Paediatrics Prize Winner:
The Neonatal Microbiome

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"The supreme act of war is to subdue thy enemy without fighting" – Sun Tzu, The Art of War

Introduction

"The human microbiome" was termed in 2001 by Nobel Laureate Joshua Lederberg to encompass, "the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space." (Peterson et al. 2009; Sherman et al. 2013). In other words, "the sum of all microbial life in or on the human body" (Gritz and Bhandari, 2015). Afterwards, Lederberg, Relman and Falcow envisioned a, "second human genome project" - The Human Microbiome (HM) Project (Sherman et al., 2013). Thus, in the last 15 years, the world of medicine has witnessed a revolution in how we view our symbiotic relationship with our microbiome. With evidence emerging from the project regarding the implications of bacteria, viruses, fungi and parasites in obesity, allergic and autoimmune diseases, diabetes (Sherman et al., 2013), cancer, mood disorders and even therapeutic faecal microbiota transplantation (FMT) the question of whether they are friend or foe arises. In adulthood, the human body is colonized by more than 10 times as many bacteria as human cells (Peterson et al., 2009). If we are covered in the region of ten to one hundred trillion microbes (Peterson et al., 2009), this begs the philosophical question, are we even human? In the neonatal period of modern medicine, Hippocrates is credited with saying, "all diseases begin in the gut". New evidence over-turns the paradigm that the foetus is in a sterile environment as the placental microbiome has been identified, which may colonise the foetus in small numbers (Wassenaar and Panigrahi, 2014). What we will learn from this essay is the elegance of the gut microbiome of the neonate, its journey from utero to early infancy. In particular, we will acknowledge its role in disease and how to prevent such states.

Methodology: The Arms Race

Metagenomics is a rapidly evolving field of medicine born from the Human Genome Project (HGP), describing the study of the structure and function of the "microbiome", the term being coined by Handelsman et al.. Remember, that in the human, the microbiome consists of a myriad of genomes of bacteria, archaea, viruses, fungi and protists. It took 13 years to complete the HGP in 2003 and the HM consists of 150 times more genes (Cong et al., 2015). In just over a century, we've moved from Koch and colleagues' methods of culture and isolation to culture-independent Next Generation Sequencing (NGS) (Sherman et al., 2013). In 1985, polymerase chain reaction (PCR) cloning of precise genes, such as the 16S ribosomal RNA subunit, has made waves in whole genome analysis that allow us to study phylogenetics and taxonomy (Gritz and Bhandari, 2015; Till et al., 2015; Dunlop et al., 2015). It is postulated that up to 50% of the species of the HM cannot be cultured (Dunlop et al., 2015). Therefore, it is a necessity that shotgun metagenomics and amplicon sequencing (a combination of NGS and PCR), are employed in analyzing the HM (Aho et al., 2015). DNA based techniques have been proven to be needed in this field when Venter et al. studied the microbiome of the Sargasso sea (Aho et al., 2015; Venter et al., 2004). In 2004, they demonstrated the superiority of shotgun metagenomics compared to PCR rRNA studies due to its quantitative identification of the species diversity (Venter et al., 2004). Amplicon sequencing is described as a genetic region, common to the members of interest, being amplified using universal primers (Aho et al., 2015). 16S rRNA amplicon sequencing is limited to prokaryotes. With a combined approach, with these tools of sequencing and workflow, we can begin to sketch the phylogentic tree that is the HM. New technologies include the 454/Roche and Illumina/Solexa sequencing. These greatly reduce the cost and manpower analyzing whole genomes (Thomas et al., 2012). Each of these technologies boasts advantages as regards their low error rates, length of reads and insert sizes. Extensive reviews describing these are available. In the future, it is

hoped that de Bruijn-type assemblers, specifically "metagenomic assemblers" will be developed for scientific use (Thomas et al., 2012). A drawback of all these technologies, however, is that they are still in their infancy and so reference libraries do not exist for comparative analysis.

