THE EFFICACY OF ORAL SUPPLEMENTATION WITH PROBIOTIC IN PRETERM INFANTS IN THE PREVENTION OF NECROTIZING ENTEROCOLITIS: A RANDOMIZED, DOUBLE-BLIND, CONTROLLED STUDY

by

Suheir Mansour Zaghal

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APPROVED BY:

Assoc. Prof. Dr. F.Yeşim Ekici .....
(Thesis Supervisor)

Assoc. Prof. Dr. Ilke Mungen Akin .....

Assoc. Prof. Dr. Merih Çetinkaya .....

Assoc. Prof. Dr. Şirin Güven .....

Asst. Prof. Dr. Çiğdem Kaspar .....

DATE OF APPROVAL .../..../2016

To my beloved parents, husband and children…

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ABSTRACT

THE EFFICACY OF ORAL SUPPLEMENTATION WITH PROBIOTIC IN PRETERM INFANTS IN THE PREVENTION OF NECROTIZING ENTEROCOLITIS: A RANDOMIZED, DOUBLE-BLIND, CONTROLLED STUDY

Necrotizing enterocolitis (NEC) is a disease mostly seen in premature infants where part of their intestine undergoes tissue damage. Probiotics are considered attractive preventive potential to manipulate and normalize the intestinal flora of preterm neonates at risk of NEC. Probiotics exert their protective effect toward NEC prevention partly via immune-modulation, a mechanism that needs further elucidation and research. This clinical trial aims to evaluate the effect of supplementation with *Bifidobacterium animalis* subsp. *lactis* in preterm neonates on whole blood gene expression of inflammatory response associated genes, on the alterations of some gut microbiota groups present in the preterms fecal samples, and its overall effect on incidence and severity of NEC. The qPCR technique was used to test both the gene expression for *RORC, NCR1, HIF1A and IL1RL1* in blood samples and the bacterial count and colonization in stool samples for *Bifidobacterium genus, Enterococcus spp., Enterobacteriaceae, Lactobacillus group,* and *Candida albicans.* Blood and stool samples were collected from preterm group supplied with *B.lactis*, a preterm control group and full-term healthy group at two time points. Results showed that progression to NEC stage III was prevented in the preterms supplied by *B.lactis.* The presence of *B.lactis* in the intestines of probiotic-supplied group has played a major role in altering the gut microbiota competing with other species such as *Enterobacteriacae,* which include many potential pathogens that are considered risk factors of NEC. Gene expression analysis showed significant alterations in the tested genes that may elucidate the beneficial effect of *B.lactis* on preterms at risk of NEC. It is the first study, so far, to test these genes in blood samples of preterms prone to NEC before and after probiotic administration.

ÖZET

YENİ DOĞAN PREMATÜRELERE BEBEKLERDE NEKROTİZAN ENTEROKOLİT'İN ÖNLENMESİNDE AĞIZYOLUYLA PROBİYOTİK TAKVİYESİNİN ETKİNLİĞİ:RANDOMİZE,ÇİFT KÖR, KONTROLLÜ ÇALIŞMA

Nekrotizan Enterokolit (NEK), genellikle yenidoğan prematüre bebeklerin bağırsaklarının bir kısmında doku hasarı olarak görülen bir hastalıktır. Probiyotikler prematüre bebeklerde NEK riskine karşı bağırsak florasının değiştirilmesi ve normalleştirilmesi için kullanılan potansiyel önleyiciler olarak düşünülmektedirler. Probiyotiklerin NEK'e karşı koruyucu etkilerinikısmen bağışıklık sistemi modülasyonu yoluyla gösterdikleri düşünülmektedir ancak bu mekanizmanın detaylı araştırılması gerekmektedir. Bu klinik çalışmada, prematüre yenidoğanlarda *Bifidobacterium animalis* subsp.*lactis* takviyesinin, inflamatuar yanıt ile ilişkili genlerin ifade edilmesine, gaita örneklerindeki bazı mikrobiyota gruplarındaki değişimlere ve nekrotizan enterokolit şiddeti ve oranınagenel etkisininaraştırılması amaçlanmıştır. Gerçek zamanlı polimeraz zincir reaksiyonu tekniği (qPCR) kullanılarak hem kan örneklerindeki *RORC, NCR1, HIF1A* ve *IL1RL1* genlerinin ifade edilme oranlarıhem de gaita örneklerindeki *Bifidobacterium, Enterococcusspp., Enterobacteriaceae, Lactobacillus* grubu ve *Candida albicans* gruplarının sayımıyapılmıştır. *B.lactis* takviye edilmiş ve edilmemiş (kontrol) prematüre yenidoğan ilesağlıklı normal zamanda doğmuş bebeklerden iki farklı zaman dilimindekanve gaita örnekleri toplanmıştır. Elde edilen sonuçlar prematüre yenidoğanlarda *B.lactis* takviyesinin III. aşama'da NEK gelişimini önlediğini göstermiştir. Probiyotik takviyesi yapılan grupta *B.lactis*'in varlığı nekrotizan enterokolitgelişiminde etkili olduğu düşünülen *Enterobacteriac*eaeve diğer potansiyel patojenlerle rekabete girerek, bağırsak mikrobiyotasının değişiminde, önemli rol oynamıştır. Gen ifadeanaliz sonuçları test edilen genlerde önemli değişiklikler olduğunu. Bu çalışma, NEK'eeğilimli prematüre yenidoğanların, probiyotik uygulama öncesi ve sonrası, kan örneklerindeki genler test edilerek şu ana kadar yapılan ilk çalışmadır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

ATCC	American Type Culture Collection
B.lactis	*Bifidobacterium animalis subsp. lactis*
Bp	Base Pair
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
Cq	Quantification Cycle
DCs	Dendrtic Cells
dNTPs	Deoxynucleoside Triphosphates
EDTA	Ethylenediamine Tetraacetic Acid
ELBW	Extremely Low Birth Weight
FullCtr	Full-Term Control Group
G-	Gram Negative
G+	Gram Positive
GA	Gestational Age
GALT	Gut Associated Lymphoid Tissue
gDNA	Genomic DNA
GIT	Gastrointestinal Tract
HIF-1A	Hypoxia Inducible Factor- 1 Alpha Subunit
IBD	Inflammatory Bowel Disease
IECs	Intestinal Epithelial Cells
IL	Interleukin
INF-1	Interferon 1
LPS	Lipopolysaccharides
LTi cell	Lymphoid Tissue Inducer Cell
MAMPs	Microbial Associated Molecular Patterns
mRNA	Messanger RNA
M.R.S.	de Man Rogosa Sharpe
NCR	Natural Cytotoxicity Receptor

NEC	Necrotizing Enterocolitis
NF- κB	Nuclear Factor κB
NICU	Neonatal Intensive Care Unit
NK	Natural Killer Cell
NKp46	Natural Killer p46
NTC	No-Template Control
PAF	Platelet Activating Factor
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKG1	Phosphoglycerate Kinase1
PPIB	Peptidylprolyl Isomerase B
PreCtr	Preterm Control Group
ProGrp	Probiotic Group
PRRs	Pattern-Recognition Receptors
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RBC	Red Blood Cell
RCT	Randomized Controlled Trial
ROR-γt	Retinoic Acid Receptor Related Orphan Receptor Gamma t
Rpm	Round-Per Minute
rRNA	Ribosomal RNA
SCFAs	Short-Chain Fatty Acids
SGA	Sabouraud Glucose Agar
SGB	Sabouraud Glucose Broth
SFB	Segmented Filamentous Bacteria
TBE	Tris-Borate-EDTA
Th cell	T-Helper Cell
TNF	Tumor Necrosis Factor
Tregs	Regulatory T Cells
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth

VLBW	Very Low Birth Weight
WHO	World Health Organization

1. INTRODUCTION

1.1. NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis (NEC) is a disease mostly seen in premature infants where part of their intestine undergoes tissue damage or "necrosis". It is considered the most gastrointestinal disease leading to morbidity and mortality in newborn period [1]**.** NEC occurs in 10 per cent of infants born less than 29 gestational weeks and are very low birth weight (VLBW) (< 1500g). About 20 per cent to 30 per cent of those VLBW infants who develop NEC die, while those who survive become at risk for long-term complications, such as neurodevelopment impairment, short bowel syndrome, and impaired growth [2]. The incidence of NEC decreases with higher gestational age and higher birth weight [3]. Moreover, NEC is considered a financial burden on governments due to its long-term morbidity; it costs the United States millions of dollars annually [1]. NEC is known to be a difficult disease to eliminate, thus it is a dominant target for further investigation and exploration [4].

1.1.1. Signs, symptoms and diagnosis

Initial symptoms of NEC, which mainly affects premature infants, include feeding intolerance, increased gastric residuals, abdominal distension and bloody stools. Progression of NEC is consequential with signs of peritonitis , sepsis, and development of systemic hypotension, which necessitate medical attention and care [1].

NEC has been staged according to the characteristics and severity of symptoms using modified Bell's criteria [5]. In stage I, NEC is suspected with nonspecific systemic signs such as temperature instability. Abdominal signs include increased gastric residuals and abdominal distention with mostly normal abdominal X-ray. In stage II, NEC is proven with metabolic acidosis and thrombocytopeania. Abdominal X-ray will show intestinal dilation and pneumatosis intestinalis or free air in the portal vein. Stage III is a more advanced stage of

NEC. Infants in Stage III are critically ill and show hypotension, striking abdominal distention, acidosis, thrombocytopenia, disseminated intravascular coagulation and signs of intestinal perforation. Abdominal imaging confirms diagnosis of NEC and the follow-up of progression of disease. Characteristic X-ray features are seen in majority of infants with suspected NEC, mainly dilated bowel loops, paucity of gas, portal venous gas, and pneumoperitoneum ("free air" outside the bowel within the abdomen). Pneumatosis intestinalis is the hallmark of NEC and is seen in Stage II and Stage III. In order to understand the pathology of NEC, one should know the anatomy of the intestinal immune system.

1.1.2. Anatomy of the intestinal immune system

The immune system is highly established in the gastrointestinal tract (GIT). Intestines are composed of several kinds of cells that participate in the immune response. The intestinal epithelial cells (IECs) are single layer cells that line the lumen of the GIT and act as a physical wall that separate between the outside of the body which contains nutrients and trillions of bacteria and the beneath lamina propria. The lamina propria is the layer of mucosal tissue, which contains majority of the intestinal immune cells such as: lymphocytes, plasma cells, macrophages and mast cells. The epithelial stem cells located in the crypt of the villi differentiate into several cells: enterocytes, are the majority of cells and their function is to absorb nutrients; goblet cells, are those secreting the mucous layer; Paneth cells, they produce antimicrobial peptides; and enteroendocrine cells, which secrete enteric hormones. Moreover, there are microfold (M) cells that transport peptides antigens into underlying Peyer’s patches, lymphoid nodules containg dendritic cells, macrophages, B-cells and T-cells (Figure 1.1).

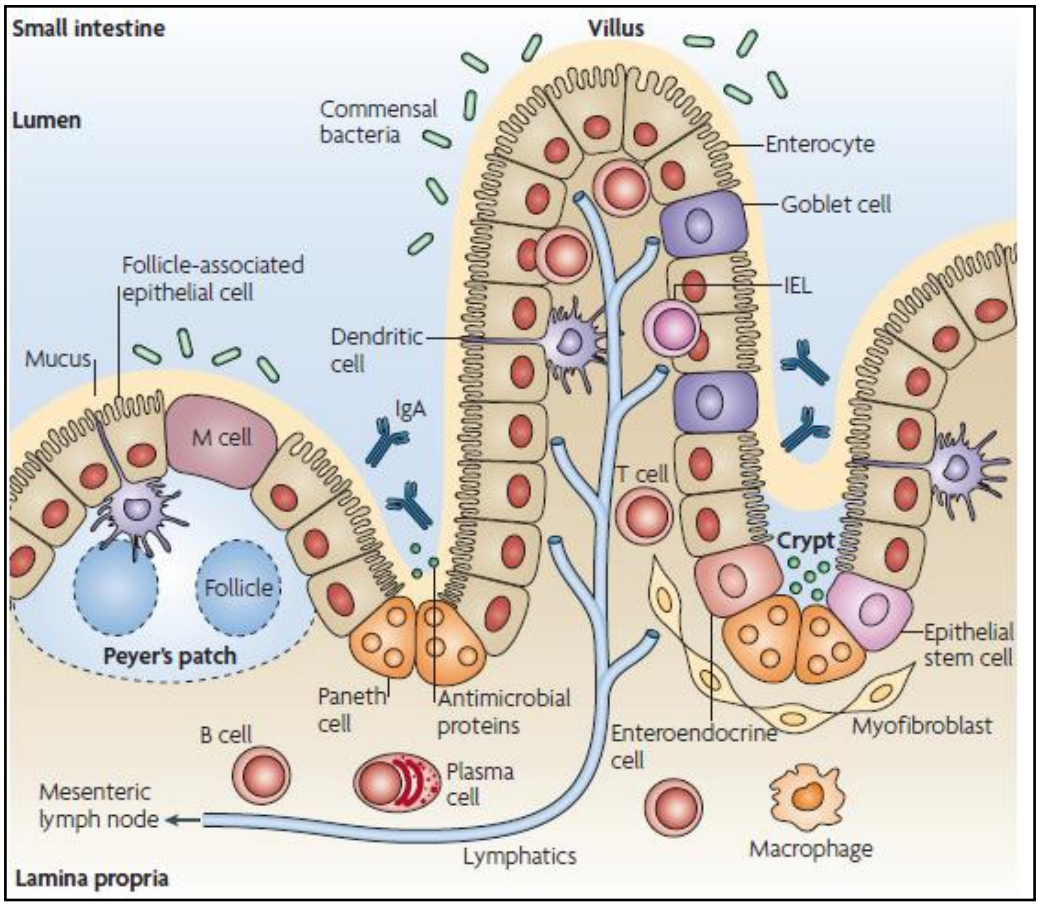

Figure 1.1. Anatomy of the intestinal immune system. [6]

1.1.3. NEC pathology

The pathophysiology of NEC is considered multifactorial and is not yet thoroughly understood. It develops in a susceptible infant, mainly a premature who generally suffers from hypoxia, intestinal immaturity, underdeveloped mucosal immune system that is unable to respond to incoming microbes [1]. Bacteria are known to be involved in the pathogenesis of NEC due to the positive blood cultures in some infants with NEC. The gastrointestinal pneumatosis contains 30 % hydrogen [7], which is produced by bacterial metabolism. Moreover, recent work suggest that analyzing the fecal volatile organic compounds that are

produced by intestinal bacteria can anticipate the development of NEC before clinical symptoms appear [8].

There are environmental factors that contribute to the pathophysiology of NEC and mainly in the alteration of the composition of gut microbiota of these infants. These factors constitute: hospitalization of infants and the presence of pathogenic organisms there, enteral feeding especially by infant formula, exposure to antibiotics, and route of birth which is mainly Caesarean section [1, 9]. All these risk factors predispose the preterm infant to mucosal inflammation, production of high levels of inflammatory factors that further develops into intestinal injury, bacterial translocation, more aggressive immune response and eventually intestinal necrosis. These NEC risk factors can be understood more if differences at the cellular level in bowel's immunity between term and preterm infants are clarified (Figure 1.2).

The newborn bowel's immunity is dependent upon innate immune system rather than adaptive immune system. Toll-like receptor 4 (TLR4), an innate immune receptor, sense the lipopolysaccharide (LPS) specific to pathogens and induce apoptosis [10]. In full-term born neonates, TLR4 surface expression is found to be less than in preterms [10], and found to be declined by breast milk [11].Another innate immune receptor, TLR9 has anatagonistic effect on apoptosis. As it senses commensal bacteria, it down-regulates TLR4 expression. Thus, colonization with commensal bacteria such as *Lactobacillus* and *Bifidobacteria* species reduce the risk of NEC [11]. Moreover, platelet-activating factor (PAF) has a crucial role in mucosal necrosis [12]. It is released in response to hypoxia and inflammation and stimulate the production of TLR4, thus increasing the severity of NEC.

Moreover, TLR4 increases the migration of T cells to intestinal tissue and skew the population of these cells in favor of the pro-inflammatory type T-helper cells 17 (Th17) over the anti-inflammatory regulatory T cells, leading to necrosis of intestinal tissue [13]. Additionally, fetal intestinal epithelial cells produce more IL-8 when they are exposed to bacterial proteins than adults' intestinal cells [11]. They further produce: TNF, IL-1, IL-6 and PAF leading to a hyper-response to inflammation [14]. NEC is also associated with reduced number of goblet cells, Paneth cells and disorganized tight junctions, [15, 16].

