Maternal Immunoglobulin A Shapes the Early Neonatal Microbiota and Protects Against the Development of Necrotizing Enterocolitis in Preterm Infants

by

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Abstract

Neonates are protected from colonizing bacteria by antibodies secreted into maternal milk. Immunoglobulin A (IgA) plays a crucial role in maintaining the homeostasis between the microbiota and intestinal epithelium. The effect of maternal IgA is potentially critical because improper handling of colonizing organisms can lead to serious complications such as sepsis, pneumonia and Necrotizing Enterocolitis (NEC). NEC is a disease of neonatal preterm infants with high morbidity and mortality that is associated with intestinal inflammation driven by the microbiota. The incidence of NEC is significantly lower in infants fed with maternal milk, though the mechanisms underlying this benefit are not clear. Here, we show that maternal IgA is an important factor in the protection against NEC.

Analysis of IgA-binding of fecal bacteria from preterm infants indicated that maternal milk was the predominant source of IgA in the first month of life and that a relative decrease in IgA-bound bacteria is associated with the development of NEC. We observed heterogeneity in the IgA repertoire of the breast milk of different mothers. Sequencing of IgA-bound and unbound bacteria revealed that, prior to disease onset, NEC was associated with an increasing domination of the IgA-unbound microbiota by *Enterobacteriaceae*.

Further, we confirmed that IgA is critical for shaping early neonatal colonization in a murine model, where pups reared by IgA deficient mothers have increased *Enterobacteriaceae* in their fecal matter and developed disease similar to formula-fed pups. Our findings show that maternal IgA shapes the host-microbiota relationship of preterm neonates and that IgA is a critical and necessary factor in maternal milk for the prevention of NEC. The public health significance of this study is to emphasize the benefits of feeding neonates with maternal milk as it reveals the successful association of maternal milk IgA binding to *Enterobacteriaceae* to a reduced incidence of NEC. In addition, this study describes the invention of a novel diagnostic test to examine the heterogeneity in the maternal milk IgA repertoire between different donors and timepoints. This test can be used to identify donor breast milk with high IgA binding to *Enterobacteriaceae*, which could be used prophylactically in preterm infants who are at risk for the development of NEC.

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Preface

My journey through the past 4 years of PhD has been intriguing and fun-filled, but there were challenging times as well. Before I begin my dissertation, I want to formally acknowledge all the people who have believed in me, helped me grow and kept me sane throughout. Words may fall short to describe the immense gratitude I have for all these people in my heart.

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Abbreviations

16S rRNA = 16S ribosomal RNA

ATCC = American Type Culture Collection

CCL28 = C-C motif Chemokine Ligand 28

CFU = Colony Forming Unit

DOL = Day of Life

EGF = Epidermal growth factor

G-CSF = Granulocyte colony stimulating factor

g/dL = grams/deciliter

HB-EGF = Heparin Binding EGF like growth factor

HMOs = Human Milk Oligosaccharides

IFN-γ = Interferon-gamma

IgA = Immunoglobulin A

IgA- = IgA unbound

IgA+ = IgA bound

IGF = Insulin-like growth factor

IgG = Immunoglobulin G

$Igha^{-/-}$ = Immunoglobulin heavy constant alpha knockout

IgM = Immunoglobulin M

J-chain = Joining chain

$Jh^{-/-}$ = Jh knockout

kcal/dL = kilo calories/deciliter

LDA = Linear Discriminant Analysis

LPS = Lipopolysaccharide

M-cells = Microfold cells

MACS = Magnetic activated cell sorting

MFG-E8 = Milk Fat Globule EGF Factor 8

MIF = Macrophage Migratory inhibitory factor

MUC1 = Mucin 1

MUC4 = Mucin 4

NEC = Necrotizing Enterocolitis

NGF = Nerve growth factor

OD = Optical Density

OUT = Operational Taxonomic Unit

PBS = Phosphate Buffered Saline

PCR = Polymerase Chain Reaction

pIgR = Polymeric Ig Receptor

$Rag1^{-/-}$ = Recombination activating gene 1 knockout

sIgA = Secretory Immunoglobulin A

TGF = Transforming growth factor

TGF-β = Transforming growth factor-beta

TLR4 = Toll like receptor 4

TNF-α = Tumor necrosis factor-alpha

VEGF = Vascular Endothelial growth factor

1.0 Introduction

One of the underlying drivers of this work was the idea that there is an immunological benefit provided by the mother's breast milk immunoglobulin A (IgA). Our goal is to understand how IgA can influence the intestinal microbiota in neonates and how this impacts health and diseases such as Necrotizing Enterocolitis (NEC). This is an understudied, but critical avenue by which to advance science. As such, the research topics detailed within this dissertation investigate the relationship of maternal milk IgA to the development of NEC in preterm infants. This focus stemmed from the rapidly growing body of literature describing how the intestinal microbiome is influenced by different components of the breast milk and how it plays a role in the prevention of NEC. In addition, ongoing research shows that the IgA produced in the body in adults can help maintain their intestinal microbial homeostasis through various mechanisms. The synthesis of these findings led to the overarching hypothesis of this thesis - maternal IgA binding of invasive intestinal bacteria is protective against the development of NEC.

1.1 Breast Milk Shapes the Intestinal Microbiota in Early Life

1.1.1 Breast milk

Breast milk is the main source of nutrition for newborn infants until approximately 6 months to 1 year of age. The American Academy of Pediatrics recommends exclusive breastfeeding to infants, as it provides all the nutrients required for the growth of the infant for the

first 4 to 6 months of life and breastfeeding with food supplementation up to 1-2 years of age[1,2]. Studies have observed that breast milk changes in nutrition and quantity over a period of time, different gestational period, diet of the mother and environmental factors[3]. The breast milk in the first few days of life is called the colostrum and it contains various proteins that provide protection for the neonates. Colostrum is rich in antibodies, antimicrobials and anti-allergens, thus protecting the neonate from several enteric and allergic diseases later in life[4]. The mature milk, produced after the first week, mainly helps in the growth and development of the infant as it is rich in fats, carbohydrates and proteins. Breast milk also contains enzymes, cytokines, growth factors and hormones, which provide protection against various diseases and promote growth of the infant[5].

1.1.2 Components of the breast milk

The nutritional components of breast milk can be categorized into macronutrients **(Table 1.1)** and micronutrients.

Table 1.1 Macronutrient Composition of Term and Preterm Breast Milk

Macronutrients	Term milk	Preterm milk
Protein (g/dL)	0.9	1.9-2.2
Fat (g/dL)	3.5	4.4-4.8
Carbohydrates (mainly lactose) (g/dL)	6.7	7.5
Energy (kcal/dL)	65 to 70	78

Table adapted from previous breast milk studies[3,5] depicting the difference in macronutrient concentration in term and preterm milk.

Maternal milk proteins can be classified into two main groups: antimicrobial or immune-stimulatory and nutritional as listed in **Table 1.2**. The anti-microbial group of proteins inhibit the

growth of pathogenic bacteria, thus preventing infections in the early life. The proteins in the nutritional group assist in the absorption of vitamins and micronutrients in the intestine of the neonate[6].

Table 1.2 Proteins present in the Breast Milk

Anti-microbial proteins	Nutritional proteins
Lactoferrin	Bile-salt stimulated lipase
Secretory IgA, IgM, IgG	Haptocorrin
Kappa-casein	Folate binding protein
Lactoperoxidase	Alpha-lactalbumin
Haptocorrin	Lactoferrin
Lactadherin	

Table created from studies[6] showing the different types of proteins in the breast milk.

The concentration of proteins is higher in the colostrum of both term and preterm milk, whereas fats and carbohydrates are at lower concentrations. The protein concentration in the colostrum of preterm milk is significantly higher than in term milk and it gradually decreases over a period of 4 weeks irrespective of the gestational age during birth **(Table 1.1)**. In the days following, the concentration of the fat, carbohydrate and energy in the breast milk increases drastically and the concentration of the proteins gradually decreases[7].

The fat concentration in the human milk is highly variable and mainly consists of high concentrations of oleic and palmitic acids in the triglycerides. During each feeding, the initial milk or foremilk, contains large amounts of water and carbohydrates, whereas the milk that comes later, called the hind milk, is rich in lipids and proteins[8]. The concentration of the lipids is influenced by

the diet, especially the fat and protein intake of the mother, thus explaining the variation in lipid concentration of the milk[3,9].

Lactose is the main source of carbohydrates in the breast milk. Variation of lactose is the least among all the macronutrients. However, another major form of carbohydrates are the oligosaccharides, which are secreted in the human milk[5]. Though non-nutritive to the infant, human milk oligosaccharides are said to protect the infants from enteric diseases and help form the initial microbiome in the neonatal gut[10,11].

The micronutrients in the breast milk consist of vitamins A, B1, B2, B6, B12 and D. It also consists of non-protein, nitrogen-containing compounds and minerals such as sodium, potassium, magnesium and zinc[5]. The concentrations of these micronutrients vary significantly with the diet of and availability of these components in the mother.

The components of the breast milk that are non-nutritional but have a significant role in biological processes such as preventing infections, development of the mucosal surfaces and overall protection of a neonate, are the bioactive factors. Bioactive factors primarily consist of anti-microbials, growth factors and white blood cells which are produced by the mother and transferred to the neonate through the breast milk. Their source can vary between the maternal mammary gland epithelium to the maternal serum. The bioactive factors are listed in **Table 1.3**.

Table 1.3 Bioactive factors in the Breast Milk

Cells	Macrophages, Stem cells
Anti-microbials	Immunoglobulins- Secretory IgA, IgM, IgG Lactoferrin, Lactadherin/MFG E8, Lysozyme, Complement C3, Antiviral mucins- MUC1, MUC4
Growth Factors	Epidermal growth factor (EGF), Nerve growth factor (NGF), Insulin-like growth factor (IGF), Transforming growth factor (TGF), taurine, polyamines, Heparin Binding EGF like growth factor (HB-EGF), Vascular Endothelial growth factor (VEGF), Erythropoietin
Cytokines, Chemokines and Anti-inflammatory factors	Tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ), Transforming growth factor-beta (TGF-β), prostaglandins, α_1-antichymotrypsin, α_1-antitrypsin, platelet-activating factor: acetyl hydrolase Interleukins- IL-6, IL-7, IL-8, IL-10 Chemokines- Granulocyte colony stimulating factor (G-CSF), Macrophage Migratory inhibitory factor (MIF)
Hormones	Calcitonin, Somatostatin, Adiponectin, Leptin, Ghrelin
Digestive enzymes	Amylase, Bile acid-stimulating esterase, Bile acid-stimulating lipases, Lipoprotein lipase
Transporters	Lactoferrin, Folate binder, Cobalamin binder, IGF binder, Thyroxine binder, Corticosteroid binder
Oligosaccharides and Glycans	Human Milk Oligosaccharides (HMOs), Gangliosides, Glycosaminoglycans

Adapted from previous studies explaining the importance of bioactive factors[5,12]

1.2 Intestinal Microbiota from Birth to Adult Life

The intestinal microbiome is comprised of various bacteria, viruses and fungi that are residing in the intestine and help in the absorption of complex carbohydrates. The intestinal microbiome has about 10 times more cells than the human body, whereas microbial genes form about 99% of the total genes[13]. The microbiota provides us with the genetic material necessary to digest complex carbohydrates and lipids. The intestinal microbiota develops from being sterile during birth to a complex set of obligate and facultative organisms during adult life[14]. This evolution is brought about by various factors such as prenatal exposure to antibiotics or toxins, mode of delivery, antibiotic exposure after birth, diet (breast feeding versus formula feeding), genetic factors, introduction of solid food and environmental factors such as geography or climate[14].

Recent studies have identified differences in the microbial composition in infants that were delivered vaginally and by caesarean section[15]. They identified infants that had antibiotic exposure during birth or soon after delivery and noticed an increase in facultative anaerobes such as *Enterobacteriaceae* and *Enterococcaceae* in their microbiota. Whereas, infants born vaginally and without antibiotic exposure had an increased abundance of obligate anaerobes such as *Bacteroides* in their intestine. Similarly, literature studying the diet of the infant, whether the infant was fed breast milk or formula, have illustrated the difference in the microbial composition. The infants that were breastfed had an increased abundance of *Bifidobacterium* in their microbiota, as compared to formula fed infants who had an increased abundance of *Proteobacteria* and *Firmicutes*[16-19].

The newborn infant is born sterile and soon after birth, the intestine is oxygenated. This leads to growth of facultative anaerobes such as *Enterobacteriaceae* and *Enterococcaceae.* Within

the first few weeks after birth, these organisms utilize the available oxygen, thus creating an anaerobic environment for the growth of anaerobes such as *Bacteroides*[14]. However, it takes about 3 years of age for a child to develop and maintain a microbial composition similar to adults[14]. One of the major factors that play a role in the evolution of the microbiome is the feeding of the neonate. Infants fed with breast milk of the mother have shown increased abundance of *Bifidobacterium* and *Bacteroides*. The bioactive factors, mainly the human milk oligosaccharides, lactoferrin and immunoglobulins present in the breast milk are crucial for the development of the healthy intestinal microbiome in infants. These factors help in the development of the intestinal epithelium and limit the growth of the intestinal microbiota.

1.2.1 Human Milk Oligosaccharides

Human milk oligosaccharides (HMOs) are a group of glycans with a lactose backbone at the reducing end, with a monosaccharide core, which is mostly glucose, galactose, fucose, N-acetylglucosamine, N-acetylneuraminic acid or sialic acid, with chain length varying from 3 to 15 carbohydrates present at the non-reducing end[20,21]. The lactose backbone of the HMOs in the breast milk can be fucosylated, glycosylated or sialylated, thus forming different structural isomers[21]. There is a high number of HMOs in the breast milk, among which about 200 have been identified through mass-spectrometry[21].

Glycosylated or fucosylated HMOs have similar structure to glycoproteins and glycolipids found in the epithelial cell wall as the glycosylated moieties are similar to those found in the epithelium[22]. Before the structural assembly of HMOs, they were thought to be glycoproteins or glycolipids secreted from the mammary epithelium[20]. These glycosylated motifs of the HMOs are similar to cell surface receptors and thus interfere with the binding of bacteria to the epithelial

surface[23]. Studies have shown that the presence of HMO's inhibit the growth of pathogenic bacteria like *Streptococcus pneumoniae* and *Campylobacter jejuni*, by interfering with the adhesion of the bacteria to epithelial cells, both in vitro and in vivo[23-25]. HMOs have been demonstrated to bind to the stable toxin of *Escherichia coli* and inhibit their growth in mice fed with human milk[26]. Previous studies have demonstrated the role of glycosylated HMOs in the prevention of diseases such as Necrotizing Enterocolitis (NEC) and rotavirus infection in experimental murine models[11,27,28].

HMOs are present in large amounts in the breast milk, about 1g/dL[5]. They are not broken down by the digestive enzymes of the infant and thus can reach to the distal ileum and colon[29]. Organisms such as *Bifidobacterium* and *Bacteroides*, metabolize these HMOs and utilize it as their energy source. Since the only source of HMOs is the breast milk of the mother, studies have shown that infants who are fed with mother's milk have increased abundance of obligate anaerobes such as *Bifidobacterium* and *Bacteroides* in the first few weeks of life as compared to formula-fed infants[30,31]. HMOs are variable in each mother depending on the genetic composition, diet, body weight of the mother during pregnancy and stages of lactation[32-35]. Difference in the concentration of HMOs has been observed in mother's with preterm infant and term infant[36,37]. This variation in the HMOs has been proposed to cause the difference in the protective effects of breast milk.

1.2.2 Lactoferrin

Lactoferrin, referred to as the "red protein from milk", is an iron binding protein, known for its bacteriostatic properties[38]. It is said to be produced from the glandular epithelial cells of mammals and secreted in granules of neutrophils[39]. It is a member of the transferrin family of proteins and is secreted in the breast milk[39]. Colostrum contains a high concentration of lactoferrin

and it slowly decreases in the first month of lactation[40]. The amount of lactoferrin in the breast milk varies with maternal age, diet, gestational age at birth, ethnicity and stage of lactation[41].

The iron binding ability of lactoferrin led to the discovery of its anti- microbial and immunomodulatory functions[42]. It was believed that lactoferrin sequesters iron by binding to it with high affinity, thus making it unavailable to bacteria in the gut, which require iron as an essential component for its growth[43]. This bactericidal action was demonstrated in various bacteria such as *Streptococcus* and *Vibrio* in-vitro, but was not seen in *Escherichia coli*[43]. However, an iron-independent mechanism in which lactoferrin interacts with the lipopolysaccharide (LPS) of Gram- negative organisms and inhibits its growth, was demonstrated in further studies[44]. Through its immunomodulatory capacity, lactoferrin enhances phagocytosis of Gram-positive bacteria, by macrophage activation[42]. It prevents the formation of biofilms in the intestinal tract and the interaction of microbes with the host epithelial cells[42,45]. The reduced digestive capacity of the infant intestine protects breast milk lactoferrin from degradation and maintain its bactericidal and bacteriostatic properties, thus restricting the growth of aerobes and facultative anaerobes in early life.

1.2.3 Secretory Immunoglobulin A

One of the most important anti-microbial bioactive factors in the breast milk are the immunoglobulins. The main antibodies present in the breast milk are IgA, IgM and IgG. IgA constitutes about 90-95% of all antibodies, with IgM accounting for 2-5% and IgG is less than 1%[46]. Colostrum contains the highest amount of IgA and is essential for providing protection and immune development in the early days of life. Colostrum is required for sustenance of life in mammals such as piglets. Increased mortality rates within the first few days of life is seen in piglets

who do not receive adequate amounts of colostrum[47,48]. Antibodies provide passive immunity from infections to the newborn infant. IgG can cross the placenta and provide protection to the fetus in-utero. However, IgA and IgM cannot cross the placenta and thus are provided to the neonate through the maternal breast milk to facilitate protection against various infections.

The structure of IgA consists of polymers of 2-4 IgA monomers bound together by disulphide linkages between the constant end of one monomer and J-chain (joining chain), which is formed in the IgA-secreting cells[49]. IgA exists in dimeric form in the laminal surface of the cells and is transported across the epithelium by polymeric Ig receptor (pIgR). During transcytosis, a portion of the polypeptide chain of pIgR gets attached to the IgA and is called the secretory component[50]. Thus, the dimeric IgA after transcytosis across the epithelial layer is known as the secretory IgA (sIgA) **(Figure 1.1)**. The secretory component prevents the proteolytic degradation of IgA and also prevents IgA from activating effector mechanisms[49].

