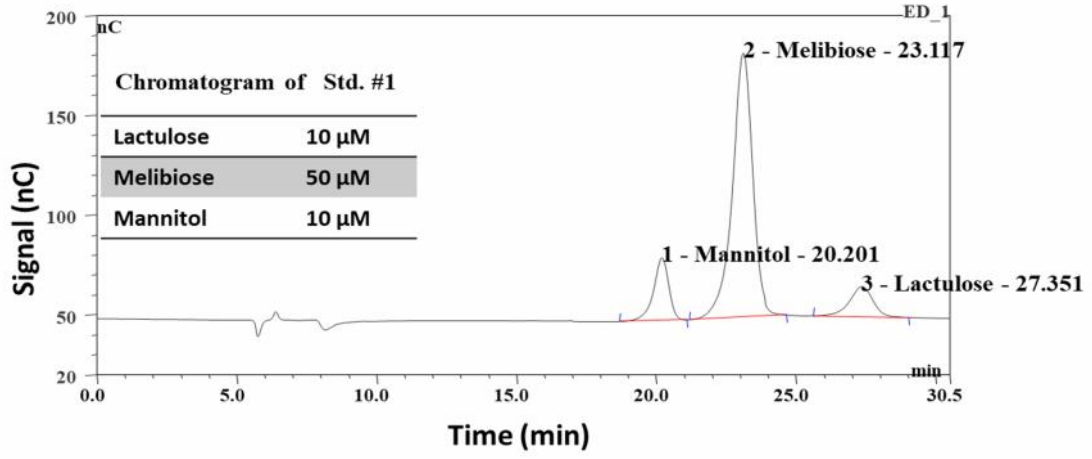
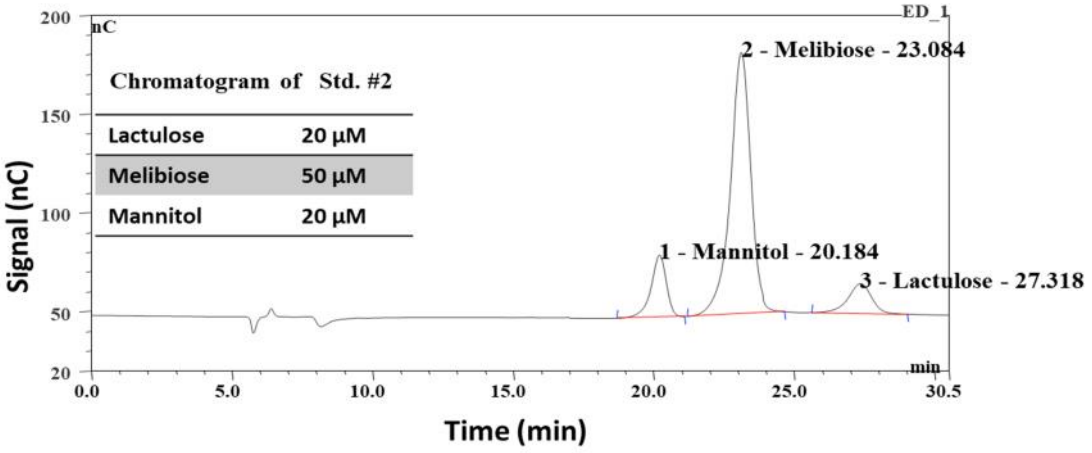
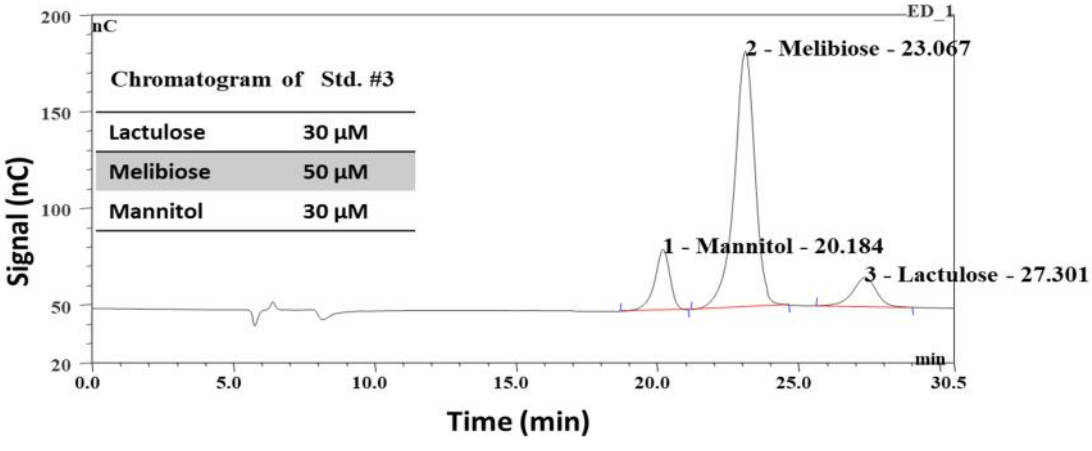


Figure Error! No text of specified style in document.**.3** Standard curve of Mannitol, Lactulose and Melibiose


ED_1
nC
Chromatogram of Std. #1
Lactulose 10 µM
Melibiose 50 µM
Mannitol 10 µM
2 - Melibiose - 23.117
1 - Mannitol - 20.201
3 - Lactulose - 27.351
min
Signal (nC)
Time (min)


ED_1
nC
Chromatogram of Std. #2
Lactulose 20 µM
Melibiose 50 µM
Mannitol 20 µM
2 - Melibiose - 23.084
1 - Mannitol - 20.184
3 - Lactulose - 27.318
min
Signal (nC)
Time (min)


ED_1
nC
Chromatogram of Std. #3
Lactulose 30 µM
Melibiose 50 µM
Mannitol 30 µM
2 - Melibiose - 23.067
1 - Mannitol - 20.184
3 - Lactulose - 27.301
min
Signal (nC)
Time (min)

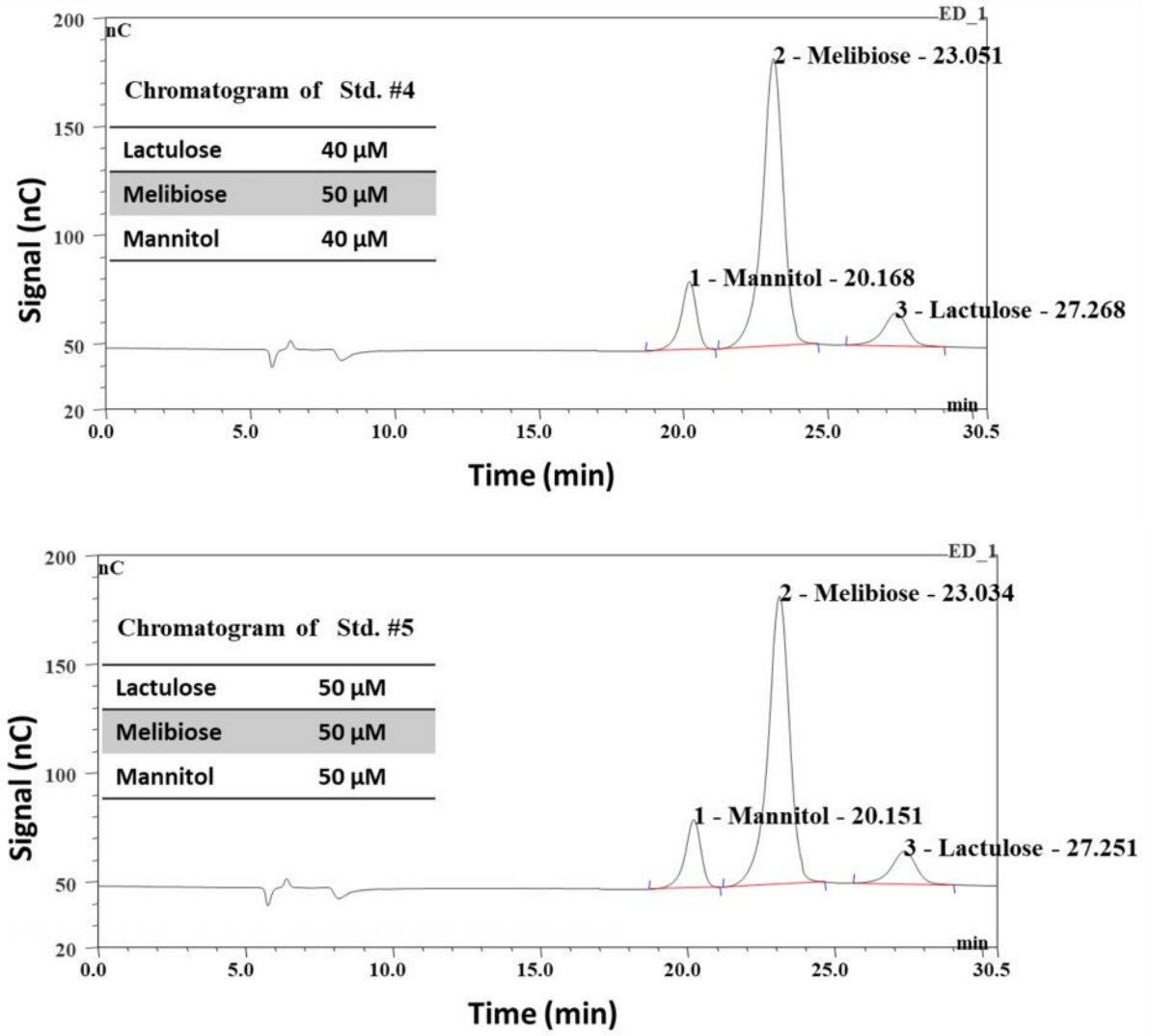


Figure Error! No text of specified style in document.**.4** Chromatogram of Lactulose Mannitol & Melibiose (standard 1,2,3,4,5)

1.5 microRNA Extraction in Stool Specimen

1. At first, about120 mg of frozen stool samples (thawed at room temperature) were transferred to 1.5 ml micro-centrifuge tubes
2. Stool (120 mg) was homogenized for 30 seconds after being mixed with RNAse-free water in the presence of Qiazol lysis reagent using a vortex mixer
3. In a 2 ml RNAse-free tube, chloroform was added to precipitate the RNA
4. The sample was vortexed for 15 s
5. The suspension was incubated for two to three minutes at room temperature
6. The sample was centrifuged at 4°C for 15 min at 12,000 g

7. Using RNase free water total stool RNA was eluted
8. Finally, Nanodrop 2000 was used to determine the RNA concentration (Thermo Fisher Scientific, Wilmington, DE, United States).

1.6 Reverse Transcription (First Strand cDNA Synthesis) and Real-Time PCR

1.6.1 First Strand cDNA Synthesis

Reverse transcription (RT) was performed according to the manufacturer's instructions using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, United States). The RT reaction was carried out using whole treated RNA and an RT universal stem loop primer (214).

A sterile, nuclease-free tube was placed on ice, and the following reagents were added in the specified order.:

1. 0.1 ng - 5 µg/1 µL of treated RNA was used
2. 1 µL of gene specific primer was then added {stem-loop RT primer (5 µM)}
3. This was then finalized to a volume of 12 µL by adding water
4. The sample mixture was then vortexed gently, briefly centrifuged and incubated for 5 min at 65°C. Following incubation, the sample was chilled on ice, spinned down and the vial was placed back on ice
5. Then the following reagents were added in the indicated order:
    a. 5X Reaction Buffer- 4 µL
    b. RiboLock RNase Inhibitor (20 U/µL)- 1 µL
    c. 10 mM dNTP Mix- 2 µL
    d. RevertAid M-MuLV RT (200 U/µL) 1 µL
6. The sample was mixed gently and centrifuge briefly.
7. The mixture was incubated at 42°C for 60 min.
8. The reaction was finally terminated by heating for 5 min at 70°C

1.6.2 Real time PCR

The miRNAs in the fecal nucleic acid samples were quantified using SYBR Green [EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen)].