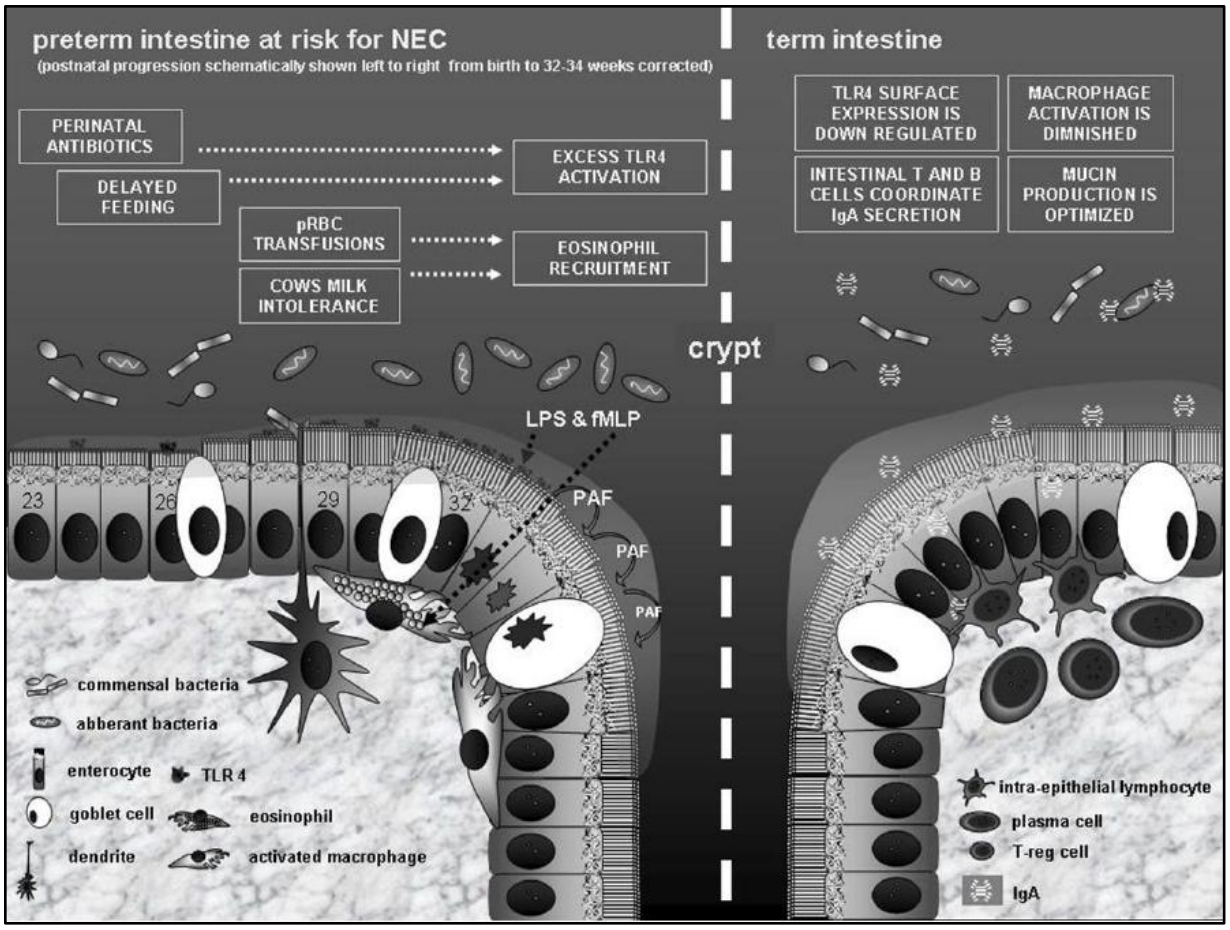

Figure 1.2. Differences in NEC risk between preterm and term intestines [17].

1.2. THE GUT MICROBIOTA

"Microbiota," "microflora," or "normal flora" are the group of microorganisms living with the host [18]. It is estimated that the human microbiota contains 100 trillion (10^{14}) bacterial cells, that is 10 times the number of human body cells [19].There are more than 2000 species of commensal bacterial organisms most of which reside in the gut (Figure 1.3) [20]. There are two main divisions of bacteria in the distal part of the gut, i.e. *bacteroidetes* and *firmicutes* [21]. The large intestine is the most condensed niche worldwide; a trillion (10^{12}) bacteria in each gram of fecal material [22].

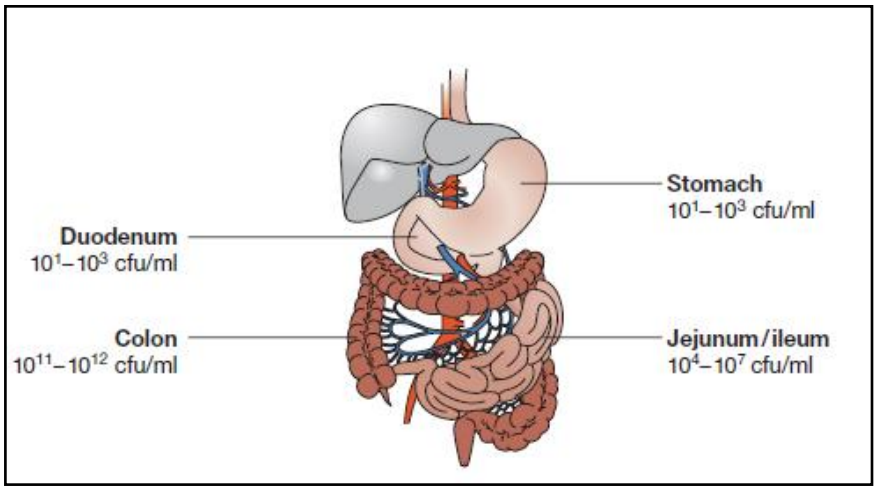

Figure 1.3. Intestinal microflora [23].

1.2.1. Main functions of gut microbiota

The interaction between microbiota and humans creates several advantages for both and thus it is called "commensal bacteria", which originates from the Latin cum mensa or 'sharing a table'[24]. The massive number and high variability of the gut microbiota play an important role in human's health and disease. It is actually acting as an organ within an organ due to its various structural, protective and metabolic activities [23]. The main advantages of the microbiota on the host are: 1) carbohydrate metabolism; 2) production of short chain fatty acids (SCFAs) , metabolites that play important physiological functions in fermentation 3) resistance to colonization of pathogenic bacteria 4) production of vitamins: especially those of group B and K; 5) degradation of xenobiotics; 6) development, regulation and interactions with the intestinal immune system. Gut microbiome rather than human genome plays a crucial role in the metabolism of dietary carbohydrates. Consumption of carbohydrates leads to increase in the proportion of *Bacteroidetes*; a phylum that encode for carbohydrate-active enzymes such as: glycotransferases and polysaccharide lysases [25, 26].

In the colon anaerobic bacteria such as *Bifidobacteria* ferment indigestible carbohydrates to SCFAs such as: butyrate, acetate, and propionate. Butyrate is the primary energy source for

colonic epithelial cells. Acetate act as a substrate for lipogenesis and gluconeogenesis [27]. SCFAs modulate electrolyte and water absorption in intestines and regulate several leukocyte functions including production of cytokines and chemokines as part of the effect of gut microbiota on intestinal immune function. Butyrate, for instance, suppresses the LPS and cytokine induced production of pro-inflammatory cytokines (Figure 1.4) [28].

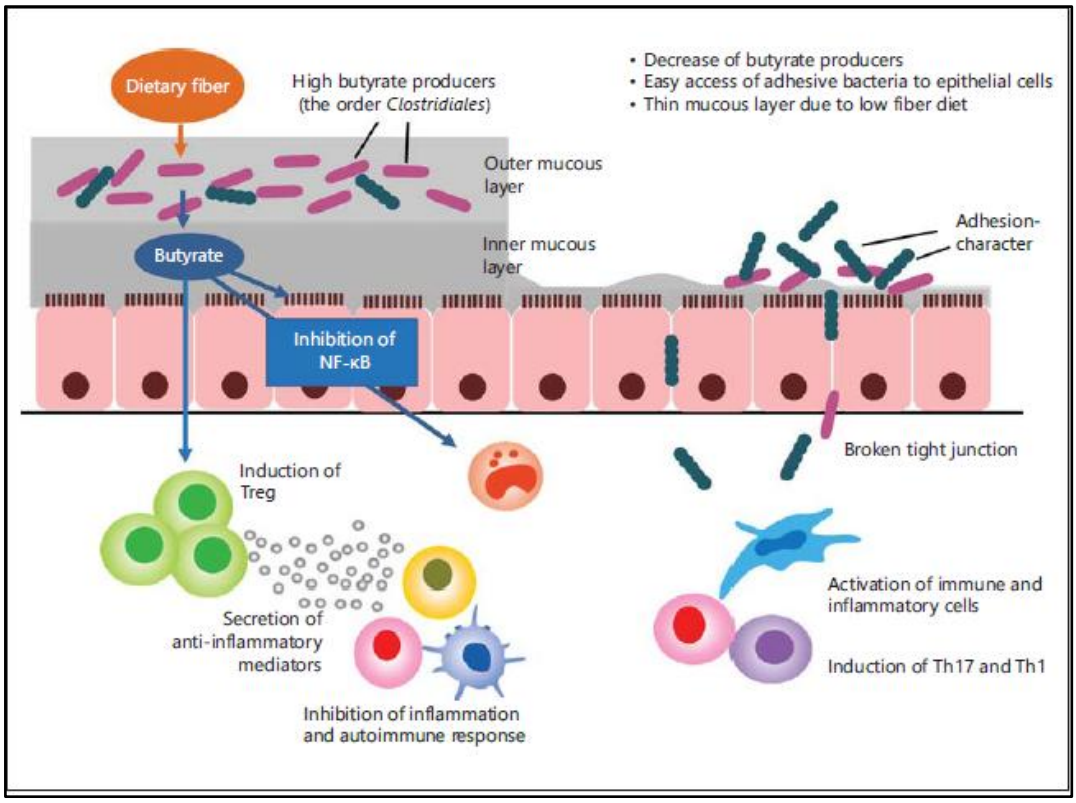

Figure 1.4. Role of gut microbes and SCFAs in mucosal immune responses [27]

1.2.2. Gut microbiota and the immune system

Gut microbiota modulate the maturation of the gut associated lymphoid tissue (GALT) and prevent pathogens colonization by both directly interacting with them or by stimulating the immune cells. Bacterial fermentation products such as SCFAs act as a link between microbiota and immune system (Figure 1.5). They even modulate the gene expression of the host by

increasing the acetylation of histone and non-histone proteins of certain transcription factors [28].

Regulatory T cells (Tregs) are a subpopulation of T cells that modulate the immune system and maintain tolerance to self-antigens [29]. These cells down regulate the induction and proliferation of effecter T cells. Studies showed that *Clostridium* is a strong inducer of Tregs through butyrate production [30], and reduced concentration of SCFs is accompanied by impaired development of Tregs cells [31]. Moreover, gut microbiota is involved in Th1 and Th17 cell development [32]. Th17 cells are a novel class of helper CD4+ T cells characterized by secretion of several interleukins such as IL-17 and IL-22, potent pro-inflammatory cytokines that amplify ongoing inflammation [33]. Germ-free mice models are used in several experiments to understand the relationship between microbiota and the immune system. These mice that completely lack microbiota show several problems in the development of their immunity [34].

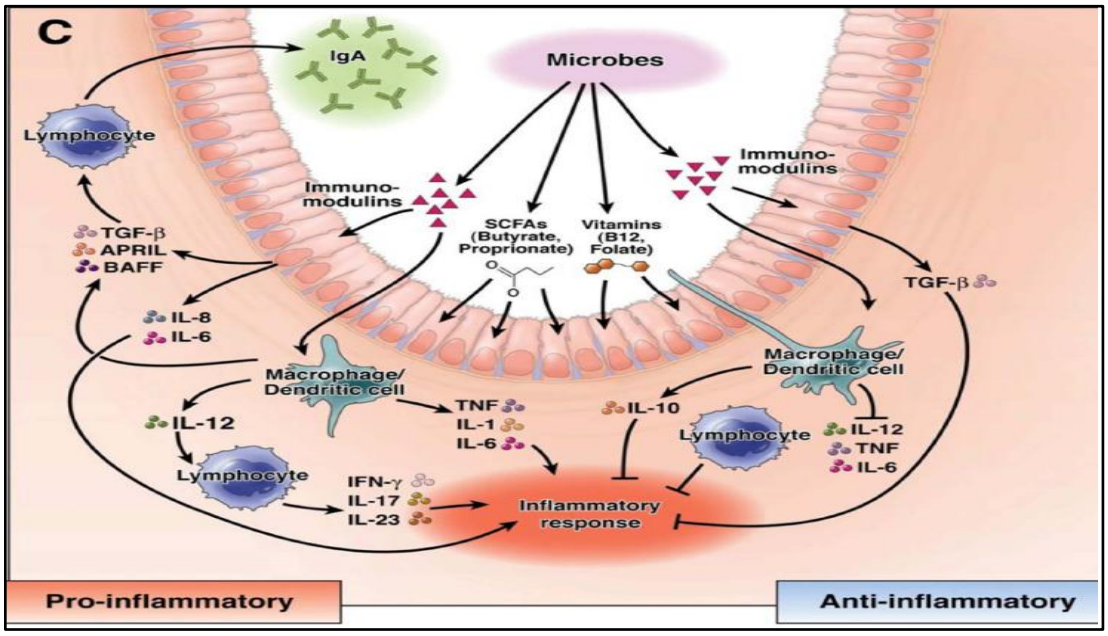

Figure 1.5. Immunomodulation by the intestinal microbiome [25]

1.2.3. Early microbiota in neonates

Contradictory to what has been believed previously that the neonates are born sterile, colonization of microbiota starts before birth. Neonates are found to be colonized early in uterus by microbes originating mainly from the placenta and the amniotic fluid which they swallow large amounts of it especially in the third trimestor. Maternal intestinal bacteria are transferred to the fetus via dendritic cells (DCs) which are able to carry live commensal bacteria and cross epithelial barriers [35]. Accordingly, maternal flora can be found in mammary glands, breast milk, placental tissue and in the neonates meconium samples [36].

Perinatal exposure to microbes occurs during delivery. The mother's vaginal tract microbes, which are influenced by maternal infection and peripartum antibiotic therapy, will be transferred to the newborn during normal delivery [37]. Neonates born by caesarian section will primarily be exposed to their mothers' skin microbiota. During early postnatal period, bacterial species rarely exceed 10 and their composition is unique in each neonate. Adult microbiota, however, is highly diverse with more than thousand bacterial species.

Streptococcus, *Staphylococcus*, *Escherichia. coli*, *Lactobacillus*, and *Enterobacter* were found to be the leading species that colonize infants [38]. During the first week of life, transformations of the intestinal flora take place; aerobic bacteria is replaced by facultative anaerobic bacteria and then eventually the latter will be displaced by strict anaerobes, such as *Bifidobacteria* species, *Clostridia* and *Firmicutes* phyla [39]. The most frequently found bacteria are *Staphylococci, E. coli, Enterococci, Clostridia, Bacteroides, and Streptococci* and mostly are commensals acquired through parental contact [40]. Initial bacterial colonizing in infants is also diverse in population density, since numbers of 10^3–10^{10}cfu/g of feces have been reported. Moreover, changes in the intestinal microbiota are traced over time in the same infant [40]. The first colonizing bacteria could be outcompeted by another species within weeks and keep on in this pattern of variation until about 1 year of age when the adult microbiota is determined.

1.2.4. Full-terms vs preterms gut microbiota

Preterms microbiota colonization is disrupted by various external factors leading to atypical, less diverse profile of microbiota. Preterms are usually born by Caesarean section rather than normal delivery and have a lower birth weight than full-terms. After delivery and due to their low birth weight, preterms last prolonged period in the neonatal intensive care unit (NICU) where they are exposed to antibiotics for treatment of infections. In NICU, preterm infants are fed breast milk, breast milk with fortifiers that are derived from cow's milk or formula.

Full-terms, however, leave the hospital within few days and spend increased skin-to-skin contact with their mothers. Compared to preterms, full-terms get earlier and larger quantities of breast milk that contains immunostimulatory and antimicrobial constituents as well as ingredients that promote growth of commensal microorganisms [41]. Actually, diet- being breast milk or formula- appears to influence the gut microbiota composition. Analysis of microbiota in solely breastfed infants revealed the presence of mostly *Bifidobacteria* with lower counts of species of *Escherichia* and *Clostridia* [42]. Moreover, the gut microbiome differs in infants fed exclusive breast-milk versus those fed cow's milk based formula [43].

According to the previously mentioned differences between preterm and full-term environments, it is not surprised to find out that preterm infants have lower percentage of the favorable *Bifidobacterium* and *Lactobacillus* bacteria; and higher percentage of *Enterobacteriaceae*, *Enterococcaceae*, and *Staphylococcus* that include pathogenic species [41]. Overall, the lower bacterial diversity in the gut of preterms makes them more prone to invasion by pathogenic microbes.

Beside the formerly external factors, inherent differences in immune function between preterm and full-term infants affect the interaction between microbiota and the host's GIT immune system. The adaptive and innate immunity is more robust and potent in full-term neonates.

1.2.5. Intestinal colonization in NEC

Premature infants in NICUs develop a different intestinal colonization compared with healthy full-term infants. Accordingly, many studies suggest that NEC is associated with these abnormal colonization patterns [44, 40]. Since most preterm infants are delivered by Caesarean section and are less likely to acquire normal flora in the birth canal through delivery, or from breast milk feeding, the colonization of beneficial bacteria such as *Bifidobacteria, Lactobacillus, and Bacteroides* is decreased and the risk of developing NEC is increased [45]. Moreover, the hospital environment with its pathogenic microorganisms and the exposure to antibiotics of both the preterms and their mothers negatively affect the intestinal flora composition.