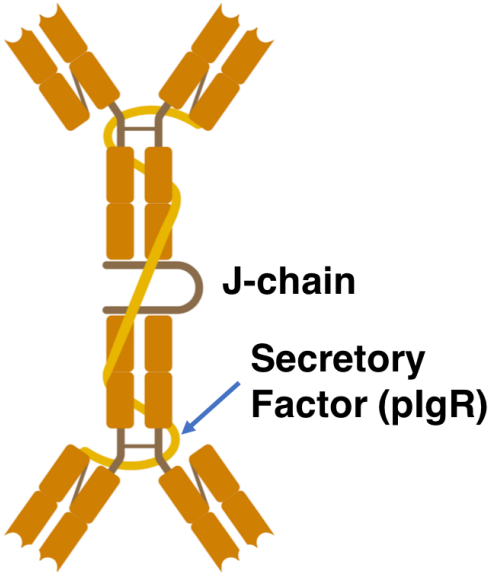

Figure 1.1 Structure of Secretory IgA (sIgA)

Secretory IgA is produced in the mucosal surfaces and provide anti-bacterial effects by binding to the bacteria and preventing it from invading the mucosal epithelium. Unlike IgG or IgM, the anti-bacterial activity of sIgA is not through cytolysis by activating complement or through opsonization or phagocytosis. The antibody-based immunity against the bacteria is through a) steric hinderance of bacterial surface molecules, b) increased uptake or sampling by the M-cells in the Peyer's patches, c) through modification of bacterial transcription or d) "enchaining" the bacteria to prevent gene transfer[51-53].

The importance of entero-mammary circulation of IgA-producing B-cells has been illustrated by various studies suggesting that, during pregnancy, the B-cells activated in the Peyer's patches are transported to the mammary gland, with the help of chemokine CCL28[54,55]. These B-cells secrete IgA into the mammary gland and transferred to the infant through the breast milk[56]. Recent studies have confirmed the presence of B-cells with the same variable regions in both the intestine and mammary glands, in mice[57]. Thus, the IgA secreted by the B-cells in the mammary gland may be specific to particular bacterial taxa.

IgA is necessary to maintain the intestinal microbial homeostasis, as it binds to the bacteria and prevents the adhesion or invasion of the bacteria into the intestinal epithelium. Studies have observed the increase in the relative abundance of *Enterobacteriaceae* in the intestinal microbiota of patients with selective IgA deficiency as against to controls[58]. This study also noticed that the overgrowth of *Enterobacteriaceae* was not controlled even with compensatory increase of IgM in these patients. Similar data has been perceived in mice models, where mice deficient in IgA ($Igha^{-/-}$ or $Jh^{-/-}$ mice), had similar total 16S rRNA counts as C57BL/6 mice but their microbiota was dominated by *Enterobacteriaceae*[59].

Maternal breast milk is the main source of IgA in the first few weeks of life in neonates since it takes about 3-4 weeks for the neonatal intestine to be populated by B cells[60]. As infant formula does not contain IgA, formula-fed infants have an increase in the relative abundance of *Enterobacteriaceae* in their microbiota[19]. This suggests that IgA is required to regulate the growth of *Enterobacteriaceae* in the intestine and promote the growth of obligate anaerobes such as *Bacteroides*.

The microbiota in early life plays an important role in the development of infections and inflammatory conditions in later life. The microbial composition in early life is integral for the development of epithelial barrier functions, development of immune activation and tolerance. Early perturbation of the intestinal microbiota due to feeding differences, exposure to antibiotics, mode of delivery, genetic and environmental factors can lead to long term health consequences such as inflammatory bowel disease, asthma and allergy[61]. Studies in germ-free mice have depicted that in the absence of the microbiota, there is enlarged colon due to accumulation of degraded mucus, which would have been digested by the microbes and decreased intestinal peristalsis[62,63]. These mice also had smaller crypts and absence of anti-microbial peptides[64].

The bioactive components of the breast milk discussed above support the growth of a complex microbiota and help in maintaining the diversity of the intestinal microbial ecosystem. Maternal milk provides nutrition for the physical growth and development of the neonate and also delivers bioactive components to maintain a physiological balance between the immune system and the microbiota. This aids the neonate to develop defenses against invasion by the bacteria and prevents infections later in life.

2.0 Maternal Immunoglobulin A Protects Against the Development of Necrotizing Enterocolitis

2.1 Foreword

This chapter was adapted from a previously published manuscript in Nature Medicine in 2019: **Gopalakrishna KP**, Macadangdang BR, Rogers MB, Tometich JT, Firek BA, Baker R, Ji J, Burr AH, Ma C, Good M, Morowitz MJ, Hand TW. Maternal IgA protects against the development of necrotizing enterocolitis in preterm infants. Nature medicine. 2019 Jun 17:1

2.2 Necrotizing Enterocolitis

2.2.1 Incidence and Pathophysiology

Necrotizing enterocolitis affects about 7% of preterm very low birth weight infants, resulting in both high mortality (>20%) and frequent lifelong complications amongst infants who recover[65,66]. The exact etiology of NEC is unknown, but the disease is believed to occur subsequent to intestinal damage due to stress or hypoxia associated with prematurity and bacteria-driven immune-mediated inflammation, causing necrosis of the intestine **(Figure 2.1)**[67-69].

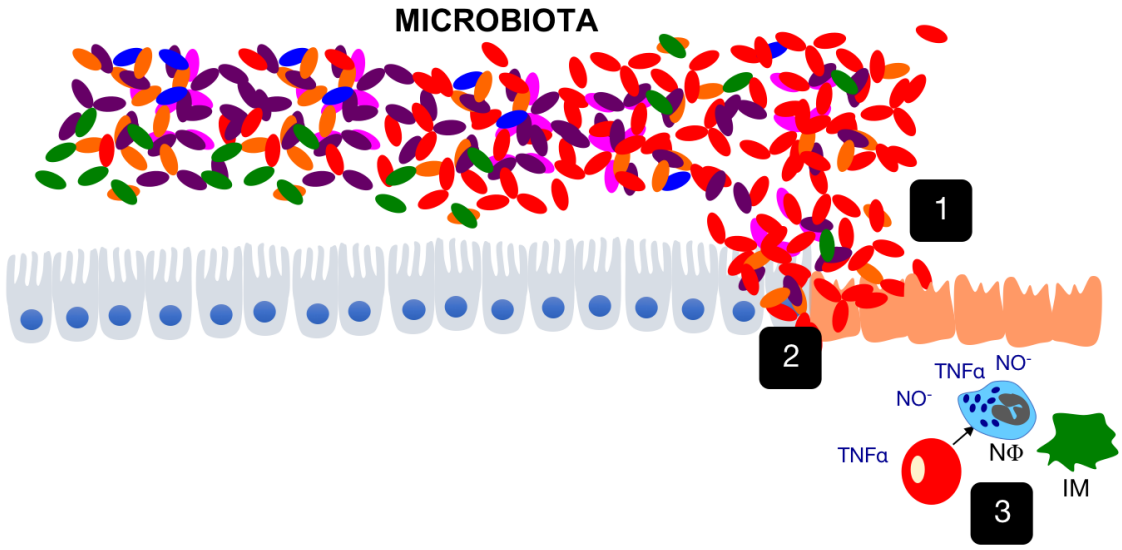

Figure 2.1 Paradigm explaining the pathophysiology of NEC

Figure showing the intestinal epithelium and the microbiota in the lumen of the intestine. 1. Damage to the intestinal epithelium (stress, hypoxia), 2. Invasion of intestine by bacteria (*Enterobacteriaceae, Enterococcaceae*), 3. Inflammation and immune activation due to TLR4 activation causing necrosis of the intestinal epithelium.

2.2.2 Disease Manifestations, Diagnosis and Treatment

The clinical features associated with NEC are increased abdominal girth due to abdominal distension, visible intestinal loops, decreased bowel sounds, frank blood in the stools and erythema on the abdominal wall. Systemic manifestations such as respiratory failure, decreased peripheral perfusion and circulatory collapse can occur leading to adverse consequences in the infant[65].

Diagnosis of NEC is based on Modified Bell's Criteria consisting of both clinical findings and imaging of the abdomen[70]. In Stage 1 or suspected disease, there are obvious clinical signs but no radiographic evidence. The infant is closely monitored for signs of distress or progression of the clinical signs, along with decompression of the bowel and brief discontinuation of oral feeding. Broad-spectrum antibiotics are also given prophylactically. In Stage 2 or definitive disease, there

is radiologic evidence of pneumatosis in the intestinal wall. The feeds are completely restricted and abdominal decompression is carried out, along with broad-spectrum antibiotic treatment. The white blood cells and the platelet counts are closely monitored. In advanced cases or Stage 3, there is free gas in the intraperitoneal region and dilated loops of the bowel along with progression of the clinical signs. Laparotomy is advised sometimes along with drain placement. Surgical resection of the necrotized parts of the intestine is conducted[70]. This may lead to long term complications such as short bowel syndrome, malabsorption, strictures, adhesions and failure to thrive[71,72]. Therefore, strategies for the prevention of NEC would be of great benefit to neonatal health. The approximate healthcare expenditure for hospitalization for NEC is $5 billion in the United States alone. This excludes the cost of surgery, which when performed also extends the duration of hospital stay, thus contributing an additional $6.2 billion burden annually to the healthcare expenditure[73].

2.2.3 Maternal milk feeding is associated with decreased risk of NEC

The incidence of disease is significantly higher among infants fed with artificial formula than in those receiving maternal milk, but the mechanism(s) for these differences remain unknown[74-77]. NEC has been associated with shifts in the intestinal microbiota, specifically a decrease in diversity and a modestly increased relative fraction of *Enterobacteriaceae*[78,79]. However, these changes are not sufficient for disease and thus lack predictive power. Thus, NEC appears to be caused by a failure of the preterm infant to properly contain colonizing bacteria, due in part to an immature mucosal immune system.

The diverse components of breast milk such as milk oligosaccharides and antimicrobials have been shown to modify the neonatal microbiota and reduce the incidence of NEC[80-84]. Breast

milk is also enriched with maternal antibodies[85]. IgA, the most abundant antibody class in human milk, is particularly important in shaping the development of the pediatric microbiota by promoting maturation of the community away from *Proteobacteria* and towards anaerobic *Firmicutes* and *Bacteroidetes*[19,59]. Interestingly, B cells that traffic to the mammary gland and secrete IgA into maternal milk originate in the small intestine, indicating that the IgA repertoire of breast milk is primarily targeted against intestinal bacteria and may be biased towards the most common organisms of the maternal microbiota[54,55,57,85]. For various reasons, including exposure to antibiotics and an underdeveloped gastrointestinal tract, preterm infants harbor gut microbial communities that are distinct from those of term infants[86]. Specifically, the preterm gut is enriched in facultative anaerobes (*Enterobacteriaceae, Enterococcaceae* and *Staphylococcaceae*) that are relatively rare in the maternal intestinal microbiota and may be under-represented in the maternal secretory IgA repertoire[78]. Therefore, we hypothesized that the failure of maternal IgA to bind intestinal bacteria is central to NEC pathogenesis **(Figure 2.2)**.

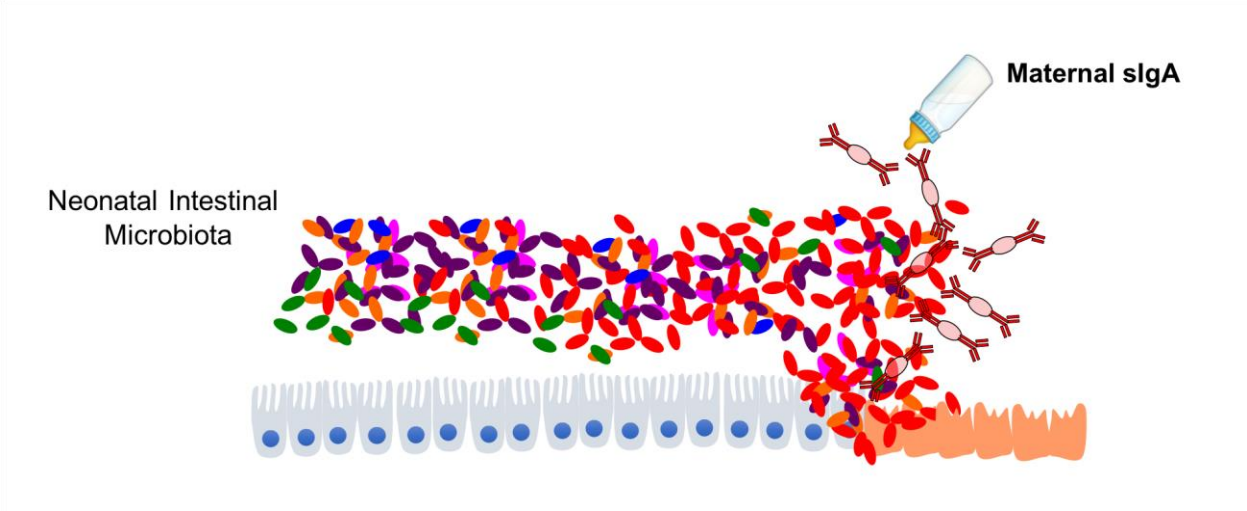

Figure 2.2 Hypothesis

Maternal IgA binding of invasive intestinal bacteria is protective against the development of NEC.

2.3 Methods

2.3.1 Experimental Model and Subject Details

Mice

All mice were maintained at and all experiments were performed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at the University of Pittsburgh and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh (protocol #16099226). C57BL/6 mice were purchased from Taconic Biosciences (Germantown, NY). $Rag1^{-/-}$ mice were obtained from Jackson Laboratories (Maine). $Igha^{-/-}$ mice were kindly provided by Dr. Yasmine Belkaid (NIH/NIAID). Both male and female age-matched mice were used for all experiments in order to account for sex differences. All the mice were housed in specific pathogen-free (SPF) conditions in the University of Pittsburgh.

Human Fecal Samples

The human study protocol was approved by the Institutional Review Board (Protocol Nos. PRO16030078, PRO09110437) of the University of Pittsburgh. Fecal samples were collected fresh or from the diaper of preterm infants at UPMC Magee-Womens Hospital and frozen immediately at -80°C. The samples were later divided into age-matched controls and NEC depending on the incidence of NEC.

Experimental Models

Murine model of experimental NEC was undertaken as detailed in induction of NEC.

2.3.2 Method Details

Fecal IgA Flow Cytometry and Magnetic Sorting of IgA+ and IgA− Bacteria

Either fecal pellets collected from mice after sacrifice or ~50 mg of frozen human fecal material was placed in 1.5ml Eppendorf tubes and 1ml Phosphate Buffered Saline (PBS) was added. The fecal material was disrupted by a combination of vortexing and pipetting and passed through a 40μm filter to remove food/fibrous material. The fecal material was diluted with PBS until the mixture's absorbance at 600nm is ~0.4 to maintain similar bacterial counts between samples and to prevent the magnetic columns from clogging. A volume of 200μl of the suspended bacterial material was then frozen at -80°C as an 'unsorted'control. An additional 200μl of the suspended material was divided equally on a 96-well plate for anti-IgA and isotype control staining for each sample to account for non-specific binding. The fractions were washed with twice with staining buffer (1% Bovine Serum Albumin (Sigma) in PBS sterile-filtered through a 0.22μm filter). The bacteria were stained with Syto BC (Green Fluorescent nuclear acid stain, Invitrogen-1:400), APC Anti-Human IgA (Miltenyi Biotec clone IS11-8E10) (1:10)/ Anti-Human IgA APC (Miltenyi Biotec clone REA1014) (1:50), Anti-Human IgM BV421 (BD Biosciences clone G20-127) (1:30)/ BV421 Mouse Anti-Human IgG (BD Horizon clone X40) (1:30) or PE-conjugated Anti-Mouse IgA (eBioscience clone mA-6E1) (1:500), Anti-Mouse Rat IgM BV421 (BD Horizon clone R6-60.2) (1:30)/ Anti-Mouse Rat IgG2a isotype (BD Horizon clone R35-95), Anti-Mouse IgG FITC (BioLegend clone Poly4060) (1:30) and blocking buffer of 20% Normal Mouse Serum for human or 20% Normal Rat Serum for mouse samples (ThermoFisher). The isotype control was stained similarly using APC Mouse IgG1 isotype control (Miltenyi Biotec clone-IS5-21F5) (1:10) or PE-conjugated Rat Anti-Mouse IFNγ (eBioscience clone XMG1.2). The stained samples were

incubated in the dark for an hour at 4°C. Samples were then washed three times with 200µl of staining buffer before flow-cytometric analysis (LSRFortessa-BD Biosciences).

For magnetic activated cell sorting (MACS), we used 500µl of the suspended fecal material to compensate for the loss of material during sorting and scaled our staining volume accordingly. Anti-IgA stained fecal bacterial pellets were incubated in 1ml per sample of staining buffer containing 45µl of anti-APC or anti-PE MACS Microbeads (Miltenyi Biotec) (20 min at 4°C in dark), washed twice with 1 ml Staining Buffer (8000 x *rpm*, 5 min, 4°C), and then sorted using MS columns (Miltenyi Biotec). The flow-through was collected as IgA-unbound (IgA-negative) fraction and the IgA-bound fraction was eluted from the column. Columns were washed with 70% ethanol and sterile PBS between separations and the IgA-bound fraction of each sample was run five times to increase purity. 100µl each of the IgA-bound and IgA-unbound fraction was used for post-sort flow cytometric analysis (along with the unsorted sample). Absolute bacterial counts were determined by adding a known number of AccuCheck Counting beads (Life Technologies) to antibody stained fecal samples of a given mass, which allows for the calculation of the total number of SYTO (DNA)+ events in any given sample. This can then be multiplied by the measured abundance of any OTU (Operational Taxonomic Unit) to represent the number of bacteria of that taxon/mass in any sample.

DNA Extraction

All microbial DNA was extracted using the MO BIO PowerSoil DNA Isolation kit (single tube extractions). The unsorted, IgA-bound and IgA-unbound pellets were resuspended in Solution TD1 by pipetting and vortexing and ~200µl of 0.1mm diameter Zirconia/Silica beads (Biospec) were added and shaken horizontally on a lab mixer for 12-18 min at maximum speed using a MO

BIO vortex adaptor. All remaining steps followed the manufacturer's protocol. The DNA extracted was stored at -20°C for further 16s amplicon PCR and sequencing.

16S Amplicon PCR and Sequencing

PCR amplification of the small subunit ribosomal RNA gene (16S rRNA) was performed in triplicate 25µl reactions. Reactions were held at 94°C for 3 min to denature the DNA, with amplification performed for 30 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; followed by a final extension of 10 min at 72°C[87]. Amplicons were produced utilizing primers adapted for the Illumina MiSeq. Amplicons target the V4 region and primers utilized either the Illumina adaptor, primer pad and linker (forward primer) or Illumina adaptor, Golay barcode, primer pad and linker (reverse primer) followed by a sequence targeting a conserved region of the bacterial 16S rRNA gene as described[88,89]. The only deviation from the protocol was that PCR was run for 30 cycles. Amplicons were cleaned using the Qiagen UltraClean 96 PCR Cleanup Kit. Quantification of individual amplicons was performed with the Invitrogen Quant-iT dsDNA High Sensitivity Assay Kit. Amplicons were then pooled in equimolar ratio. Agarose gel purification was performed to further purify the amplicon pool and remove undesired PCR products prior to submission for paired-end sequencing on the Illumina MiSeq.

Deconvolution and microbiome data

Flow cytometry was used to determine the percentage of IgA positive and IgA negative bacteria in each sample (unsorted, IgA positive, IgA negative) post magnetic separation. We assumed that contamination affected each OTU equally and that the IgA positive and IgA negative samples are reciprocal (fractions of the same whole). The raw reads from sequencing of 16S rRNA

genes were then deconvolved by summing the proportion of IgA bound or IgA unbound (as measured by flow cytometry) across the paired (infant and time point [Day of life]) IgA positive and IgA negative samples for each OTU.