The protocol below was followed:

1. Reactions were setup on ice

2. Volumes for a 12 -µl reaction size was prepared:

a. EXPRESS SYBR® GreenER™ qPCR SuperMix Universal- 5 µL

b. 10 µM forward primer (200 nM final)- 0.4 µl

c. 10 µM reverse primer (200 nM final)- 0.4 µl **(Table 3.1)**.

d. Template DNA

e. DEPC-treated water

3. No-template control (NTC) reactions were prepared to test for DNA contamination of the enzyme/primer mixes
4. A Bio-Rad iCycler® CFX96 real-time PCR detection instrument was used to measure the amount of reverse-transcribed RNA.
5. Reactions were placed in a real-time instrument (A Bio-Rad iCycler® CFX96 and programmed as follows: 50°C for 2 minutes (UDG incubation); 95°C for 2 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; Melting curve analysis: 60°C–95°C
6. Data was collected and the results were analyzed. The miRNA expression levels were determined using the quantification cycle values (Cq values). For data normalization, the endogenous control/housekeeping gene used was U6 RNA and the $2^{-\triangle\triangle^{Cq}}$ method was used for assay quantification (215-217). The relative expression of miR-21 and miR-122 [ΔΔCT = ΔCT (a miRNA of interest)-ΔCT (U6 RNA as a normalizer accounting for sample-to-sample variation)] was analysed using the $2^{-\Delta\Delta CT}$ method.

Table 3.1 Primers used for quantitative RT-PCR

RNA	5' to 3'
hsa-miR-122	Forward 5' - GGGAACGCCATTATCACAC - 3' Universal Reverse 5'- GTGCAGGGTCCGAGG - 3'
U6	Forward 5′- CTCGCTTCGGCAGCACA-3' Reverse 5′-AACGCTTCACGAATTTGCGT-3'
hsa-miR-21	Forward 5′- GTTTGGTAGCTTATCAGACTGA-3' Universal Reverse 5′- GTGCAGGGTCCGAGG -3'

1.7 TaqMan Array Card for Enteric Pathogen detection

The TaqMan Array Card system real-time polymerase chain reaction format, developed by Applied Biosystems ViiA7 Real-Time PCR system (Life Technologies, Foster City, CA, United States), was used to detect multiple enteropathogens using total nucleic acid isolated from faecal samples. This system is capable of quickly detecting and quantifying 72 enteropathogens. With a quantitative detection, TAC makes it possible to quickly and accurately identify a variety of enteropathogens. The TaqMan Array Card (TAC) is a 384-well real-time PCR format for TAC-compatible instrument platforms. The enteric TAC targets include viruses, bacteria, fungi, protozoa, and helminths. During nucleic acid extraction, PhHV and MS2 are added to the faecal samples as extrinsic controls to check extraction and amplification.

- All reagents were kept on wet ice or on a cold block during assay set up
- The enzyme mix was viscous because of glycerol;
- Master Mix was prepared for only one card (8 samples) at a time

1. Master mix was prepared for 8 samples:

Ag-Path-ID 2X RT-PCR buffer	425µL
Ag-Path-ID Enzyme mix	34µL

2. The 54µL of master mix was aliquot into each of eight (8) 1.5mLmicrocentrifuge tubes.
3. To each tube,20µLof total nucleic acid extract from stool specimens was added then supplemented with 26µL nuclease-free water, or extraction blanks, or nuclease-free water (for NTC).

Mix gently, then centrifuge the tubes to eliminate air bubbles from the mixtures

1.7.1 Loading the TAC

1. The TAC was allowed to reach room temperature in the original packaging.
2. The card was placed on a lab bench, with the foil side down.
3. The 100µL of each PCR reaction mix was transferred into the fill port:
4. Centrifuge 1 minute. Wait for the centrifuge to stop, then repeat so that the cards are centrifuged for a total of two consecutive, 1-min spins.
5. The card was sealed (“staked”). The sealer used a precision stylus

assembly (“carriage”) to seal the main fluid distribution channels of the array.

6. The sealed card was placed in the instrument with well A1 at the top left corner of the tray and the notched corner at the top right, with the bar code facing toward the front of the instrument.
7. Within the software,“Run” was clicked on the left panel, then the arrow next to “START RUN”
8. Cycling Conditions was confirmed in the Run Method Screen **(Table 3.2**):

Reaction volume: 1µL

Table 3.2 Thermo cycling conditions

Step	**Temperature**	**Time**
Activation	45°C	20min
RT (1cycle)	95°C	10min
Denaturation (1 cycle)	95°C	15sec
PCR (40cycles)	60°C	1min

1.7.2 Analysis

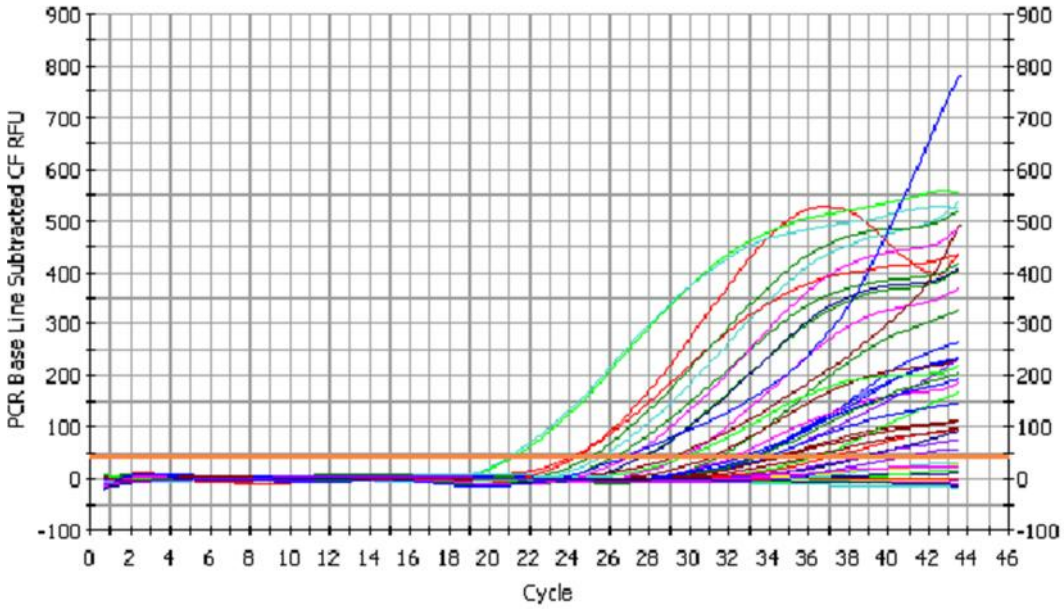
1. Amplification curves was reviewed **(Figure 3.5)**. A sample was deemed positive when an appropriately shaped curve crosses the threshold and all controls worked properly.
2. Fluorescence growth curves that cross the threshold shouldn't be seen in NTC reactions.
3. Positive control should result in reaction fluorescence curves (amplification plots) that cross the threshold for each target assay

4 A sample was considered positive if the signal exceeded a preset threshold (Threshold cycle, CT)

1.

A sample was considered positive if the signal exceeded a preset threshold (Threshold cycle, CT)

Figure Error! No text of specified style in document.**.5** Amplification signal of TaqMan Array Card



1.8 Stool Enzyme-Linked Immunosorbent Assay

Using ELISA kits that are available commercially (Calprotectin ELISA, BÜHLMANN Laboratories AG, Basel, Switzerland (218), the gut inflammatory biomarker fecal

calprotectin was quantified in accordance with the manufacturer's instructions (219). Standard curve was generated using the kit standards to quantify faecal calprotectin using the kit standards and was used to calculate the amount of faecal calprotectin, which was then represented as µg/g stool.

The ELISA technique by TECHLAB, Inc., Blacksburg, VA, United States, was used to measure the concentration of REG1B (µg/g), an inflammatory biomarker that may be an indicator of intestinal damage and repair, in fecal samples to assess epithelial health (220).

1.8.1 Calprotectin Assay Procedure

1.The stool extracts 1:150 with incubation buffer (e.g., 20 µL extract and 2980 µL incubation buffer) was diluted and mixed well. The samples were equilibrated for at least 5 minutes at 18-28 °C

2. The coated wells were washed twice using at least 300 µL of wash buffer per well. The wells were emptied and the plate was firmly tapped onto blotting paper. (Allow wash buffer to remain in the wells for a minimum of 20 seconds during each wash step)

3a. 100 µL of incubation buffer (blank) and 100 µL of calibrator A-E were pipetted into the respective wells

3b. 100 µL of the controls low and high were pipetted into the respective wells.

3c. Now 100 µL of each diluted sample was pipetted into the subsequent wells.

4. The plate was covered with a plate sealer, and incubated for 30 ±5 min on a plate shaker set to ~450 rpm at 18-28 °C

5. The plate sealer was removed and discarded. Wells were emptied and washed three times using at least 300 µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

6. 100 µL of enzyme label was pipetted to all the wells

7. The plate was covered with a plate sealer, and incubated for 30 ±5 min on a plate shaker set to ~450 rpm at 1828 °C

8. plate sealer was then removed and discarded and the wells were emptied and washed five times using at least 300 µL of wash buffer per well. Now the plate was firmly tapped onto a blotting paper.

9. 100 µL of the TMB substrate solution was then pipetted to all wells

10. The plate was covered with a plate sealer and protected from direct light and incubated

for 15 ±2 min on a plate shaker set to ~450 rpm at 18-28 °C

11. To each well pipet 100 µL of stop solution. Using a pipette tip remove air bubbles. Proceed to step 12 within 30 min

12. Using a microtiter plate reader, the absorbance was measured at 450 nm **(Figure 3.6)**

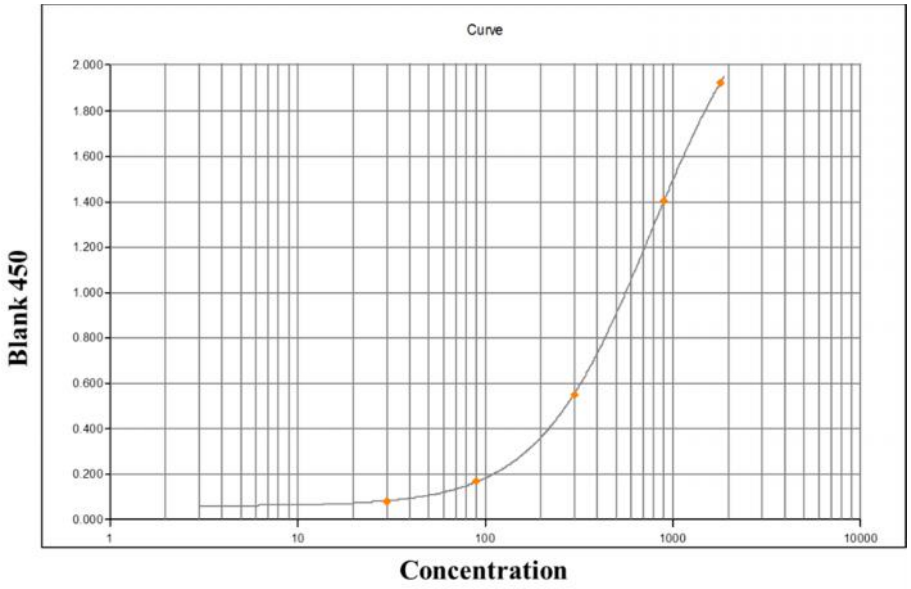


Figure Error! No text of specified style in document.**.6** Standard curve for

1.8.2 Regenerating gene 1B (Reg 1B) ELISA Assay Procedure

The samples and all reagents were brought to room temperature (15-30ºC) and mixed well. The positions of STD (Standard)/Sample/ CTRL (Controls) were marked on a protocol sheet**.**