The association of NEC to bacterial colonization can be also deduced from the observations of bacteremia and endotoxinemia in affected neonates, and the presence of Pneumatosis intestinalis; the presence of gas in the intestinal wall due to bacterial fermentation. However, none of the NEC related bacteria has been found to meet Koch's postulates [46], because they were found in neonates without NEC as well.

Several studies compared intestinal microbiota in NEC vs. non-NEC infants taking into consideration other parameters such as mode of delivery and type of feeding. In a study by Wang and colleagues, patients with NEC were found to have less bacterial diversity with increase in Gammaproteobacteria and decrease in other bacteria species as compared to the control group [47].

In some studies, NEC was associated with the prevalence of non-*E.coli* Gram-negatives [9, 48, 49], *Clostridia* [50], *Enterococcus* [51], *Staphylococcus* [51], *Candida albicans* [52], *Lactobacillus* [49], and lower species diversity. Other studies found no correlation between NEC and intestinal microbiota [53, 54]. Smith *et al.*, studied the microbiota in NEC diagnosed preterms that were either diagnosed as Stage II or Stage III according to Bell's criteria and compared the findings to corresponding control group [50]. He found that infants developing NEC were mainly colonized with Gram-positive (G+) bacterial species such as *Staphylococcus spp.*, *Enterococcus spp.* and *Clostridium spp.*. Whereas the control group was

colonized by Gram-negative (G−) species such as *E.coli*, *Klebsiella spp.*, *Pseudomonas spp.* and *Enterobacter spp.* along with the G+ species [50].

1.3. PROBIOTICS

Diverse methods have been proposed to prevent NEC. These methods include delaying enteral feedings; feeding the perterms with merely breast milk; antenatal glucocorticoids, administering lactoferrin, probiotic agents, prebiotic agents, or both. Wang *et al.*, measured the effect of probiotic administration to preterms for NEC prevention by studying 20 randomized controlled trials [55]. He concluded that "Probiotic supplement was associated with significantly decreased risk of NEC in preterm VLBW infants" [55]. This section discusses probiotics as a potential for NEC prevention.

The term "probiotic" meaning "*for life*" was firstly used in 1965, by Lilly and Stillwell. They used the term to describe the beneficial effects exerted by an organism's secretions on the health of another. The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations have defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" [56]. Probiotics consist of bacteria or yeasts that provide health benefits by reestablishing the microflora of the intestinal tract [56]. Simply, Probiotics are those bacteria found in the normally delivered breastfed infants that help them accommodate with solid food digestion, protect them from several diseases and boost their immune system.

For a strain to be used as a probiotic, it must possess certain criteria besides being from human source; it must be: 1) able to attach to the intestinal wall, multiply and colonize there competing with the pathogenic bacteria. 2) able to produce acids, hydrogen peroxide and bacteriocins against any pathogenic growth; 3) able to restore the balance of the gut microbiota; 4) stable during manufacturing and refrigeration; 5) identified to the strain level; 6) acquire a sufficient viable number of cells that can exert the required function; 7) evaluated in *in vitro* and *in vivo* trials to prove being safe, non-invasive, non-carcinogenic and non-pathogenic [57].

Since the preterm gut microbiota colonization is delayed and disrupted, restoration of the normal commensal flora by probiotic supplementation can be helpful in reducing the incidence and severity of NEC. Several probiotic organisms have been studied in preterm infants, at varied dosages and durations of therapy with strains from *Bifidobacteria* and *Lactobacillus* being remarkably used.

1.3.1. Probiotics mechanism of action

There is accumulating evidence for benefits of probiotics in preventing NEC. The advantages offered by probiotics have been concluded from data of either clinical trials or animal models. The mechanisms by which probiotics exert their effects toward NEC can be by immunomodulation, competitive exclusion, and enhancement of the epithelial barrier (Figure 1.6).

Probiotics express microbial associated molecular patterns (MAMP) that bind to the same receptors as pathogens do to prevent their attachment by competitive exclusion [58]. They also stimulate mucus secretion which protect intestines from pathogenic bacteria [59]. Moreover, probiotics support intestinal barrier function by antimicrobial peptide production and secretion of immunologic defensins [60], enhancing tight junction protein expression and localization [61, 62], preventing epithelial cell apoptosis by inducing bioactive molecules and increasing IgA responses [63].

IECs were found to differentiate between commensal bacterial DNA and pathogenic bacterial DNA by Toll-like receptor 9 (TLR9) ; its expression and localization is increased upon recognition of pathogenic bacterial DNA [64]. Accordingly probiotics do not exert inflammatory response in our GIT, yet decrease inflammation and restore epithelial wall permeability through released soluble peptides [65]. Moreover, ingestion of probiotics enhance existing commensal flora in fermentation of intestinal nutrients [66], and modulate the immune system through stimulation of the GALT. Actually, probiotics are found to be highly involved in the promotion of the immune cascade by inducing cytokine production, playing a major role in differentiation of Th17 and Treg cells by stimulating an interleukin

necessary for this process [67]. They interact with several immune cells such as dendritic cells, macrophages and natural killer (NK) cells and influence intracellular inflammatory pathways through TLRs [68].

Lactobacillus and *Bifidobacterium* strains are the mostly used probiotics in clinical studies, because they are the predominant bacteria in the healthy microbiome. A study by Khailova et al. found that *B. bifidum* as a probiotic restore intestinal integrity and decrease mucosal injury by downregulating apoptosis in a rat NEC model [69].

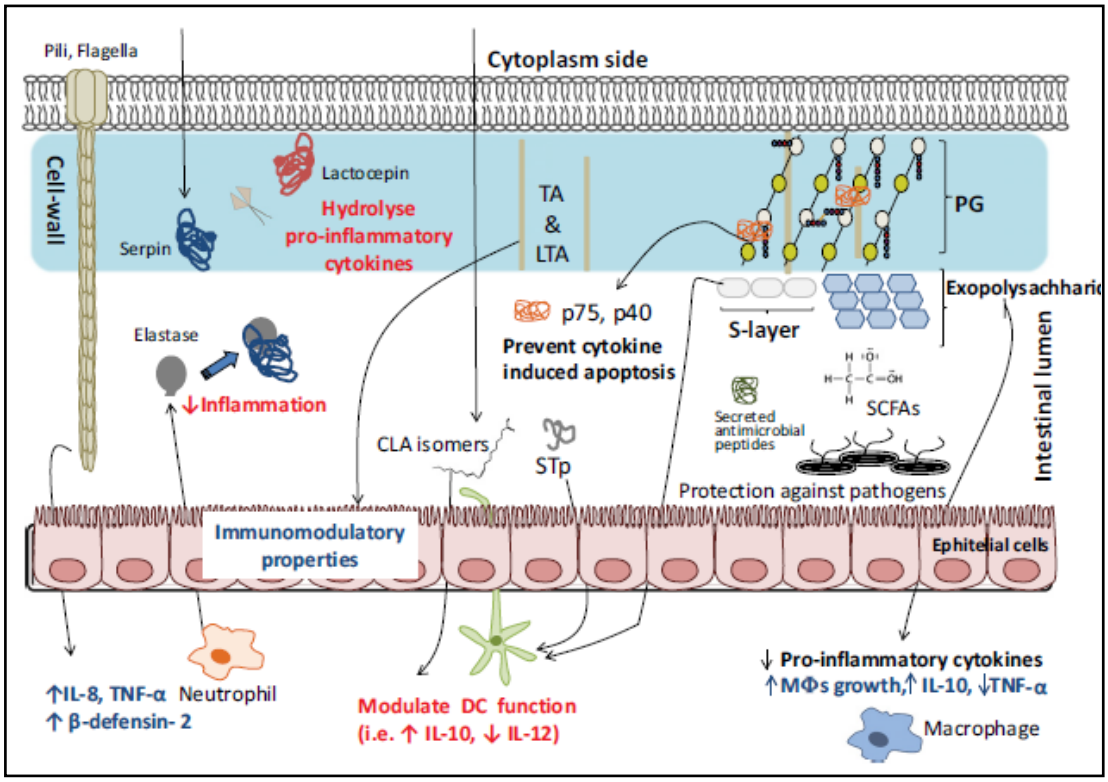

Figure 1.6. Mechanism of action of probiotics [70].

1.3.2. *Bifidobacterium lactis* as a probiotic:

Strains of *Bifidobacteria*, are the most frequently used probiotic because they are normal constituents of the healthy intestinal flora. They were firstly isolated from the feces of a breast fed infant in 1899. *Bifidobacteria* are Gram-positive, non-spore forming, anaerobic bacteria

that produce lactic acid and live in symbiotic relationship with human cells in the intestinal tract [71]. *Bifidobacterium animalis* and *Bifidobacterium lactis* were firstly described as two different species. Modern molecular classification techniques reclassified them to become *B. animalis* with the subspecies *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium animalis* subsp. *Lactis* (*B.lactis*) [72]. *B.lactis* is added to infant formula and fermented milk products as a dietary supplement. Attractively, it does not affect the taste, texture or appearance of the food beside being able to survive during storage and colonize in the GIT [73].

B.lactis shows several beneficial effects all over its use in clinical trials. In comparison to other bifidobacterial species of human source, *B.lactis* expresses the most relevant probiotic characteristics and proves its usefulness for the immune function in the GIT.

Studies show that majority of *B.lactis* can tolerate gastric acid and bile better than other bifidobacteria after being consumed, a crucial characteristic that makes it able to survive and provide healthy effects to the host [74]. Indeed, *B.lactis* is found to have a gene coding for bile salt hydrolase enzyme which is needed for enduring the high bile salt concentrations in the small intestine [73]. In intestines, *B.lactis* fermentation products increase tight junction strength as found in some studies and enhance immune response by inducing the secretion of various interleukins and cytokines [73].

Furthermore, *B.lactis* has high adherence properties and thus confer better colonization, immune interaction and displacement of pathogenic bacteria [75]. Studies show that *B.lactis* is further capable of inhibiting potential gastrointestinal pathogenic bacteria through production of antimicrobial peptides besides competing for mucosal adhesion [76].

B.lactis survives during passage through the GIT and colonizes the colon as proved by data from a placebo-controlled study that was conducted to evaluate the effect of yogurt supplemented by *B.lactis* and inulin as a prebiotic on the rest of inhabitant microbiota [77]. Analysis of the fecal samples using a differentiation method between live and dead bacterial cells indicated that >90 per cent of the detected *B.lactis* was alive.

Conclusively, the formerly mentioned characteristics of *B.lactis* make it an attractive choice for use as probiotic in clinical studies.

1.3.3. Clinical trials for using probiotics in NEC prevention

Taking into consideration the high mortality and morbidity of NEC and the lack of specific medication or treatment, there is a great appeal to develop preventative strategies to control this disease. Probiotics are considered lately an attractive preventive potential to manipulate and normalize the intestinal flora of preterms at risk of NEC. According to the American Pediatric Surgical Association Outcomes and Clinical Trials Committee, they concluded that: "substantial data support routine supplementation of enteral intake with probiotics in premature infants to reduce the incidence of severe NEC (Grade A/B)" [78].

In 1990s, outstanding researches on probiotic colonization in preterms GIT emerged [24]. Then shortly, several randomized-controlled trials were held to test the efficacy of probiotics for the prevention of NEC in preterms. Results, discussions, analysis and summaries on the topic were published in several systematic-reviews and meta-analyses [79–81, 82]. The Cochrane systematic review, for instance, analyzed 1,425 preterm infants born earlier than 37 gestational age and/or less than 2500 g birth weight. The review included nine trials that vary with reference to inclusion criteria, stage of NEC in the control groups, timing, dose, constituent of the probiotics, and feeding criteria. In this systematic review they found that enteral probiotics supplementation significantly reduced the incidence of severe NEC [79].

In another systematic review and meta-analysis published in 2015, Aceti *et al.* reviewed and analyzed twenty-six randomized controlled trials and set the main outcome to be incidence of NEC stage ≥II. Aceti *et al.* found that probiotics prevented NEC in preterm infants with a significant effect for *Bifidobacteria* [81].

In 2016, Olsen *et al.* published another systematic review and meta-analysis to investigate the efficacy of probiotics in preterm neonates involved in observational studies [82]. They included 12 studies with 10,800 premature neonates. Their results showed a significantly decreased incidence of NEC as well as mortality. Thus, they concluded that probiotics had

beneficial effect on NEC in preterm infants at risk. Similar to other meta-analysis, they noted that there are still inadequate data on the specific probiotic strain to be used, the optimal strain, dose and timing and on the effect of probiotics in high-risk populations such as extremely-low-birth- weight (ELBW) infants.

Guidelines of the Canadian Paediatric Society indicate that "probiotics may help prevent NEC and that the administering of live microorganisms to preterm newborns should be approached with caution" [83]. They recommended that breastfeeding along with probiotics could be considered for the prevention of NEC in preterm infants that weigh more than one kilogram and are at risk for NEC.

Based on the previously mentioned systematic review studies, the recommendation for prophylaxis against NEC should be for premature infants born in approximately 27–37 weeks gestational week and weighing less than 1500 g at birth. The dose should range from 1×10^8 to 6×10^9 CFU/d for at least 17 days to six weeks. The common probiotic strains that were used in these publications included *Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium bifidus, Bifidobacterium lactis, Bifidobacterium longum, Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactobacillus casei, Streptococcus thermophilus,* and *Saccharomyces boulardii.* In table 1.1, there are examples of randomized clinical trials on probiotic administration for preterms for NEC prevention.

Table 1.1. Examples of randomized-controlled trials on probiotic administration for preterms for NEC prevention.

Study	**Neonates On Probiotic, (N)**	**Controls, (N)**	**Birth Weight/ Gestational Age**	**Probiotic Agent**	**Dose**	**Outcome**
Dilli, 2015 [84]	100	100	<1500g, <32 weeks	*Bifidobacterium lactis*	5×10^9 CFU	NEC Bell ≥2
Patole, 2014 [85]	79	80	<1500g, <33 weeks	*Bifidobacterium breve* M16-V	3×10^9 CFU	NEC Bell ≥2

Rojas, 2012 [86]	372	378	≤2000g	*Lactobacillus reuteri*	3×10^9 CFU	Death and nosocomial infection
Saengtawesin, 2014 [87]	31	29	≤1500g ≤34 weeks	*Lactobacillus acidophilus Bifidobacterium bifidium*	1×10^9 CFU Of each strain	NEC Bell ≥2
Totsu, 2014[88]	153	130	<1500 g	*Bifidobacterium bifidium*	2.5×10^9 CFU	Enteral feeding , incidence of morbidity
Stratilki, 2007[89]	41	34	27-32 weeks	*Bifidobacterium lactis*	2×10^7 CFU	Decrease of intestinal permeability and other outcomes as NEC

1.3.4. Safety of probiotics use in preterms

Establishing and confirming that probiotics are safe and tolerable by preterms is highly significant regarding their immature intestinal tract and underdeveloped immunity. The possible serious outcomes of inappropriate administration of probiotics include sepsis, antibiotic resistance, disturbed immune responses, lactic acidosis, diarrhea, and distension of the abdomen. Therefore, clinicians should register and follow any possible distressing or abnormal sign during supplementation of the probiotic such as feeding intolerance and infection, and stop the probiotic administration immediately if any adverse effects are noticed. A review of the level of evidence for probiotics in preterm infants concluded that "so far no side effects have been reported and long-term outcomes are promising" [90]. Data from 25 randomized controlled trial (RCT) that included around 5,000 infants indicate that serious detrimental side- effects of probiotics are unlikely [91].

In a six year-follow- up study that reviewed the complete data of 743 patients who received *Lactobacillus rhamnosus GG* for four to six weeks during their stay in NICU and until discharge, supplementation of probiotic resulted no sepsis or other possible adverse effects and proved microbiologically safe and clinically well tolerated [92]. Parker *et al.* , reviewed the best available evidence and developed a probiotic practice guideline for NEC prevention in

VLBW neonates [90]. Their inclusion criteria included systematic reviews, meta-analyses, RCTs, practice guidelines, and case studies from preterms weighing 1000 to 1500 g and ELBW (< 1000 g). The main guidelines they discussed can be summarized in the following points:

- Probiotic strain: *Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Bifidobacteria infantis, Bifidobacteria bifidum, and Lactobacillus casei.* proved to significantly reduce the risk in the incidence of NEC.

- Probiotic dose: a dosage range of 0.056 to $6 \times 10^7$to 10^9CFU/day of probiotics proved to be both beneficial and safe for NEC prevention in VLBW neonates and no serious effects were mentioned in the studies that used these probiotic dose ranges.

- Onset of the probiotic administration: the probiotic administration may be started if the patient has no symptoms of abnormal abdominal examination and is clinically ready to start enteral feedings. It is preferred to start probiotic supplementation in the first two days of birth [93].

- Duration of therapy: the literature supports continuing probiotic therapy for four to six weeks and up to 36 weeks of adjusted gestational age or until the neonate's discharge.

NEC is considered an economic burden for NICUs due to its multiple complications and prolonged hospital stay. Probiotics, on the other hand, cost less than one dollar a day and are a globally available intervention. Thus, the use of probiotics for preterms would become an attractive strategy to reduce the risk of this destructive disease.