Example to solve for IgA positive from one time point and one OTU 'X':

Total IgA positive 'X' = 'X' correctly bound to IgA positive + 'X' contaminating IgA negative

Total IgA positive 'X' = (%IgA+ in bound) * (# 'X' reads in bound) + (%IgA+ in unbound) * (# 'X' reads in unbound)

Quantitative PCR for 16S rRNA

PCR amplification of the small subunit ribosomal RNA gene (16S rRNA) was performed in triplicate 10µl reactions. Reactions were held at 95°C for 3 min to denature the DNA, with amplification performed for 35 cycles (95°C for 10 s and 60°C for 30 s). The forward primer sequence of 16S was ACTCCTACGGGAGGCAGCAGT and the reverse primer sequence of 16S was ATTACCGCGGCTGCTGGC.

Quantitative PCR for Enterobacter spp.

PCR amplification of the small subunit ribosomal RNA gene (23S rRNA) was performed in triplicate 10µl reactions. Reactions were held at 95°C for 3 min to denature the DNA, with amplification performed for 35 cycles (95°C for 10 s and 60°C for 30 s). The forward primer sequence of *Enterobacter* 23S was AGTGGAACGGTCTGGAAAGG and the reverse primer sequence of *Enterobacter* 23S was TCGGTCAGTCAGGAGTATTTAGC[90].

Induction of NEC

NEC was induced in 7- to 8-day old mice by hand-feeding mice formula via gavage 5 times/day (22-gauge needle; 200µl volume; Similac Advance infant formula [Ross Pediatrics, Columbus, Ohio]/ Esbilac canine milk replacer 2:1). The formula was supplemented with 10^7 CFUs of *Enterobacter spp*. (99%) and *Enterococcus spp*. (1%) and mice were rendered hypoxic (5%O2, 95% N2) for 10 minutes in a hypoxic chamber (Billups-Rothenberg, Del Mar, CA) twice daily for 4 days[74,91]. We used males and females in all experiments. Disease was monitored by weighing mice daily prior to the second feed.

2.3.3 Quantification and Statistical Analysis

Flow Analysis

All the data from flow cytometry was derived from samples that were run through the LSR Fortessa flow cytometer from BD Biosciences. The raw data was analyzed through the software FlowJo V10.4.2 (FlowJo, OR, USA). The cell population abundance was measured by SytoBC+ events that range from 60% to 80% of all events; Of SytoBC+ events the range of IgA+ bacteria was <1% to 95%.

Antibody Validation

All antibody stains were compared to an isotype control which was specific for an antigen irrelevant to the intestinal microbiome (Keyhole Limpet Hemocyanin or 4-Hydroxy-3-nitrophenylacetyl).

Human: Positive antibody binding was confirmed early on by staining of pediatric IBD patients for IgA, IgG and IgM on the fecal bacteria. These patients are known to have increased

binding of such antibodies. Cross-reactivity for IgA was tested for by staining fecal bacteria of formula fed infants 10-21 days post-delivery.

Mouse: Mouse antibodies were tested for cross-reactivity by staining fecal samples from adult Rag1-/- mice that lack all antibodies.

All the antibodies used were commercial antibodies with validation procedures described on the websites of the companies.

16S rRNA Gene Sequencing Analysis

Data was collected through high-throughput sequencing- MiSeq Reporter Software v2.6. Read pairing, clustering and core diversity statistics were generated through PEAR, UPARSE and QIIME and R[92,93]. LEfSe was used to compare family level relative abundances between NEC and control groups[94]. Raw 16S rRNA data has been uploaded to SRA and is available under accession number PRJNA526906.

Deconvolution Analysis

The deconvoluted data was processed through the QIIME2 workflow to create alpha diversity metrics with sampling depth chosen based on alpha rarefaction plotting. Abundance of individual or 'pooled' (Anaerobes) OTUs was then calculated using the deconvoluted values. The algorithm for deconvolution is available on GitHub (https://github.com/handlab/IgA_Seq_Deconvolution)

We categorized all of the following OTUs as 'Anaerobes': *Bifidobacteriaceae, Prevotellaceae, Bacteroidiales_S24-7, Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae, Veillonellaceae, Tissierellaceae.*

Histology Scoring of NEC

The severity of disease was determined on histologic sections of the entire length of the small intestines stained with hematoxylin and eosin by trained personnel who were blinded to the study conditions according to previously published scoring system from 0 (normal) to 4 (severe)[95].

Statistical Tests

Statistical tests used are indicated in the figure legends. Lines in scatter bar charts represent the mean of that group. Group sizes were determined based on the results of preliminary experiments. Mice studies were performed in a non-blinded fashion. Statistical significance was determined with the two-tailed unpaired Student's t-test or non-parametric Mann-Whitney test when comparing two groups and one-way ANOVA with multiple comparisons, when comparing multiple groups. All statistical analyses were calculated using Prism software (GraphPad). Differences were considered to be statistically significant when $p < 0.05$.

Analysis software

Raw data from flow cytometry was analyzed through FlowJo V10.4.2 (FlowJo, OR, USA); QIIME2, PhyloSEQ version 1.23.1, R version 3.4.2, PEAR v0.9.0, UPARSE v10.0.0240, LEfSe Galaxy version 1.0 http://huttenhower.sph.harvard.edu/galaxy was used to analyze 16S rRNA sequencing data; GraphPad PRISM 7 was used for all statistical analyses.

2.3.4 Data and Software Availability

Raw 16S rRNA sequencing data has been uploaded to SRA and is available under accession number PRJNA526906. The algorithm for deconvolution is available on GitHub (https://github.com/handlab/IgA_Seq_Deconvolution).

2.4 Results

2.4.1 IgA binding to the intestinal bacteria of preterm infants is positively correlated to breastfeeding and negatively correlated to the development of NEC

To determine whether there was a correlation between antibody binding and the incidence of NEC, we analyzed the level of immunoglobulin (Ig) binding on intestinal bacteria found naturally on fecal samples obtained from 30 NEC patients and 39 matched preterm controls **(Table 2.1)**. This initial cohort contained samples from NEC infants that were collected within 36 hours after diagnosis.

Table 2.1 Post-diagnosis preterm NEC/Control Cohort

	NEC	**Control**
No. of patients	30	39
Avg. gestational age at birth (in weeks)	29 2/7	28 5/7
Mean age at NEC diagnosis (DOL)	22.2	NA
Sex	Females- 11	Females- 15
	Males- 19	Males- 24
Mean birth weight (grams)	1176.52	1202.26
Mode of Delivery	Vaginal- 11	Vaginal- 9
	Caesarean- 19	Caesarean - 30
Feeding	Maternal milk only- 6 Combination of maternal milk and formula- 13	Maternal milk only - 5 Combination of maternal milk and formula- 26
	Formula-fed- 11	Formula-fed- 8

The post-diagnosis preterm cohort of patients with NEC and controls included single fecal samples taken from patients with NEC and controls within 36 h of NEC diagnosis. Samples were analyzed from day of life (DOL) 4 to 70. NA-not applicable.

Fecal samples were stained with anti-human IgA antibodies and the Ig-bound populations were measured by flow cytometry[51,96-98]. Surveyed across all samples, the percentage of IgA-bound bacteria was high **(Figure 2.3a)**. To elucidate the relative roles of IgA secreted by infants and their mothers, we compared the abundance of IgA-bound fecal bacteria in samples from infants either fed exclusively with infant formula or with maternal milk (often supplemented with formula) **(Figure 2.3b)**. These analyses identified that intake of maternal milk was associated with a much higher abundance of IgA-bound bacteria than formula feeding, as a majority (11/19) of formula-

fed infants had <1% of their intestinal bacteria bound by IgA irrespective of disease status **(Figure 2.3b).** However, some formula-fed infants harbored substantial amounts of IgA-positive bacteria. Since it takes about 4 weeks for the intestine to become populated with B cells, we hypothesized that the presence of IgA-bound bacteria in formula-fed infants would be limited to later time points post-delivery[99]. Indeed, we found a significant relationship between age post-delivery and increased IgA binding in formula-fed infants but not in maternal milk-fed infants, implying that during at least the first 4 weeks of life, the primary source of IgA is maternal milk **(Figure 2.3c)**. To focus our analysis on the effect of maternal IgA while not discarding too many NEC samples and limiting experimental power, we analyzed samples collected within 40 days of birth. We discovered that infants who develop NEC have, on average, a significantly lower proportion of IgA-bound bacteria in their intestine **(Figure 2.3d)**. Thus, NEC is inversely correlated with bacterial IgA binding. However, because the group that developed NEC was much more likely to be formula fed (and thus lack intestinal IgA altogether) and these fecal samples were acquired post-NEC diagnosis and treatment, it was important to confirm our findings in a prospective cohort where all infants were fed maternal milk and not complicated by disease-associated inflammation and treatment.

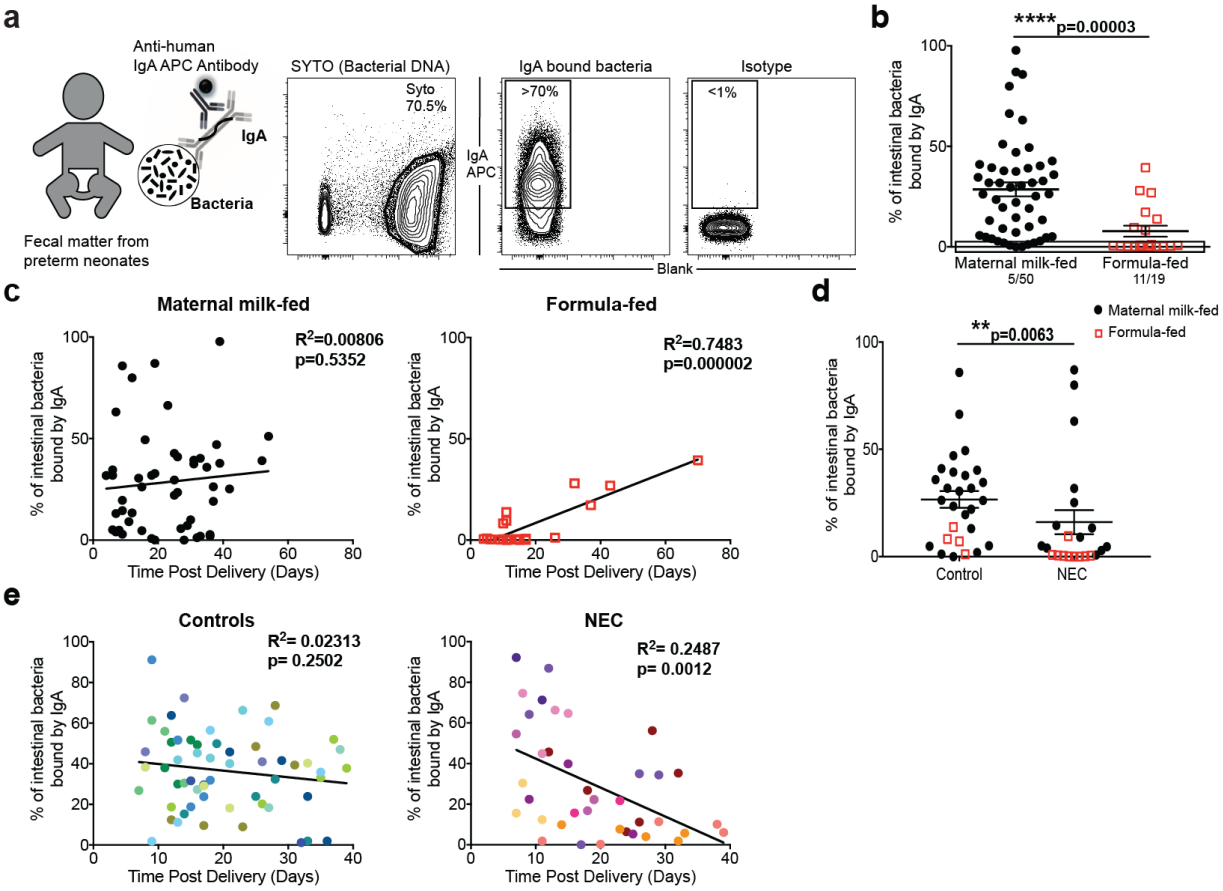

Figure 2.3 IgA binding to the intestinal bacteria of preterm infants is positively correlated to feeding with maternal milk and negatively correlated to the development of NEC

Flow cytometry analysis of bacterial IgA binding on fecal samples from preterm infants. **a**, Example of IgA staining of samples from preterm infants. **b**, Percentage of IgA-bound bacteria from infants fed with maternal milk fed ($n = 50$) or formula ($n = 19$). The box represents the number of samples with <1% IgA binding of intestinal bacteria. Two-sided Mann–Whitney *U*-test, data are mean ± s.e.m. **c**, Percentage of IgA binding was correlated by linear regression with time after delivery for infants fed with maternal milk ($n = 50$) and formula ($n = 19$); Pearson's correlation coefficient. **d**, Percentage IgA-bound bacteria from controls ($n = 28$) or infants diagnosed with NEC ($n = 23$), from samples collected before DOL 40. Two-sided Mann–Whitney *U*-test, data are mean ± s.e.m. **b**–**d**, Samples from infants fed with maternal milk are indicated by black circles, samples from infants fed with formula are indicated by open red squares. **e**, Percentage IgA-bound intestinal bacteria from prospectively collected longitudinal samples of patients that will develop NEC ($n = 10$ patients, $n = 39$ samples) and controls ($n = 13$, $n = 59$) for each analyzed DOL. Patients are shown in different colors. Linear regression and Pearson's correlation coefficient.

To address whether a relative lack of IgA bound bacteria is associated with NEC prior to the development of disease we identified 23 preterm infants (10 NEC, 13 age and gender-matched controls) present in a previously collected cohort from whom we could analyze multiple longitudinal samples prior to the onset of disease **(Table 2.2)**.

Table 2.2 Prospective/longitudinal preterm NEC/Control cohort

	NEC	**Control**
No. of patients	10	13
Avg. gestational age at birth (in weeks)	27 1/7	27 3/7
Mean age at NEC diagnosis (DOL)	27.4	NA
Sex	Females- 5	Females- 8
	Males- 5	Males- 5
Mean birth weight (grams)	873.1	932.92
Mode of Delivery	Vaginal- 0	Vaginal- 8
	Caesarean - 10	Caesarean - 5
Feeding	Maternal milk- 5 Combination of maternal milk and formula- 5	Maternal milk- 5 Combination of maternal milk and formula- 8
	Formula-fed- 0	Formula-fed- 0

The prospective preterm cohort of patients that will progress to NEC and controls consisted of longitudinally collected samples from infants (gestational age 24 to 31 weeks) captured prospectively (from DOL 7 to 40) and assigned to groups after the diagnosis of NEC or discharge from the neonatal intensive care unit. NA- not applicable.

Flow cytometric analysis of the samples **(Table 2.2)** revealed a lower percentage of IgA bound bacteria over time in infants that went on to develop NEC, which was not observed in controls **(Figure 2.3e and Figure 2.4)**.

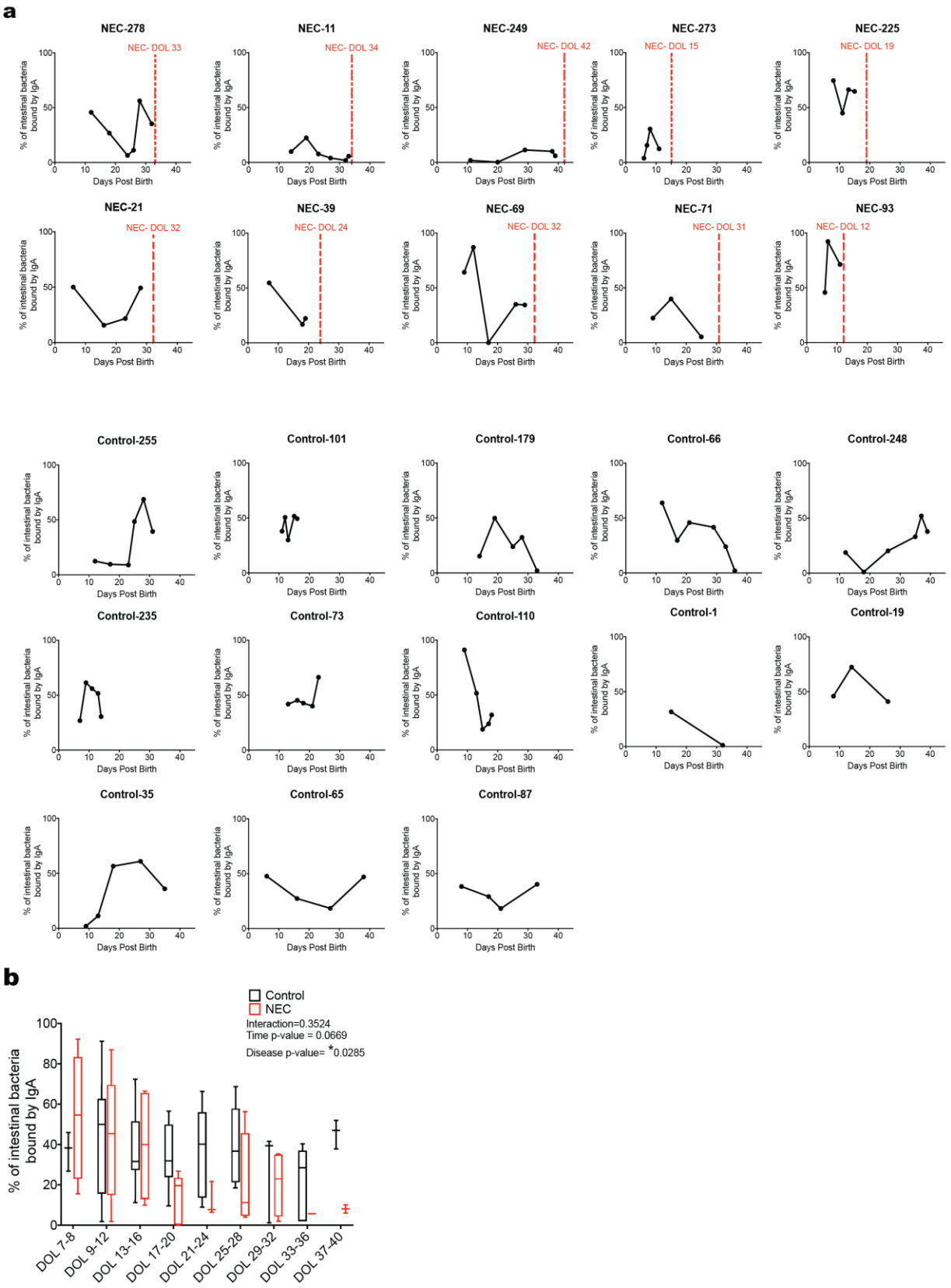

Figure 2.4 Fraction of intestinal bacteria bound by IgA in preterm infants

a, Percentage IgA-bound bacteria from longitudinally collected fecal samples from preterm infants in our study. The dotted red lines indicate the date of NEC diagnosis. **b**, Percentage IgA-bound intestinal bacteria from prospectively collected samples of patients that will develop NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). Samples from multiple patients are pooled and represented in four-day windows post-delivery to increase the number of samples available for each time window. Box and whisker plots depict the mean (line) the 25th and 75th percentiles (box) and range (whiskers) for each time window. Two-way ANOVA.