1. All reagents, samples and working standards were prepared as directed.
2. Excess microplate strips were taken out from the plate frame, sealed after being reinserted into the foil pouch containing the desiccant pack.
3. The 100 ul of Assay Diluent RD1W was added to each well.
4. The 100 ul of control, standard or sample were added per well and covered with the adhesive strip and incubated for 3 hours at room temperature. A plate layout was provided as a record of samples and standards assayed.
5. Three times, the procedure was repeated after aspirating and washing each well for a total of four washes. Each well was washed by filling with Wash Buffer (400ul) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and bloted against clean paper towels.
6. Each well received 200 µL of Reg1b Conjugate. A fresh adhesive strip was placed over the plate, and it was let to incubate at room temperature for an hour.
7. As in step 5, the aspiration/wash procedure was repeated.
8. To each well, 200 µL of the substrate solution was added. The plate was kept at room temperature and shielded from light for 30 minutes.
9. Each well received a 50µL of Stop Solution. A change in color from blue to yellow should take place. The plate was lightly tapped to ensure full mixing if the wells were green or the color change did not appear to be uniform.
10. Within 30 minutes, the optical densities of each well were calculated, using a microplate reader set to 450 nm. If no reference wavelength was available, read only at 450 nm. If the extinction of the highest standard exceeded the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference **(Figure 3.7)**

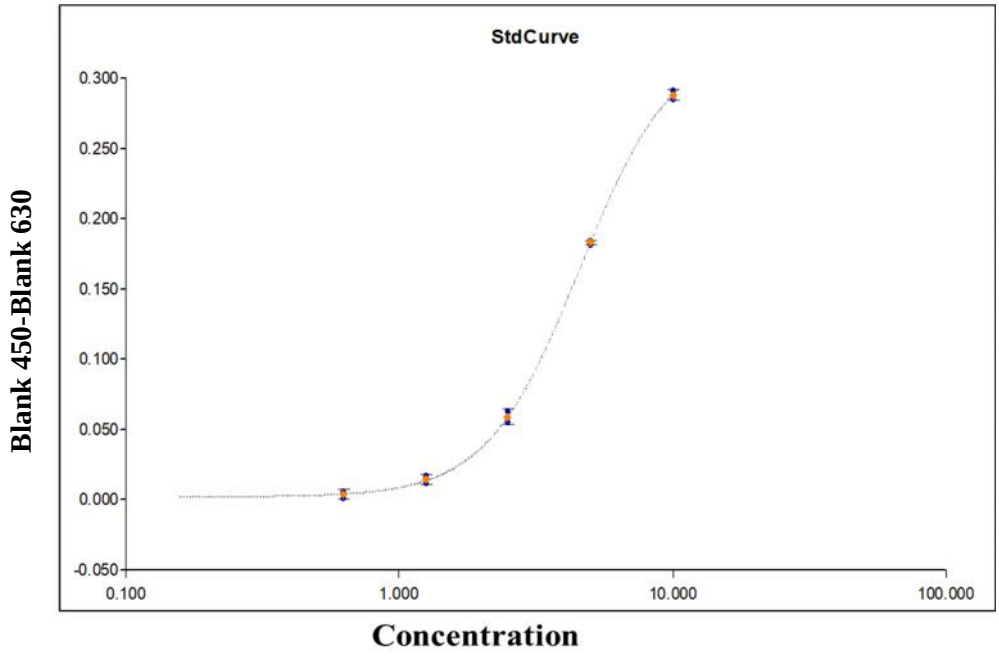


Figure Error! No text of specified style in document.**..7** Standard curve for Reg1B

3.9 Cytokine Measurements

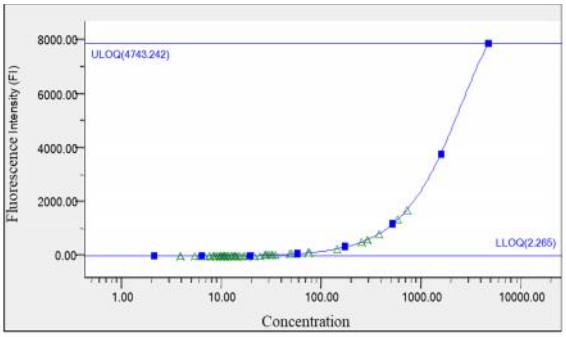
Human Bio-Plex Pro Assays (Bio-Rad, Hercules, CA) were used to analyze the serum samples for the presence of IL-2, IL-1β, IL-10, IL-5, IFN-γ, IL-13, and TNF-α utilizing a Bio-Plex 200 platform (Bio-Rad, Hercules, CA, United States). For data processing, Bio-Plex Manager software version 6.0 was employed (221).

Bio-Plex Pro™ tests are sandwich immunoassays formatted on magnetic beads. The sandwich ELISA assay works on a similar premise. The desired biomarker-specific capture antibodies are covalently linked to the beads. Coupled beads react with the target biomarker-containing sample.

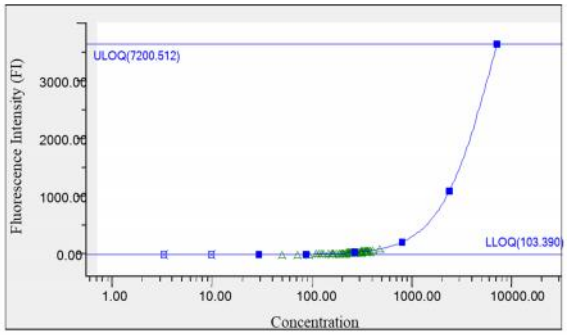
A sandwich complex is made by adding a biotinylated detection antibody after a series of washing steps to remove unattached protein. With the addition of streptavidin-phycoerythrin (SA-PE) conjugate, the ultimate detection complex is created. Here a fluorescent indicator or reporter is phycoerythrin.

Assay Procedure:

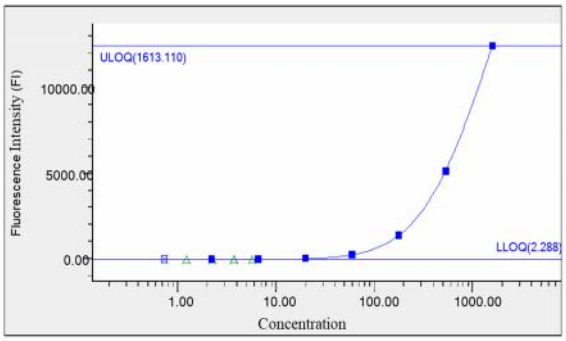
1. Vortex the diluted (1x) beads at medium speed for 30 sec. Pour into a reagent reservoir and to each well transfer 50 µl of the assay plate.
2. Using the preferred wash technique, wash the plate two times with 100 µl of Bio-Plex Wash Buffer for each well.
3. Vortex the diluted samples, standards, blank, and controls at medium speed for 5 sec. Transfer 50 µl of each to the appropriate well of the assay plate, changing the pipet tip after every volume transfer
4. Apply a fresh sheet of sealing tape over the plate. Incubate on shaker for 30 min at room temperature at 850 ± 50 rpm
5. Add the required volume of detection antibody diluent HB to a 5 ml polypropylene tube.
6. Vortex the 10x or 20x stock detection antibodies at medium speed for 5 seconds, then perform a 30 sec spin to collect the entire volume at the bottom of the tube.
7. Into the 5 ml tube dilute detection antibodies to 1x by pipetting the required volume Vortex the tube.
8. After detection antibody incubation, slowly remove and discard the sealing tape.
9. With 100 µl of wash buffer per well wash the plate 3 times.
10. Vortex the diluted (1x) SA-PE at medium speed for 5 sec. Pour into a reagent reservoir and transfer 50 µl to each well using a multichannel pipet.
11. with a new sheet of sealing tape cover the plate. At 850 ± 50 rpm incubate on shaker for 10 min at RT.
12. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.
13. Use 100 µl of wash buffer per well to wash the plate 3 times.
14. Add 125 µl of assay buffer into each well to resuspend the beads for plate reading. With a new sheet of sealing tape cover the plate. For 30 sec shake at room temperature at 850 ± 50 rpm. Slowly remove and discard the sealing tape before placing the plate on the reader.
15. Acquire the data on Bio-Plex Manager Software **(Figure 3.8)**



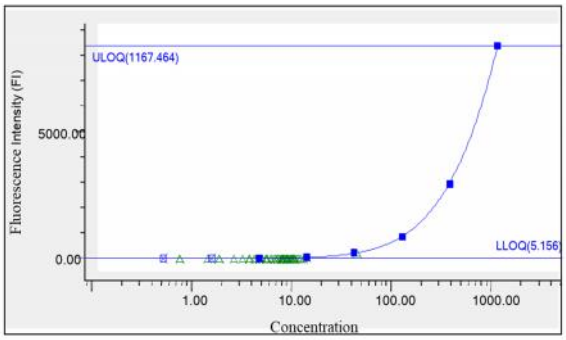
IL-1β



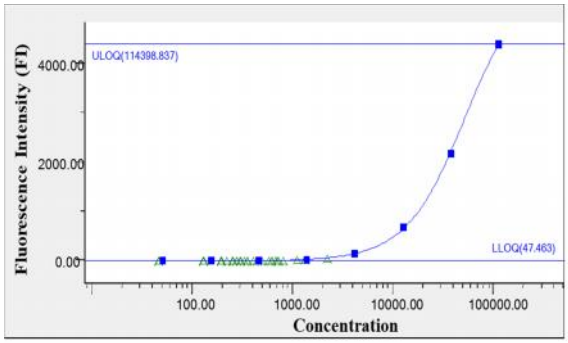
IL-2



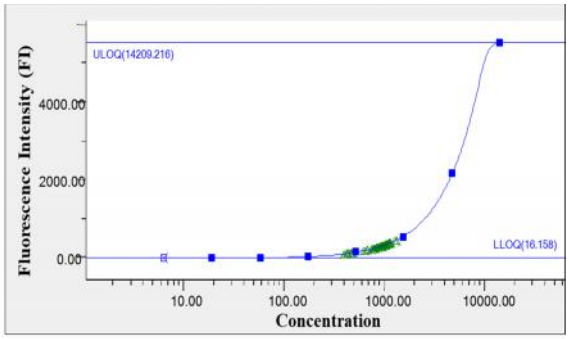
IL-5



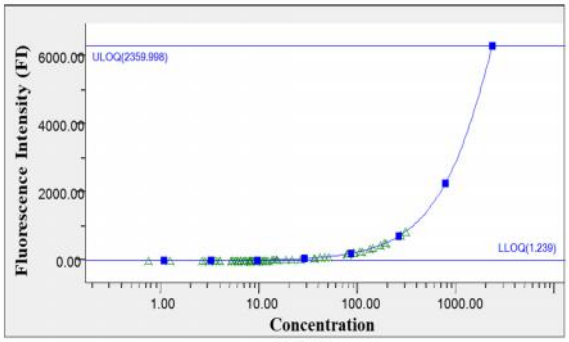
IL-10



IL-13



IFN-γ



TNF-α

Figure Error! No text of specified style in document..**8** Standard curve for cytokines: for IL-1β, IL-2, IL-5, IL-10, IL-13, IFN-γ, and TNF-α

3.10 Statistical Analysis

GraphPad Prism 8.0 (San Diego, CA, United States) or SPSS (version 17.0) were used to conduct statistical analysis. The mean ± standard deviation (SD) was used to express the results (SD). if $p \leq 0.05$, differences were deemed significant. Using Mann-Whitney U tests and the t-test the association between microRNAs and pathogenic infections and intestinal permeability was examined. Bivariate correlations between variables, such as microRNA levels, and faecal inflammatory biomarkers, cytokines, were studied using Pearson's correlation coefficient (r). The specificity and sensitivity of miRNAs for detecting children with Increased intestinal permeability (L:M > 0.09) were determined using receiver operating characteristic (ROC) curve analysis.

1.1 Nutritional Status of Children

For this study we selected 442 children in total;47.2% of the children of them were female (**Figure 4.1)** 37.5% of the children (166/442) had higher L:M ratio (> 0·09) **(Figure 4.2)** which may indicate increased intestinal permeability (IIP). Of the total 442 children present, 30.3% of them had less than -2 WAZ and 32.3% of them had less than -2 HAZ **(Figure 4.3)**. Only 23.5% (65/276) with normal intestinal permeability (NIP) children were less than -2 WAZ compared to 42 % (69/166) of those with IIP, which is statistically significant (*P* =0.0001). Similarly, 27.8% (77/276) of children with NIP were less than -2 HAZ, compared to 40% (66/166) of children with IIP which is also statistically significant (*P* =0.012). 25.1% (111/442) children were moderately underweight and 5.2% (23/442) were severely underweight, whereas 23% (102/442) were moderately stunted and 9.2% (42/442) were severely stunted **(Figure 4.4 and Table 4.1)**. The nutritional status of 85 children was also checked for miRNA-122 expression analysis; 27% of the children had less than -2WAZ and 28.2% of the children had less than -2HAZ.