A more pressing limitation is sample collection. Till et al. calls us to question whether faecal sampling is an accurate representation of the intestinal microbiome and whether the transient luminal bowel in its diseased state is synonymous with the mucosal microbiome (Till et al., 2015). Are we required to take direct endoscopic samples from various areas of the bowel to get a more precise representation of the gut microbiome? Sample sizes and stratification into sub-cohorts may hinder progress in this field for some time.

Finally, it is important to remember there are a number of ethical issues in the study of neonates in intervening or not intervening.
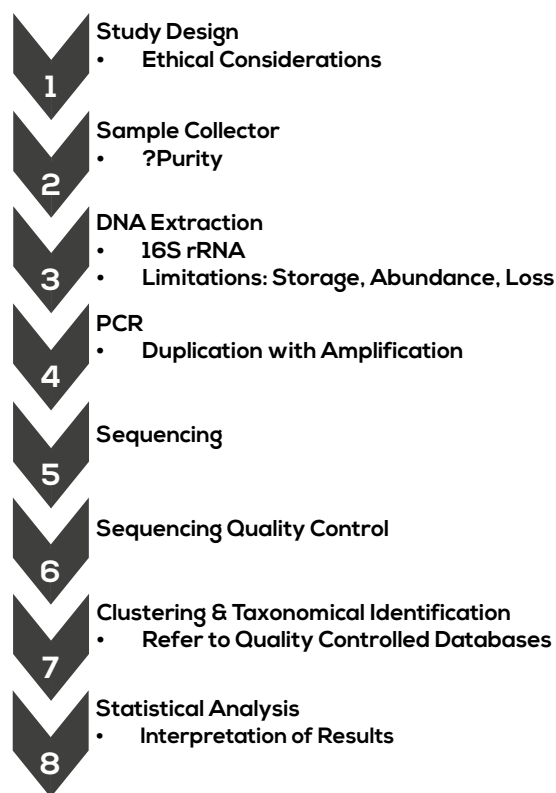

Study Design
• **Ethical Considerations**
1

Sample Collector
• **?Purity**
2

DNA Extraction
• **16S rRNA**
• **Limitations: Storage, Abundance, Loss**
3

PCR
• **Duplication with Amplification**
4

Sequencing
5

Sequencing Quality Control
6

Clustering & Taxonomical Identification
• **Refer to Quality Controlled Databases**
7

Statistical Analysis
• **Interpretation of Results**
8

Figure 1. Potential workflow for a Microbiome Study. Adapted from Aho et al., 2015.

The Neonatal Microbiome

"Il faut cultiver notre jardin" (We must cultivate our garden) – Voltaire

A neonate's microbiome is influenced by a host of different factors. Genetic factors, the maternal microbiome, mode of delivery, diet, environmental factors and a dynamic interplay between the developing immune and metabolic systems all play a part (Figure 3). The neonate's microbiome launches into action in the first year of life, resembling a mature "adult-like" microbiome from 1-4 years to population equilibrium (Stewart et al., 2015; La Rosa et al., 2014). The average adult has a more individualized microbiome than genome as it harbours a mere 15% of the growing number of intestinal bacterial species already documented (Gritz and Bhandari, 2015; Madan et al., 2012). What this means is that there is huge diversity between the microbiota that colonize you and your neighbour. In neonates and infants, it plays a quintessential and dynamic part in prevention of pathogen invasion, and immune and metabolic programming.