Thus, it appears that in infants that will develop NEC, a change occurs in either the intestinal microbiota or the maternal IgA repertoire that leads to the 'escape' of intestinal bacteria from binding.

To assess the binding of IgM and IgG antibodies, we stained fecal samples with anti-IgM and anti-IgG antibodies and measured the Ig-bound populations through flow cytometry. However, inverse to IgA-bound bacteria, there were minimal IgM- and IgG-bound bacteria **(Figure 2.5a and b).** Furthermore, a separate longitudinal analysis of a preterm infant fed formula over the first 4 weeks of life revealed no IgA bound bacteria, strongly supporting the contention that the primary source of IgA in early life (first 30 days post-delivery) is maternal milk **(Figure 2.5c)**.

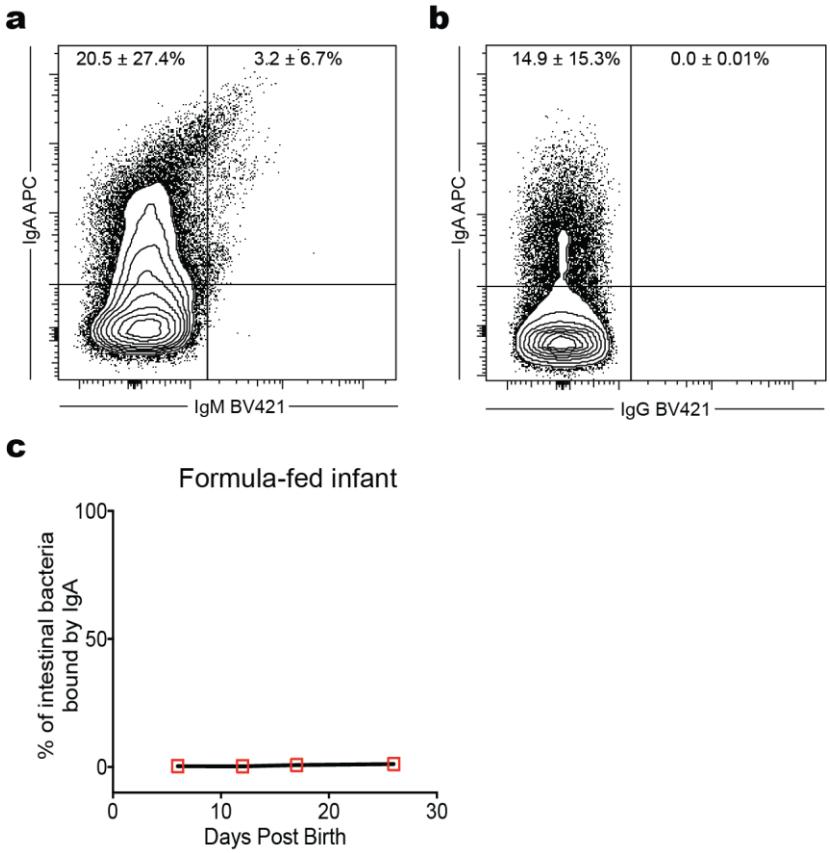

Figure 2.5 Maternal milk-derived antibodies binding to intestinal bacteria from preterm infants

a, b, Representative flow plots show binding of intestinal bacteria derived from preterm infants to IgM (**a**; $n = 20$) and IgG (**b**; $n = 12$) relative to IgA. Numbers in quadrants show the mean percentage ± s.d. of data collected from the cohort in Table 1. **c**, Percentage IgA staining of intestinal bacteria of a single preterm infant fed exclusively with formula

2.4.2 The IgA unbound fraction of the microbiota lacks diversity and becomes dominated by *Enterobacteriaceae* prior the development of NEC.

To determine the mechanism of the loss of IgA binding on the intestinal bacteria of NEC patients, we then sought to correlate shifts in the composition of the microbiota with our observations of maternal IgA. To do so, we identified bacterial taxa by sequencing the

hypervariable regions of the 16S rRNA genes from each sample in our longitudinal cohort **(Table 2.2)**. Grouped together, over all timepoints, we observed a modest enrichment for *Enterobacteriaceae* and reduction in Gram positive anaerobes such as *Lachnospiraceae* in infants who will go on to develop NEC, similar to what has been seen in previous studies **(Figure 2.6a and Figure 2.6b)**[78].

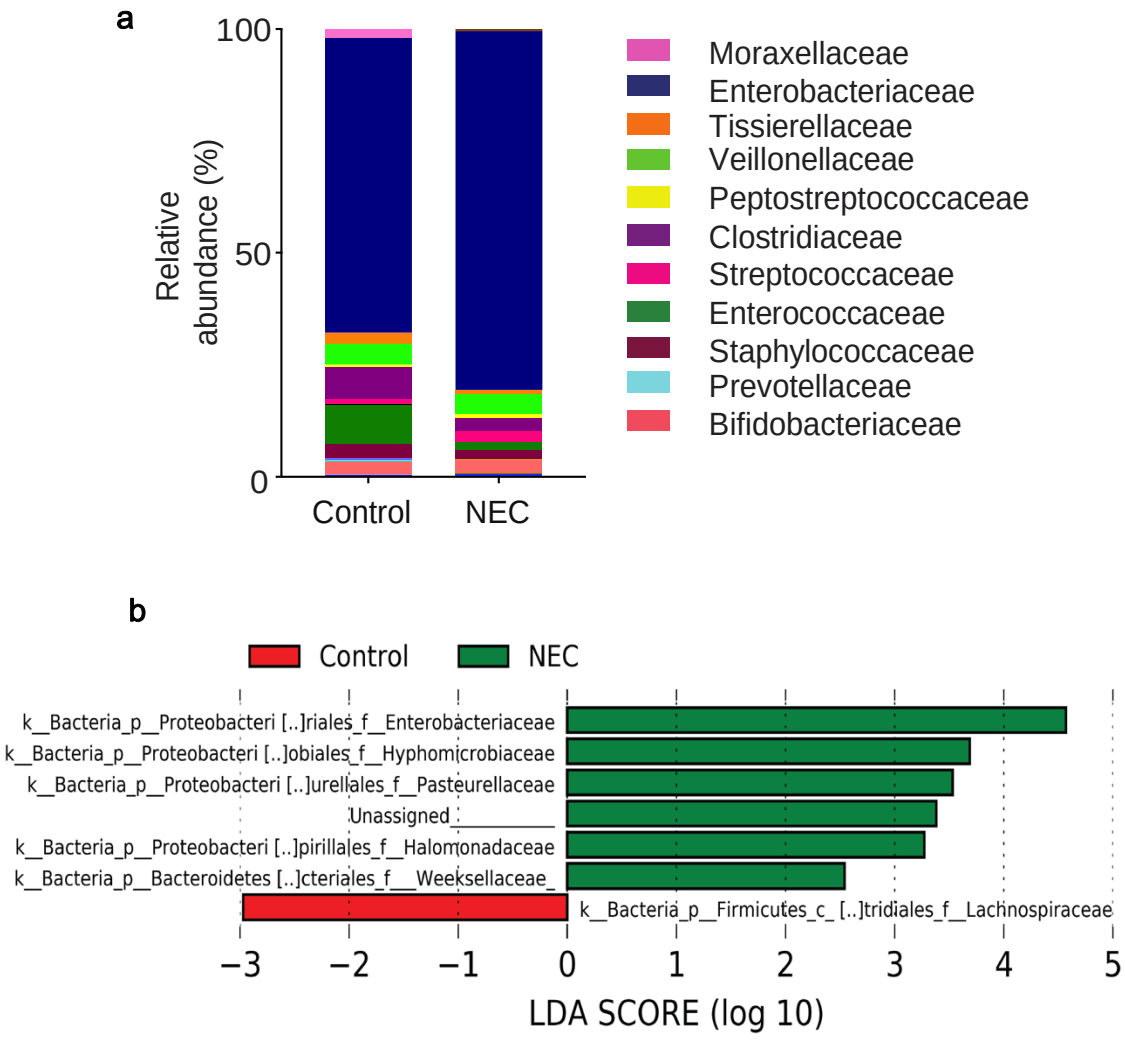

Figure 2.6 *Enterobacteriaceae* is the most abundant family in preterm infants

Longitudinal fecal samples from preterm infants before the onset of NEC were selected from infants with NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). **a**, Samples were analyzed for the relative abundance of different bacterial OTUs by targeted sequencing of 16S rRNA genes. The mean relative abundances of different taxa are shown for patients that will develop NEC and controls (pooled from all time points and infants), **b,**

Linear discriminant effect size analysis comparing pooled samples from controls or infants that will develop NEC before the diagnosis of disease. OTUs that discriminate between patients that will progress to NEC and controls before disease onset are ranked by the effect size (represented by the linear discriminant analysis (LDA) score).

Given this data and the fact that *Enterobacteriaceae* was the most abundant Operational Taxonomic Unit (OTU) in the majority of samples within the cohort, we hypothesized that the observed reduction in IgA binding in infants that develop NEC should be associated with this taxon. To test this, we plotted the relative abundance of IgA bound bacteria against *Enterobacteriaceae* within each of our samples **(Figure 2.7)**.

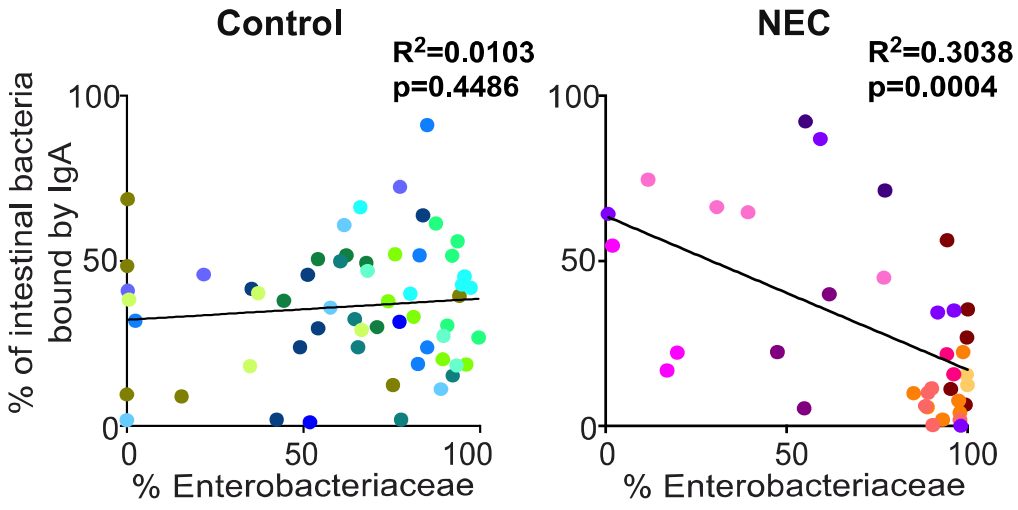

Figure 2.7 Decrease in IgA binding in NEC patients is related to the increase in *Enterobacteriaceae*

Longitudinal fecal samples from preterm infants before the onset of NEC were selected from infants with NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). Relative abundance of *Enterobacteriaceae* in preterm fecal samples compared to the percentage of IgA-bound bacteria from controls and patients that will develop NEC. Each patient is shown in a different color and the R^2 value is based on a linear regression and Pearson's correlation coefficient.

This analysis reveals a striking inverse relationship only in infants who will develop NEC in the future, which is consistent with the idea that loss of IgA binding of *Enterobacteriaceae* is

driving the reduction in IgA binding seen in NEC patients and may contribute to the development of the disease **(Figure 2.3e** and **2.7).**

We next sought to directly measure which bacterial taxa might be more or less bound by maternal antibodies in our longitudinal prospective cohort **(Table 2.2)**. To identify the IgA positive and negative bacteria, we separated IgA-bound bacteria using magnet-activated cell sorting (MACS) and measured the bacterial taxa via the abundance of 16S rRNA genes (IgSeq)[100]. In accord with multiple published reports[24,26,29], we did not achieve >99% purity in our IgA positive/IgA negative separations and we could not accurately describe either the IgA positive or IgA negative populations without significant cross-contamination (**Figure 2.8a**). Post-sort flow cytometric analysis of each sample for IgA binding allowed us to deconvolute the contamination of the other fraction **(Figure 2.8a** and **b**). In support of the necessity and accuracy of the deconvolution protocol we examined how the relative abundance of bacteria in IgA positive samples (as a fraction of the sum of paired IgA positive and negative reads) corresponded to the percent IgA binding measured in the unsorted sample from which they were derived. Prior to deconvolution the relative percentage of IgA positive 16S rRNA reads could be correlated to the percent IgA positive bacteria by flow cytometry in the parental unsorted sample (**Figure 2.8c**). However, the relationship was weakest amongst sample groupings where percent IgA bound unsorted bacteria were lowest, indicating a high level of contamination of these IgA positive samples with IgA negative bacteria and providing impetus for deconvolution (**Figure 2.8c**). After deconvolution, we saw a much more significant correlation between IgA positive reads and the abundance of IgA positive bacteria in the parental sample, particularly amongst samples with low levels of IgA positive unsorted bacteria. This validates that our technique is correcting for contamination introduced by imperfect sorting (**Figure 2.8d**).

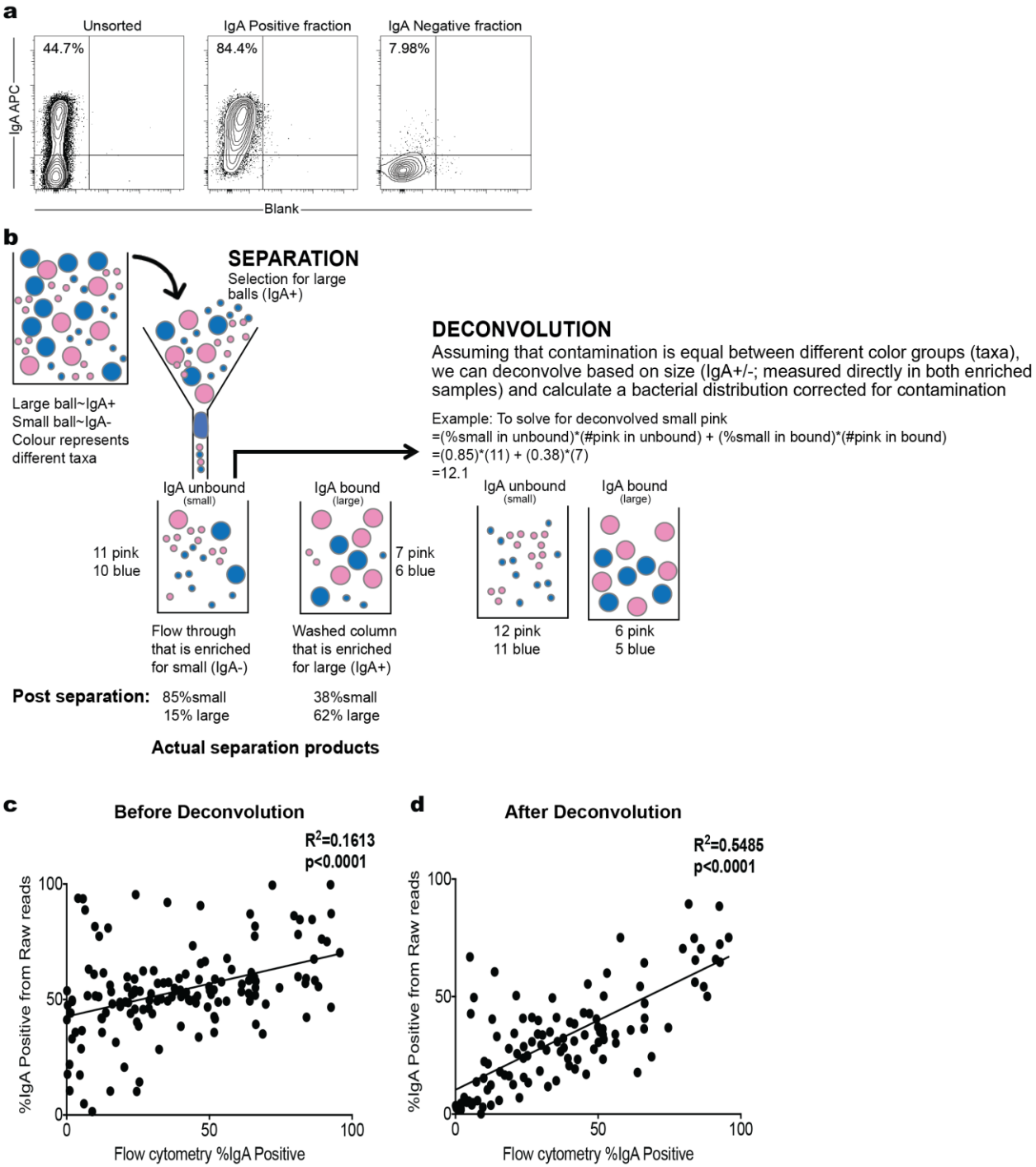

Figure 2.8 Deconvolution method to decrease the effect of contamination in IgSeq

a, Representative staining of the abundance of IgA-bound bacteria before (unsorted) or after magnetic separation (IgA^+ and IgA^-) measured by flow cytometry of all samples (NEC samples, $n = 39$; control samples, $n = 59$). **b**, Graphical depiction of our deconvolution methodology. In this schematic, size represents IgA binding (large, IgA^+; small, IgA^-), which can be measured on intact cells, and colors represent two different taxa, which can only be

measured by sequencing. **c**, Percentage of IgA^+ bacteria (measured by flow cytometry of unsorted fecal samples) compared to percentage of 16S rRNA reads found in the IgA^+ sample ($IgA^+/IgA^+ + IgA^-$). Each dot represents a paired sample derived from the same fecal sample ($n = 140$). **d**, The same analysis as in **c** was carried out on samples after deconvolution ($n = 140$). **c**, **d**, Data were analyzed by linear regression with Pearson's correlation coefficient and contain samples ($n = 42$) that were excluded from our prospective NEC analysis as they were collected after the NEC diagnosis, after DOL 40 or complicated by non-NEC illness and/or treatment.

The power of our study was in being able to correlate differences in the identity of IgA positive and negative bacteria from the intestine longitudinally as infants progressed (or not) to NEC. Longitudinal IgSeq analysis of intestinal microbial diversity revealed that control infants showed a significant and consistent increase in bacterial diversity amongst IgA negative bacteria over time, while no significant changes were detected in the IgA positive samples (**Figure 2.9**). Conversely, among infants who will progress to NEC, the diversity of both IgA positive and negative fecal bacteria significantly decreases over time and many NEC samples have microbiomes of very low diversity during the critical window before NEC development (3 to 5 weeks of age) (**Figure 2.9**)[101].