On comparing the post urinary L:M ratio with nutritional status of children, the median value of L:M ratio for children who were stunted and normal was 0.1250 and 0.06612 respectively. Both the groups showed a statistical difference (p= 0.041[a]). Similarly, the median value of L:M ratio for children who were malnourished and well-nourished was 0.1030 and 0.0643 respectively with a statistical difference of p= 0.001[b]**(Table 4.2)**

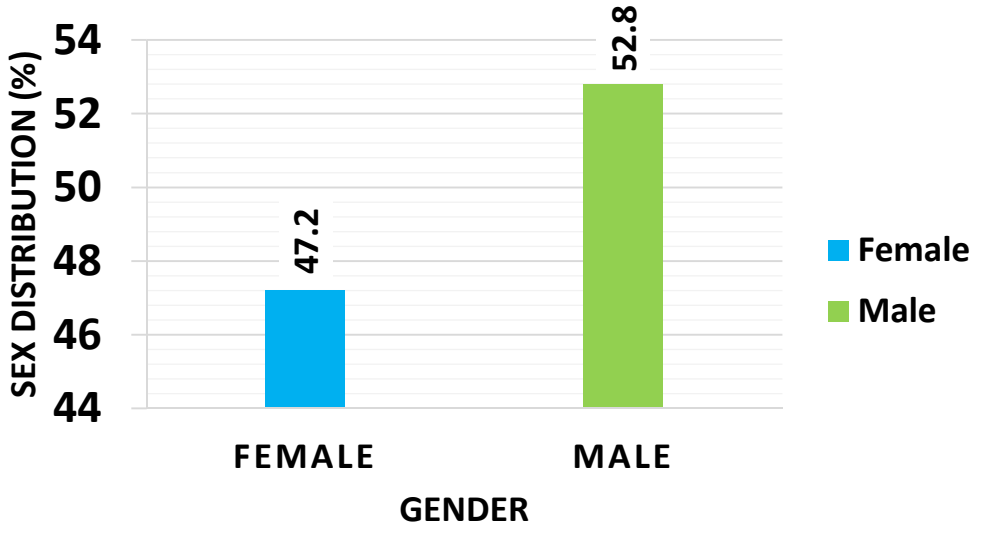


Figure Error! No text of specified style in document.**..1** Demographic data: Bar graph showing sex distribution of the study population

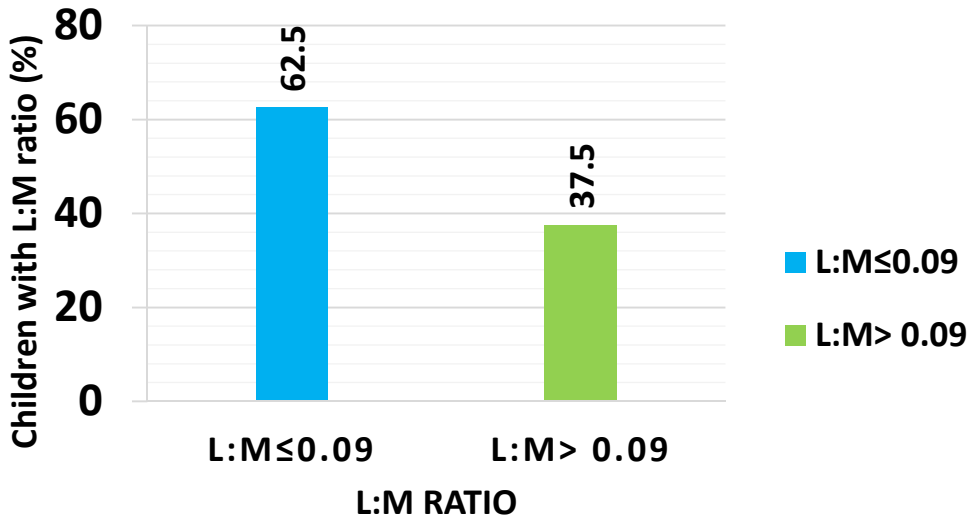


Figure Error! No text of specified style in document.**..2** Children with L:M ratio: Bar graph showing the percentage of children with normal and increased intestinal permeability (> 0·09)

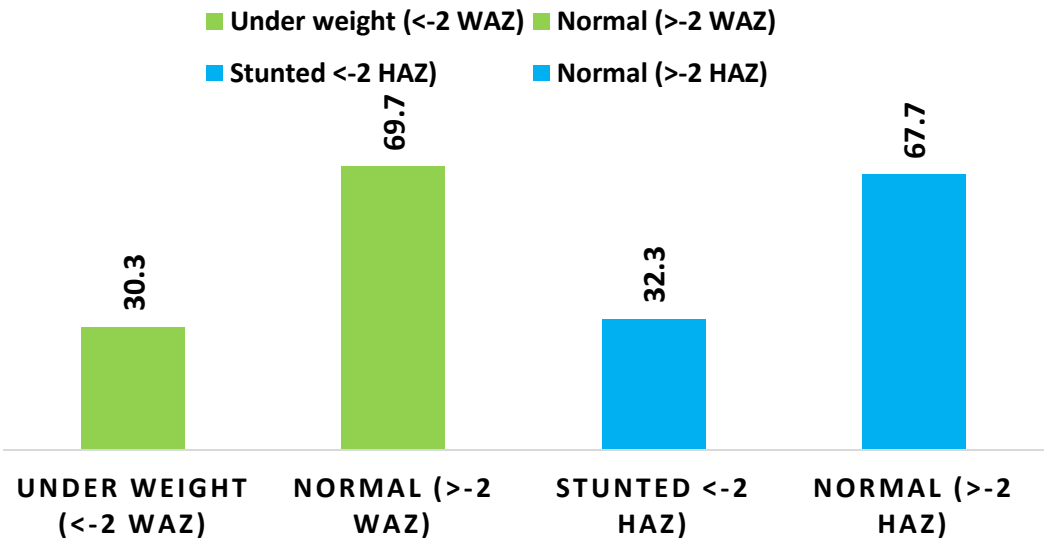


Figure Error! No text of specified style in document.**.3** Distribution of HAZ and WAZ in children

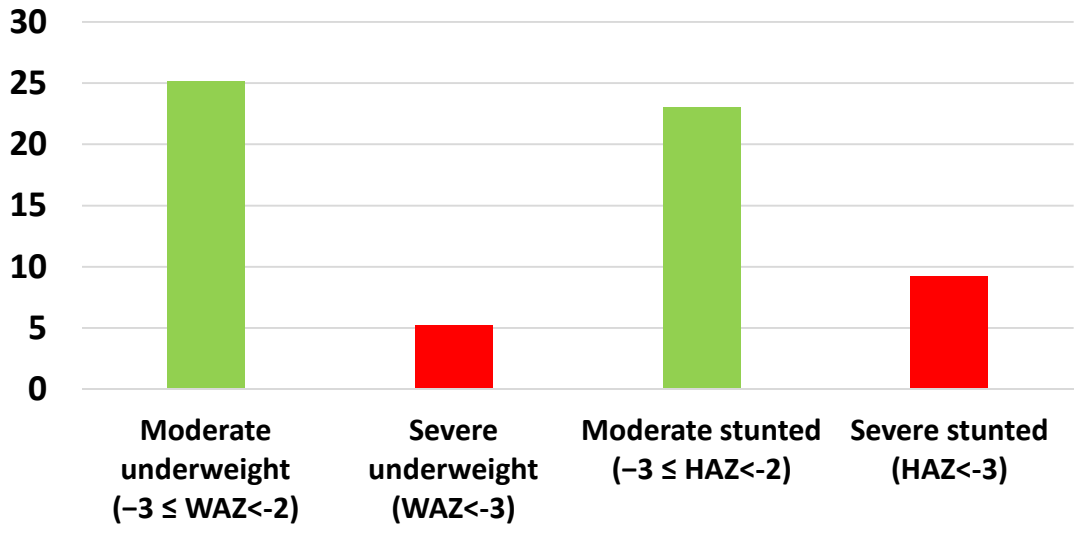


Figure Error! No text of specified style in document.**.4** Distribution of HAZ and WAZ (Severe and Moderate)

Table 4.1 Clinical features of the study population in Mirpur slum area, Dhaka< Bangladesh

Characteristics (442 Children)	Frequency, n	Percent
Moderate underweight (−3 ≤ WAZ<-2)	111	25.1
Severe underweight (WAZ<-3)	23	5.2
Moderate stunted (−3 ≤ HAZ<-2)	102	23
Severe stunted (HAZ<-3)	41	9.2

Table 4.2 Comparison of post urinary LM ratio with nutritional status of children

Grouping Variable	Group		Lactulose Recovery Rate Median	Mannitol Recovery Rate Median	Lactulose/ Mannitol Ratio Median
HAZ	HAZ ≤-2	Stunted (n=143)	0.00085 (0.00047-0.00187)	0.01182 (0.00485-0.02479)	[a] 0.1250 (0.0501-0.1830)
	HAZ>-2	Normal (n=299)	0.00102173 (0.00046-0.00176)	0.01445 (0.00546-0.02965)	[a] 0.06612 (0.0414-0.1119)
WAZ	WAZ≤-2	Malnourished (n=134)	0.00104 (0.00056-0.00246)	0.01283 (0.00550-0.02935)	[b] 0.1030 (0.0552-0.1545)
	WAZ>-2	Well nourished (n=308)	0.00092 (0.00047-0.00171)	0.01435 (0.00556-0.02920)	[b] 0.0643 (0.0426-0.1058)

A typical SRM chromatogram for lactulose, mannitol, and their internal standard, melibiose, in a urine sample is shown in **Figures 4.5 and 4.6.** The illustration clearly illustrates that the three molecules were adequately separated by chromatography and that no interferences were seen.

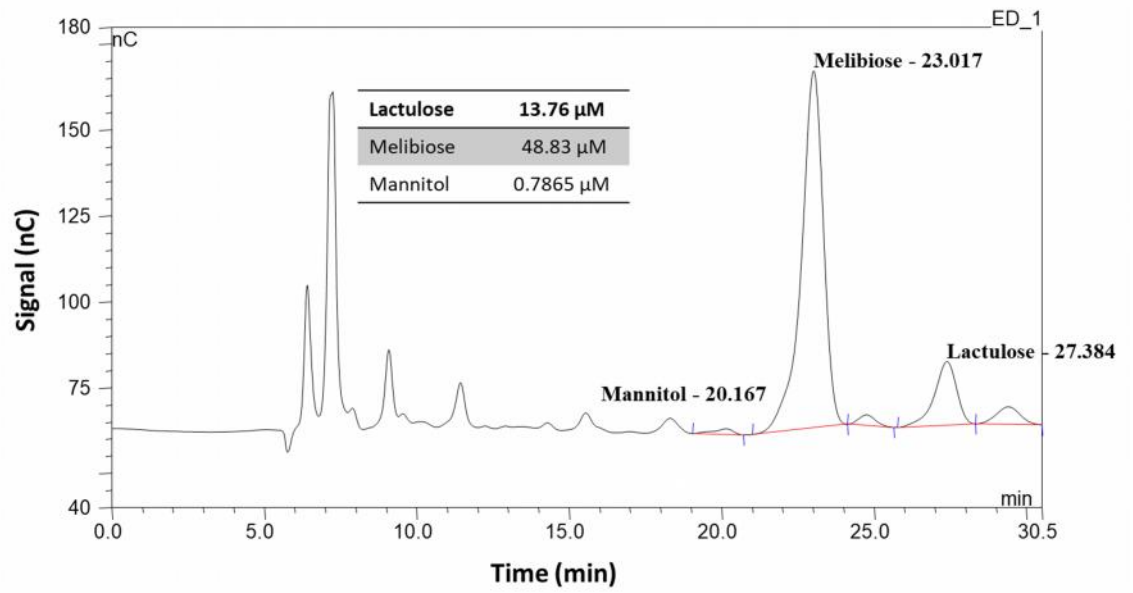


Figure Error! No text of specified style in document.**.5** Chromatogram of urine specimen before lactulose mannitol solution intake