Prenatal Microbiome

Despite popular opinion that the uterus and foetus are in a sterile environment and that our first encounter with the microbe corps is at birth, recent studies disprove this with the identification of the placental microbiome (Wassenaar and Panigrahi, 2014; Satokari et al., 2009). We have seen in many disease states (and indeed normal states), that commensal bacteria translocate to various locations such as the mesenteric lymph nodes, portal venous system and beyond (Satokari et al., 2009; Berg and Garlington, 1979; MacFie, 2004; Romano-Keeler and Weitkamp, 2014). In 2014, in a study by Aagard et al. involving 320 women, it was reported that "a unique placental microbiome niche, composed of nonpathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla," was found amongst study participants (Aagaard et al., 2014). This "garden" was similar to the oral microbiome (Aagaard et al., 2014). Investigators were unconvinced that these were the fruits of contamination (Wassenaar and Panigrahi, 2014). Instead it was suggested that Fusobacteria nucleatum may permit haematogenous transmission by increasing permeability with FadA binding vascular endothelial cadherin
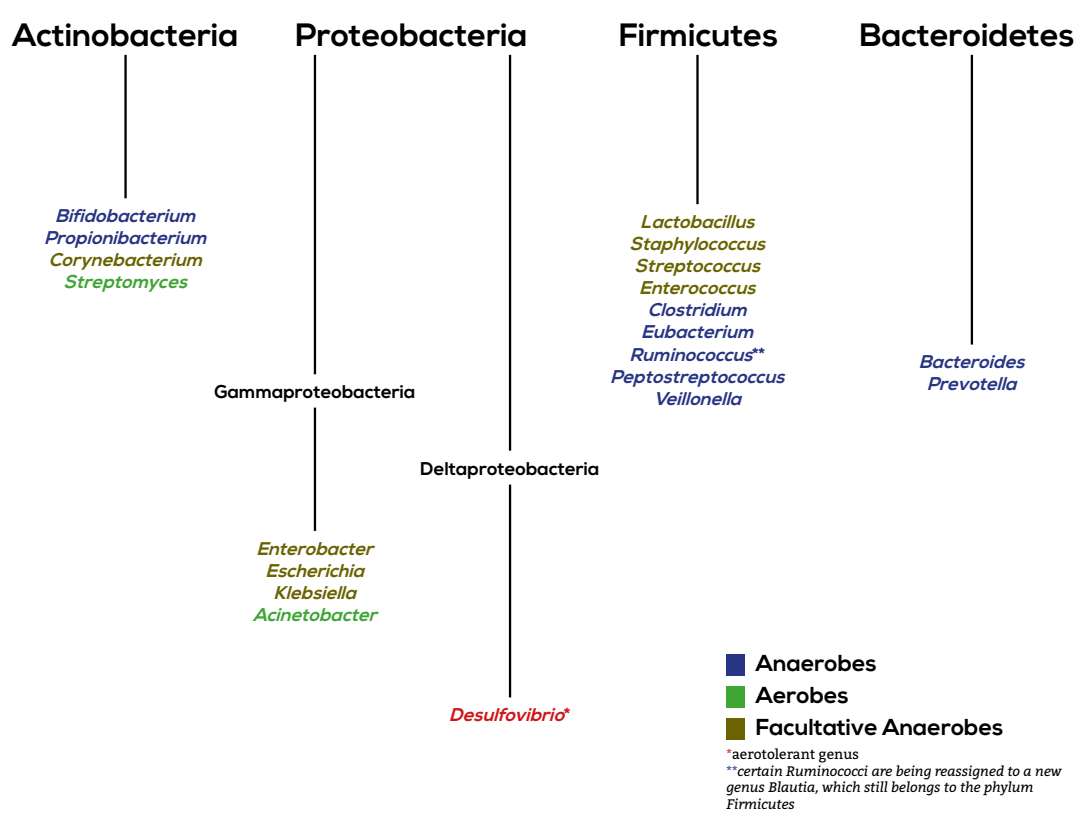
Actinobacteria **Proteobacteria** **Firmicutes** **Bacteroidetes**

Bifidobacterium
Propionibacterium
Corynebacterium
Streptomyces

Lactobacillus
Staphylococcus
Streptococcus
Enterococcus
Clostridium
Eubacterium
*Ruminococcus***
Peptostreptococcus
Veillonella

Gammaproteobacteria

Bacteroides
Prevotella

Deltaproteobacteria

Enterobacter
Escherichia
Klebsiella
Acinetobacter

■ Anaerobes
■ Aerobes
■ Facultative Anaerobes

*aerotolerant genus
**certain Ruminococci are being reassigned to a new genus Blautia, which still belongs to the phylum Firmicutes*