1.4. INTERACTIONS BETWEEN PROBIOTICS, NEC AND INFLAMMATORY GENES

The beneficial effects of probiotics are partially exerted by immune-modulatory interactions. The exact molecular mechanisms responsible for the protective effect of probiotics are not fully understood and still under investigations. Most of the inflammation-related genes have not been studied in newborns supplemented with probiotics. Thus, gene expression analysis of

inflammatory genes that have not been analyzed in this group before may contribute in better understanding of the mechanisms beneath the effect of probiotics in clinical study. The genes analyzed in the present study for mRNA levels are *IL1RL1, HIF-1A, RORC* and *NCR1 (NKp46).*

1.4.1. IL1RL1 gene

IL1RL1 (Interleukin 1 Receptor-like 1) gene encodes for the IL1RL1 protein, an IL-1 family receptor that is selectively expressed on T-helper 2 (Th-2) cells and mast cells and is also known as ST2. IL33, a cytokine belonging to the IL-1family, is the ligand for IL1RL1. IL1RL1/ IL-33 has the ability to induce several cytokines production such as Th2, Th1 as well as Th17 [94]. Overstimulation of ST2/IL-33 has been detected in allergic and autoimmune diseases such as arthritis , airway hyperactivity and asthma , demonstrating an important role of IL1RL1in the development of Th2- dominant inflammatory pathologies [95]. In a study by Latiano *et al.* on inflammatory bowel disease (IBD), they found that mRNA expression of *IL-33* and *IL1RL1* was significantly increased in inflamed IBD biopsy samples [96]. Moreover, a recent study determined the role of the commensal flora in inducing IL-33 and Th-2 immune response, and its impact on intestinal fibrosis in an experimental model of IBD [97]. Their data showed that IL-33 expression and production was significantly increased in the IBD model and that it is greatly dependent on the presence of microbiota and may serve as a primary mediator in the events leading to intestinal fibrosis in IBD.

1.4.2. *HIF-1A* gene

HIF-1A (hypoxia-inducible factor-1A) gene encodes the alpha subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1), which is a heterodimer composed of an alpha and a beta subunit. The main function of HIF-1as a transcription factor is to accommodate the cells to hypoxia. This is achieved by stimulating the transcription of other genes that are responsible for the formation of proteins involved in angiogenesis, energy metabolism, apoptosis and any other proteins relevant in the oxygen delivery cascades.

In NEC, intestines suffer from hypoxia, increased permeability, and necrosis. The decrease in oxygen supply to the IECs contributes well to the pathogenesis and severity of the disease. Experiments performed on mouse models suffering from colitis showed that HIF-1 expression is inversely related to the severity of colitis [98]. Accordingly, increased expression of HIF-1 is a positive or protective sign during intestinal mucosal inflammation.

Additionally, HIF-1 has a critical role in stimulating the innate immune function. It supports the function of several immune cells such as mast cells, dendritic cells and phagocytic cells in bacterial infections. Molecular studies on mucosal inflammation of intestines revealed that the epithelium tissue suffers from hypoxia and that HIF-1 functions to restore the barrier integrity by regulating the expression of genes involved in barrier protection [98].

1.4.3. RORC gene

RORC (Retinoic Acid Receptor-Related Orphan Receptor C) is a protein-coding gene. RAR-related orphan receptor gamma (RORγ) is the protein encoded by this gene and is a member of the nuclear receptor family of transcription factors. Two isoforms are produced from the same *RORC* gene, RORγ (also referred to as RORγ1) and RORγt (also known as RORγ2) [99]. RORγt is mainly expressed in the thymus and uniquely in immature $CD4^+/CD8^+$ thymocytes and in lymphoid tissue inducer cells. RORγ is found to be required for the development of lymph nodes and Peyer's patches in the intestinal wall and has a crucial role in lymphoid organogenesis [100].

1.4.4. NCR1(NKp46) gene

NCR1 (Natural Cytotoxicity Triggering Receptor 1), which is also known by *NK-p46* is a protein-coding gene. The Natural cytotoxicity receptor protein that is encoded by *NCR1* is expressed on Natural Killer cells (NK), which belongs to the group of innate lymphoid cells (ILC). ILCs are newly identified population of immune cells prevalent in mucosal tissues such as the GIT. ILCs are divided into several cell subsets. ILC3 lineage express the RORγt, and is

characterized by production of IL-17 and IL-22. They can be further subdivided by the expression of NCRs; NCR^+ ILC3s secrete IL-22 and NCR^- ILC3s express both IL-22 and IL-17 [13].

Emerging evidence suggests that cross talk between ILC3s and components of the intestinal microflora has the ability to support both maintenance of gut homeostasis and induce chronic intestinal inflammation. Central to this process is IL-22, which promotes gut health by inducing the production of epithelial-derived antimicrobial peptides and mucins. ROR ILC3s are major producers of IL-22 in response to microbial products, mucosal antigen-presenting cells, as well as the local cytokine milieu. Flagellin, the principal component of bacterial flagella, induces TLR5 in dendritic cells and promotes IL-23 secretion, which enhances IL-22 from ILC3s. IECs produce IL-7 in response to elements of the gut microbiota, which also stimulates IL-22 from ILC3s. Intestinal macrophage-derived IL-1β has the ability to induce IL-22. However, commensal bacteria may also induce IECs to produce IL-25, decreasing IL-22 production. In the absence of ILC3- derived IL-22, segmented filamentous bacteria (SFB) demonstrate unrestricted growth, which encourages pathogenic Th17 immune responses that can promote colitis in mice [101]. SFB promote the differentiation of Th17 cells in the gut, which produce IL-17, IL-22 and IL-10 and may play a homeostatic role in the gut (Figure 1.7).

Hence, the microbiota is involved in either the differentiation or function of RORγt+ ILCs by the induction of IL-7 that maintains the expression of RORγt+ in ILCs. The absence of microbiota-induced IL-7 signaling or the presence of IL-12 and IL-15 facilitates the conversion of RORγt+ ILCs into RORγt− IFN-γ-producing pathogenic ILCs.

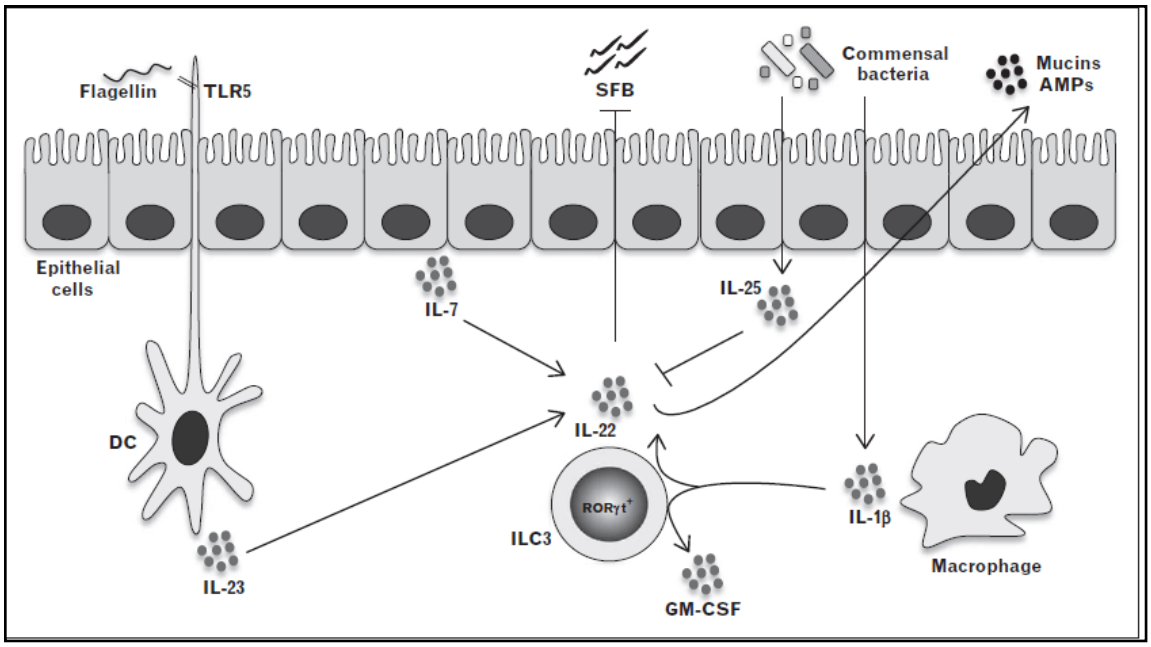

Figure 1.7. Interactions between gut microbiota and ILC3 [101]

2. AIM OF THE STUDY

The primary objectives of this clinical trial were to explore the effect of probiotic supplementation, specifically *Bifidobacterium animalis* subsp. *lactis* on whole blood gene expression and on alterations of some gut microbiota groups in preterm neonates at risk of NEC and comparing their outcomes to preterm and full-term control groups. The inflammatory response associated genes aimed to be tested in whole blood were *RORC, NCR1, IL1RL1* and *HIF1A*. The gut microbiota groups aimed to be tested in fecal samples were *Bifidobacterium genus, Enterococcus spp., Enterobacteriaceae, Lactobacillus group,* and *Candida albicans.*

The secondary objectives of this study were to test the effect of *Bifidobacterium animalis* subsp. *Lactis* on incidence and severity of NEC in the supplemented preterm neonates as well as sepsis, length of hospital stay, discharge weight and mortality.

3. MATERIALS

3.1. BACTERIAL STRAINS

Escherichia coli ATCC 10231(ATCC, USA)
*Enterococcus faecalis*ATCC 19433 (ATCC, USA)
Candida albicans(ATCC 10231) (ATCC, USA)
*Bifidobacterium bifidium*ATCC 11863 (ATCC, USA)
Lactobacillus acidophilus ATCC 4356(ATCC, USA)
Bifidobacterium animalis ssp lactis B94 (Maflor, Mamsel Pharmaceutical Company, Turkey)

3.2. CHEMICALS and BUFFERS

- Brain Heart Infusion Agar (BHIA) (Salubris, USA)
- Tryptic Soy Agar (TSA) (OXOID,UK)
- Tryptic Soy Broth (TSB) (OXOID,UK)
- Sabouraud Glucose Agar (SGA)(OXOID,UK)
- Sabouraud Glucose Broth (SGB)(OXOID,UK)
- De Man Rogosa Sharpe Agar (M.R.S) (bio LAB, UK)
- De Man Rogosa Sharpe Broth (M.R.S) (MERCK, Germany)
- Reinforced Clostridium Medium (RCM)(OXOID,UK)
- Sheep blood
- Glycerol(Pancreac, Spain)
- Lysozyme (Bio Basic INC, Canada)
- Lyticase, 2000 units/g (SIGMA, Germany)
- Sorbitol buffer
- Zirconia beads, 0.1 mm and 0.5mm (BioSpec, USA)
- Phosphate Buffer Saline (PBS) (Thermo scientific, USA)

- β-mercaptoethnanol (Merck, Germany)
- Triton-100 (SIGMA, Germany)
- Tris-EDTA buffer (Santa Cruz Biotechnology INC, USA)
- Tris Boric-EDTA Buffer (SIGMA,Germany)
- Bovine Serum Albumin (BSA)(Ambion, USA)
- Taq polymerase (5 units/µl), (Fermentas, Canada)
- Taq polymerase buffer, (Fermentas, Canada)
- Magnesium chloride (Fermentas, Canada)
- dNTP mix (Promega, USA)
- Bacterial primers (MacrogenInc,Korea)
- Gene expression primers (QIAGEN, Germany)
- Tris Base (Sigma, USA)
- Ethidium bromide (Merck, Germany)
- Loading dye (Sigma, Germany)
- DNA ladder (Invitrogen, USA)
- SYBR Green qPCR master mix (SABiosceinces, Germany)
- Agarose gel (Sigma, Germany)

3.3. KITS

- Gram staining kit(Salubris, USA)
- QIAamp DNA Mini and Blood (QIAGEN, Germany)
- QIAamp DNA Stool Mini kit (QIAGEN, Germany)
- QIAamp Fast DNA Stool Mini kit (QIAGEN, Germany)
- Gene JET PCR Purification kit (Thermo scientific, USA)
- High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA)
- Tempus Spin RNA isolation kit (Applied Biosystems, USA)
- Power SYBR Green PCR master mix (Applied Biosystems, USA)

3.4. LABORATORY EQUIPMENTS

- Anaerobic workstation (Don Whitley, UK)
- Autoclave (HV-85 (HICLAVE, Hirayama, Japan)
- Centrifuge (Hettichmikro 22R and SIGMA 2-5 centrifuge, Germany)
- Centrifuge 5810 R (Eppendorf, USA)
- Biological Safety Cabinet (NuAire, USA)
- CO2 incubator (Nuaire NU5510/E/G, USA)
- Microwave (Arçelik, Turkey)
- NanoDrop 2000 (Theromo scientific,)
- Light Microscopy (Olympus, USA)
- pH meter (Hanna instruments PH211, Germany)
- Real Time Thermal Cycler (BIO RAD, Germany)
- Thermocycler (BIO RAD, Germany)
- UV Illuminator (BIO RAD, Germany)
- Vortex (Stuart SA8, UK)
- -80 °C freezer (Thermo Forma -86 C ULT Freezer, USA).
- Hot plate and stirrer
- Bead beater ProH-6000 Homogenizer (INOVIEA tech,
- Molecular Imager ChemiDoc XRS+ (BIO RAD, Germany)
- Water bath (Wisebath, Korea)
- -20 °C freezer and refrigerator (Arçelik, Turkey)
- Incubator (Binder, USA)
- Analytical balance Explorer Pro (OHAUS, USA)

4. METHODS

4.1. STUDY DESIGN

A prospective, blinded, randomized controlled multi-center trial was conducted in which the study group received the probiotic *Bifidobacterium animalis* ssp *lactis* B94 (Maflor, Mamsel Pharmaceutical Company, Turkey) during their first month of life. All infants included in this study were born in Istanbul in one of the following hospitals: Kanuni Sultan Suleyman Training and Research Hospital, Umraniye Training and Research Hospital or Medeniyet University Goztepe Training and Research Hospital. The laboratory analyses were performed at Yeditepe University, Istanbul. The study was approved by the local Ethics Committee of the hospitals. The probiotic (ProGpr) and preterm control (PreCtr) groups contained 20 and 22 preterm infants respectively. Each group was recruited from a different hospital to prevent probiotic cross-contamination. The nurses feeding the neonates were blinded to the treatment. A full-term neonates control group (FullCtr) containing 21 infants was included in the study to represent the healthy fully developed neonates.

4.2. INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria include gestational age (GA) of ≤32 weeks and/or ≤1500g birth weight preterms that survived to feed enterally. Exclusion criteria include major congenital and chromosomal abnormalities, fetal distress, preterms not starting enteral feeds and lack of parental consent for participation in the study. The full-term babies include healthy breast-milk fed infants with no complications.

4.3. SAMPLE SIZE

In order to achieve a 0.8 power to detect a mean difference of 30% in colonization of *Bifidobacteria* with a significant difference of $p<0.05$, 13 infants were required for each group. We enrolled 20, 22, and 21 infants for probiotics, preterm control and full-term control groups respectively to account for potential dropouts.

4.4. PROBIOTIC PROTOCOL

Half of the Probiotic sachet that contains $5x10^9$ CFU of *B.lactis* was suspended in 4ml of sterilized water and 1ml was administered enterally twice a day that is each neonate received 2ml (one in the morning and one at night) and accordingly 1.25 $x10^9$ CFU/day. Then the probiotic suspension was added to either breast-milk or mixed feeding (breast milk and preterm formula for infants with insufficient production of mother's breast-milk).The addition of *B.lactis* to breast-milk or formula was under sterile conditions. Supplementation of probiotic to perterms started in the first 72h of enteral feeding and continued at least 28 days and/or until discharge. Infants were started to be fed as soon as they had stable vital signs, active bowel sound without any abdominal distension and no bile or blood from the gastric tube. Breast-milk and/or formula feeding were started as 10-20 ml per kg depending on gestational age and the prenatal history (hypoxia, preeclampsia, chorioamnionitis, etc.). The amount of enteral feeding was gradually increased with an amount of 10-30 ml per kg as it was tolerated. Total parenteral nutrition was also started from the first day of life for all infants and gradually increased. Feeding was stopped in all groups if there were at least two signs of feeding intolerance: presence of gastric residual $\geq$ 50% of the previous feeding, abdominal distension, or heme-positive stools.

4.5. NEC DIAGNOSIS PROTOCOL

Diagnosis of NEC was performed clinically along with the abdominal radiographic results. The stages of the disease were classified as indicated in the modified Bell's criteria [5].

Preterms that were suspected of NEC (stage I) were not included in the final analysis; only stage II and stage III cases were counted. In preterms diagnosed with NEC, abdominal radiographs were performed every six hours for the first 48 hours.

Preterms were given the appropriate medical intervention when needed, such as mechanical ventilation, antibiotic treatment, gastric decompression or volume expansion with fluid or blood products. The antimicrobial therapy was stopped and enteral feeding was started when clinical signs and laboratory results show improvement.