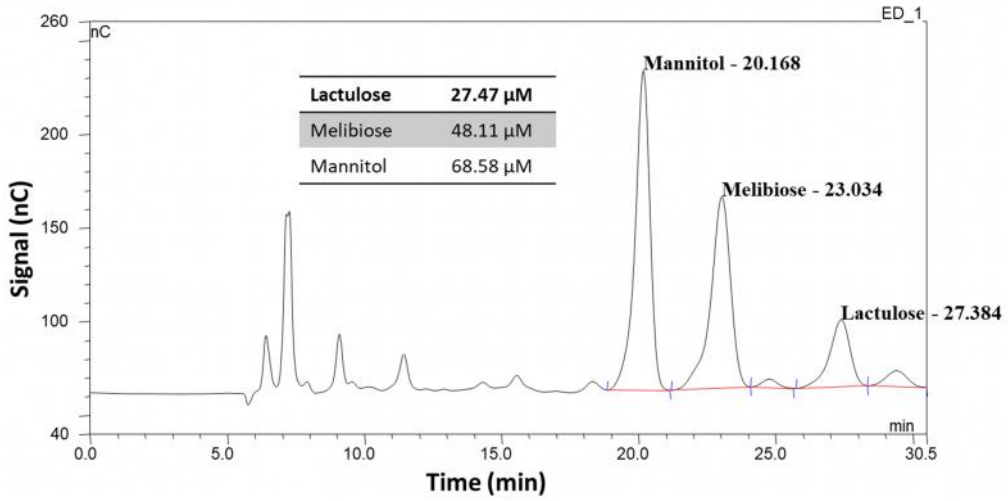
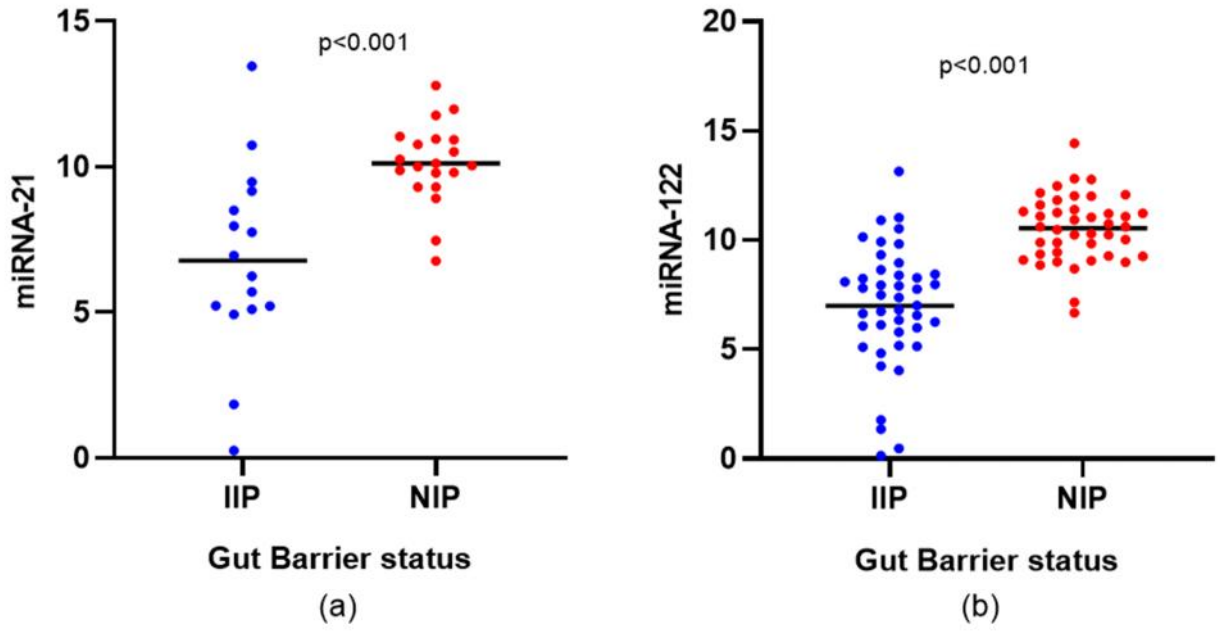


Figure Error! No text of specified style in document.**.6** Chromatogram of urine specimen after lactulose mannitol solution intake

1.2 miRNA-21 and miRNA-122 expression in feces

The real time quantitative PCR data are displayed as normalized ΔCT values. miRNA-122 showed a significant difference in expression between children with IIP and those without IIP in stool samples ($P < 0.001$). In a similar way, miRNA-21 expression in fecal samples from children with increased intestinal permeability was significantly different from that in children with NIP ($P<0.001$). There were significant differences in the mean ± standard deviation of the normalized ct values for miRNA-21 and miRNA-122 in children with IIP compared to children with NIP (miRNA-21: 6.78± 3.24 vs. 10.12 ± 1.40; miRNA-122: 6.99 ± 2.75 vs. 10.53 ± 1.51). miRNA-21 and miRNA-122 expression levels in feces were upregulated in children with IIP when compared to children with NIP 10;($P < 0.001$, 95% CI:5.055-10.78); and 11.6; ($P < 0.001$, 95% CI: 6.146-11.01) times respectively. These

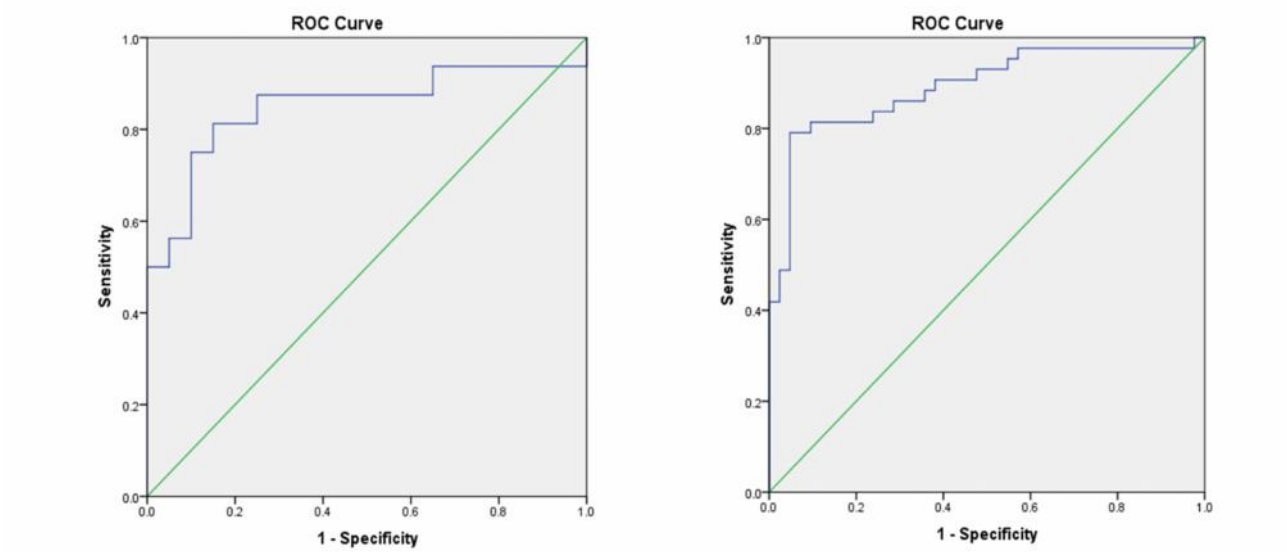


results are displayed graphically **(Figure 4.7)**

Figure Error! No text of specified style in document.**.7** microRNA-21 and microRNA-122 expression level comparison between increased intestinal permeability and normal intestinal permeability respectively

1.3 Receiver operating characteristic (ROC) curve analysis for miRNA-21 and miRNA-122

Biomarkers are crucial for detecting diseases early, since doing so can help patients avoid serious complications and improve their prognosis. The detection of miRNA in stool has a good sensitivity and specificity (222). ROC curve analysis was performed to evaluate the diagnostic capability of fecal microRNA-21 and microRNA-122 levels in order to study the possibility that these miRNAs may serve as new and prospective potential biomarkers for IIP. The AUC values for miR-21 was 0.850 (95% confidence interval [CI] 0.706-0.94, *P*=0.0001) and the AUC values for miR-122 was 0.893 (95% confidence interval [CI] 0.822-0.964, *P*=0.0001) which are shown in **Figure** Error! No text of specified style in document.**.8**. This finding showed that miR-21 and miR-122 has the potential to detect IIP. As a result, the levels of miRNA-21 and miRNA-122 in the stool could be seen as potential biomarkers for



(a) Area under ROC curve=0.850

(b) Area under ROC curve=0.893

the diagnosis of increased intestinal permeability in children.

Figure Error! No text of specified style in document.**.8** The relative expression of miR-21 and miR-122 in fecal samples. It was analyzed using receiver operating characteristic (ROC) analysis to determine the area under the curve (AUC) for differentiating between increased and normal intestinal permeability. According to ROC curve analysis stool miR-21 and miR-122 relative expression has significant sensitivity and specificity to distinguish between children with IIP and NIP. **(Figure 4.8a):** Area under the curve (AUC) for miR-21 yield a value of 0.850. (*P*-value = 0.0001). **(Figure 4.8b):** Area under the curve (AUC) for miR-122 yield a value of 0. 893 (*P*-value = 0.0001).

Additionally, we compared the expression levels of fecal microRNA with the children's L:M ratios, in order to determine the cut-off values of miRNA-122 and miRNA-21 that suggest IIP. When L:M ratio is > 0.09 intestinal permeability is considered to be increased and when L:M ratio ≤ 0.09 intestinal permeability is considered to be normal. Using a cut-off of 9.8 for miRNA-122 (normalized ct value) 84% (36/43) of children with increased intestinal permeability were below this cut-off. As a result, microRNA-122 had a specificity of 71.4% and sensitivity of 84% to detect increased intestinal permeability at this cut-off value. Using the same cut-off value for microRNA-21, 88% (14/16) children with increased intestinal permeabilty were below this cut-off, with a specificity of 75% and sensitivity of 88% (**Table 4.3 and 4.4)**

Table 4.3 Crosstabulation of Normalized microRNA-122 ct value (DCT) *L:M ratio Status: Children with L:M status 1 indicates IIP (L:M >.09); whereas L:M status 0 indicates children with NIP (L:M ≤ .09). DCT 1 denotes normalized microRNa-122 ct value ≤ 9.8 and DCT 2 denotes normalized microRNA-122 ct value >9.8.

Count				
		L:M status (Lactulose:Mannitol status)		**Total**
		0	**1**	
DCT (normalized miR-122 ct value)	**1**	12	36	48
	2	30	07	37
Total		42	43	85

Table 4.4 Crosstabulation of Normalized microRNA-21 ct value (DCT) *L:M ratio Status: Children with L:M status 1 indicates IIP (L:M >.09); whereas L:M status 0 indicates children with NIP (L:M ≤ .09). DCT 1 denotes normalized microRNA-21 ct value ≤ 9.8 and DCT 2 denotes normalized microRNA-21ct value >9.8.