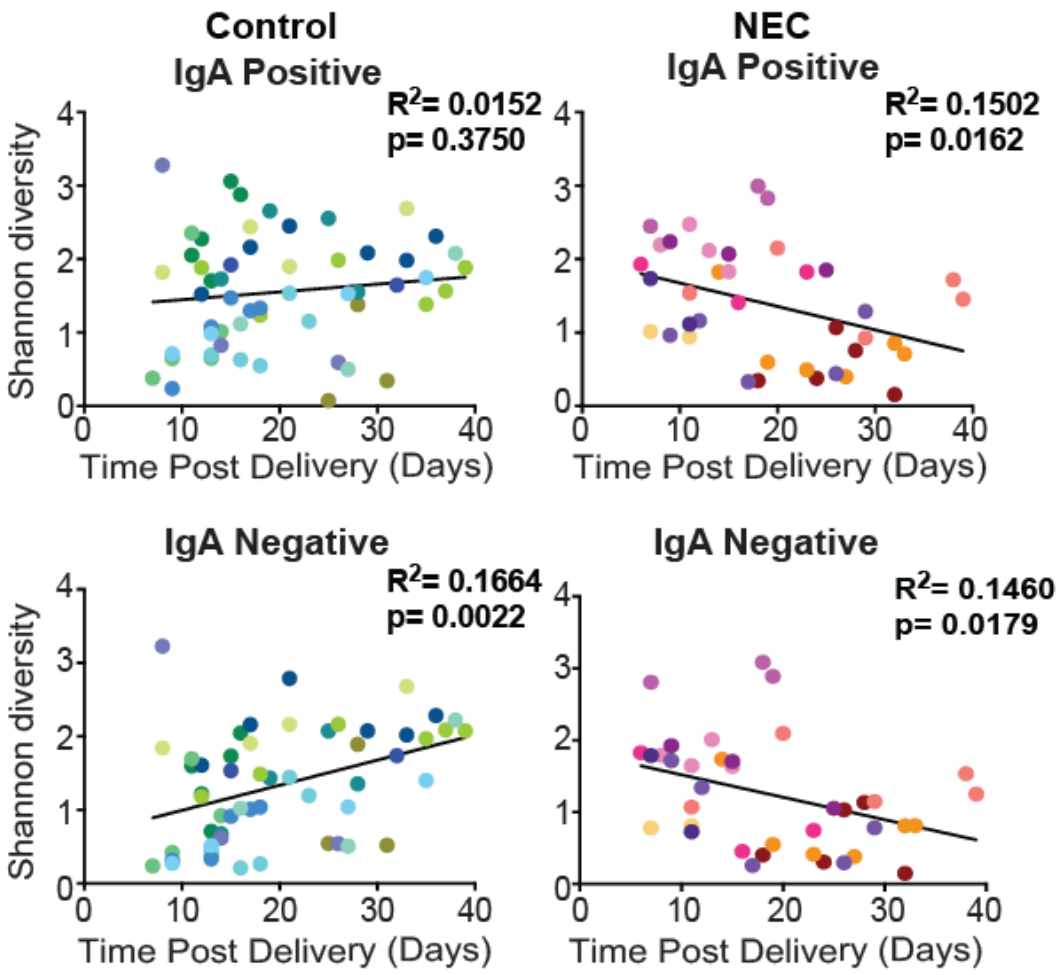

Figure 2.9 Decrease in diversity is observed prior to the incidence of NEC

Longitudinal fecal samples from preterm infants before the onset of NEC were selected from infants with NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). Fecal samples were separated into IgA^+ and IgA^- pools before 16S rRNA sequencing and deconvolution based on post-sort analysis. Shannon diversity scores of IgA^+ and IgA^- samples from patients that will develop NEC and controls for each analyzed DOL. Each patient is shown in a different color and the R^2 value is based on a linear regression and Pearson's correlation coefficient.

IgSeq can also identify which taxa are associated with the IgA positive and negative fractions of the microbiota. We revealed that over time the IgA negative intestinal microbiota of infants who go on to develop NEC become significantly dominated by *Enterobacteriaceae*, while the fraction of the microbiota composed of anaerobes (predominantly *Clostridiales* and *Bifidobacteriales* with few *Bacteroidiales*) is almost undetectable in many infants (**Figure 2.10a,**

b and **Figure 2.11**). Conversely and in accord with diversity measurements, amongst controls the relative abundance of fastidious anaerobes increases while *Enterobacteriaceae* decreases in the IgA negative fraction, confirming that this taxon is bound at relatively higher frequencies over time in control infants compared to infants that will develop NEC (**Figure 2.8a, b** and **Figure 2.9**).

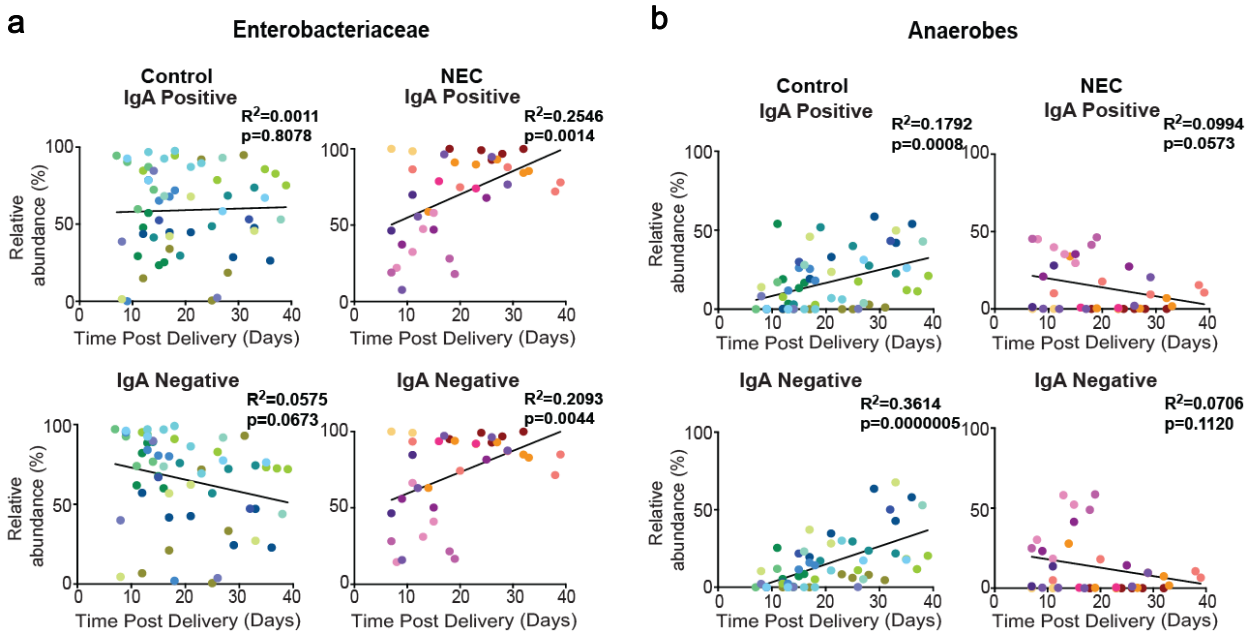

Figure 2.10 Infants who develop NEC have increased *Enterobacteriaceae*

Longitudinal fecal samples from preterm infants before the onset of NEC were selected from infants with NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). Fecal samples were separated into IgA^+ and IgA^- pools before 16S rRNA sequencing and deconvolution based on post-sort analysis. **a**, Relative abundance of *Enterobacteriaceae* from IgA^+ and IgA^- samples from patients that will develop NEC and controls for each analyzed DOL. **b**, Relative abundance of combined anaerobic bacterial OTUs from IgA^+ and IgA^- samples from patients that will develop NEC and controls for each analyzed DOL. Each patient is shown in a different color and the R^2 value is based on a linear regression and Pearson's correlation coefficient.

Interestingly, while the IgA positive and negative fractions from controls differed in both diversity and the relative abundance of *Enterobacteriaceae*, in NEC patients they are not discernibly different (**Figure 2.9** and **2.10a**). An explanation for the lack of difference between the IgA positive and negative fractions of NEC infants is that they often show very low diversity and

are dominated by *Enterobacteriaceae*, so essentially there are few taxa available for IgA to bind (**Figure 2.11).**

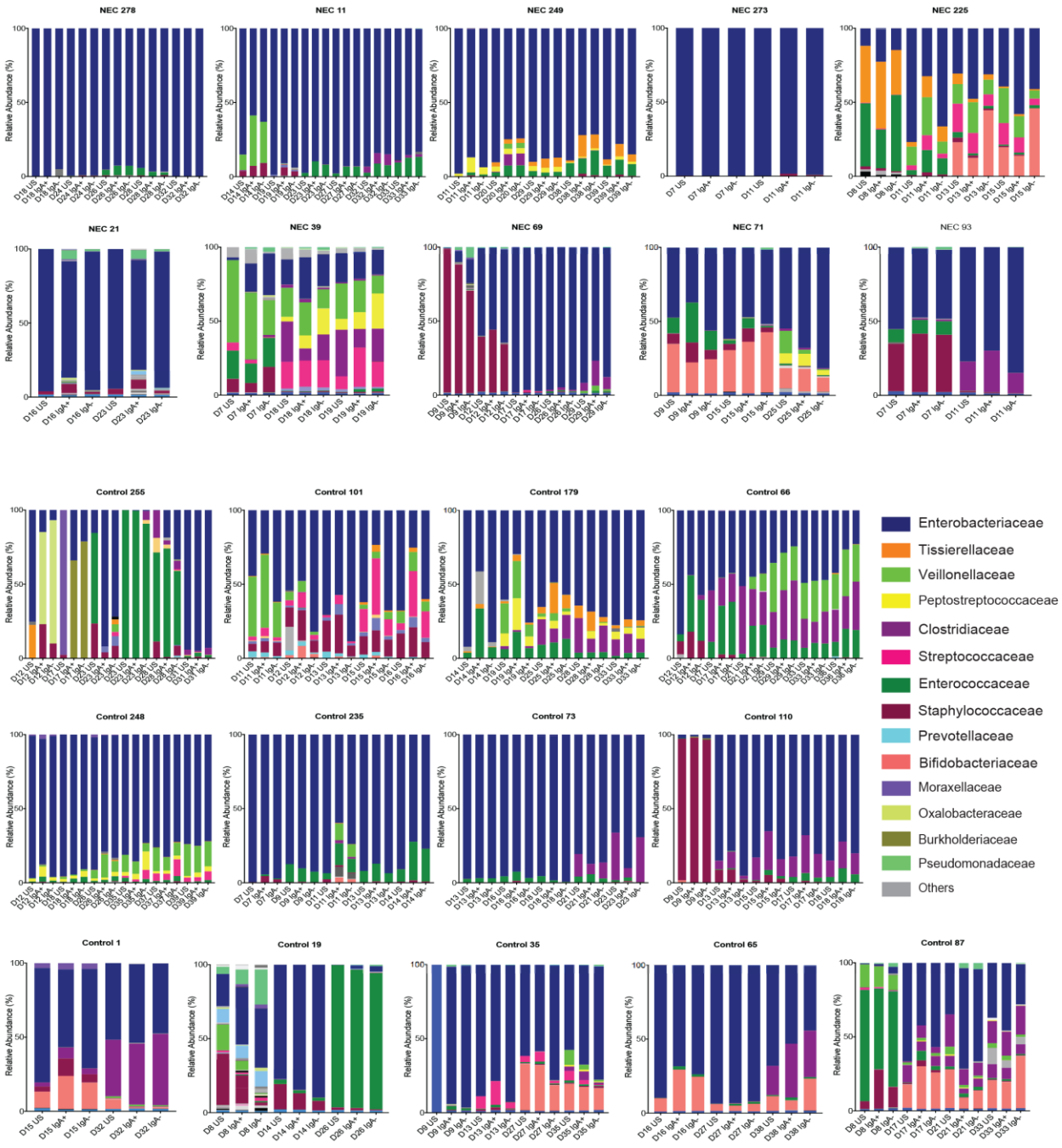

Figure 2.11 Longitudinal analysis of the intestinal microbiota of preterm infants

Stacked bar charts depict the relative abundances of OTUs (unsorted sample, deconvolved IgA^+ and IgA^- fractions) at the family level, from all patients in our study, at all time points analyzed. Patients will develop NEC, $n = 10$, 39 samples combined; controls, $n = 13$, 59 samples combined.

To further test whether *Enterobacteriaceae* is uniquely enriched in the IgA negative fraction of infants prior to NEC we calculated the relative total abundance of different OTUs, compared between IgA positive and negative samples derived from the same 'parental' fecal sample. Our evidence for the validity of this approach is the significant correlation between the percent IgA positive bacteria (measured by flow cytometry) and the relative percent IgA positive reads [IgA^+reads/(IgA^++ IgA^- reads)] which indicates that post-deconvolution the ratio of reads between the two samples corresponds well to the actual ratio in the parental source sample (**Figure 2.8d**). By calculating the ratio of the paired IgA negative and positive reads (IgA^- reads/IgA^+ reads; Log2 transformed), we observed a unique increase over time in the proportion of IgA negative total and *Enterobacteriaceae* reads in infants who went on to develop NEC (**Figure 2.12**).

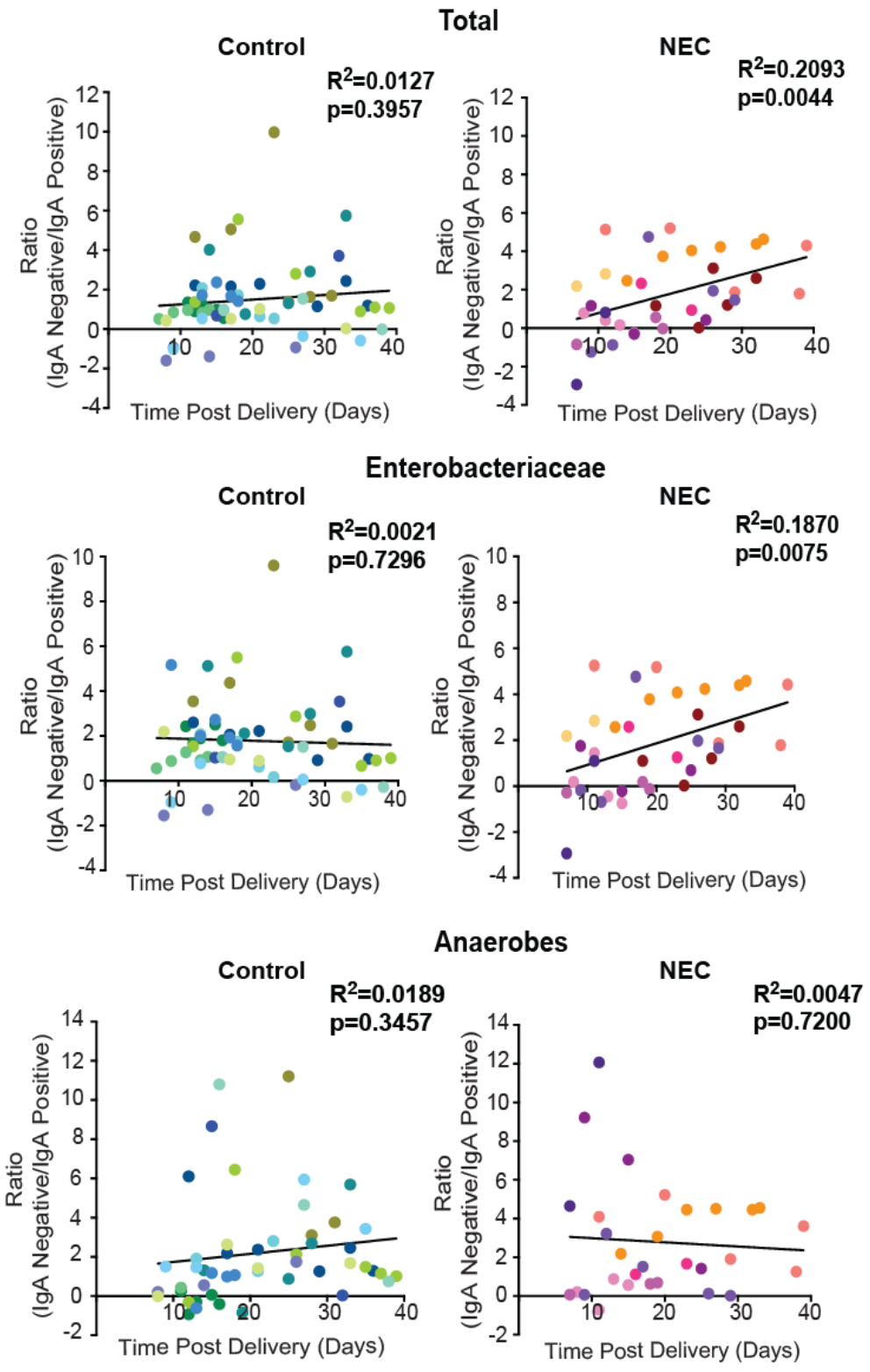

Figure 2.12 Increase in the proportion of IgA negative fraction of total bacterial and *Enterobacteriaceae* counts precceding NEC

Longitudinal fecal samples from preterm infants before the onset of NEC were selected from infants with NEC ($n = 10$, 39 samples combined) and controls ($n = 13$, 59 samples combined). Fecal samples were separated into IgA^+ and IgA^- pools before 16S rRNA sequencing and deconvolution based on post-sort analysis. Ratio of reads (IgA^-/IgA^+; log_2-transformed values) from paired IgA^+ and IgA^- samples for each analyzed DOL. The ratios of total number of bacterial reads, *Enterobacteriaceae* reads and combined anaerobe reads are shown. Each patient is shown in a different color and the R^2 value is based on a linear regression and Pearson's correlation coefficient.

As *Enterobacteriaceae* is the most abundant OTU in preterm infants and the only taxa uniquely increasing in the IgA negative fraction of the microbiota prior to disease, the increase in IgA negative *Enterobactericeae* is the most likely driver of the reduced IgA bound bacteria seen preceding NEC (**Figure 2.3e**, **2.6a, 2.12** and **Figure 2.13**). In contrast, control infants show no significant shifts in the relative abundances of IgA positive and negative total bacteria, *Enterobacteriaceae* or anaerobes and instead only show significant shifts in the less abundant OTUs such as *Staphylococcaceae*, *Streptococcaceae* and *Enterococcaceae* (**Figure 2.12** and **Figure 2.13**).