Maternal risk factors for NEC such as: preeclampsia, prolonged rupture of amniotic membranes that may lead to infection, and chorioamnionitis which is inflammation of the fetal membranes (amnion and chorion) due to a bacterial infection were recorded. Clinical parameters that are controversial for NEC development such as antenatal steroid use and surfactant use that are needed for the fetal lung development and function were also recorded. In addition, relevant clinical information about the mode of delivery, antibiotic use, type of feeding and age at which feeding started were registered.

4.6. BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strains used as positive and negative controls in this study were obtained from the American Type Culture Collection (ATCC) and included *Escherichia coli* (ATCC 10231), *Enterococcus faecalis* (ATCC 19433), *Bifidobacterium bifidium* (ATCC 11863), *Lactobacillus acidophilus* (ATCC 4356) and the yeast *Candida albicans* (ATCC 10231). *E.coli* and *E.faecalis* were grown on Trypticase soy agar (TSA) (OXOID, UK) or in Trypticase soy broth (TSB) (OXOID, UK) at 37^0C overnight. *C.albicans* was grown on Sabouraud Glucose Agar (SGA) (OXOID, UK) or in Sabouraud Glucose Broth (SGB) (OXOID, UK) at 37^0C overnight. Bacteria requiring anaerobiosis for growth were cultured at 37°C in an anaerobic chamber (85% N_2, 5% CO_2, 10% H_2, by volume) (MACS-MG-1000 anaerobic workstation, dw scientific, UK) using the following media: TSA (OXOID, UK) supplemented with 5% sheep blood for *B.bifidium*, De Man Rogosa Sharpe (M.R.S) agar (bioLAB, UK) and M.R.S broth (MERCK, Germany) for *L.acidophilus*. *L.acidophilus* was incubated at 37^0C with 5% CO_2 in

CO_2 incubator (Nuaire NU5510/E/G, USA). Aerobic microorganisms were cultured at 37°C (Binder incubators, USA). Purity of bacterial cultures was checked by Gram staining and slides were examined under light microscope (Olympus, USA). Media and culture conditions are summarized in Table 3.1.

Table 4.1. Bacterial strains and culture conditions

Bacteria	**Media**	**Broth**	**Time and Condition of Culture**
E.coli	TSA	TSB	24h / aerobic
E.faecalis	TSA	TSB	24h/ aerobic
C.albican	SGA	SGB	24h / aerobic
B.bifidium	TSA + 5% sheep blood	RCM	48 h/ anaerobic
B.lactis	M.R.S	M.R.S broth	48 h/ anaerobic
L.acidophilus	M.R.S	M.R.S broth	48h/ CO_2 incubator

4.7. PROBIOTIC ASSESSMENT

A quality and quantity assessment of the probiotic organism (*B.lactis*) was performed prior to the clinical trial in order to confirm the identity of the species used, to check for the viable bacterial count indicated in the sachet and to check for any microbial contamination present. The content of a sample sachet was reconstituted and plated on M.R.S agar (Table 4.1). Gram staining using a Gram staining kit (Salubris, USA) was performed for the grown bacteria. DNA isolated from a broth cultures was subjected to PCR using *B.lactis* specific primers to confirm the identity of the bacteria.

4.8. EXTRACTION OF DNA FROM BACTERIAL CULTURES

Chromosomal DNA was isolated from individual bacterial reference strains at the stationary growth phase of all cultures. Genomic DNA of all the strains was extracted from 1ml overnight cultures of known CFU contents using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for G+ and G- bacteria. Overnight cultures of 10ml of each bacterium were used for DNA isolation. The count of colonies

(CFU/ml) was determined using the serial dilution plate method; count is indicated in Table 4.2. To harvest bacterial cells, each 1ml was pipetted into 1.5ml microcentrifuge tube and centrifuged for 10 min at 5000xg (Hettichmikro 22R, Germany). Once harvested, the cells were washed twice with phosphate buffered Saline (PBS) (Thermo scientific, USA), and for G- bacteria the pelleted cells were suspended directly in 180 µl ATL buffer (supplemented in the kit) and the protocol for the kit was followed according to the manufacturer guidelines. For G+ bacteria the harvested washed cells were suspended in 20 mg/ml lysozyme solution that was prepared as follows: 20 mg lysozyme (Bio Basic INC, Canada) , 1.2% Triton-100 (SIGMA, Germany) in 1 mM disodium EDTA and 10mM Tris-HCl pH=8.0 (Santa Cruz Biotechnology INC, USA). Bacterial cells were incubated for 30 min at 37°C in the lysozyme solution. Then, 20 µl of proteinase K provided in the kit was added as well as 200 µl of AL buffer, mixed by vortexing and then incubated at 56°C for 30 min and then for a further 15 min at 95° C. Then the protocol of the kit was followed as for the rest of bacteria. Finally, elution was performed using 100 µl AE buffer for all bacterial DNA.

For *C.albicans* DNA isolation was done according to the protocol for yeast of the same kit. The cells' pellet was suspended in sorbitol buffer that was prepared using 1M sorbitol, 100 mM EDTA , and 14 mM β-Mercaptoethanol (Merck, Germany). The cell pellet was suspended in 600 µl sorbitol buffer and 200U of lyticase (SIGMA, Germany) was added and incubated at 30°C for 30 min, then the protocol was continued according to the kit protocol. DNA quantification was performed using NanoDrop 2000 (Thermo Scientific, USA).

Table 4.2. Bacterial strains concentration (CFU/ml) from which DNA was isolated

Bacterial Strain	**Bacterial Population (CFU/ml)**
E. coli	2.01×10^9
E. faecalis	1.33×10^9
B. bifidium	6.7×10^5
B.lactis	2.4×10^8
C. albicans	9.1×10^6
L. acidophilus	5.8×10^6

4.9. FECAL SAMPLES

A total of 110 fecal samples, 36 from the ProGrp, 38 from the PreCtr group, and 36 from the FullCtr group were included in this study for bacterial quantitative real-time PCR analysis. Two fecal samples were collected from each neonate: the first sample collected was in the first week after birth and was called baseline sample and the second sample was collected one month later. Samples were frozen after collection and stored at -80° C until analysis.

4.10. EXTRACTION OF DNA FROM FECAL SAMPLES

DNA was extracted from 150-250 mg frozen fecal samples using the QIAamp® Fast DNA Stool Mini kit (QIAGEN, Germany). DNA extraction was performed according to the instructions of the manufacturer with the following modifications: fecal samples were initially homogenized and washed using four to five glass beads along with one ml sterile 1X PBS buffer. The suspension was vortexed thoroughly and centrifuged at full speed (16.000 rpm) for three minutes. The stool pellet was saved and then the process repeated twice. Upon the addition of 1ml InhibitEX Buffer, samples were vortexed thoroughly and then incubated for 5min at 95^0 C. Samples were subsequently mixed with 0.3g and 0.1g Zirconia beads of 0.1mm and 0.5mm in diameter respectively (BioSpec, USA). The mix was then subjected to a bead-beating step for three minutes at a speed of 7 m/s using ProH-6000 homogenizer (INOVIA, Turkey). Moreover, the amount of Proteinase K was increased to 25 µl, mixed with 400µl of the supernatant and 400 µl AL buffer. DNA was then purified according to the manufacturer instructions and finally eluted in a preheated 50 µl of the provided elution buffer.

4.11. BACTERIAL PRIMERS

The primers' sets used in this study are listed in Table 4.3. The primers were checked using the database similarity search program BLAST for possible cross-hybridization with genes from *Eucarya* and *Archaea* as well as for self-hybridization and hetero-hybridization, percentage of GC content and annealing temperature (Tm). The specificity of each primer set

was experimentally tested by running conventional PCR using DNA of the target strain and a negative control. The primers were synthesized commercially by Macrogen Inc., Korea.

4.12. PCR AMPLIFICATION

Determining the specificity of each primer set and the optimum Tm was done by gradient PCR using MyCycler™ thermal cycler (BioRad, Germany). Each PCR mixture (100 µl) contained 10µl 10X PCR buffer (Fermentas, Canada), 0.2 mM concentration of deoxynucleoside triphosphates (dNTPs) (Promega, USA), 1.5 mM of $MgCl_2$ (Fermentas, Canada), 50 pmole primer, 2.5 U *Taq* DNA polymerase (Fermentas, Canada), and 50 ng of bacterial template DNA. The gradient PCR program consisted of 33 cycles with a DNA denaturation step at 94°C for 3 min, followed by an annealing step at a range of 55°C- 65°C for 30 sec and elongation step at 72°C for 30 sec. The PCR was completed with a final elongation step at 72°C for 5 min.

4.13. GEL ELECTROPHORESIS

The PCR products were subjected to electrophoresis to check the product size and sharpness of the product for each primer set. Agarose gel (1.5%) was prepared by dissolving 1.5 mg of agarose (Sigma, Germany) in 100ml of 1X TBE buffer (Sigma, Germany). Agarose was heated in a microwave (Arçelik, Turkey) for 2 min. The solution was then left to cool to 50°C-55°C. Ten microliters of ethidium bromide (Sigma, Germany) was added to the solution and the solution then poured into the casting tray. Seven microliters of the PCR product was mixed with 3µl of 6X Loading dye (Sigma, Germany), then loaded onto the agarose gel and run for 1h at 80V. Gels were visualized and documented using a Molecular Imager ChemiDoc XRS+ (BIO RAD, Germany).

4.14. BACTERIAL QUANTIFICATION BY REAL-TIME PCR

The amplification reactions were carried out in duplicate in a volume of 12.5 µl of 2X Power SYBR Green PCR Master Mix (Life technologies, UK), 10 pmole of each primer, and either 2µl of template DNA, positive control, negative control or deionized sterile water as no-template control (NTC). The qPCR analyses were performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using PCR-96-FLT-C 96-well PCR plates (AXYGEN, USA) covered with Ultra Cruz^{TM} PCR Plate Seals (Santa Cruz Biotechnology,INC, USA). The optimal Tm was determined for each assay. The amplification involved one cycle at 95°C for 10 min for initial denaturation followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 60°C for 1 min. To determine specificity of the PCR reactions, a melt curve analysis was carried out in conjunction with each amplification run by slow cooling from 95°C to 65°C, with fluorescence collection at 0.5°C intervals and a hold of 5 sec at each decrement.

4.15. DNA STANDARDS USED FOR DETERMINING BACTERIAL NUMBER BY qPCR

For fecal bacteria quantification, genomic DNA from different pure cultures were used to generate a standard curve for the mathematical conversion of quantification cycle (Cq) values into genomic equivalents of bacteria. For construction of standard curves, 10-fold dilution series of between 0.02 pg and 20 ng from target species genomic DNA preparations were applied for PCR. Two microliters of each fecal DNA preparation in conjunction with 20 ng of non-target bacterial DNA as a negative control and sterile water as NTC were analyzed in the same qPCR run. Each fecal sample was run in duplicate. The limit of detection for each assay was determined with concentrations of purified DNA of the reference strains ranging from 0.00002–20 ng.

4.16. DETERMINTATION OF BACTERIAL NUMBER PER GRAM OF FECAL SAMPLE

For each assay, PCR results were converted to the average estimate of target bacterial genomes present in 1g of feces (wet weight). PCR tests, except for *B.lactis*, were designed to detect a wide range of phylogenetically related bacterial species that have diverse ribosomal DNA (rDNA) copy numbers and different genome sizes. Thus, estimated average genome size for each target bacteria group was used while differences in the ribosomal RNA copy numbers were ignored. The following genome sizes were used: 2.3 Mb for *Lactobacillus spp.*, 2 Mb for *Bifidobacterium spp.*, 3 Mb for *Enterococcus spp*. [102], 15.6 Mb for *Candida albicans* [103], 4.6 Mb for *Enterobacteriacea spp*. [104].

To calculate genomic equivalents per gram of stool, the target genomes calculated from 2 µl of gDNA (the volume used per qPCR reaction) were extrapolated into genomic equivalents per gram of stool by multiplying them by the total volume of DNA extracted from the weighed amount of stool which was 50µl, and then extrapolating this value into grams. Data was presented as log10 genomic equivalents of 16S rRNA gene per gram of feces.

4.17. VALIDATION EXPERIMENT (SPIKING EXPERIMENT)

To validate the quantification method and to evaluate the reliability of the DNA purification method, fecal samples from first-day born neonates that were cultured and found to contain no bacterial growth were spiked with serial dilution of measured quantity of *B.lactis*. Serial dilution of *B.lactis* were used to spike 200 mg of bacteria-free fecal samples in duplicate at six different concentration levels ranging from 7×10^7 CFU/ml to 7×10^2 CFU/ml. As a negative control, one of the fecal samples was spiked with sterile water. To mimic the experimental design, the feces were mixed with the bacterial dilutions and then stored at -80°C until extraction and real-time PCR analysis. Spiked samples were then extracted as described above, and analyzed by real-time PCR using specific *B.lactis* primers (Table 4.1).

Table 4.3 Target groups or species and sequences of the PCR primers used in this study

Target Group or Species	Primer Name	Primer Sequence (5'-3')	Product Size (Bp)	Tm	Ref
Bifidobacterium genus	Bif-F	GGGTGGTAATGCCGGATG	441	59	[105] [106]
	Bif-R2	TAAGCGATGGACTTTCACACC			
Bifidobacterium lactis	Blact-F	CCCTTTCCACGGGTCCC	194	60	[107]
	Blact-R	AAGGGAAACCGTGTCTCCAC			
Lactobacillus group	Lact-F	AGCAGTAGGGAATCTTCCA	341	58	[102]
	Lact-R	CACCGCTACACATGGAG			
Enterococcus spp.	Ent-F	CCCTTATTGTTAGTTGCCATCATT	144	61	[102]
	Ent-R	ACTCGTTGTACTTCCCATTGT			
Enterobacteriaceae	ENTbac-F	GTTGTAAAGCACTTTCAGTGGTG AGGAAGG	424	59	[108]
	ENTbac-R	GCCTCAAGGGCACAACCTCCAAG			
Candida albicans	Calb-F	C(C/T)GGCTCTTGTCTATGTT(C/T) C	411	55	[109]
	Calb-R	GTCTA(A/G)GCTGGCAGTATCG			

4.18. BLOOD SAMPLES

A total of 114 blood samples, 40 from the ProGrp, 34 from the PreCtr group, and 40 from the FullCtr group were included in this study for gene expression analysis by qPCR. Two blood samples were collected from each neonate: the first sample collected was in the first week after birth and was called baseline sample and the second sample was collected one month later. Around 2ml of blood was collected from each neonate into Tempus™ Blood RNA Tube (Applied Biosystems,USA). These tubes contain stabilizing reagent, which effectively lyses blood cells and inactivates cellular RNases. Blood samples were then frozen and stored at -80° C until analysis.

4.19. RNA ISOLATION

As soon as blood samples were drawn in Tempus™ Blood RNA Tube (Applied Biosystems, USA) and mixed with the stabilizing reagent present, the gene expression profile of the genes was immediately conserved. The cellular RNases became inactivated by the stabilizing

reagent, which also selectively precipitated RNA while keeping genomic DNA (gDNA) and proteins in solution. RNA isolation chemistry was used to purify high quality RNA without sample pretreatments such as leukocyte isolation or selective red blood cell (RBC) lysis. RNA concentration and its quality were measured using a Nanodrop spectrophotometer; NanoDrop 2000 (Thermo Scientific, USA).To further test RNA integrity of the isolated RNA, 2-3 µl of each RNA sample was run on 1% TBE agarose gel for 1h.

4.20. CONVERSION OF RNA TO cDNA

RNA conversion into cDNA was done using High Capacity cDNA Reverse Transcription kit (Applied Biosystems,USA). RNA (500ng) was converted into cDNA according to the manufacturer guidelines in a final volume of 20 µl; the produced cDNA had a concentration of around 25 ng/µl. Each cDNA tube was aliquoted into two micro-tubes containing 10 µl each in order to reduce the freeze-thawing effect and thus decrease degradation of cDNA during experiments. Upon the addition of 500ng of total RNA to each nuclease-free micro-centrifuge tube, 10 µl of the 2X RT Master mix, and a variable volume of RNase-free water were added to attain a final volume of 20 µl. The mixture was then incubated at 25° C for 10 min, followed by another 120 min at 37° C. The reaction was terminated by heating at 85° C for 5 min, and cooled to at 4° C. cDNAs were stored at -20 C until use in qPCR.

4.21. QUANTITATIVE REAL-TIME PCR FOR GENE EXPRESSION

Quantitative Real-time PCR was carried out for four selected genes: hypoxia-inducible factor-1A (*HIF-1A*), Retinoic Acid Receptor-Related Orphan Receptor C (*RORC*), natural cytotoxicity receptor (*NCR1*), and Interleukin 1 receptor-like 1 (*IL1RL1*). The amplification reactions were carried out in duplicate in a volume of 12.5 µl of 2X Power SYBR Green PCR Master Mix (Life technologies, UK), 2 µl of 1:20 dilution of cDNA and which is around 10 ng of the appropriate cDNA, 10 µM primers, deionized sterile water as NTC. The qPCR analyses were performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using PCR-96-FLT-C 96-well PCR plates (AXYGEN, USA) covered with Ultra Cruz^{TM} PCR Plate

Seals (Santa Cruz Biotechnology,INC, USA). qPCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles 15 sec at 95°C, 1min annealing at 60°C. A melting curve analysis was used to determine amplification specificity. The primers were obtained from QIAGEN, Germany.