Count				
		L:M status (Lactulose:Mannitol status)		**Total**
		0	**1**	
DCT (normalized	**1**	05	14	19

miR-21 ct value)	**2**	15	02	17
Total		20	16	36

1.4 Association of Enteric pathogens with intestinal permeability

Enteric pathogens were compared in children with increased intestinal permeability and children with normal intestinal permeability. Children with increased intestinal permeability were more often infected with *C. jejuni*, rotavirus, adenovirus, *B.fragilis*, astrovirus, norovirus, and *E.coli* strains (*Enterotoxigenic E. coli-STp*, *Enterotoxigenic E.coli-STh*, Enteroaggregative *E. coli*-aatA Enteroaggregative *E. coli*-_aaiC) when compared to children with increased intestinal permeabilty (P-value < 0.001) **(Figure 4.9)**

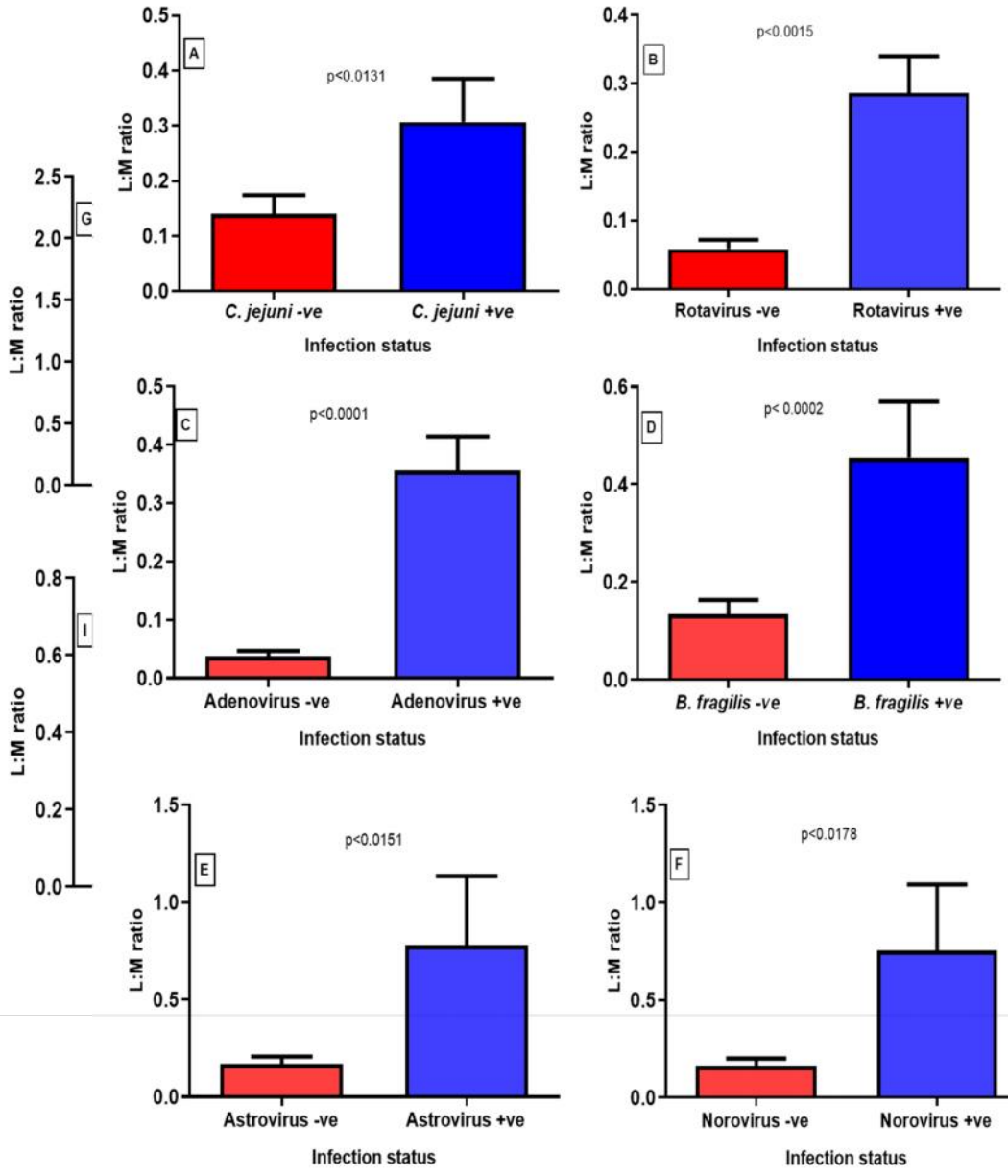
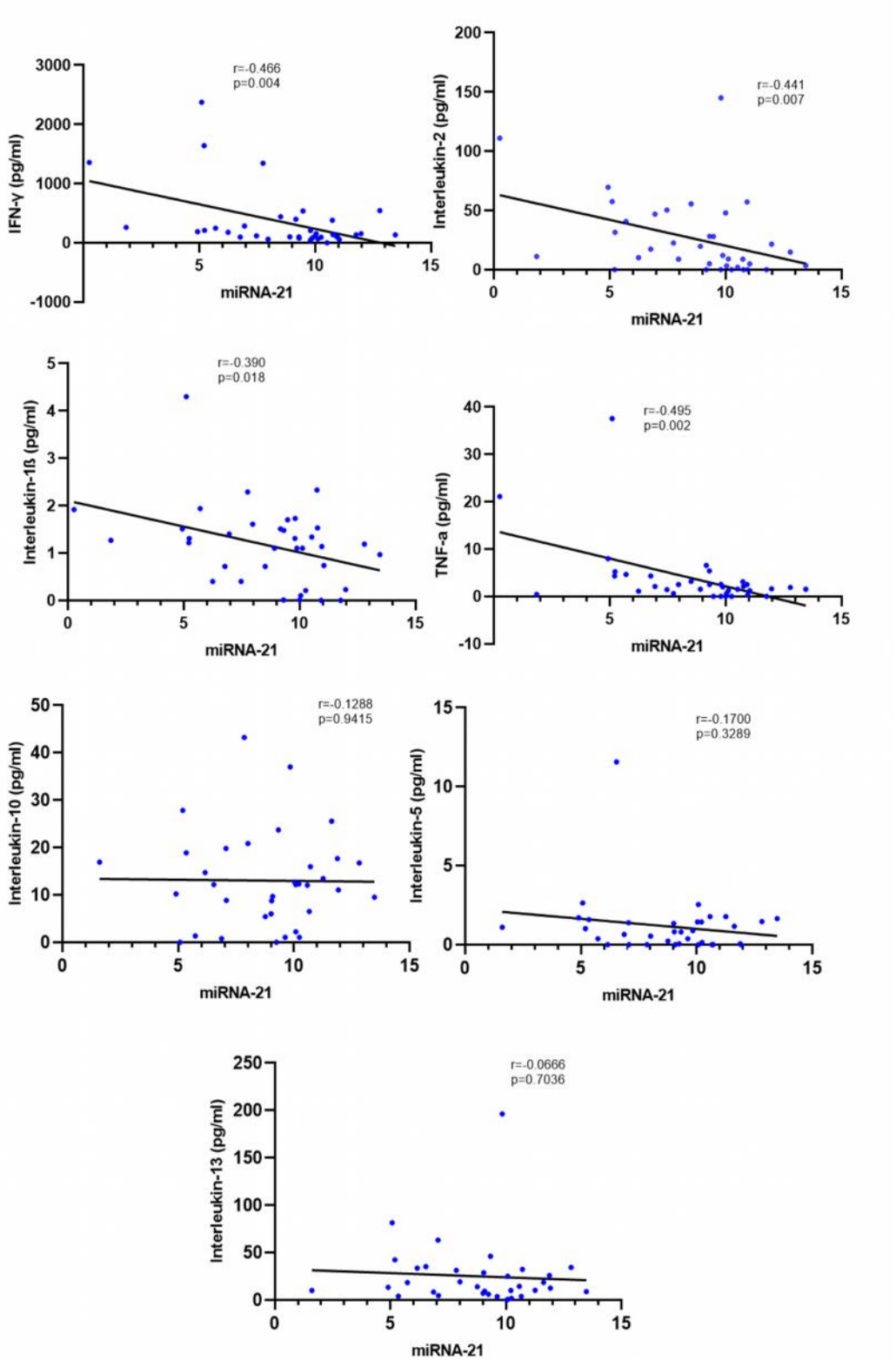


Figure Error! No text of specified style in document.**.9** Comparison of Intestinal permeability with Enteric pathogens in children. Statistical analysis was carried out by independent t-test

1.5 Correlation of inflammatory cytokines with miRNAs

The correlations between the inflammatory cytokines (IFN-γ, IL-2, IL-1β, TNF-α, IL-10, IL-5, and IL-13), with the levels of miRNAs (miRNA-21 and miRNA-122) was also assessed. The levels of cytokines IFN-γ, IL-2, IL-1β and TNF-αlevels were significantly correlated with the levels of the miRNAs. miRNA-21 expression levels were correlated with the levels of IFN-γ ($r = -0.466$, $P = 0.004$), IL-2 ($r = -0.441$, $P = 0.007$), IL-1β ($r = -0.390$, $P = 0.018$),

and TNF-α ($r = -0.495$, $P = 0.002$). Similarly, miRNA-122 expression levels correlated with the levels of IFN-γ ($r = -0.2683$, $P = 0.05$), IL-2 ($r = -0.385$, $P = .008$), IL-1β ($r = -0.3939$, $P = 0.007$), and TNF-α ($r = -0.3222$, $P = 0.030$) (**Figures 4.10 and 4.11**). Inflammatory



cytokines IL-10, IL-5, or IL-13 did not show any significant correlations with the levels of miRNA-21 and miRNA-122

Figure Error! No text of specified style in document.**.10** miRNA-21 expression levels correlation with inflammatory cytokines. The X-axis represents the miR-21 relative expression, while the Y-axis represents cytokines. Pearson's correlation test for statistical assessment was performed

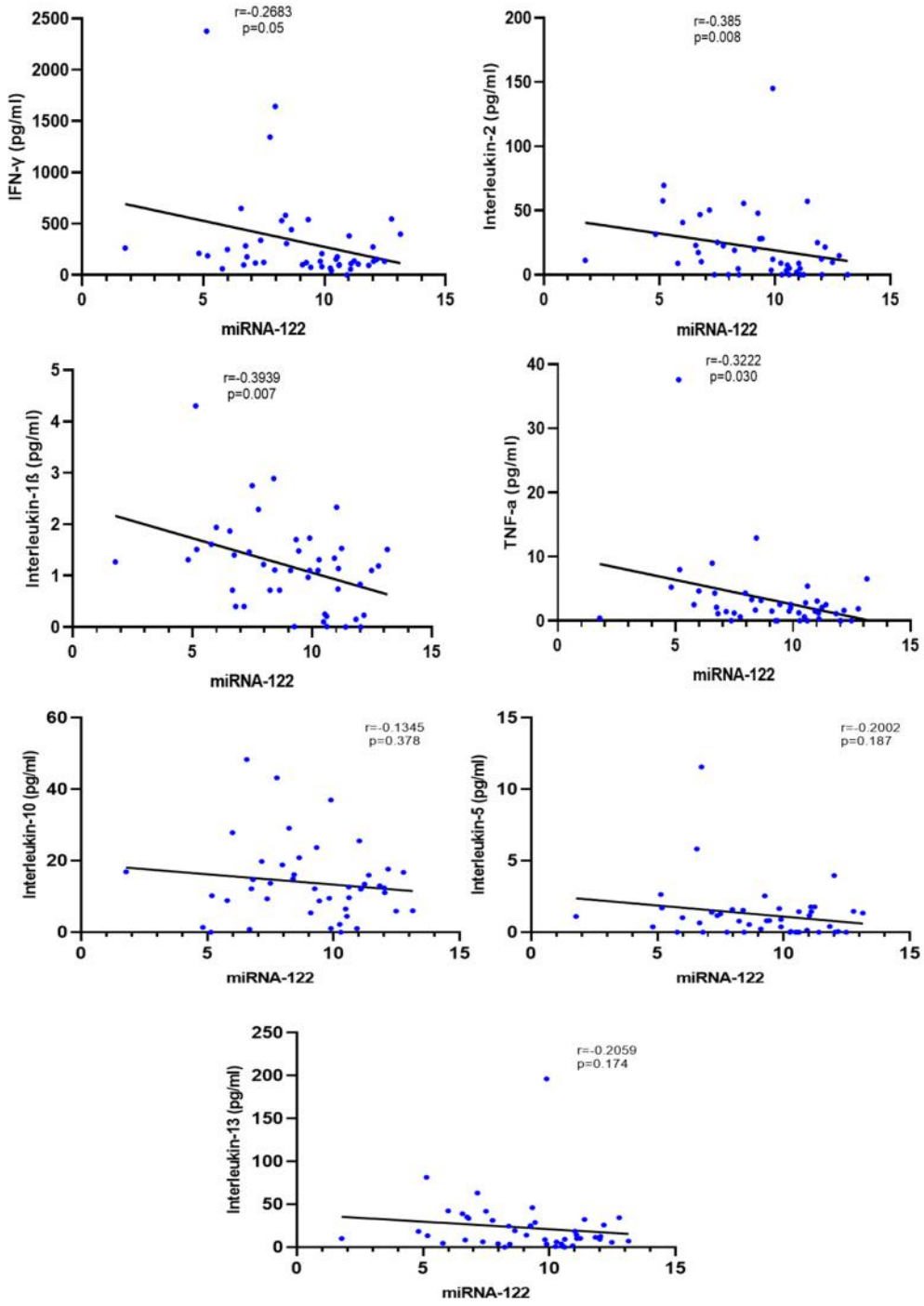


Figure Error! No text of specified style in document.**.11** miRNA-122 expression levels correlation with inflammatory cytokines. The X-axis represents the miRNA-122 relative expression, while the Y-axis represents cytokines. Pearson's correlation test for statistical assessment was performed.