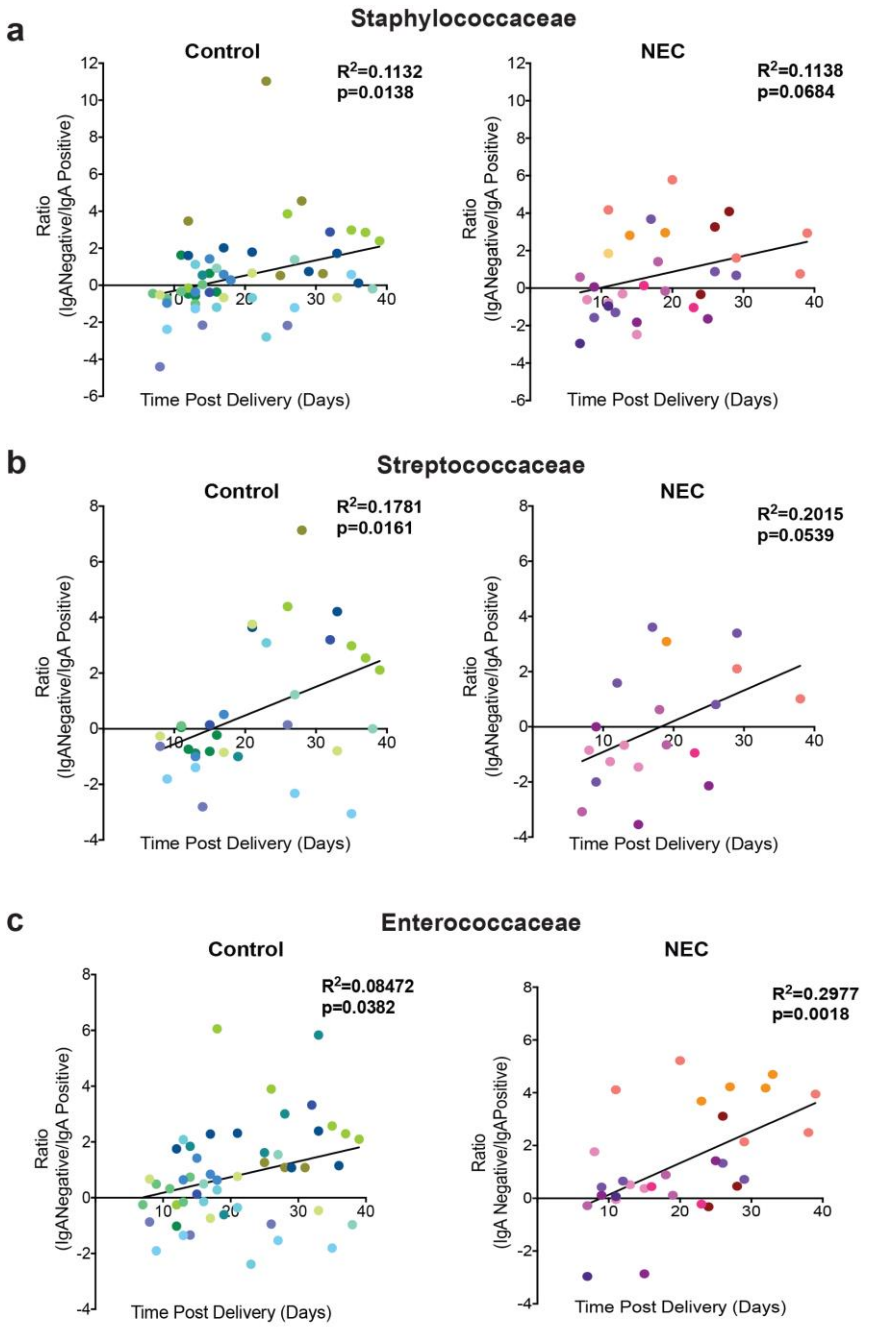

Figure 2.13 Ratio of IgA- to IgA+ reads for low abundance taxa

a–**c**, Ratio of reads (IgA^-/IgA^+; log_2-transformed values) from paired IgA^+ and IgA^- samples shown for each analyzed DOL. **a**, *Staphylococcaceae* reads. **b**, *Streptococcaceae* reads. **c**, *Enterococcaceae* reads. For all graphs, each patient is shown in a different color and the R^2 value is based on a linear regression and Pearson's correlation coefficient. The number of samples used in each graph varies because instances in which the IgA^+ and/or IgA^- samples had zero reads were excluded.

Taken together, our data indicates that the development of NEC is uniquely preceded by a domination of the intestinal microbiota by *Enterobacteriaceae* that lack IgA binding. The increased frequency of *Enterobacteriaceae* is accompanied by a failure of the IgA negative fraction of the preterm microbiome to diversify via the acquisition of fastidious anaerobic bacteria (*Clostridiales* and *Bifidobacteriales*). One explanation for this phenomenon would be a selective rapid outgrowth of *Enterobacteriaceae* in infants that later develop NEC, as seen previously in other inflammatory contexts[102]. To test this possibility, we used a combination of flow cytometry and counting beads combined with 16S rRNA sequencing to determine the number of each bacteria per unit mass in each fecal sample[103]. Although we saw increased *Enterobacteriaceae* amongst some infants developing NEC, inter-individual variation was high and there were no statistically significant differences between cases and controls and no observation of a rapid expansion of this taxon **(Figure 2.14)**. Many infants who will go on to develop NEC have almost no anaerobes, so while total number of *Enterobacteriaceae* is not increased in these infants the relative fraction of the microbiota derived from this taxon is very significantly different **(Figure 2.12** and **Figure 2.13)**. Also, our analysis may not well represent the site of the disease (small intestine) so we cannot rule out that there are focal expansions of *Enterobacteriaceae* associated with NEC. Nonetheless, we favor a model where loss of IgA binding of the microbiota is induced either by mutation or by transcriptional modifications that allow various bacterial clones from the *Enterobacteriaceae* family to escape the maternal IgA repertoire but do not lead to acute increases in the total number of these bacteria present in the intestine.

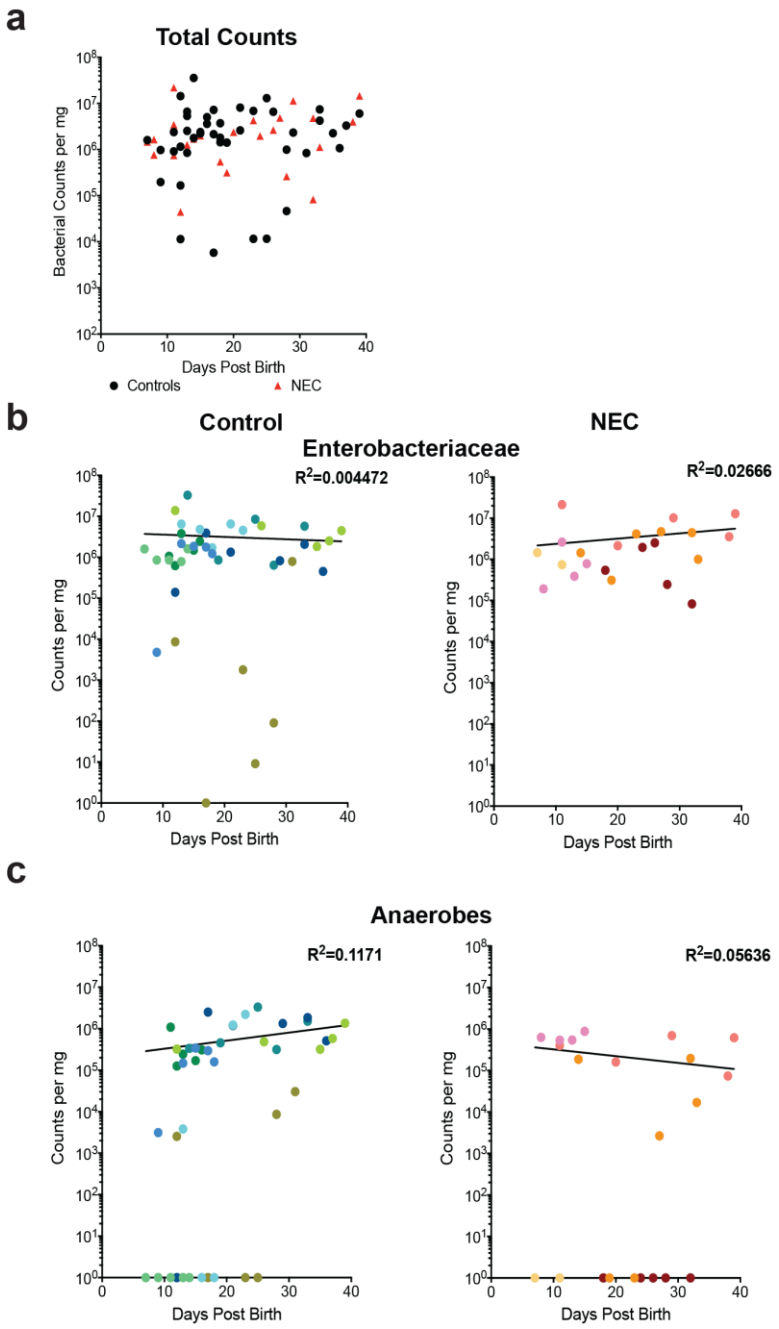

Figure 2.14 Absolute number of bacteria and number of bacteria associated with the dominant taxa in preterm infants

a, Total number of bacteria in each fecal sample determined by bead-based flow cytometry analysis. Black circles represent controls ($n = 8$, 43 samples combined); red triangles represent infants that went on to develop NEC ($n = 5$, 24 samples combined). **b**, Total number of *Enterobacteriaceae* in patients that will develop NEC (right) and controls (left). Semi-log non-linear regression. **c**, Total number of anaerobes in patients that will develop NEC (right) and controls (left). Semi-log non-linear regression.

2.4.3 IgA is a necessary component of breast milk for the prevention of the development of experimental NEC.

Based upon our results, we turned to a commonly used murine model of experimental NEC to further define the importance of maternal IgA in the pathogenesis of the disease. In this model, NEC-like pathology is frequently observed in 10-12 day old formula-fed pups exposed by oral gavage of bacteria derived from the intestine of a NEC patient (99% *Enterobacter spp.* and 1% *Enterococcus spp.*), whereas maternal milk-fed pups exposed to the same bacterial gavage do not develop disease [104,105]. To investigate the importance of maternal IgA in NEC, we set up a breeding program where heterozygote wild-type pups were fed by mothers that either can (C57BL/6) or cannot produce IgA (Rag1$^{-/-}$ or Igha$^{-/-}$) **(Figure 2.15)**.

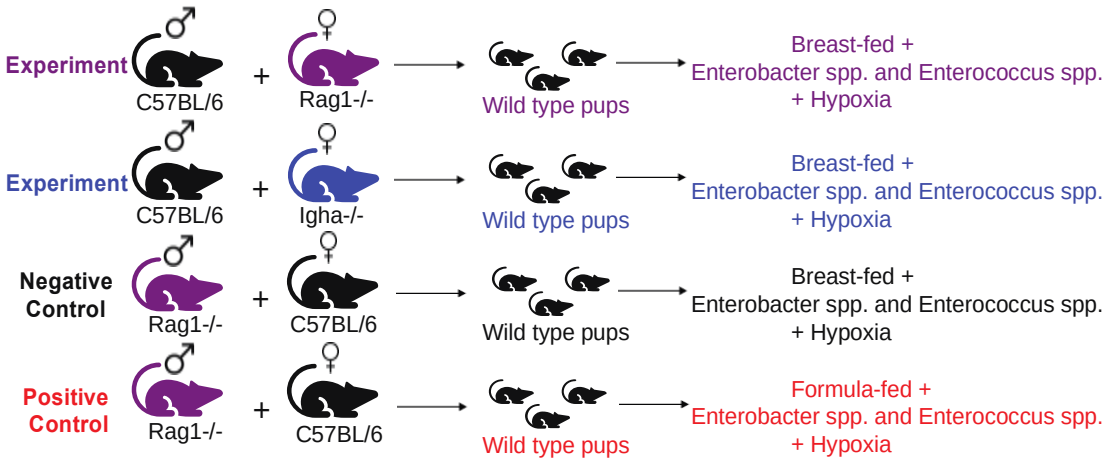

Figure 2.15 Breeding scheme for murine model of experimental NEC

Experimental NEC mouse model in which wild-type pups are fed by dams that either can (C57BL/6 mice ($n = 31$)) or cannot produce IgA (*Rag1*$^{-/-}$ mice ($n = 22$) or *Igha*$^{-/-}$ mice ($n = 14$)). Formula-fed mice ($n = 48$) are used as a positive control.

This scheme allows us to focus on maternally-derived intestinal IgA as pups born to wild-type C57BL/6 and Igha$^{-/-}$ mothers will receive normal amounts of maternal IgG via the placenta, which has been shown to be critical for control of neonatal bacteremia[106,107]. We confirmed that mice, like humans, produce little IgA during their first two weeks of life and that mothers are the primary source of neonatal IgA[99,108,109] **(Figure 2.16a and b)**. To determine the role of IgM and IgG on the intestinal bacteria, we also stained ileal contents along with fecal samples from the pups **(Figure 2.16a and b)**.

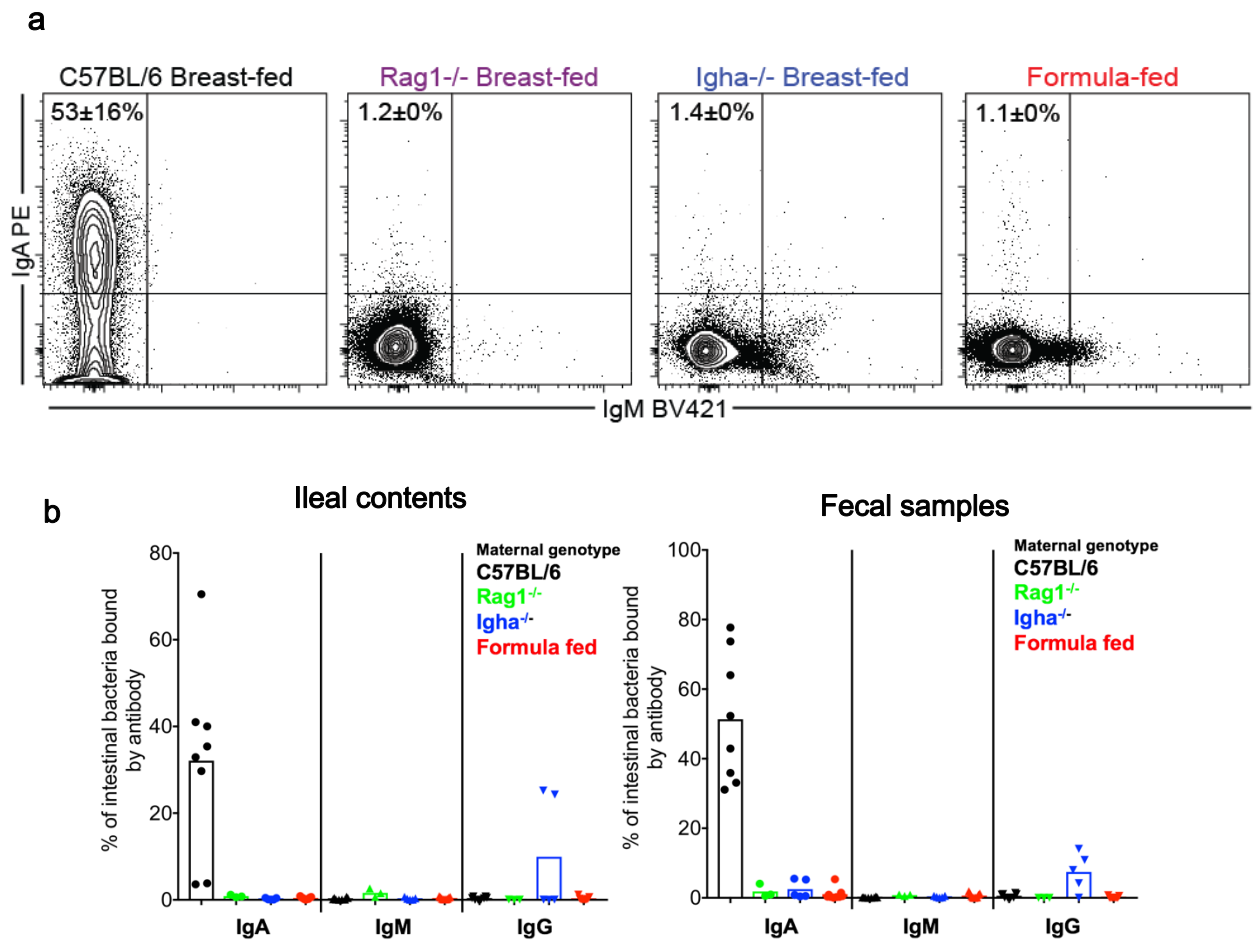

Figure 2.16 Percent of intestinal bacteria bound by different Ig classes in wild type pups with dams of various genotypes

Graph showing the Ig bound bacteria in pups from **Figure 2.15**. **a,** Representative images of IgA staining of the fecal matter of 12-day-old pups from **Figure 2.15**. Images show the absence of IgA-bound bacteria in dam breast-fed and

formula-fed pups. **b,** High percent IgA binding to bacteria is seen in pups fed by C57BL/6 mother. Increased percent of IgG binding to bacteria seen only in pups fed by Igha$^{-/-}$ mothers may be reciprocal to the absence of IgA. C57BL/6 ($n = 8$), *Rag1*$^{-/-}$ ($n = 3$), *Igha*$^{-/-}$ ($n = 3$) mothers or that were formula-fed ($n = 4$), repeated over 3 different experiments.

However, we noticed that some mice fed by Igha$^{-/-}$ mothers had IgG bound bacteria, but this result was inconsistent and did not appear to affect the incidence of NEC. Strikingly, pups undergoing the NEC protocol that were breast-fed by mothers lacking IgA (Rag1$^{-/-}$ or Igha$^{-/-}$) showed a phenotype consistent with the formula-fed pups. Specifically, they exhibited increased mortality, severe intestinal damage characterized by shortened and necrotic villi, and mucosal sloughing that was indistinguishable from formula-fed controls **(Figure 2.17a-c)**.