Phosphoglycerate kinase 1 (*PGK1*) and Peptidylprolyl isomerase B (*PPIB*) were used as reference genes to normalize for variation in the amount of cDNA template. Several publications were checked for the best reference genes to be used for testing gene expression in blood. Finally, two publications concluded that both *PGK1*and *PPIB* are reliable for normalization method to monitor the expression of target genes in peripheral whole blood. Gene expression was calculated as fold-change relative to the average of expression in the controls. Results were analyzed using the comparative critical threshold (ΔΔ Ct) method to compare differences between samples.

Table 4.4. Reference genes primers sequence

Primer	**Sequence (5'-3')**	**Product Size (bp)**
PGK1-F	CAAGAAGTATGCTGAGGCTGTCA	68
PGK1-R	CAAATACCCCCACAGGACCAT	
PPIB-F	CAGCAAATTCCATCGTG	132
PPIB-R	CCGTAGTGCTTCAGTTT	

5. STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS software version 23. The variables were first investigated using Kolmogorov-Smirnov Test and showed that data was not normally distributed and thus, further statistical analysis was done using non-paramatric tests. Study groups characteristics were presented using mean ± standard deviation for continuous variables and frequency percentages for categorical variables. Baseline demographic characteristics, feeding data and selected outcomes were compared between ProGrp and PreCtr groups using Chi-square or Fisher's exact test for categorical data and Mann-Whitney test for continuous data.

Bacterial count was presented as median and interquartile range (25^{th} -75^{th}). The colonization of bacteria was calculated as the percentage of the number of neonates with the detected bacteria with regard to the total number of neonates in the tested group. Bacterial counts were transformed to the base 10 logarithm for analysis. The difference between baseline and one month levels of bacteria were compared using Wilcoxon sign test. Blood genes expression levels are normalized to Phosphoglycerate kinase 1 (PGK1) and Peptidylprolyl isomerase B (PPIB) genes. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method and the average ΔCt values of the baseline point served as the calibrator when compared after one month. Pairwise comparisons between the baseline and one month time points for each gene were made using paired sample t test. Comparisons of gene expression levels between tested groups at different time points were made using One way ANOVA . Levene test was used to assess the homogenity of the variances. When overall significance was observed, pairwise post hoc tests were performed using Tukey's test. If the variances are not homogenous, Welch ANOVA was used to compare the parameters among groups. When overall significance was observed, pairwise post hoc tests were performed using Dunnet's T3 test. An overall p value of less than 0.05 was considered a statistically significant result.

6. RESULTS

A total of 63 newborns took part in this study between October 2014 and June 2015. Twenty preterms were in the probiotic group (ProGrp), 22 preterms were in the control group (PreCtr) and 21 full-term newborns comprised the full-term control group (FullCtr). Due to the small number of neonates in each group, analyzing subgroups was limited.

6.1. STUDY GROUPS CHARACTERISTICS

The demographic and maternal characteristics of the ProGrp and PreCtr group are presented in Table 6.1. Both groups were similar in the maternal characteristics registered such as, antenatal steroid use, surfactant use, prolonged rupture of amniotic membrane, chorioamnionitis, preeclampsia, and mode of delivery. They also showed similarity in other baseline characteristics such as, gestational age, birth weight, sex, and sepsis. The significant difference between ProGrp and PreCtr group was detected in the total days members of both groups were subjected to antibiotics (p=0.000).

Regarding the FullCtr group, they were born between 38 to 42 gestational weeks and their mean weight was 3308±466 g. The group consisted of 7 (33.3%) males and 14 (66.7 %) females who were born either vaginally 15 (71.4 %) or by caesarian section 6 (28.6 %) Fourteen (66.7 %) were breast-milk fed and 7 (33.3 %) were fed by both breast-milk and formula.

In table 6.2, the feeding data of ProGrp and PreCtr group showed significant differences mainly in the mode of feeding (p=0.000). All the PreCtr group neonates were fed breast milk compared to 50% in the ProGrp that were fed both breast milk and formula and 5% that were only breastfed. Moreover, the age at which enteral feeding was initiated showed statistical significance (p=0.018). The differences between both groups concerning the feeding data parameters are attributed mainly to the different protocols implemented in each hospital's NICU each group was recruited from.

Table 6.1. Probiotic group and preterm control group demographic and maternal characteristics

	Probiotic Group N=20	**Preterm Control Group N=22**	**p-Value[1]**
Gestational age (mean±SD)	28.8±1.02	30.5±1.14	0.93
Birth weight (g) (mean±SD)	1356.3±138.2	1350.2±348.1	0.941
APGAR (1min)	6.3±1.3	6.0±1.8	0.673
APGAR (5min)	8.1±1.1	7.8±1.4	0.602
sex			0.121
Male (n,%)	13 (65)	8 (36.4)	
Female (n, %)	7 (35)	14 (63.6)	
Mode of delivery			0.315
Vaginal (n,%)	4 (20)	8 (36.4)	
C/S (n/%)	16 (80)	14 (63.6)	
Total days on antibiotics (d)	17±5.8	7.7±4.2	0.000*
Proven sepsis; n (%)	7 (35)	4 (18.2%)	0.298
clinical sepsis; n (%)	19 (95)	18 (81.2%)	0.346
Preeclampsia (n,%)	3 (15)	9 (40.9)	0.091
Chorioamnionitis (n,%)	1 (5)	0	0.476
Use of antenatal steroid (n,%)			0.803
None	8 (40)	11 (50)	
Complete dose	9 (45)	8 (36.4)	
Incomplete dose	3 (15)	3 (13.6)	
Use of surfactant (n,%)			0.79
None	11 (55)	13 (59.1)	
Complete dose	1 (5)	2 (9.1)	
Incomplete dose	8 (40)	7 (31.8)	

[1] p-value was calculated using Mann-Whitney test for continuous data and Chi-square test for categorical data
*statistically significant

Table 6.2. Feeding data for Probiotic and preterm control groups

	Probiotic Group N=20	**Preterm Control Group N=22**	**p-Value[1]**
Mode of feeding (n, %) Breast milk Formula Mixed	 1 (5) 9 (45) 10 (50)	 22 (100) 0 0	0.000*
Age when enteral feeding began (d)	2.85±1.2	2.05±0.8	0.018*
Full enteral (d)	20.6±12.2	16.0±9.4	0.179

[1] p-value was calculated using Mann-Whitney test for continuous data and Chi-square test for categorical data

*statistically significant

6.2. NEC and SECONDARY OUTCOMES

In Table 6.3, NEC incidence besides the other secondary outcomes showed no significant statistical difference between the study group and the control group. Two neonates in the ProGrp were diagnosed with NEC stage II and one neonate in the PreCtr group was diagnosed with a more severe NEC stage (stage III). None of the neonates in the study group died of either NEC or sepsis, and those diagnosed by NEC were treated and finally discharged without any complications. Probiotic was continued for both babies until discharged. However, one preterm in the PreCtr group died due to NEC and another died due to sepsis.

Secondary outcomes such as length of hospital stay, the discharge weight or the difference in weight gain between both groups showed no statistical significant difference ($p>0.05$).

Table 6.3. selected outcomes in the Probiotic group and preterm control group

	Probiotic Group N=20	**Preterm Control Groups N=22**	**p-Value[1]**
NEC ≥ Stage II; n (%)	2 (10)	1 (4.5)	0.607
Death; n (%)	0	2 (9.1)	N.C[2]
Death due to NEC; n (%)	0	1 (4.5)	N.C
Death due to sepsis; n	0	1 (4.5)	N.C

(%)			
length of hospital stay (d); mean (SD)	37.3±11.9	32 ±14.3	0.179
Discharge weight (g), ; mean (SD)	1925.8±268.1	1980 ±397.9	0.61
difference of weight gain (g); mean (SD)	569.5±244.2	591±351.9	0.529

[1] p-value was calculated using Mann-Whitney test for continuous data and Chi-square test for categorical data
[2] not calculated

6.3. PROBIOTIC ASSESSMENT

Results of the qualitative and quantitative assessment supported the information provided by the manufacturer (5×10^9 CFU/g active *B. lactis* in 1.74 g weight). The product did not contain any contamination; only one kind of bacteria grew that showed to be Gram positive upon Gram staining. The product of the PCR using its isolated DNA and the specific *B. lactis* primers was sharp and in the expected size.

6.4. SPECIFICITY OF PRIMERS

All primer sets were specific and resulted in products merely with the corresponding target bacteria and in the expected size.

6.5. *Bifidobacterium lactis* COUNTS IN FECAL SAMPLES

A total of 18 neonates in the ProGrp, and 19 in the PreCtr group had two stool samples available for analysis. These samples were collected at two different time points referred to as baseline sample and one-month sample respectively. In the FullCtr group, 17 baseline stool samples and 19 one-month samples were available for analysis. As shown in Table 6.4, *B.lactis* was not detected in any of the baseline samples in the three groups. One month after the probiotic supplementation, *B.lactis* was detected in 12/18 (67 %) of the ProGrp preterms which is the colonization percentage in this group. Three out of the 19 (13 %) FullCtr neonates

found to have *B.lactis* as well. On the other hand, none of the PreCtr group contained the probiotic in their stool samples.

Table 6.4. Fecal *Bifidobacterium lactis* colonization and counts in the three tested neonatal groups at baseline and one-month time points.

Bifidobacterium lactis	**Probiotic Group N=18**	**Preterm Control Group N=19**	**Full Term Control Group[1]**	**p-Value[2]**	
				Probiotic vs. Preterm	**Probiotic vs. Full Term**
Proportion colonized					
Baseline	0	0	0	N.C [3]	N.C
One-month	12 (67%)	0	3(16%)	0.00001*	0.002*
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	0	0	0	N.C	N.C
One-month	6.16, 6.3 (5-7.1)	0	7.3, 6 (5.12-10.8)	0.000*	0.84

[1] Full-term control group number is 17 samples at baseline and 19 samples at one-month point.

[2] p-value calculated using Wilcoxon sign test.

[3] not calculated

*statistically significant ($p<0.05$)

6.6. SELECTED MICROBIOTA COMPOSITION AMONG TESTED GROUPS

The baseline and one-month stool samples for the ProGrp, PreCtr, and FullCtr groups were also analyzed using real-time PCR for selected microbiota such as: *Bifidobacterium genus, Enterococcus spp., Enterobacteriaceae, Lactobacillus group,* and *Candida albicans.* The colonization of each group, its median and interquartile range in both baseline and one-month stool samples are shown in Table 6.5.

Table 6.5.Selected fecal microbiota colonization and counts in the three tested neonatal groups at baseline and one-month time points.

Bifidobacterium genus	**Probiotic Group N=18**	**Preterm Control Group N=19**	**Full Term Control Group[1]**	**p-Value[2]**	
				Probiotic vs. Preterm	**Probiotic vs. Full Term**
Proportion colonized					
Baseline	1 (6%)	7 (37%)	5 (29%)	0.042*	0.087
One-month	12 (67%)	13 (68%)	15 (79%)	1.000	0.475
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	4.41,4.41	5.1, 4.7 (4.4-4.9)	7.4, 7.4 (6.6-8.1)	0.750	0.333
One-month	6.52, 6.3 (5.9-7.4)	7.6, 8.3 (5.4-9.2)	8.6, 9.3 (7.3-9.7)	0.150	0.004*
Enterococcus spp.					
Proportion colonized					
Baseline	3 (17%)	7 (37%)	6 (35%)	0.269	0.443
One-month	13 (72%)	18 (95%)	15 (79%)	0.713	0.089
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	6.9, 5.5 (5.2-10.1)	7.7, 7.8 (6.9-9.1)	5.9, 5.5 (4.6-7.5)	0.833	0.548
One-month	7.4, 7.5 (6.8-8.4)	7.6, 7.8 (7.3-8.9)	6.6, 7 (4.6-8)	0.332	0.294
Enterobacteriaceae					
Proportion colonized					
Baseline	3 (17%)	5 (26%)	6 (35%)	0.692	0.443
One-month	11 (61%)	18 (95%)	18 (95%)	0.0187*	0.0187*
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	6.7, 6.7 (5.1-8.3)	8.8, 9.1 (7.8-9.5)	5.5, 5.4 (4.4-6.5)	0.143	0.262
One-month	6.8, 7.2 (5.8-7.6)	7.9, 7.8 (7.3-8.9)	7.3, 7.6 (6.3-8.2)	0.028*	0.238
Lactobacillus group					
Proportion colonized					
Baseline	1 (6%)	8 (42%)	7 (41%)	0.018*	0.017*
One-month	6 (33%)	14 (74%)	14 (74%)	0.0217*	0.0217*
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	6.7, 6.7	6.3, 6.4 (5.8-6.5)	6.7, 6.6 (6.1-7.4)	0.444	1.000
One-month	6.4, 6 (5.8-7)	7.1, 6.8 (5.8-8)	8, 8 (6.7-10)	0.494	0.062

Candida albicans					
Proportion colonized					
Baseline	0	2 (11%)	0	0.486	n.c[3]
One-month	2 (11%)	1 (5%)	5 (26%)	0.603	0.404
Count (log 10)Mean, Median (25^{th}-75^{th} percentile)					
Baseline	0	4.8, 4.8	0	n.c	n.c
One-month	6.1, 6.1	5.1, 5.1	7.2, 6.9 (6.3-8.2)	1.000	0.571

[1] Full-term control group number is 17 samples at baseline and 19 samples at one-month point.

[2] p-value calculated using Wilcoxon sign test.

[3] not calculated

*statistically significant ($p<0.05$)

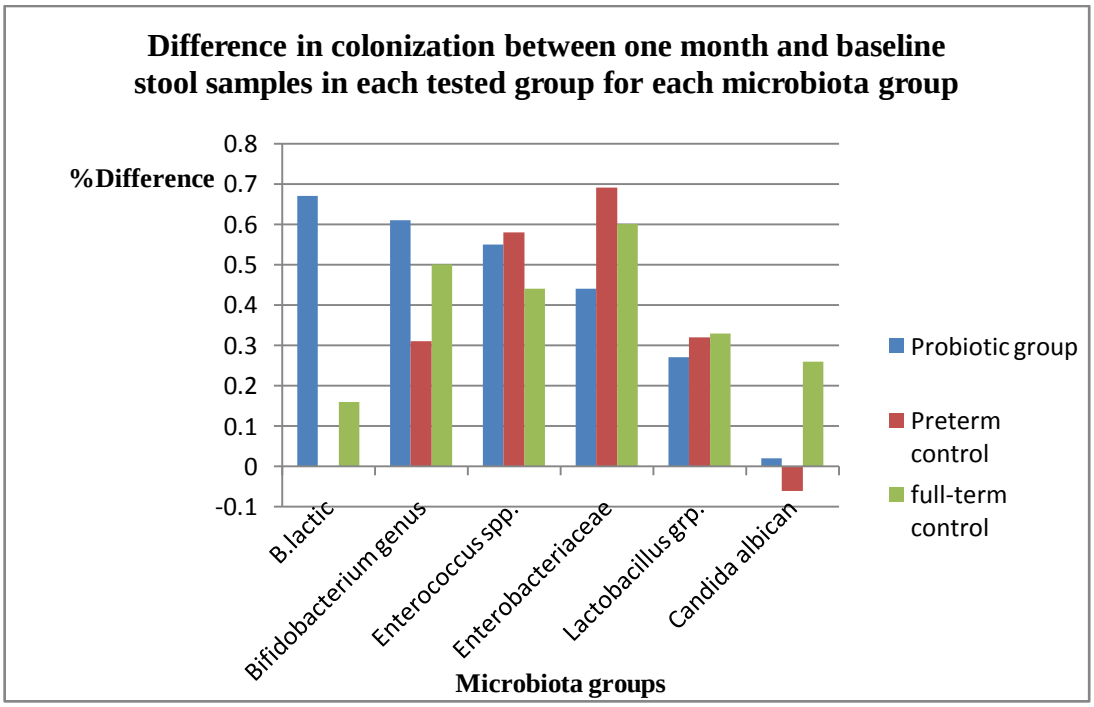

Figure 6.1. The difference in colonization percentage of the analyzed microbiota groups

Figure 6.1 presents the change in colonization during one month in neonates of the three tested groups. This change is assessed by comparing the percentage of neonates harboring a specific bacterial group at baseline and one-month time points. For instance, as shown in Table 6.5 *Bifidobacterium genus* was detected in 6% of the ProGrp neonates at the baseline point and in

67% at one-month point resulting a 61% increase in the number of neonates colonized. In PreCtr group, 37% of neonates were colonized by *Bifidobacterium genus* at baseline point compared to 68% becoming colonized after one month; 31% increase in the number of neonates was detected (Figure 6.1). In FullCtr group, 29% of neonates tested positive for *Bifidobacterium genus* at baseline compared to 79% became positive for the same bacterial group after one month; thus a 50% increase in colonization was detected (Figure 6.1).

Generally, all microbiota groups showed increase in colonization after one month in variable percentages, yet *C.albicans* colonization decreased in PreCtr group as it was detected in 11% of neonates at baseline compared to 5% after one month (6% decrease as shown in Figure 6.1).