*Desulfovibrio**

Figure 2. Common microbes in the Neonate. Adapted from Gritz and Bhandari, 2015.

at placentation (Wassenaar and Panigrahi, 2014). This potentially allows troops of microbes to cross the border to the amnion. However, this has not been demonstrated in a lab. Is the Placental Microbiome a friend or a foe? Or a symbiotic evolutionary natural occurrence that allows the fetal "gut barrier" and immune and alimentary immune system to develop? The precise timing of fetal or neonatal intestinal colonization is not known or proven (Romano-Keeler and Weitkamp, 2014). Infection and spontaneous preterm labour are almost synonymous with cause and effect with the origin thought to be from a vaginal or urinary tract infection (Goldenberg et al., 2000). Despite this, Goldenberg et al. showed in preterm labour with intact membranes, bacteria can still be cultured in a sterile procedure from chorioamniotic tissue (Goldenberg et al., 2000). Thus, in terms of the maternal-fetal microbiome the species, population and density of bacteria in the placenta directly play a part for pregnancy outcomes and

fetal health (Romano-Keeler and Weitkamp, 2014; Epstein et al., 2000). In the first trimester, CD4+ and CD8+ cells can be identified (Spencer et al., 1986). Throughout fetal life we see Peyer's patches, Paneth cells and goblet cells developing from 9-17 weeks gestation (Brugman et al., 2015). T-regulatory cells develop in the second trimester possibly suggesting the idea that peripheral tolerance and exposure to microbial antigens occurs in utero (Brugman et al., 2015) and the maternal microbiome is the source of the fetal microbiome (Collado et al., 2012).

Perinatal Microbiome
At birth, facultative anaerobes are first to colonize, followed by anaerobic Bifidobacterium, Bacteroides and Clostridium (Cong et al., 2015). The microflora in the neonate and even to adult life is influenced by its mode of delivery. Born via the vaginal canal, one is graced with maternal vaginal and perineal microbes, but if born by Caesarian

section (CS) or in hospital, one carries with them nosocomial microbes (Penders et al., 2006). In the KOALA Birth Cohort Study (Netherlands) involving 1032 infants, Penders et al. showed that the CS group had decreased populations of Bifidobacteria and Bacteroides (100 fold) with increased Clostridum difficile (100 fold) and Escherichia coli compared with the vaginally delivered at home group (Penders et al., 2006). Vaginally delivered infants will be colonized by the vaginal microbiome (Lactobacilli and Prevotella) while CS infants will culture Staphylococcus from the skin Propionibacterium and Corneybacterium (Madan et al., 2012). CS delivered infants with lower Bifidobacteria mount a stronger humoral response at 1 month and then have higher rates of allergy, auto-immune, metabolic disorders (diabetes and obsesity) and GI dysfunction in later life (Brugman et al., 2015; Borre et al., 2014).

Postnatal

Breast fed infants have a greater and more stable population of Bifidobacteria that is beneficial to immune development (Collado et al., 2012; Borre et al., 2014). Skin flora such as Staphylococcus, Corynebacterium, and Propionibacterium will also begin to colonize the neonate as it begins to suckle. The bounty of Staph. epidermidis and Staph. aureus in breast-fed neonates' faeces versus formula fed infants' indicates an adventitious advantage as they are involved in lactose and galactose metabolism through the D-tagatose-6-phosphate pathway and play a part in the metabolism of milk oligosaccharides (Rodríguez, 2014; Schleifer et al., 1978; Hunt et al., 2012). Solely formula fed infants were associated with higher incidence of E. coli, C. difficile, Bacteroides and Lactobacilli and earlier introduction of non-maternal products being linked with infections (Collado et al., 2012). The microbial conquest of the baby via breast feeding also has a dynamic and interlinked role with passive immunity. Prematurity, Hospital and NICU admission also saw a spike in Clostridium species count, especially with hospital stay (Collado et al., 2012; Hartz et al., 2015). Antibiotics are the weapons we use to prevent and treat infections, but caution must be taken when doing an "airstrike" on the microbiome to wipe out the pathogenic enemies. Commensal innocent casualties are seen with depleted Bifidobacteria and Bacteroides, which we've already noted to be important for microflora stability and immune development. (Collado et al. 2012).