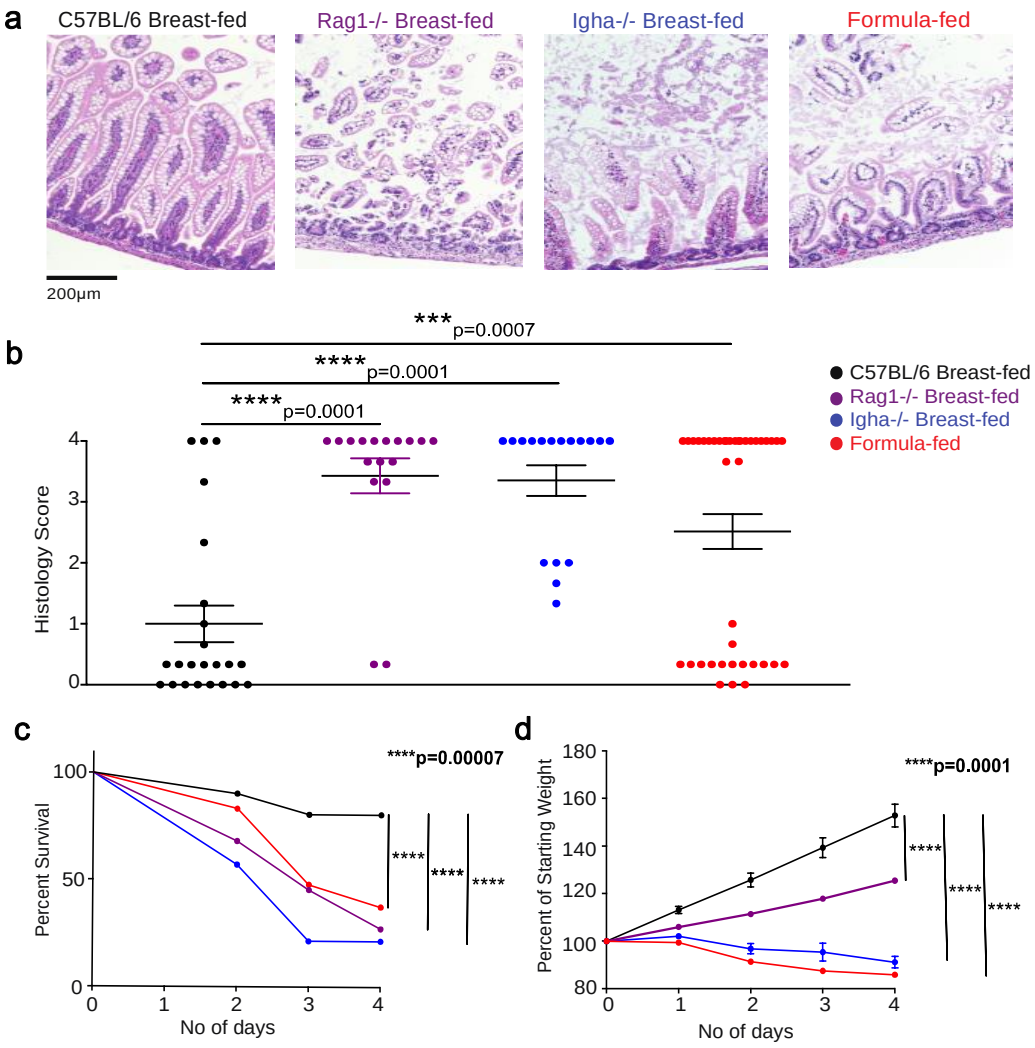

Figure 2.17 Pups fed by mothers deficient in antibodies in breast milk had disease severity similar to formula fed pups

a, Representative images of hematoxylin and eosin staining of small intestines of pups that underwent the mouse protocol that induces NEC; pups were fed by C57BL/6 ($n = 23$), *Rag1*$^{-/-}$ ($n = 17$), *Igha*$^{-/-}$ ($n = 17$) mothers or were formula fed ($n = 40$). **b**, Histology scores of the small intestines of pups from **a** on day-5 of the NEC protocol. One-way ANOVA with multiple comparisons; data are mean ± s.e.m. **c**, Percentage of pups, from **Figure 2.15,** that survived the NEC protocol at different time points. Statistics determined by log-rank (Mantel–Cox) test. ****$P = 0.00007$. **d**, Weights of pups from **Figure 2.15**. Statistical difference calculated by one-way ANOVA with multiple comparisons on weights at experimental completion point; data are mean ± s.e.m. ****$P = 0.0001$. Data shown in **a-d** are grouped from three individual experiments with minimum $n = 5$ pups in each group for every experiment.

Furthermore, pups fed by Igha$^{-/-}$ mothers exhibited a significant reduction in weight gain compared to pups fed by wild type mothers **(Figure 2.17d)**. Indeed, our analysis of the murine model of NEC was limited to gross physiological and pathological analysis of the disease and we would argue that it matches perfectly with our human data. Specifically, the bacteria that are necessary to induce murine disease, *Enterobacteriaceae* and *Enterococcaceae* are the exact taxa identified to be most significantly 'unbound' prior to the development of NEC **(Figure 2.7, 2.12 and 2.13)**. However, in the murine model of experimental NEC, mice are receiving relatively large amounts of bacteria (10^7 CFU of *Enterobacter spp.* and *Enterococcus spp.*) as part of the protocol necessary to induce NEC-like disease, potentially obscuring our ability to see the effect of IgA on resident bacteria. Nevertheless, we sought to examine the microbiota of mice in undergoing the NEC protocol. Using 16srRNA gene-based sequencing we analyzed the fecal microbiota of mice undergoing the NEC protocol being fed formula or maternal fed by Rag1$^{-/-}$, Igha$^{-/-}$ or C57BL/6 controls. Analysis of the OTUs revealed few substantial differences, many of which might be outside of the effects of IgA. For example, the strongest difference that we saw was a complete loss of *Lactobacillaceae* in formula fed pups, but this was not dependent upon a lack of IgA as all Igha$^{-/-}$ fed and some Rag1$^{-/-}$ fed pups had substantial amounts of this taxon (**Figure 2.18**).

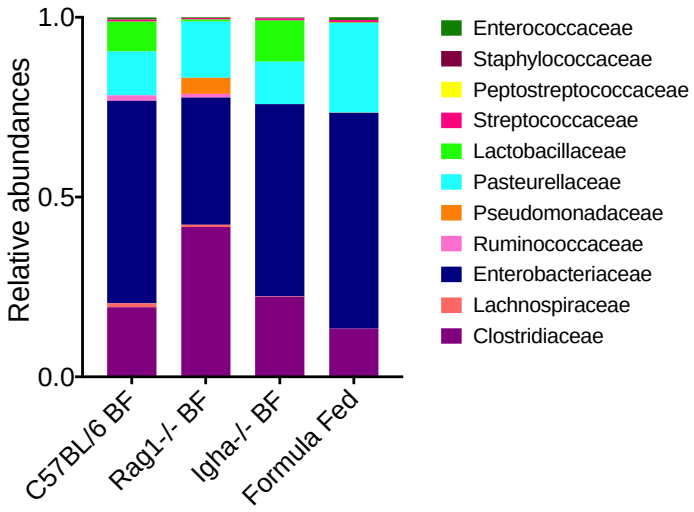

Figure 2.18 Analysis of the intestinal microbiota from fecal samples of pups from Figure 2.13

Stacked bar charts of relative OTU abundance at the family level in fecal samples of mice pups on day-5 of the NEC protocol, being maternal fed by different dams C57BL/6 ($n = 18$), *Rag1*$^{-/-}$ ($n = 3$), *Igha*$^{-/-}$ ($n = 10$) mothers or hand-fed with formula ($n = 21$), samples from 3 different experiments combined together.

Perhaps the most important finding is the similarity of all of the microbiotas (dominated by *Enterobacteriaceae,* which is 99% of the bacteria provided in feeds), which we believe is driven by the daily addition of bacteria. Principal coordinate analysis bears this out. While each feeding regimen clusters separately, there is significant overlap (**Figure 2.19**).

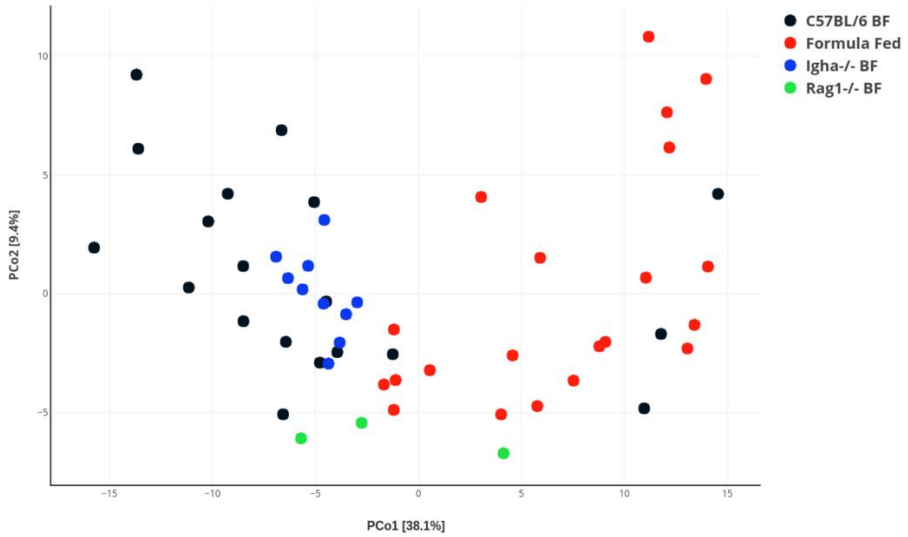

Figure 2.19 Beta diversity between different groups of mice undergoing the NEC protocol shown through Principal coordinate analysis (biomial, weighted)

Fecal samples collected on day-5 of NEC protocol from mice pups being maternal fed by different dams C57BL/6 ($n = 18$), *Rag1*$^{-/-}$ ($n = 3$), *Igha*$^{-/-}$ ($n = 10$) mothers or hand-fed with formula ($n = 21$), samples from 3 different experiments combined together.

We also determined that *Enterobacter spp.* gavaged into pups (C57BL/6 dams) to induce NEC was enriched in the IgA positive fraction, indicating that murine dams may produce protective IgA without being vaccinated **(Figure 2.20)**.

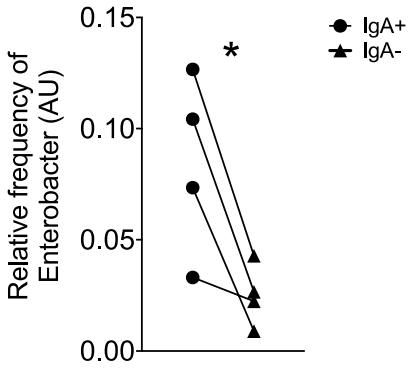

Figure 2.20 *Enterobacter spp.* is enriched in the IgA+ fraction of fecal matter of the pups breast-fed by C57BL/6 mice

Magnetically sorted IgA^{+} (left) and IgA^{-} (right) samples from fecal samples of day 12 (day 5 of NEC protocol) pups ($n = 4$). Frequency of *Enterobacter spp.* in each sample is shown as measured by qPCR for *Enterobacter spp.* (23S

rRNA expression) normalized to the relative number of bacteria in each sample (as measured by 16S rRNA expression). AU, arbitrary units. *$P = 0.0379$ by paired Student's *t*-test.

We have thus shown, using a murine model of NEC, that maternal IgA provided in breast milk is necessary for prevention of the disease. Taken together, our data from the mouse model indicates that IgA may control intestinal bacteria by mechanisms other than controlling the accumulation/proliferation of *Enterobacteriaceae*. Indeed, recent studies have revealed that IgA binding may have only modest effects on the proliferation of bacteria and may instead 'enchain' bacteria, preventing horizontal gene transfer and mediating transcriptional change[52,53,110-112]. We hope to be able to study these possibilities in the future and describe the mechanism of how maternal IgA shapes the colonization of neonatal intestine.

2.5 Discussion

NEC is a multi-factorial disease that is dependent upon the susceptibility of the preterm intestine and inflammation driven by a dysbiotic intestinal microbiota. Our work now adds the critical importance of maternal IgA binding of intestinal bacteria as a preventative mechanism against the development of NEC. However, some critical questions remain to be answered. First, the mechanism via which IgA might control and modify intestinal bacteria in preterm infants is unknown. IgA has been shown to modify bacterial transcription and in particular, motility and epithelial adhesion which could limit the ability of bacteria to gain access to the intestinal epithelium[51,52]. IgA may also limit intestinal bacteria by 'enchaining' them together, allowing for easier expulsion and preventing gene transfer[53]. The current study cannot discriminate between bacterial strains and thus we do not yet know whether the loss of IgA binding is due to colonization

with organisms that are not bound by IgA or mutation/transcriptional shifts that allow escape of resident organisms. It is also possible that local intestinal conditions may shift to allow for focal outgrowth of bacteria that overwhelms maternal IgA locally within portions of the small intestine, as maternal IgA would not increase in scale to respond to bacteria in an infant's intestine. Since all infants in our study were fed maternal milk (no donor milk) we presume that the maternal IgA repertoire is relatively stable, though temporal changes in maternal milk IgA may also contribute to shifts in bacterial binding. Future studies relying upon metagenomics and metatranscriptomics to discriminate IgA bound and unbound strains will be necessary to better understand the phenomenon[113].

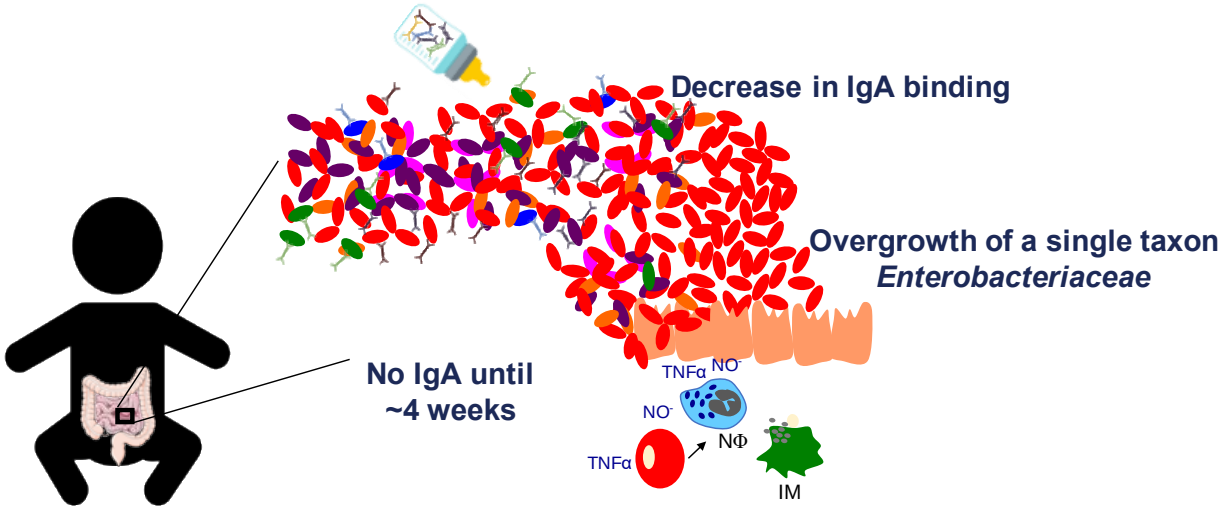

Figure 2.21 Schematic representation depicting the role of maternal IgA in the prevention of Necrotizing Enterocolitis

Our studies identify *Enterobacteriaceae* as the primary taxon associated with the loss of maternal IgA binding of the preterm intestinal microbiota that precedes the development of NEC. Previously it has been shown that an increased relative abundance of *Enterobacteriaceae* is

associated with the development of NEC[78]. Our study now shows that IgA unbound *Enterobacteriaceae* is more closely linked to NEC disease potential than total *Enterobacteriaceae* abundance and we would hypothesize that IgA binding of this taxon is protective. Indeed, animal studies have indicated that IgA is important in controlling *Enterobacteriaceae* and establishing a mature microbiota characterized by mostly fastidious anaerobic bacteria[19,59]. We have now shown that binding of bacteria by maternally-derived IgA may promote diversity in the microbiome and the acquisition of anaerobic bacteria during the critical window when infants make little or no IgA of their own, perhaps by limiting inflammation driven by *Enterobacteriaceae*[19,114,115]. The abundance of *Enterobacteriaceae* within maternal fecal samples increases during the final trimester of pregnancy[116]. One explanation for this increase may be to boost antibody responses against this class of bacteria, of which many members are prominent neonatal pathogens. Thus, since many infants who suffer from NEC are delivered before the third trimester, their mothers might lack the boosting effect of *Enterobacteriaceae* outgrowth.

We do not know the bacterial targets of maternal IgA with regard to the microbiota or the nature of the B cells that produce it. In unimmunized mice, most IgA in maternal milk appears to be 'poly-specific' and capable of binding multiple moieties[117,118]. However, mammary gland B cells are uniquely T cell dependent and intestinal bacteria induce taxa-specific plasma B cells so maternal IgA in humans living in a more diverse microbial environment may be more tightly targeted to individual taxa via affinity maturation[119,120]. Of note, previous attempts to supplement the diet of at-risk preterm infants with venous-derived immunoglobulins (delivered orally) have largely failed to protect against the development of NEC[121,122]. Our work would indicate that this is probably due to differences in the antigenic repertoire of secretory versus circulatory antibodies and the unique properties of secretory IgA (sIgA). A better understanding of interindividual

variation in the antigen-specific IgA repertoire might allow for targeting protective maternal milk to the most at-risk infants or augmenting the feeds of preterm infants with protective antibodies.

3.0 Maternal Immunoglobulin A Repertoire Varies Between Mothers

3.1 Foreword

This chapter contains data that will be published in future manuscripts. This work would not be possible without the following co-authors: Kara Coffey, Chelseá Johnson, Kelly Baumgartel, Cyndy Verardi, Justin Tometich, Timothy W. Hand.

3.2 Introduction

Feeding infants with breast milk has significantly reduced the incidence of NEC but has not been 100% protective[77]. The long-lived affinity matured B cells in the intestine produce antigen specific IgA in response to intestinal colonization[120]. The IgA-secreting B cells in the breast are derived from the intestine of the mother, suggesting a dependence on the unique intestinal microbiota of each mother[54,57]. Antigen specificity of IgA binding can contribute to escape of certain bacteria in some mothers who have never encountered the pathogen, which could explain the incomplete protection against NEC. We propose to test the variability in IgA binding of bacteria in breast milk from different mothers and combine donor breast milk with differential IgA binding to provide maximum protection. Since we have previously shown that maternal IgA binding to intestinal microbiota of infants has been correlated to be protective against NEC, it would be important to study the IgA repertoire of the breast milk.

3.3 Methods

3.3.1 Experimental Model and Subject Details

Human Donor Breast Milk Samples

The human study protocol was approved by the Institutional Review Board (Protocol Nos. PRO18090392) of the University of Pittsburgh. Frozen donor breast milk samples were collected from the Mid-Atlantic Mother's Milk Bank DBA Human Milk Science Institute and Biobank and stored at -80°C.

3.3.2 Method Details

IgA Extraction from Donor Breast milk

Donor breast milk was thawed at 4° C overnight and 2ml of the breast milk was placed in 2ml Eppendorf tubes. To separate the whey protein from the fat, the breast milk was centrifuged at 16,000g for 5 mins at 4° C. The fat forms a layer at the top of the tube. The whey protein was separated from the fat by carefully pipetting and filtered through a 0.22µm syringe filter. The filtered sample was then passed through a gravity flow column containing peptide M/agarose, after equilibrating the column with 10ml of wash buffer (1x Phosphate Buffered Saline (PBS)). Allow the sample to completely enter the matrix. Wash the column with 20ml of PBS. The column was then eluted with 10ml elution buffer (0.1 M glycine, pH 2-3). 10ml of 1 M Tris with pH of 7.5 was used to neutralize the solution[123]. The 20ml of the sample was concentrated using a protein concentrator, by centrifugation of the column at 3000g for 20 min at 4° C. The concentrated sample was collected in 1.5ml Eppendorf tubes, IgA concentration was measured through Nanodrop and stored at -80° C.

Bacterial culture and preparation of bacterial plates

The different families and strains of bacteria are purchased through American Type Culture Collection (ATCC) and grown according to guidelines provided by ATCC. Bacteria was grown to ~10^8 CFU/ml, (Colony Forming Units) as measured by OD. 25µl of $8x10^8$ CFU/ml will be added to 2 wells each in a 96-well U-bottom plate, as experiment and control. In order to preserve the integrity of the bacteria during freezing process, 2µl of sterile glycerol was added to each well and resuspended with the bacterial solution. The plates were then stored at -80° C.