6.7. GENE EXPRESSION IN WHOLE BLOOD SAMPLES AMONG GROUPS

RNA collected from whole blood samples at baseline and after one month was isolated and converted to cDNA to be used as a template for measuring the gene expression of the following genes: hypoxia-inducible factor-1A (*HIF-1A*), Retinoic Acid Receptor-Related Orphan Receptor C (*RORC*), Natural Cytotoxicity Receptor (*NCR1*) genes, and Interleukin 1 Receptor-like 1 (*IL1RL1*). In Table 4.5, the difference in gene expression levels in each tested group was compared between the two time points (baseline and one-month). Table 6.6 compared the difference in gene expression levels among groups at each particular time point.

Table 6.6. Comparison of whole blood Gene expression levels[1] between baseline and one-month time points in each group.

	Tested Group	**Baseline ΔCT**	**One-Month ΔCT**	**Fold Change**	**p-Value[2]**
RORC	ProGrp	7.77±1.22	7.15±0.75	1.5	0.025*
	PreCtr	8.74±0.79	6.7±1.61	4.1	0.00035*
	FullCtr	8.48±0.84	7.15±0.75	3	0.000006*
		Baseline ΔCT	**One-Month ΔCT**		
NCR1	ProGrp	6.03±0.99	5.57±0.64	1.4	0.89

	PreCtr	6.4±0.9	5.19±1.15	2.3	0.003*
	FullCtr	6.4±0.47	5.66±0.93	1.7	0.004*
		Baseline ΔCT	**One-Month ΔCT**		
HIF-1A	ProGrp	1.36±0.99	1.8±1.71	-1.4	0.262
	PreCtr	1.37±0.54	1.3±0.55	1.1	0.775
	FullCtr	0.86±0.23	1.64±0.64	-1.7	0.0001*
		Baseline ΔCT	**One-Month ΔCT**		
IL1RL1	ProGrp	4.84±1.66	5.06±1.14	-1.17	0.603
	PreCtr	4.44±1.23	4.51±1.57	-1.1	0.889
	FullCtr	4.73±1.02	6.18±1.21	-2.7	0.0004*

[1]Expression levels of genes are normalized to Phosphoglycerate kinase 1 (PGK1) and Peptidylprolyl isomerase B (PPIB) genes. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method and the average ΔCt values of the baseline point served as the calibrator when compared after one month.

[2]p-value calculated using paired sample t test

*statistically significant

Table 6.7. Comparison of Gene expression levels[1] in whole blood samples between tested groups at either baseline or one-month time points

Gene	ProGrp	PreCtr	FullCtr	Fold Change ProGrp vs PreCtr	Fold Change ProGrp vs FullCtr	p-Value
			RORC			
Baseline ΔCT	7.77±1.22	8.74±0.79	8.48±0.84	2	1.6	0.011*
One-month ΔCT	7.15±0.75	6.7±1.61	7.15±0.75	-1.4	-1.2	0.46
			NCR1			
Baseline ΔCT	6.03±0.99	6.4±0.9	6.4±0.47	1.3	1.3	0.452
One-month ΔCT	5.57±0.64	5.19±1.15	5.66±0.93	-1.3	1.1	0.306
			HIF-1A			
Baseline ΔCT	1.36±0.99	1.37±0.54	0.86±0.23	1	-1.4	0.002*
One-month ΔCT	1.8 ±1.71	1.3±0.55	1.64±0.64	-1.4	-1.2	0.357
			IL1RL1			
Baseline ΔCT	4.84±1.66	4.44±1.23	4.73±1.02	-1.3	-1.1	0.655
One-month ΔCT	5.06±1.14	4.51±1.57	6.18±1.21	-1.4	-1.2	0.002*

[1]Expression levels of genes are normalized to Phosphoglycerate kinase 1 (PGK1) and Peptidylprolyl isomerase B (PPIB) genes. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. The ΔCt values of either the preterm control group or the full-term control group served as the calibrator when compared to the probiotic group at each time point.

[2] p-value calculated using one way ANOVA
*statistically significant

The *RORC* gene expression was significantly increased in the ProGrp, PreCtr, and FullCtr groups after one month; 1.5 fold, p=0.025; 4.1fold, p=0.00035; 3 fold, p=000006; respectively (Table 6.6). A significant difference in gene expression of *RORC* between the three groups was also detected at the baseline time point (p=0.011), with ProGrp having a 2 fold increase compared to the PreCtr group and 1.6 fold increase compared to the FullCtr group. On the other hand, no significant change in expression was present between groups at the one-month time point.

Similarly, the *NCR1* gene had shown an increase in expression levels in the three tested groups after one month. In the PreCtr and FullCtr groups, the *NCR1* levels showed a significant increase of 2.3 fold, p=0.003 and 1.7 fold, p=0.004; respectively. Although it did not reach statistical significance, the expression of *NCR1* was also increased in the ProGrp (1.4 fold, p=0.89). No significant change was detected between the three groups at either baseline time point or the one-month time point (Table 6.6).

HIF-1A expression levels was decreased by -1.4 fold (p=0.262) in the ProGrp one month after probiotic supplementation. It was also found to be significantly decreased in the FullCtr group (-1.7 fold, p=0.0001). However, the level was not changed in the PreCtr group (1.1 fold, p=0.778). Moreover, there was a significant difference in expression levels between the three groups at baseline point with the ProGrp having -1.4 fold decrease compared to the FullCtr group (p=0.002). No significant change between groups was detected after one month.

IL1RL1 gene expression was decreased after one month in the three groups. The most significant decrease was noted in the FullCtr group (-2.7 fold, p=0.0004). At one month time point, there was a significant difference between gene expression level in the three tested groups (p=0.002), with the ProGrp having 2.2 fold increase in *IL1RL1* expression than the FullCtr group, but -1.5 fold decrease in expression compared to the PreCtr group. The *IL1RL1* expression level was the lowest in the ProGrp at baseline time point with no significant difference.

7. DISCUSSION

In spite of decades of research on NEC that included tens of clinical trials, *in vitro* and *in vivo* experiments, meta-analysis, systematic reviews and animal models, still the pathogenesis of this devastating disease is not fully understood, neither its prevention nor its treatment. NEC is considered one of the most common life-threatening diseases among neonates [1]. It is known for its high morbidity and mortality that could reach 30 per cent [2]. Thus, perception of the pathogenesis of this disease, establishment of a preventive protocol as well as finding a potent treatment to it is very urgent. A robust preventive method toward NEC will save the lives of hundreds of thousands of neonates that are yearly affected by this disease; it will also save millions of dollars that are spent for caring for NEC cases in NICUs around the world [1]. Yet, NEC is still a target for thorough research.

Intestinal microbiota along with intestinal immaturity in preterms are main factors involved in the pathogenesis of NEC. Probiotics are showing accumulating evidences for their benefits in normalizing the gut microbiota, interacting with the GIT immune system and their safety in neonates. Accordingly, probiotics are the most promising preventive approach for NEC [55].

Several studies and meta-analysis about probiotic administration to premature infants have been published [79,81,110]. Many of them focused on the beneficial effects of probiotics in NEC prevention using variable types of probiotic microorganisms, doses and time intervals [111, 112]. Moreover, various clinical trials aimed at testing the efficacy of probiotics on prevention of NEC by observation of clinical outcomes such as stages of NEC or sepsis [84,113,114], gastrointestinal complications and feeding tolerance [115], length of hospital stay [116], or by measuring inflammatory markers and cytokines [117]. Other studies analyzed the gut microbiota in the stools of the vulnerable groups and traced the way they change before being diagnosed as NEC [118], or explored the relationship between pathogen microorganisms and NEC [119] .

Other investigators created animal models for NEC to study the pathogenesis of this disease or the effect of probiotics on the GIT immune pathways [120]. A group of researchers examined the activation status of NF-κB perinatally in the small intestine of a neonatal rat model of NEC

[121], others ran a genome microarray experiment to show the beneficial effect of probiotics on gut in neonatal pig [122]. In some animal experiments, researchers analyzed inflammatory genes expression [48], or knocked out others [123] to elucidate the molecular basis of the protective effect of probioitcs in prevention of NEC. Others quantified the number of Paneth cells present in infants with NEC [15]. Furthermore, Tremblay *et al.* worked on ileal samples of NEC affected neonates and tested their histology and/or gene expression of some inflammatory genes [124]. In some studies, researchers examined samples of the intestinal tissue from preterms that were diagnosed with acute NEC and looked for levels and localization of mRNA of some cytokines [125]. None of the clinical studies interested in NEC and probiotics so far, tested gene expression in the blood samples of preterms prone to NEC before and after probiotic administration.

This prospective blinded randomized controlled trial is unique in that it investigated blood gene expression and gut microbiota of preterms prone to NEC; either on probiotic or not at baseline and one month later. Besides, full-term born neonates that represent the healthy, normal, and fully developed group were analyzed for the same genes and gut microbiota and at the same time points. Analysis of gene expression and gut microbiota was performed using real-time PCR technique on blood and stool samples of neonates in the three groups, respectively.

Study groups characteristics:

Baseline characteristics of the preterms in both groups such as gestational age, birth weight, mode of delivery, APGAR score (a score that summarizes the health of a newborn by considering its **A**ppearance, **P**ulse, **G**rimace, **A**ctivity and **R**espiration), sex and sepsis showed no statistical difference. Moreover, both groups were similar in maternal characteristics such as preeclampsia, chorioamnionitis, use of antenatal steroid and use of surfactant as shown in Table 5.1.

However, there was significant difference in duration of antibiotic use between both groups (Table 5.1). In addition, significant difference was found in their feeding data especially in the mode of feeding since all preterms of control group were fed exclusive breast milk besides starting enteral feedings earlier than ProGrp (Table 5.2).

The small sample size in each tested group and the recruitment of the ProGrp and PreCtr groups from different hospitals are most probably the reasons behind the imbalance in distribution of these characteristics. The intention of separating the ProGrp from the PreCtr group was to overcome cross-contamination that was a limitation of other studies [126]. In a randomized controlled phase 3 study held by Costeloe *et al.*, cross-contamination was documented to be the major limitation of the study that masked any benefit of the probiotic intervention [126]. High colonization in the placebo group has been also documented in other studies and reached around 44% of their placebo group [127]. Accordingly, the two groups were separated in our study. However, variations in clinical services and feeding protocols of each hospital led to other differences between groups that were later recognized. The parameters such as, breast feeding and antibiotic use, which are important factors affecting risk of NEC were taken into account while discussing the results of this study.

The difference in incidence of NEC ≥ Stage II was not statistically significant between ProGrp and PreCtr groups and found as 10% and 4.4%, respectively (p=0.607). This rate of NEC in the ProGrp was actually similar to rates found in placebo group of other studies (10%) [126], and within the range rated in large observational studies (11%) [128]. In a data from 3324 probiotic group and 3281 control group preterms, NEC ≥2 was 2.65% vs. 5.73%, respectively [81]. On the other hand, the mortality in the PreCtr group was 9% compared to zero mortality in the ProGrp. Even though, two preterms were diagnosed by NEC stage II in the ProGrp, none of them died and both were discharged as normal, oral feeding. In one of the meta-analysis, the mortality rate was found to be 5.5% in the probiotic group compared to 8.4% in the control group [111].

Consequently, our data show that although NEC incidence was not prevented in the ProGrp, its severity was reduced and progression to NEC stage III was prevented. Besides, none of the ProGrp died of sepsis, while one preterm had died of sepsis in the PreCtr group.

Secondary outcomes such as the length of stay in hospital, the discharge weight of infants and the difference in weight gain were not significantly different between groups. Another study also reported no significant difference in length of stay in hospital and the discharge weight of

infants as well [85]. In a meta-analysis, they noted that infants on probiotics stay less in the hospital than corresponding infants [111].

Probiotic choice:

There has been no preference in literature to the optimal strain of probiotic that should be administered to premature infants. Wang *et al.*, attempted to further analyze data regarding the species of probiotics and found that both *Lactobacillus* and *Bifidobacteria* were effective [55]. A recent meta-analysis combining data from twenty-six studies was unable to draw definite conclusion on which single-strain of probiotic would be more effective in decreasing incidence of NEC [81]. Different strains, variable doses, and time intervals have been used across trials. Although, it has been noticed that multiple probiotic strains showed better NEC prevention results [81,110], we decided to use a single strain instead, in order not to complicate interpretation of our results.

Bifidobacterium lactis was chosen for this trial for the following reasons: first, it has been shown to have a high colonization capacity; the highest among all Bifidobacteria examined [75]. Second, it has proved across previous clinical studies to be safe for preterms [84, 89, 129], and no sepsis was registered due to its use. Third, this probiotic has been available from manufacturer along with its details. In the present trial, *B.lactis* raised no concerns about its safety in the dose used.

Microbiota in preterms:

The microbial DNA of the stools of premature neonates was tested using quantitative real time PCR method by targeting the 16S rRNA region of each bacteria [130].

The presence of microbial DNA in meconium of our tested preterms groups suggest an intrauterine origin, similar to other studies [131]. Previous studies detected the presence of microbes in amniotic fluid that was not ruptured using both culture and non culture based techniques [132]. Regardless of being in ProGrp or PreCtr group, we were able to detect *Bifidobacterium genus, Enterococcus spp.,Enterobacteriaceae, Lactobacillus group,* and *Candida albicans* in the meconium samples of the investigated preterms.

B.lactis counts in fecal samples:

Results in Table 6.4 indicate that *B.lactis* was successfully colonized in the ProGrp infants one month after supplementation. Twelve out of eighteen (67%) infants in the ProGrp harbored *B.lactis* in their one-month stool samples. *B.lactis* was tolerated by all supplemented preterms with no adverse effects such as abdominal distention, diarrhea or vomiting. On the other hand, *B.lactis* was not detected in any of the PreCtr group members' stool samples; neither in baseline nor in one-month stool samples. Moreover, *B.lactis* was detected in 16% of the one-month stool samples of the FullCtr group and not in their baseline samples, an indication of its presence in breast milk as the normal source of this species.

Importantly, the detection of *B.lactis* in most of the ProCtr group members suggests that concomitant antibiotic treatment that lasted seventeen days in average did not prevent colonization of *B.lactis*. Prolonged early antibiotic use in preterms was found to be associated with increased risk of NEC and death [133]. Additionally, early antibiotic use in preterms decreases the proportion of commensal bacteria and promotes colonization of pathogenic ones. Accordingly, the high colonization of the probiotic *B.lactis* in the intestines of the study group inspite of being subjected to early-prolonged antibiotic use is considered a significant health benefit and a crucial result of this study.

The evaluation of *B.lactis* colonization was important in order to correlate its presence to the beneficial effects detected, which is a strong point of this study. Few studies checked the colonization in their study groups after probiotic supplementation. Coseloe *et al.,* for instance, reported a colonization of 84% for the supplemented *B.breve* in the study group and 35% in the placebo group two weeks after intervention [126]. Patole *et al.* reported 91% colonization in the probiotic group and 38% in the placebo group three weeks after intervention with *B.breve* as well [85]. Stratiki Z *et al.*, checked the increase in the median numbers of fecal *Bifidobacteria* upon supplementation with *B.lactis* during the study period which lasted thirty days and found that it increased significantly with time, especially in the study group as compared to the control group [89].

Microbiota composition among tested groups:

Bifidobacterium genus:

The ProGrp was significantly less colonized with *Bifidobacterium* genus than the PreCtr group in the baseline stool samples. This higher colonization in the PreCtr group is most probably attributed to earlier enteral feeding with breast milk when compared to the ProGrp. After one month of supplementation with *B.lactis,* it was prominent that the difference in colonization of *Bifidobacterium* genus between ProGrp and PreCtr groups disappeared.

The high colonization of *Bifidobacterium* genus in the PreCtr group in comparison to zero colonization of *B.lactis* in the same group implies that this group was colonized with other species of *Bifidobacterium* genus. The interquartile range of the count of *Bifidobacterium* genus in ProGrp in one-month stool samples (log 5.9-7.4 cfu/g) compared to (log 5-7.1 cfu/g) count of *B.lactis* indicates the presence of other *Bifidobacterium* species as well.

The significant difference in *Bifidobacterium* count between ProGrp and FullCtr group is consistent with other publications that used the same technique and validated that the prevalence level of *Bifidobacteia* are low in ELBW infants [134]. Actually, the FullCtr group has the highest colonization and count of *Bifidobacterium* genus compared to the preterm groups. Similarly, Butel *et al.*, showed that when the GA at birth is < 33 weeks , the gut colonization by *Bifidobacteria* is decreased [135].

Enterobacteriacae:

Data in this trial detected a significant difference in colonization among the ProGrp , PreCtr and FullCtr group with regard to *Enterobacteriacae* in one month stool samples, with ProGrp having the least colonization . The difference was significant in the log counts as well, which was significantly less in the ProGrp. Our findings are consistent with those of Mohan *et al.* who studied effects of *B.lactis* supplementation on intestinal microbiota of preterm infants and found that infants supplemented with *B.lactis* had lower viable counts of *Enterobacteriaceae* than the infants in the placebo group [129]. Some studies showed a bloom of *Enterobacteriacae* associated with NEC [136], and that the inflammatory host response selectively enhances the growth of commensal *Enterobacteriaceae* [137]. Moreover, Stewart *et al.* found that antibiotic treatment reduce the abundance of *E.coli* and increase the

abundance of other members of *Enterobacteriacae* [51]. Beside the significantly lower colonization and count of *Enterobacteriaceae* in ProGrp, we noticed that in some stool samples when *B.lactis* was detected *Enterobacteriacae* was abscent and vice versa. Consequently, the presence of *B.lactis* in the intestines of ProGrp preterms has played a major role in altering the gut microbiome competing with other species such as *Enterobacteriacae* that include many potential species that are considered risk factors of NEC. Consequently, these findings imply that supplementing preterms at risk for NEC with *B.lactis* has created a positive change in the composition of their gut microbiota toward a decrease in potential pathogens.