Neonatal Disease States

Neonatology and microbiology are interlinked with the peri- and postnatal immature immune system and gut being exposed to an abundance of microbes. Necrotising enterocolitis (NEC), short bowel syndrome (SBS), Hirschsprung's disease associated enterocolitis (HAEC) and late onset sepsis (LOS) all have preceding microbiome changes - a dysbiosis, often resulting in catastrophic consequences for the fetus. A particular knowledge chasm leaves us questioning the pathogenesis of such diseases and how to prevent them.

NEC is still a prevalent disease seen by paediatric surgeons and the neonatal intensive care unit (NICU), yet outcomes remain dismal despite advancements in medicine. It is a syndrome delineated by abdominal distension, bilious aspirates, blood stools and intramural air (pneomatosis intestinalis) on abdominal X-ray (Lissauer et al., 2015). The severity of which is classified using the Bell's staging (Lin and Stoll, 2006). A most grave gastrointestinal disease of preterm infants, affects 2-10% of very low birthweight infants (<1500g) with a mortality of 25-30% (Till et al., 2015; Lissauer et al., 2015).

Risk factors for NEC include preterm birth, formula feeding, hypoxic-ischemic insult to the gut, flawed intestinal motility and disproportional microbial colonization (Till et al., 2015; Lissauer et al., 2015; McElroy et al., 2012). Studies show that NEC does not manifest in germ-free mice and so we can deduce that the microbiome plays a role in its aetiology (Musemeche et al., 1986; Jilling et al., 2006). This corroborates the statement from the 2006 NICHD workshop on NEC research, "NEC can be thought to arise from an uncontrolled exuberant inflammatory response to bacterial colonization that characterizes the intestine of premature infant" (Gritz and Bhandari, 2015). 90% of the infants with NEC are premature and have a premature "gut barrier" and immune system (Lin and Stoll, 2006). The mature gut epithelial barrier has tight junctional complexes that permit protective mechanisms such as secretary diarrhea (Lin and Stoll, 2006). When underdeveloped, pathogens and toxins such as LPS are left interacting with the premature mucousa (Neu, 2014). The interaction between Toll like receptor 4 and lipopolysaccharides from gram negatives (such as Proteobacteria e.g. Klebsiella, E. coli) allows translocation to take place and promotes the inflammatory cascade

Figure 3. Influences on the Neonatal Microbiome.

that we see in NEC (McElroy et al., 2012; Neu, 2014). Whether it is a "top-down" or "bottom-up" hypothesis that occurs in coagulation necrosis at the mucosa in NEC is yet to be elucidated but a combination of both is probable (McElroy et al., 2012). Morrow et al. demonstrated that a week and <72 hours preceding a NEC diagnosis, 11 patients (Total n=35 preterm infants, <29 weeks Gestational age and <1,200 g) had an overabundance of Proteobacteria (Enterobacter and Escherichia) and decreased Firmicutes (Enterococcus and Staphylococcus) and diversity (Morrow et al., 2013). Bacteroidetes and Actinobacteria were minimally detected (Morrow et al., 2013).

Short bowel syndrome is caused by a number of paediatric surgical conditions from NEC, congenital defects, volvulus or anything that may result in resection of the intestine (Till et al., 2015). Lactobacillus overgrowth and limited Clostriudium leptum, Clostridium coccoides and Bacteroidtes was found in a small study of these patients (Till et al., 2015). Lactobacillus, a facultative anaerobe, may be an advantageous adaption as it can ferment carbohydrates (Till et al., 2015). Dysbiosis seems to be related more to parenteral nutrition in SBS with Enggstran et al. finding an overgrowth of Enterobacteriaceae in these patients (Lilja et al., 2015). Small sample sizes of SBS patients with

dissimilar profiles results in difficulties in standardizing and treating these patients.