1.6 Correlation of inflammatory cytokines with L:M ratio

We also assessed the correlations between the inflammatory cytokines (IFN-γ, IL-2, IL-1β,

TNF-α IL-10, IL-5, IL-13, and), with L:M ratio levels. The cytokines IFN-γ, IL-1β, TNF-α, and IL-5 levels were correlated significantly with L:M ratio levels. The cytokine level of IFN-γ correlated with the levels of L:M ratio ($r = 0.4713$, $P = 0.0011$), IL-1β ($r = 0.5327$, $P = 0.0002$), TNF-α ($r = -0.2006$, $P = 0.0062$) and IL-5 ($r = 0.2722$, $P = .0500$ **(Figure 4.12). I**nflammatory cytokines IL-2, IL- 13 or IL-10 did not show any significant correlation with L:M ratio.

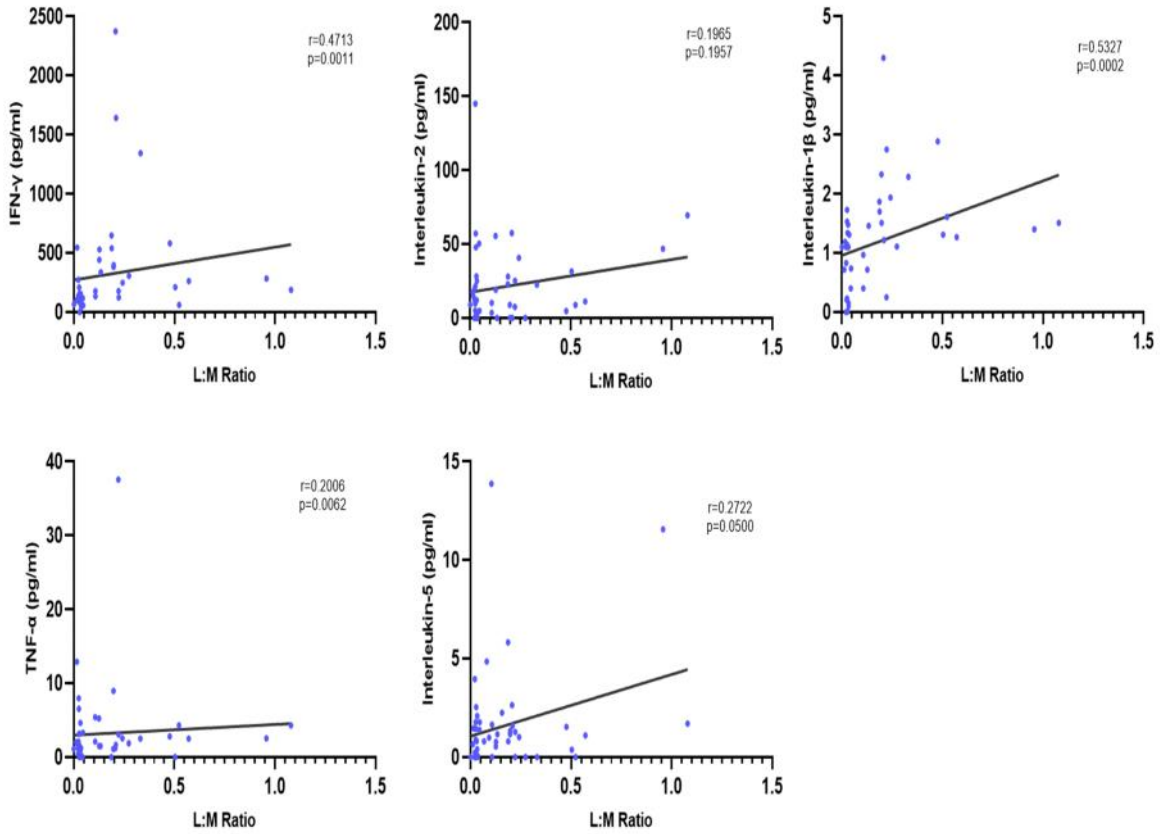


Figure Error! No text of specified style in document.**.12** Correlation of inflammatory cytokines with L:M ratio. The X-axis represents L:M ratio, while the Y-axis represents cytokine levels.Pearson's correlation test for statistical assessment was performed

1.7 Comparison of fecal Inflammatory biomarkers with intestinal permeability

We compared the concentration of fecal Inflammatory biomarkers in children with increased intestinal permeability and children with normal intestinal permeability. Calprotectin (P-value < 0.0121) and Reg1B (P-value < 0.0001) were significantly higher in children with increased intestinal permeability compared to children with normal intestinal permeability **(Figure 4.13).**

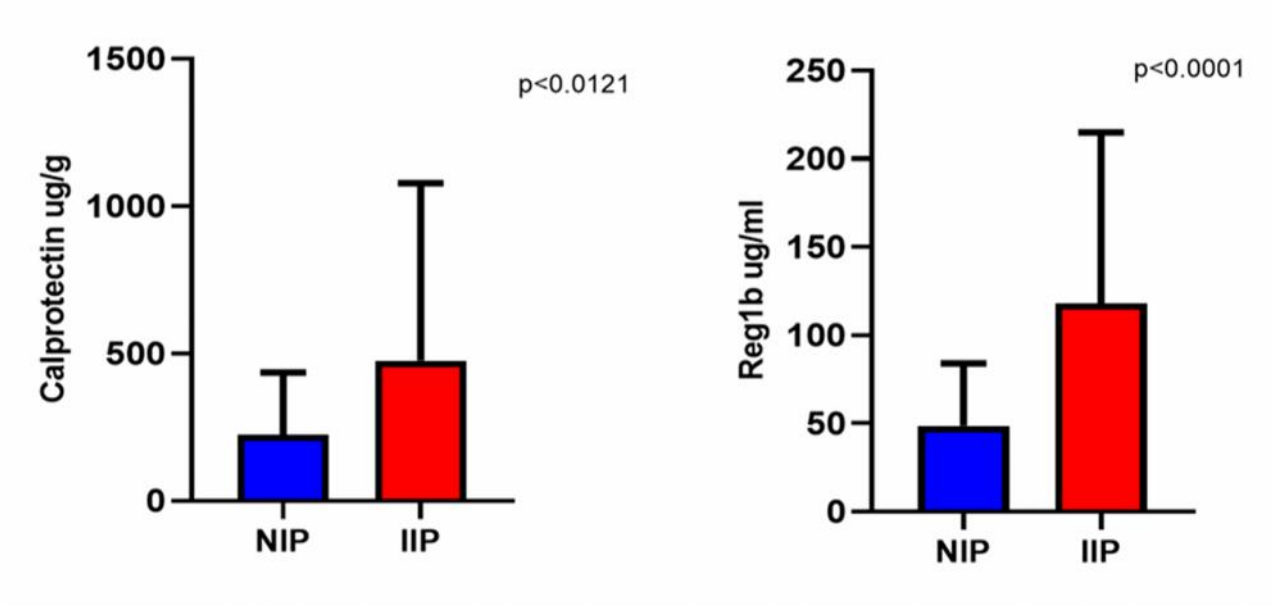


Figure Error! No text of specified style in document.**.13** Comparison of fecal inflammatory biomarkers with intestinal permeability

1.8 Correlations between miR-122 and miR-21 and fecal inflammatory biomarkers

Significant correlation between miRNA-122 and calprotectin, the most prevalent inflammatory biomarker during disease progression was observed (r=-0.278, $P < 0.030$). miRNA-122 and REG1B were also found to be significantly correlated. When compared to children with low concentrations of REG1B, children with high concentration of REG1B had higher expression levels of miRNA-122 in their feces (r=-0.259, $P < 0.015$). The fecal biomarkers Calprotectin and REG1B, however, did not significantly correlate with miR-21**(Figure 4.14).**

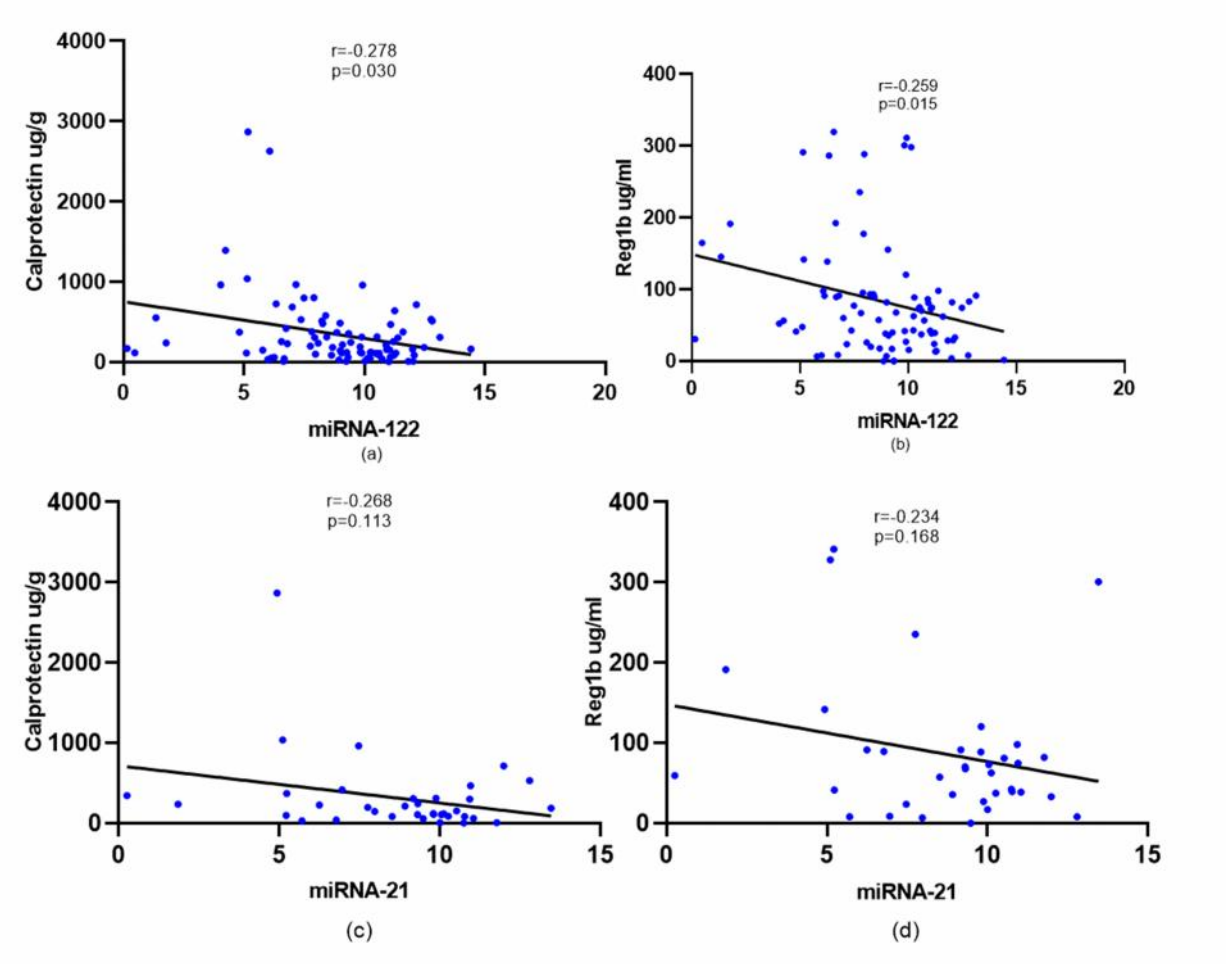


Figure 4.14 Correlations between miRNA-122 and miRNA-21 and fecal inflammatory biomarkers. The X-axis represents the miRNA relative expression, while the Y-axis represents fecal inflammatory biomarker concentration. Pearson's correlation test for statistical assessment was performed

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Infection with enteropathogens has been linked to stunted growth in early children in low-resource settings, which has been linked to long-term consequences with serious consequences for health and human capital. The considerable mucosal damage and intestinal inflammation brought on by exposure to several enteric infections (223) increases intestinal permeability and microbial translocation. we evaluated intestinal permeability in 442 children by analysing the urine samples for Lactulose: Mannitol ratio. Of 442 children, 166 children had a L:M ratio > .09, which indicated altered intestinal permeability. Research from previous studies suggests an IIP is associated with enteric pathogen presence which may indicate systemic and intestinal inflammation(224, 225). In this work, we showed that children with IIP had considerably higher expression levels of microRNA-21and microRNA-122 than children with normal intestinal permeability.