Lactobacillus group:

The low colonization of *Lactobacillus* group in the ProGrp was significantly prominent compared to the PreCtr and FullCtr groups both in baseline and one-month samples. The high rate of breastfeeding in the PreCtr group had most probably led to the increase of *Lactobacillus* group and become comparable to the FullCtr group. The *Lactobacillus* group is known to be one of the first bacteria to colonize normal infants GIT along with *Streptococcus, Staphylococcus, E.coli,* and *Enterobacter* species [39]. This group is typically colonized in infants born vaginally [138], since *Lactobacillus* is the dominant species of the maternal vaginal flora by the end of gestation [139]. Moreover, collected colostrums were found to contain *Staphylococcus, Streptococcus, Lactobacillus*, and *Weissella* species [140], which means that breast milk has a direct role in adding bacterial species to the infant's GIT. Conversely, in a study on vaginally delivered full-term infants, Satokari *et al.* found that "there was no conspicuous difference in the distribution of *Bifidobacterium* or *Lactobacillus* species between breast-fed and formula-fed infants" [141].

Hall *et al.* as well found a noticeable deficiency of *Lactobacilli* compared with coliform organisms in preterm infants and he regarded this lower colonization to treatment with antibiotics [142]. Thus, once more the prolonged antibiotic use in the ProGrp may contribute to the low colonization and count of *Lactobacillus* in this group. Arboleya *et al.,* observed higher levels of *Lactobacillus* group microorganisms in preterm infants and attributed this partially for the primers used that do not amplify only *Lactobacilli* but also related microorganisms from the genus *Weissella* [143].

Enterococcus spp.:

Our data show that *Enterococcus spp.* was detected in the preterm groups as well as the FullCtr group. They were detected in 17% in ProGrp and 37% in PreCtr group baseline meconium samples. In a study that evaluated bacterial diversity in preterm meconium samples, Moles *et al.* found that *Enterococcus spp.* was the most abundant genera detected in 64% preterm fetuses [144]. The lower percentage of colonization in our preterm groups could be due to the prolonged antibiotic use (17 days on average) compared to that in Moles *et al.* tested group, which was on average 3.5 days. Arboleya *et al.* compared full-term breastfed vaginally delivered infants with preterm infants regarding the diversity in microbial groups within gut flora and found that preterm infants showed increased populations of facultative anaerobes such as *Enterococcus* and *Enterobacter* [143]. Similarly, *Enterococcus spp.* were detected in higher number and colonization rate in the PreCtr group than the FullCtr group, although it did not reach significant statistical difference.

NEC diagnosed cases in our study did not contain *Enterococcus* in their stool samples, similarly the finding of Mai *et al.* who did not observe an association of NEC risk with the frequency of *Enterococcus* [9]. Hallstrom *et al.* and Mshvildadze *et al.* , in contrast, detected higher numbers of *Enterococcus* in cases with NEC compared with controls [52,131].

Candida albicans:

The presence of *C.albicans* was checked in our study groups since in a study by Hallstrom *et al. C.albicans* along with *Enterococcus* species were detected in fecal samples of infants diagnosed with NEC more than controls samples [52]. However, *C.albicans* was not detected in the NEC diagnosed infants in our study.

Although *C.albicans* was only detected in two neonates of the PreCtr group and not present both in the ProGrp or FullCtr in the baseline stool samples, after one month *C.albicans* was detected in two (11%) of the ProGrp, one (5%) of the PreCtr group and five (26%) of the FullCtr group. In comparison to other studies on preterms, Mohan *et al.* isolated *C.albicans* from 5.7% preterm infants [129] , *Candida* species were also isolated from preterms suffering from late-onset sepsis; with estimated incidences of 1.6%–9% among VLBW neonates [145].

Moreover, Baley *et al.* determined fungal colonization rate and mainly *Candida spp.* to be 26.7% in preterms that weigh less than 1500 g [146].

In addition, in a study by Manzoni *et al.* on the effect of probiotics on prevention of colonization of *Candida* species in preterms, it was found that *L. casei* significantly reduced the incidence and the intensity of colonization by *Candida* species among VLBW neonates [147]. However, in our study insignificant difference between ProGrp and PreCtr group in *C.albicans* colonization does not suggest an effect of the probiotic *B.lactis* on colonization of *C.albicans*.

Difference in colonization between baseline and one month stool samples in the tested groups:
Figure 6.1 shows that the supplementation of *B.lactis* caused a rapid increase in the colonization of other bacterial groups over a period of one month. This figure is strong evidence that colonization of *B.lactis* in the ProGrp preterms caused an increase in *Bifidobacterium genus, Enterococcus spp.* and *Lactobacillus* group, making the overall composition of their gut microbiota comparable to that of the healthy full term group. Besides, the figure shows that the increase in colonization of *Enterobacteriacae* is the least after one month compared to the other tested groups; an added value to the colonization of *B.lactis*.

It is worth mentioning here that extra factors contributed to the different counts and colonization rates of the previously mentioned bacterial groups. For example, mother's diet and her exposure to antibiotics affect the composition of her breast milk and the normal flora in it and subsequently the composition of her baby's gut microbiota. Analyzing the microbiota of the breast milk for the mothers of tested neonates would have been better for thorough understanding of the make-up of the newborns gut microbiota.

Genes expression:
The mechanisms by which probiotics, *B.lactis* in our case, affect the preterms gene expression are not fully explored. It has been found in previous studies that gut bacteria up regulate the expression of host gene that encourage commensal bacteria rather than pathogenic species [148]. Moreover, probiotics have been shown to up regulate markers of immune system function in human studies [149]. Though limited studies have investigated the effects of

probiotics on proinflammatory factors among neonates, the present study aimed to investigate the effect of supplementation with *B.lactis* on gene expression of some inflammatory genes in preterm groups and compare these findings to control preterms and healthy full-term neonates. It is worth noting that this gene investigation was performed in whole blood samples and not using samples of the intestines of the neonates, since gene expression in white blood cells reflects gene expression in the peripheral tissue.

Given the complex pathology of NEC, it is difficult to determine the exact mechanism by which the probiotic reduce its severity as shown in our study. Hopefully, the results of genes expression levels will shed light on some mechanisms, suggest some correlations or confirm findings of non-clinical studies.

RORC and *NCR1 (NKp46):*

The current investigation shows significant increase in *RORC* expression after one month in the three tested groups. *NCR1* expression showed a similar pattern as well, since it significantly increased in FullGrp and PreCtr groups as well as ProGrp, although it did not reach statistical significance in the ProGrp. There was significant difference in *RORC* expression among the groups in baseline blood samples with ProGrp having the highest expression. However, no significant difference was noticed among the groups in one-month blood samples. Concerning *NCR1*, significant difference in expression was not detected among the groups in either baseline or one-month blood samples.

The increase in expression of both *RORC* and *NCR1* in the ProGrp may indicate an increase in the presence of NCR^{+}ILC3 cells that are known to express both genes. $ROR\gamma t^{+}$ NCR^{+} ILC3 cells are key producers of IL-22, which promotes gut health by inducing production of epithelial derived antimicrobial peptides and mucins, and reduced number of these cells may lead to weakened gut mucosal protection in these individuals [101]. Thus, the increase in *RORC* and *NCR1* expression after one month of *B.lactis* administration in the ProGrp could partly explain the protective role in the prevention of NEC progression.

Furthermore, RORγt is found to be responsible for the differentiation of Th17 cells that are involved in autoimmune diseases. Mice lacking RORγt develop autoimmune disease demonstrating its role in immune homeostasis [150].

Comparison of the expression of *RORC* in this study with other published articles targeting neonates indicates high expression as well. Black *et al.*, found that neonates expressed higher levels of *RORC* than in adults in a study about the development of Th17 cells by assaying CD4 T cells from extremely preterm infants, term infants, and adults [151]. In addition, Weitkamp *et al.* examined the development of intraepithelial lymphocytes in the ileum of human infants and their role in NEC pathogenesis, and found that loss of intraepithelial lymphocytes in human NEC was associated with downregulation of *RORC* [152].

Moreover, RORγt$^+$ NCR$^+$ ILC3 are affected by both commensal and pathogenic bacteria through interaction of NKp44 and NKp46 proteins with bacterial components [153]. Differentiation of Th17 cells is also highly correlated to the composition of gut microbioa. Actually, RORγt$^+$ deficient mice were found to develop severe intestinal inflammation [154]. Besides, Sanos *et al.* found that signals from commensal flora contribute to *RORC* expression that is needed for differentiation of ILC producing IL-22, which in turn maintain the mucosal immune system homeostasis [155]. Interestingly, *Lactobacillus spp.* was shown to regulate RORγt$^+$ NCR$^+$ ILCs to induce IL-22 through specific signaling pathway [156]. This may explain in part the reason why both *RORC* and *NCR1* genes were expressed in PreCtr and FullCtr groups more than ProGrp. Besides, the high expression of *RORC* in PreCtr group might be attributed to the high colonization of *Enterococcus spp*. that was found to be mostly colonized in this group; however further investigation is needed.

HIF1A gene:

The main function of the product of *HIF1A* gene is to accommodate the cells to hypoxia by regulating proteins relevant in the oxygen delivery cascades. Epithelial decrease in oxygen plays a critical role in intestinal inflammation and demands the up regulation of this gene to restore intestinal homeostasis.

Our data showed statistically significant down regulation of this gene in the healthy full-term neonates control group (FullCtr). Similarly, preterms at risk of NEC that were supplied by *B.lactis* showed a down regulation of *HIF1A* one month after intervention, although it was not significant statistically. On the other hand, there was no change in the gene expression of the PreCtr group. This may indicate that the colonization of *B.lactis* in the ProGrp group intestines

might have been contributed to intestinal homeostasis by partially manipulating *HIF1A* expression.

Studies on newborns with stage III NEC observed significant up regulation of *HIF1A* in these patient's intestinal tissues [157]. Furthermore, in some animal studies *HIF1A* has been associated with disease severity of NEC [158, 159]. The up regulation of *HIF1A* was considered as beneficial on disease outcomes and barrier function as implicated by studies in animal models of intestinal inflammation. For instance, Cummins *et al.* found that knocking out of either *HIF-1* or (IKKβ-dependent) NF-κB pathways in intestinal epithelial cells promotes inflammatory disease in murine models of colitis [160]. Similarly, Keeley *et al.* showed that induction of *HIF1A* resulted in an overall beneficial influence on clinical symptoms in murine colitis, most likely due to barrier protective function and wound healing during severe tissue hypoxia at the site of inflammation emphasizing the role of *HIF1A* during inflammatory diseases in the colon [161].

A significant difference of *HIF1A* expression was detected among tested groups in baseline blood samples, while no significant difference was present in their one-month blood samples. Variability in composition and number of the gut microbiota in each group could be a reason behind this difference. Some researches pointed to a correlation between *HIF1A* expression and some bacteria or *Candida*. For example, Hirota *et al.* assessed the effects of *HIF1A* signaling in response to *C. difficile* toxin and found that *HIF1A* protects the intestinal mucosa from *C. difficile* toxins [158]. Moreover, Fan *et al.* found that activation of colonic *HIF1A* results in a significant reduction of *C. albicans* GI colonization [159].

IL1RL1 gene:

IL1RL1 has been found to be up regulated in relation to some inflammatory conditions and diseases. A study on inflamed IBD biopsy samples, found that mRNA expression of *IL1RL1* was significantly increased [96]. The healthy full term neonates group in the present study showed a significant down regulation of *IL1RL1* gene in the one-month blood samples, suggesting that this might be the normal condition of this gene. *IL1RL1* expression was

similarly down regulated in ProGrp one month after *B.lactis* supplementation, although it did not show any statistical significance.

The present results are in agreement with results reported in literature. Troost *et al.* showed that continuous injection of *L. plantarum* for one hour into the proximal duodenum induced *IL1RL1* (ST2) down regulation in healthy intestinal mucosa, an exploration that they found it could provide a molecular support for probiotic activity of this strain [164] .

Several other studies focused on the IL-33/ ST2 axis in the intestinal mucosa of IBD patients [165]. Other studies showed that intestinal tissue expression pattern of *IL1RL1* is different in healthy mucosa compared to that found in chronically inflamed IBD patients [166]. Moreover, in a gene expression study on duodenal biopsies of adults and children with Celiac disease, an immune-mediated systemic disease, it was found that *IL1RL1* was the uniquely up-regulated gene in adults [167]. The IL1RL1 receptor and its IL-33 have been also implicated in the onset of spontaneous preterm labor in the context of infection [168].

When expression levels at baseline blood samples of the tested groups were compared, no differences were noted. However, the expression in one-month blood samples showed a significant difference with FullGrp being the least expressed followed by the ProGrp and the PreCtr group, respectively. To our knowledge, there are limited data about the expression of *IL1RL1* in neonates. Our data provided a novel addition to literature by testing *IL1RL1* expression in whole blood samples of neonates and comparing it in probiotic supplied preterms with preterm controls and full-term neonates.

In summary, we noticed that the selected candidate genes expression tested in whole blood samples showed the same regulation pattern both in the preterms that were supplemented by *B.lactis* and the healthy normal full-term group. This indicates that the beneficial effect of *B.lactis* in prevention of NEC progression might have been exerted partly throughout the manipulation of these genes. It is worth mentioning here that our prediction was that the effect of *B.lactis* would have been more pronounced on the gene expression level if no significant differences were present in the feeding data of preterms groups, especially that breast milk is found to contain microRNAs that are involved in control of immunologic reactions [170].

8. CONCLUSION

Data presented in this thesis showed that NEC severity was reduced, and progression to NEC stage III was prevented in the ProGrp preterms supplied by *B.lactis.* Moreover, *B.lactis* was shown to be successfully colonized in most of this group members one month after supplementation in spite of concomitant prolonged antibiotic treatment and without sepsis or mortality cases registered.

Gene expression analysis for the ProGrp preterms' whole blood samples showed for the first time significant alterations that may partly played role in the control of NEC pathogenesis due to the probiotic. This is the first study to test the expression of selected inflammatory related genes in preterms before and after *B.lactis* probiotic administration and recognizing that, they follow the same trend as the healthy full-term control group.

Further work could be undertaken to do protein level analysis that is needed to confirm the expression of the studied genes. Further investigations of other genes in relation to *B.lactis* in preterms would also be recommended. Besides, the testing of IL-22 and IL-17 serum levels as they are the products of *RORC* and *NCR1* expressing cells will be beneficial.

Moreover, the gut microbiota groups analysis suggested that the presence of *B.lactis* in the intestines of ProGrp caused a rapid increase in the colonization of other bacterial groups over a period of one month competing with other species such as *Enterobacteriaceae*, which include many potential pathogens that are considered risk factors of NEC. Actually, the increase in the count and colonization of *Bifidobacterium* genus in the ProGrp and having the lowest count and colonization of *Enterobacteriaceae* in comparison to other tested groups is one of the significant results of this trial. We suppose that if the breast milk feeding had been lower in the PreCtr group, greater differences would have been observed concerning the microbiota composition results as well as gene expression levels.

Even though other gut microbiota groups that could have been affected by the colonization of *B.lactis* were not in the scope of this study, the evaluation of other bacterial groups as well is advised.

An important strength of this study was the incorporation of a full-term control group that act as a healthy control group and subjecting it for the blood gene expression and the fecal microbiota analysis performed at the baseline and one month time points. Another strength of this study was the measurement of *B.lactis* colonization percentage in the supplemented group in order to consider when interpreting the alterations detected in either the gut microbiota or the gene expression.

Main limitations of this trial were the small sample size and the significant differences in the feeding data and antibiotic duration among the preterm groups that are confounding factors affecting gut microbiota composition. Given that this was a multicentre study, it was difficult to standardize feeding strategies and antibiotic use among groups. Although this imbalance may masked the real effect of *B.lactis* on both gut microbiota and gene expression, it actually demonstrated the considerable effect of exclusive breast feeding in obtaining outcomes similar to the full-term neonates regarding both gut microbiota and gene expression.

Although more genes need to be studied and evaluated, this study provides a valuable addition to literature concerning the manipulations in gene expression levels detected in *B.lactis* supplemented group as well as the controls that apparently constitute an advantage to preterms prone to NEC.

Interestingly, the present study raises questions about some correlations that need further investigation such as *Enterococcus* spp. presence and expression of *RORC* and *NCR* as well as the effect of breast milk on the expression of the tested genes in the neonates.

In conclusion, we think that the findings of this study send a strong message to neonatologists to implement *B.lactis* probiotic beside exclusive breast-feeding to preterms in their NICUs to improve their immune function and consequently prevent NEC.

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