Bacterial Flow Assay

The bacterial plates, stored at -80° C, were thawed at room temperature and washed twice with staining buffer (0.5% Bovine Serum Albumin (Sigma) in PBS-filtered through a 2.2µm filter). The concentrated IgA from the breast milk was thawed at 4° C and normalized to 0.1mg/ml by diluting the sample with sterile 1x PBS. 25µl of the normalized IgA was added to all the bacteria in the experimental wells. For controls, 25µl of sterile 1x PBS was added. The plate was incubated for 1 hour, in dark at 4° C. After incubating for an hour, the plate was washed 2.5x times with the staining buffer. All the wells in the 96-well plate were stained with Syto BC (Green Fluorescent nuclear acid stain, Invitrogen-1:400), APC Anti-Human IgA (Anti-Human IgA APC (Miltenyi Biotec clone REA1014) (1:50) and blocking buffer of 20% Normal Mouse Serum for human samples (ThermoFisher). The stained samples were incubated in dark for an hour at 4°C. Samples were then washed three times with 200µl of staining buffer before flow-cytometric analysis (LSRFortessa-BD Biosciences).

3.3.3 Quantification and Statistical Analysis

Flow Analysis

All the data from flow cytometry was derived from samples that were run through the LSR Fortessa flow cyometer from BD Biosciences. The raw data was analyzed through the software FlowJo V10.4.2 (FlowJo, OR, USA). The cell population abundance was measured by SytoBC+ events that range from 60% to 80% of all events; Of SytoBC+ events the range of IgA+ bacteria was <1% to 95%.

Antibody Validation

All antibody stains were compared to an isotype control which was specific for an antigen irrelevant to the intestinal microbiome (Keyhole Limpet Hemocyanin or 4-Hydroxy-3-nitrophenylacetyl).

Human: Positive antibody binding was confirmed early on by staining of pediatric IBD patients for IgA on the fecal bacteria. These patients were known to have increased binding of such antibodies. Cross-reactivity for IgA was tested for by staining fecal bacteria of formula fed infants 10-21 days post-delivery.

Statistical Tests

Spearman Rank Correlation was used to identify the difference in IgA binding between different donor breast milk IgA in the heatmap.

Analysis software

Raw data from flow cytometry was analyzed through FlowJo V10.4.2 (FlowJo, OR, USA); The heat map was created using the software Heatmapper[124].

3.4 Results

3.4.1 Difference in the repertoire of IgA binding to a single bacterium in different donor breast milk samples.

To study the difference in the repertoire of IgA binding to bacteria, we collected donor breast milk from the Mid-Atlantic Mother's Milk bank and concentrated the IgA by passing the filtered whey protein from the breast milk through peptide-M agarose columns **(Table 3)**. The concentration of IgA was measured via Nanodrop and normalized for downstream applications.

Table 3.1 List of donor breast milk samples collected from the Mid-Atlantic Breast Milk Bank

Donor Number	Gestational age at birth of the infant	Sample collected (Days postpartum)
1	41 weeks	14-36
2	38 weeks 5 days	37-48
3	38 weeks 5 days	66-70
4	40 weeks	276-469
6	37 weeks	38-48
7	40 weeks 3 days	89-101
8	40 weeks 2 days	161-164
9	40 weeks 4 days	66-123
10	40 weeks	6-93
12	40 weeks 5 days	52-61
13	33 weeks 6 days	14-18
14	Not known	261

One breast milk sample per donor was collected, from which IgA was extracted.

Bacteria were grown to ~10^8 CFU/ml (as measured by OD) and $2x10^7$ CFU was added to each well in a 96-well plate. The IgA purified from the donor breast milk was combined with the bacteria. Secondary staining was conducted with anti-human IgA, and IgA binding to the bacteria was measured through flow cytometry **(Figure 3.1)**.

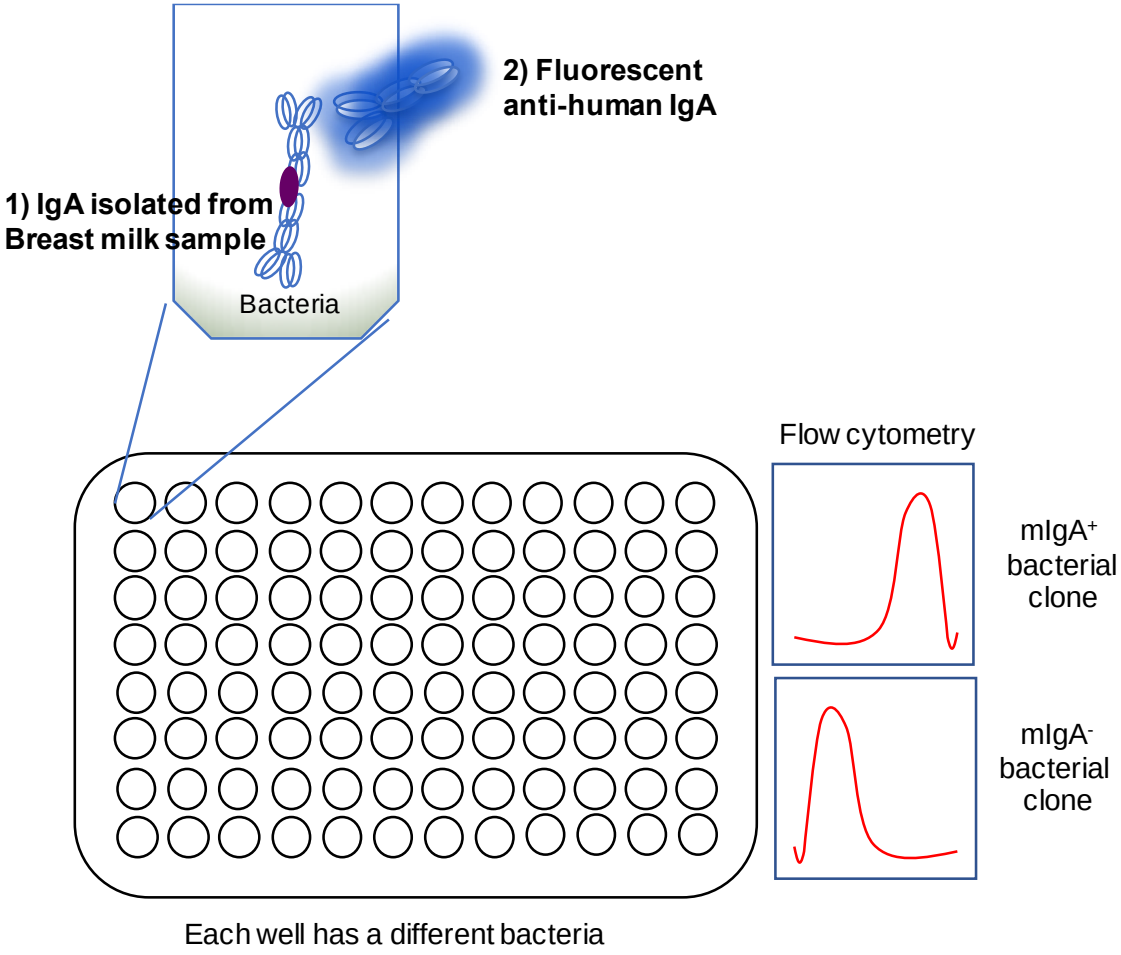

Figure 3.1 Schematic representation of the experimental procedure

We have validated the difference in IgA binding to a single bacterium with four different donor breast milk IgA isolates **(Figure 3.2)**, in which we observed differences in IgA binding repertoire.

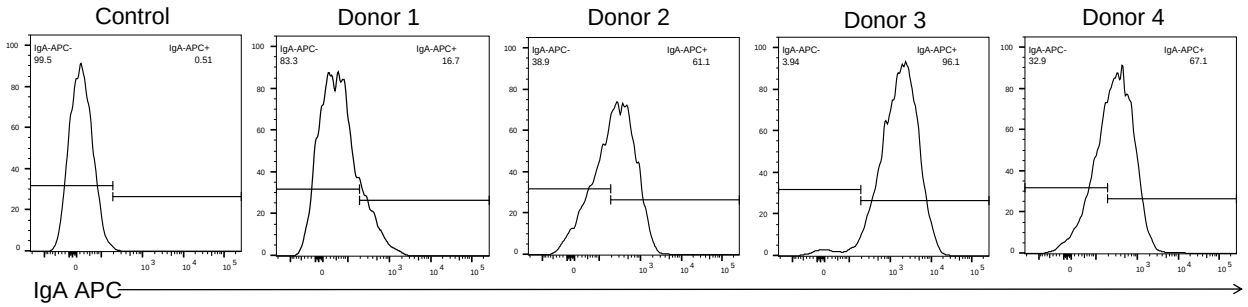

Figure 3.2 Histogram depicting the differences in IgA binding to *Enterococcus faecalis* from four different donor breast milk

3.4.2 Variation in the repertoire of IgA binding to bacterial array in different donor breast milk samples.

With this as a basis, we tested the IgA extracted from donor breast milk from **Table 3.1** to an array of bacteria **(Table 3.2)**, which have been previously recognized in the infant microbiota[125].

Table 3.2 List of bacteria identified from preterm neonates

Number of bacteria	Strain Name	Family Name
1	*Citrobacter rodentium*	*Enterobacteriaceae*
2	*Enterobacter aerogenes*	
3	*Enterobacter cloacae*	
4	*Escherichia coli 587*	
5	*Escherichia coli 596*	
6	*Escherichia coli 605*	
7	*Escherichia coli 909*	
8	*Escherichia coli 910*	
9	*Escherichia coli 4185*	
10	*Escherichia coli ECO2A*	
11	*Escherichia coli ECMB*	

Table 3.2 Continued

12	*Escherichia coli ECT5*	
13	*Escherichia coli MT8*	
14	*Salmonella typhimurium SL3261*	
15	*Enterobacter spp.*	
16	*Klebsiella aerogenes 13048*	
17	*Klebsiella oxytoca 43165*	
18	*Klebsiella oxytoca K405*	
19	*Klebsiella pneumoniae*	
20	*Serratia marcescens 855*	
21	*Serratia marcescens 853*	
22	*Proteus mirabilis*	
23	*Proteus vulgaris*	
24	*Pseudomonas aeruginosa*	*Pseudomonadaceae*
25	*Moraxella nonliquefaciens*	*Moraxellaceae*
26	*Lactobacillus casei*	*Lactobacillaceae*
27	*Streptococcus agalactiae*	*Streptococcaceae*
28	*Staphylococcus aureus RS*	*Staphylococcaceae*
29	*Staphylococcus aureus TWH*	
30	*Staphylococcus capitis*	
31	*Staphylococcus epidermidis LM087*	
32	*Staphylococcus epidermidis LM088*	
33	*Staphylococcus epidermidis RS*	
34	*Staphylococcus saprophyticus*	
35	*Enterococcus faecalis 2649*	*Enterococcaceae*
36	*Enterococcus faecalis 19433*	
37	*Enterococcus faecium*	

To standardize the protocol, we used frozen 96-well plate, containing the array of bacteria, to test different donor breast milk IgA samples. We noticed a significant variation in IgA binding between different donors, particularly amongst bacterial strains from *Enterobacteriaceae* and *Enterococcus*, where the binding of IgA was stronger in some donors compared to the rest. We observed the same difference in IgA binding to Gram-positive organisms **(Figure 3.3)**. We have also noticed that some donors exhibit a strain level difference of IgA binding in the family *Enterobacteriaceae.*

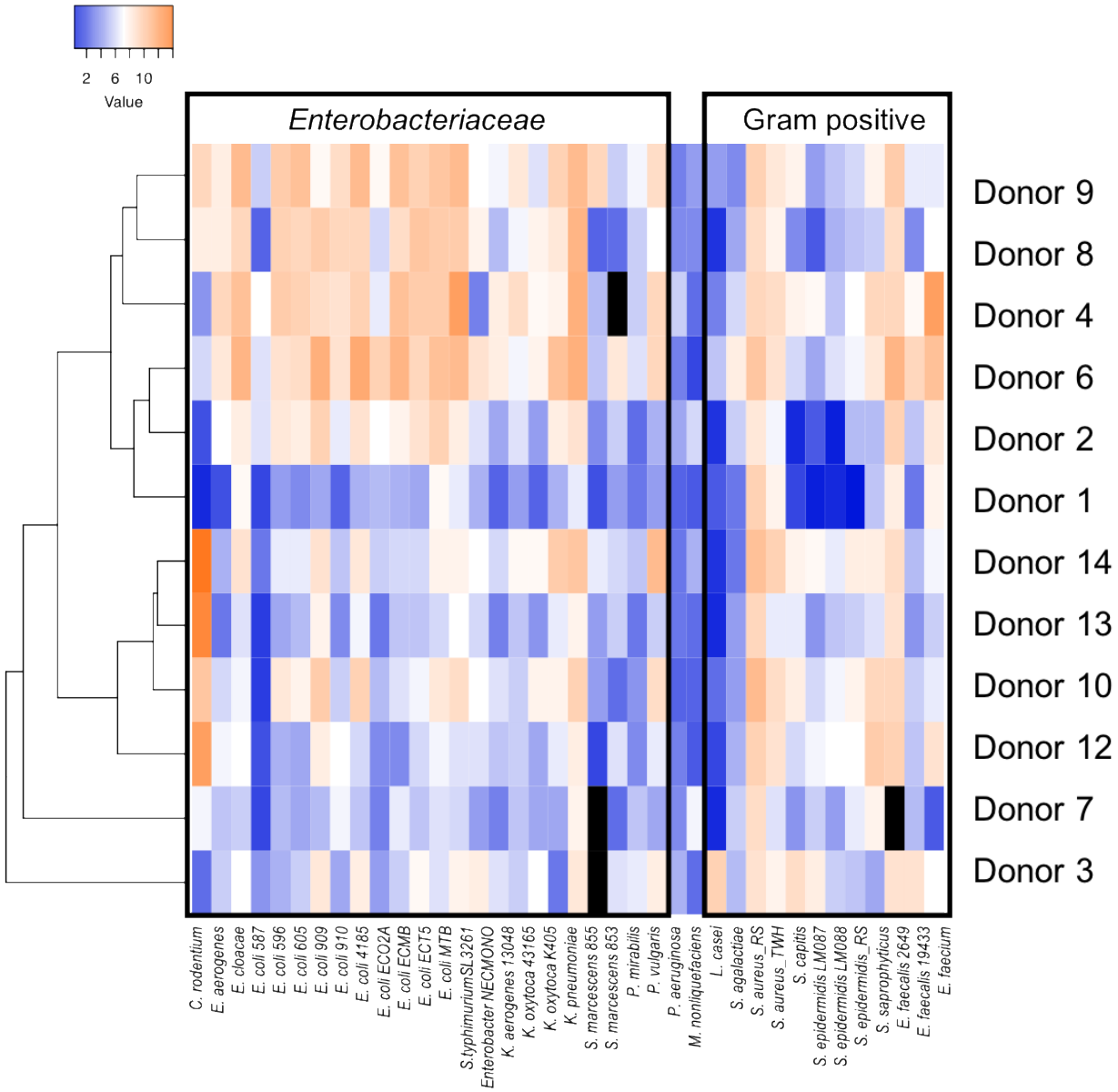

Figure 3.3 Variation in the repertoire of IgA binding to bacterial array in different donor breast milk samples

Heat map depicting the difference in IgA binding to different bacteria (n=37) in a single donor breast milk sample. This heat map also shows the differences in the IgA repertoire of different mother (n-=12). The binding of IgA was measured by the log 2 value of the product of percent IgA binding and normalized mean fluorescence intensity (MFI) as calculated from flow cytometry. Least binding is depicted in blue and maximum binding is shown in orange. The samples where data was not available are shown in black color.

3.5 Discussion

Breast milk is the best source of nutrition to a newborn infant. It is rich in carbohydrates, fats and proteins required for the growth and development of the neonate. However, along with being a source of nutrition, breast milk is also immunologically enriched with live white blood cells, cytokines, growth factors, human milk oligosaccharides and anti-microbials. Milk oligosaccharides and anti-microbials, such as lactoferrin, have been two of the most promising possibilities showing protection against NEC in animal models[27,82,83]. Antimicrobials in the breast milk consist of large amounts of antibodies comprised primarily of IgA with smaller amounts of immunoglobulin M (IgM) and immunoglobulin G (IgG)[85]. The role of maternal breast milk IgA in the prevention of NEC has not been previously studied.

Maternal IgA, which does not cross the placenta, is provided to the infant through breast milk. IgA has been shown to be important in shaping the development of the pediatric microbiota by promoting maturation of the community away from *Proteobacteria* and towards anaerobic *Firmicutes* and *Bacteroidetes*, thus preventing enteric infections[19,59]. Interestingly, the IgA-secreting B cells that provide breast milk IgA are derived from the small intestine of the mother, indicating that the IgA repertoire of breast milk is primarily targeted against intestinal bacteria and may be biased towards the most common organisms of the maternal microbiota[54,57]. As the microbiota of individual mothers is different, the IgA produced in the breast milk is unique to each mother and may have varied specificity to different bacteria. For various reasons including exposure to antibiotics and an underdeveloped gastrointestinal tract, preterm infants harbor gut microbial communities that are distinct from those of healthy term infants and adults[86]. Moreover, the preterm gut is rich in facultative anaerobes (*Enterobacteriaceae, Staphylococcaceae*) that are relatively rare in the maternal intestinal microbiota[78]. This may explain the variability in protection

conferred by breast milk. Studies have shown a bloom in *Enterobacteriaceae* in the intestine of pregnant women in the third trimester[116]. We believe that this is an evolutionary mechanism as pregnant women then produce IgA antibodies against *Enterobacteriaceae*. These antibodies are transported to the mammary gland and are secreted through the breast milk. This might provide protection to newborn infants as studies have indicated that in the first few weeks of life, the intestine of the neonates is mainly colonized by *Enterobacteriaceae*[14]. However, in preterm delivery, this boosting mechanism may be absent, leading to an uninhibited growth of facultative anaerobes.

In our study, we have described a novel technique through which the IgA repertoire of the breast milk can be evaluated. This method allows us to test the difference in IgA binding to an array of bacteria and examine the breast milk of various mothers. We have observed that not only IgA extracted from a single donor's breast milk displays varied binding to different bacteria but also that IgA binding to bacteria is different in each mother. We believe this might be due to the unique microbiota of every mother, which is constantly changing due to external and internal factors that the mother may experience. However, our study is limited to a single sample from a donor. It would be interesting to examine the repertoire of IgA over a period of time by collecting multiple samples from a single donor. Furthermore, we can also test whether there are differences in IgA repertoire between mothers with a term infant vs a preterm infant, as there are studies suggesting the increase in abundance of *Enterobacteriaceae* in the third trimester of pregnancy[116]. The rational of this study is that donor breast milk from mothers with high IgA specificity to *Enterobacteriaceae* can be provided prophylactically to preterm neonates who are at risk for developing debilitating diseases like necrotizing enterocolitis.

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