1.1 Expression of microRNA-21 and microRNA-122 was upregulated in children with Altered intestinal permeability/Intestinal barrier dysfunction

Recent studies have shown that microRNAs have a role in regulating the gene expression of vital physiological processes. microRNAs are small, single-stranded, non-coding RNAs with a length of 21–23 nucleotides. They are found in a variety of tissues and take part in a number of biological processes in eukaryotes. MiRNAs serve important roles in cell differentiation, proliferation and down-regulate the target genes by either blocking their translation or by degradation of the mRNA. Previous research has demonstrated that miRNAs, like miR-21, are involved in intestinal permeability regulation. Moreover, dysregulated miRNA expression has been associated with the pathologic hallmarks of IIP. A significant pathogenic factor contributing to the emergence of intestinal inflammation in a variety of intestinal permeability disorders is a defective intestinal epithelial TJ barrier, which results in enhanced antigenic penetration. IBS, IBD, and other disorders result from damage to the intestinal epithelial barrier. Children with IIP had significant upregulation of microRNA-21 (fold change 10; $P < 0.001$) and microRNA-122 (fold change 11.6; $P < 0.001$) compared to those with NIP. Similar, earlier studies found that specific miRNA expression profile patterns in intestinal related disorders and ongoing exposure to several stool pathogens result in increased intestinal permeability and reduced barrier function, that may result in dysregulated expression of microRNA-122 and microRNA-21(136, 137, 226-228). The overexpression of microRNA-21 was linked significantly, according to Zhang et al. (2015), to in vitro study of increased tight junction permeability. Another study found that small intestine epithelial cells isolated from burns patients with IIP had changed levels of many

microRNAs, including miRNA-122, miRNA-21, miRNA-146a, miRNA-155, miRNA-874, miRNA-429 (229). According to Zhang et al., 2018 microRNA-21 is reported to be increased during intestinal barrier failure (136, 230) According to Liu et al., 2019 and Ye et al., 2011, miRNA-122 and miRNA-21 may also be crucial for maintaining intestinal barrier function. As a result, deregulation of microRNA-21 and microRNA-122 and can affect the barrier function of the intestine.

1.2 Children with Altered Intestinal Permeability infected with enteric pathogens

The Global Enteric Multicenter Study determined that the most prevalent pathogens linked to moderate to severe diarrhea in children both infants and young in low-resource settings were Adenovirus, Rotavirus, *Campylobacter* spp ,and heat-stable toxin enterotoxigenic *Escherichia coli* (ST-ETEC)) (231). However, accumulating data suggests that enteric pathogen infection poses a health risk to children. Pathogens can cause systemic inflammation by increasing intestinal inflammation and permeability. In a multisite birth cohort research, for example, a high *Campylobacter* pathogen burden was linked to a substantial decrease in height for age z-score.

Many viral and bacterial agents have defined risk factors, such as immunodeficiency or previous diseases, that predict progression to severe disease. Rotavirus infection is the largest cause of acute gastroenteritis (AGE) hospitalization in Europe, causing a significant burden on healthcare systems, society, individuals, and families each year. Rotavirus only infects the small intestine's terminally developed villus enterocytes. In human newborns, mucosal injury involves villus atrophy and necrosis of villus epithelial cells, which is followed by the replacement of immature cells with decreased absorptive capacity with immature cells. Small intestine shrinkage and decreased intestinal mucosal protection have been linked to severe malnutrition.

Campylobacter has been involved in recent research, with environmental enteric dysfunction, a syndrome characterized by impaired intestinal function and inflammation, as the possible mechanism. *Campylobacter* infection is infrequent in high-resource settings, and is linked to undercooked chicken exposure, or less frequently, common-source outbreaks, which are often caused by contaminated dairy products. In low-resource settings, on the other hand, *Campylobacter* infection is common. *Campylobacter* was linked to increased intestinal permeability as well as local and systemic inflammation in one investigation, suggesting a reason for the lower linear growth. This is consistent with mounting evidence that

Campylobacter can promote intestinal inflammation, including through altering the intestinal microbiota, impairing the intestinal barrier, and preparing the intestine for chronic inflammatory reactions.

There have also been reports of altered levels of specific miRNAs in the stool of patients with enteric pathogens (131, 232, 233), indicating that these miRNAs may play a role in the pathogenesis of intestinal barrier dysfunction and may serve as prognostic or diagnostic tools for altered intestinal permeability (137, 234, 235). Numerous enteric pathogens were found in children with IIP in our study. Children with IIP frequently had rotavirus, adenovirus, several strains of *E.coli* (ETEC STh, EAEC aaiC, EAEC aatA ETEC STp), *B. fragilis*, *Campylobacter jejuni*, astrovirus and norovirus in their stool samples.

The intestinal barrier may be damaged as a result of infections impact on miRNA expression. This idea is consistent with mounting evidence that pathogens can cause intestinal inflammation by altering the microbiota in the intestine, compromising the intestinal barrier, and setting the stage for chronic inflammatory responses (223, 224, 236, 237). Additionally, the expression of miRNAs might alter significantly as a result of a pathogen infection (238, 239). One of the most frequent causes of diarrhea in children under the age of five is viral infections like rotavirus, and there is currently no effective medication to treat this virus. According to a previous study, mimic miRNA-7 agomir may prevent the rotavirus replication when given orally to mice. This suggests that miR-7 may reduce the rotavirus replication (240). In another investigation, children who are infected with the adenovirus displayed different expression profiles of microRNA from controls (13). Similarly, as noteworthy results of our study, infections brought on by enteric pathogens were associated with abnormal levels of microRNA-21 and microRNA-122, which might have a significant effect on children's growth and development. The inflammation and dysbiosis of the host are connected to these fecal miRNAs. Infection with an adenovirus has a significant effect on cellular miRNA. Cellular miRNAs are significantly impacted by adenoviral infection. Adenovirus infection causes host-cell miRNA expression to be significantly changed, and the virus also prevents essential proteins involved in the silencing machinery from functioning.

Inflammatory bowel diseases are just one of the conditions that can result from the normal gut microbiota's dysbiosis (241). Additionally, it has been discovered that miRNAs are principally in charge of changes to the bacterial microflora and have a significant role in modifying pathogenic illnesses (158). Therefore, further research into the potential of

microRNAs as diagnostic and therapeutic tools for pathogenic infections in humans brought on by viruses, bacteria, fungi and parasites is warranted.

1.3 Fecal Inflammatory biomarkers, miRNA expression and Intestinal barrier permeability

In tissue samples from people with benign disorders such as ulcerative colitis and Crohn's disease and acute amoebic colitis (242), REG1B and Calprotectin expression is increased (243, 244). One study found a correlation between fecal Calprotectin levels and fecal miRNA levels (158). It's interesting to note that miRNA-122 expression levels in the fecal samples in this study highly correlated with the fecal biomarker REG1B, a possible indicator of intestine damage and healing Additionally, miRNA-122 and calprotectin showed a strong correlation. Calprotectin is the most widely used inflammatory biomarker of disease progression. The lack of statistical significance with miR-21 with the stool biomarkers Calprotectin and REG1B was most likely caused by the small number of subjects. Our investigation also revealed that Calprotectin and Reg1B levels were considerably higher in children with Increase intestinal permeability when compared to children with normal intestinal permeability.

1.4 Correlation of microRNA-21 and microRNA-122 with Inflammatory cytokines in children with an altered intestinal permeability

This study focused on a population where pathogen exposure and undernutrition are common. According to one study, the damage of epithelial cells by *Escherichia coli* is marked by an elevation of cytokines like TNF-α and IFN-γ (245). Our findings suggest that even while IBD is less common among children in Bangladesh than in western nations, the high infection load increases inflammatory cytokine expression, which results in aberrant microRNA-21 and microRNA-122 expression in children with Increased intestinal permeability. Given that cytokine activation regulates miRNA expression, numerous published research also points to a strong relationship between miRNAs and cytokine activity. Additionally, our analysis reveals that the serum expression levels of the inflammatory cytokines, IL-2, INF-γ, TNF-α, and IL-1β correlated negatively with the Ct values of miRNA-21 and miRNA-122 in stools ($r<0$) where increased expression of microRNA-21 and microRNA-122 is indicated by low Ct values. These results are consistent with (Yan et al., 2020; Chen et al., 2014) who discovered that miRNA-122 and miRNA-21 can enhance the inflammatory cytokines production, such as IFN-γ, IL-1β and TNF-α, (246, 247). In general, microRNA-21 and microRNA-122 target negative immune response

regulators to increase inflammation. miRNA-21 and miRNA-122 were discovered to play inflammatory roles in intestinal barrier dysfunction, which results in decreased barrier function, according to several studies (136, 137, 235). As demonstrated by (Zhang et al., 2017; Zhang et al., 2015; Ye et al., 2011), TNF-α has been shown to rapidly upregulate microRNA-21 and microRNA-122 in intestinal tissues and enterocytes, which can result in barrier disruption and IIP

To our knowledge, no prior research has investigated the levels of miRNA-21and miRNA-122 expression in the feces of children in Bangladesh or explored the association between these microRNAs and increase intestinal permeability and the presence of enteric infections. The results of our study gives important evidence that miRNA dysregulation during inflammation may play a role in the etiology of intestinal disorders. Additionally, the correlations between these microRNAs and other inflammatory biomarkers opens up new avenues for the application of miRNAs as potential disease biomarkers.

However, our study has some drawbacks. First off, there was no statistically significant association between the fecal inflammatory biomarkers Calprotectin and REG1B and microRNA-21. The correlation with miRNA-122, however, was found to be significant. Additionally, there was no correlation between stool pathogens, miRNA-21or miRNA-122. This could be as a result of the limited statistical power to detect differences caused by the small sample size. Secondly, children who live in Mirpur, Bangladesh's slum neighborhoods were the subject of this study. Large-scale clinical investigations in various geographic locations are required to validate these results. Our results imply that stool microRNA-122 and microRNA-21 may have potential to be clinically practical, non-invasive diagnostic and prognostic biomarkers for pediatric patients, but this implication still has to be confirmed in large validation groups. To reliably validate our findings, more research with bigger sample numbers is needed. Additionally, our observations suggest a biological mechanism by which dysregulation of microRNAs caused by altered intestinal permeability might result in stunted development or malnutrition, and therapeutic correction of miRNA levels may be able to assist in managing environmental enteric dysfunction.

We conclude by demonstrating that children from Bangladesh who have IIP have significantly changed miRNA levels in their feces. Two stool miRNAs were strongly associated with disease activity and readily available surrogate biomarkers such fecal Calprotectin, REG1B, and inflammatory cytokine concentrations in the blood. This work also shows that even after long-term preservation, the miRNA expression levels in feces are quite stable and may be measured in a consistent manner. Overall, our results show microRNAs merit further investigation as possible potential biomarkers of gut barrier diseases, which is consistent with earlier findings that the miRNA expression levels in feces correspond with disease activity.

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