Congenital segmental absence of the enteral nervous system results in intestinal obstruction and is treated with surgical resection (Till et al., 2015). Yet, 40% of this cohort still suffers from HAEC. Clostridium difficile is postulated to play a role, along with Proteobacteria and Firmicutes (Till et al., 2015). Faecal bacterial diversity was much greater than fungal with an overabundance of Candida species and decreasesd Saccharomyces and Malassezia in a trial by Frykman et al. comparing of 9 children with HAEC and 9 without (Till et al., 2015; Frykman et al., 2015).

NEC, SBS and HAEC all have complex and dynamic pathogeneses between the microbiome and immune and vascular systems but a similar theme is emerging - an abundance of Proteobacteria is documented in all (Till et al., 2015).

Leverage & Negotiations

Leveraging the populace of the microbiome to work in our favour could prevent not only neonatal disease states but adult diseases. Probiotics, prebiotics and successful FMT are currently being investigated in adults and neonates to alter the disease states (Sanz, 2011; Ly et al., 2011; Indrio and Neu, 2011; Gibson et al., 2004; Donovan et al., 2012; Brandt, 2012). Gibson et al. defines probiotics as "microbial food supplements that beneficially affect the host by improving its intestinal microbial balance, have been used to change the composition of colonie microbiota" (Gibson et al., 2004). Prebiotics are "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" (Gibson et al., 2004). Meta-analyses of their implementation argue strongly in favour of their use in preventing dysbiotic related disease (Aceti et al., 2015), yet more studies need to be done to corroborate findings as regards specific probiotic strains, dosage, duration and target population. Bifidobacteria species has been seen to reduce the rates of NEC (Aceti et al., 2015). However in Extremely low birth weight infants (<1000g), these probiotic effects make no difference (Aceti et al., 2015). Natural probiotics as we have seen from breast milk have a lower risk of NEC compared with formula fed infants (Aceti et al., 2015). No adverse effects were reported but we need to clarify the effects of probiotics in specific groups. In the wake of the prevalence of allergy and autoimmunity in the developed world, employing the use of probiotics may curb this rate (Collado et al., 2012). Prebiotics in maternal milk such as oligosaccharides, glycoproteins, glycolipids, glycoaminoglycans have a "bifidoegnic effect" and encourage a genetically similar and balanced microbiome in the neonate (Sherman, 2010). The glycans additionally protect the gut from microbe binding, detoxify the gut, dampen inflammation and aid development of innate immunity (Sherman, 2010). FMT which has a 90%+ cure rate could also be employed for specific SBS patients with dysbiosis (Lilja et al., 2015). The incidence of severe side effects such as inoculation with a donor infection is low; however, prudent medical and ethical consideration of the children and their health status must be taken into account before this is attempted (Lilja et al., 2015).

Conclusion

The reason I wanted to study medicine was because one day my obnoxious 4-year-old self refused to eat Weetabix and yoghurt, a prebiotic, when my father was trying to nurse me back to health. Exasperated, he told me an elaborate story of the "baddies" that had made me sick. The yoghurt and the Weetabix were teaming with the good guys, James Bond-esque figures, who would seek out and destroy those who dared cause illness to me. Essentially, I was inspired to take arms in the microbiome war. When we think that we are possibly 1% genetically human, it's quite difficult to ignore our ecological environment. Future studies, this author would recommend, should look to establish the concrete relationships between the immunological system and the microbiome. Specific biomarkers of disease states, in particular in relation to NEC and sepsis in neonatology could be developed to detect and treat diseases early so as to prevent morbidity, mortality and parental psychological/emotional pain. 20% of NICU costs in the United States, (i.e. several billion dollars), are attributable to just NEC. A bit of probiotic spilt milk to prevent this is nothing to cry over. Preventative medicine and non-operative treatment of these dysbiotic diseases would revolutionize neonatology, paediatrics, and long-term outcomes.

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