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29. MICROBIAL METABOLISM AND MAMMALIAN PHYSIOLOGY: ONE INTEGRATED SYSTEM

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UNEXPECTED BEGINNINGS: THE HUMAN MICROBIOME IN PREGNANCY AND FOETAL DEVELOPMENT

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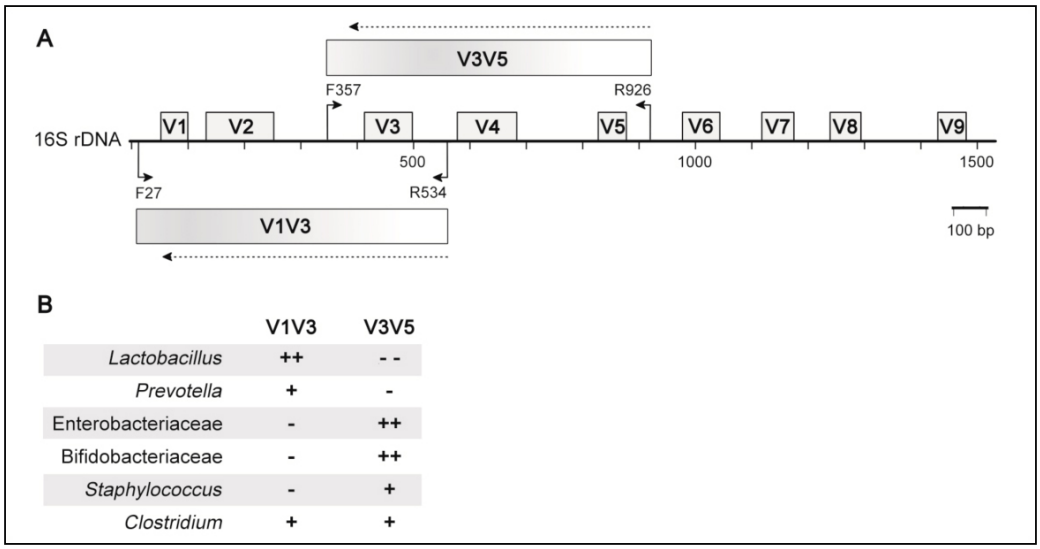
INTRODUCTION

Completed in 2012, the Human Microbiome Project (HMP) characterized the microbiome composition of multiple body sites in healthy individuals of different ethnicities located in two separate cities in the United States. Together with the European MetaHIT metagenomics of the human intestinal tract, these landmark studies document bacterial diversity, niche specificity, and microbial gene carriage patterns far exceeding what was initially suspected (*Human Microbiome Project*, 2012a,b,c; *Aagaard* et al., 2012a,b; *Huse* et al. 2012; *Gevers* et al., 2012; *Li* et al., 2012). In the interval prior and the interval since, associations between "dysbiotic" (or variation among) microbiota and disease have been suggested for obesity, type II diabetes mellitus, ulcerative colitis, Crohn's disease, and colorectal cancer (*Mangin* et al., 2004; *Ley* et al., 2005; *Gophna* et al., 2006; *Manichanh* et al., 2006; *Turnbaugh* et al., 2006; *Bäckhed* et al., 2007; *Cani* et al., 2007; *Turnbaugh* et al., 2008, 2009; *Willing* et al., 2009; *Larsen* et al., 2010; *Schwiertz* et al., 2010; *Wu* et al., 2010; *Joossens* et al., 2011; *Lepage* et al., 2011; *Marchesi* et al., 2011; *Sobhani* et al., 2011; *Qin* et al., 2012; *Wang* et al., 2012; *Devaraj* et al., 2013). However, causation has yet to be established, and a multitude of other aetiologies for these common complex disorders have been suggested over the decades (*i.e.,* human single nucleotide polymorphisms and structural genomic variation, as well as epigenomic regulation and dysregulation). It is thus critically important to first discriminate when in the course of the lifespan there is normal and anticipated variation in the human microbiome, in which body niches such variation occur in, and what other co-variates (such as host disease-susceptible genotype, host metabolic milieu and associated disorders, as well as age, gender, race/ethnicity, medications and diet) may contribute to any observed variation.

DOES THE MICROBIOME VARY DURING THE COURSE OF PREGNANCY?

Our laboratory and others have demonstrated that the vaginal microbiota vary during the course of normal pregnancy, thus providing a unique "signature" in



	V1V3	V3V5
Lactobacillus	++	- -
Prevotella	+	-
Enterobacteriaceae	-	++
Bifidobacteriaceae	-	++
Staphylococcus	-	+
Clostridium	+	+

Figure 1: The 16S rRNA gene is an ideal target for classifying bacteria.
(A) The 16S rRNA gene of bacteria contains nine hypervariable regions (V1-V9) that are flanked by conserved regions, which makes this gene an ideal target for PCR amplification and bacterial classification. The V1V3 and V3V5 primers sets utilized by the HMP consortium are outlined. The dotted line indicates the direction of amplification.
(B) Advantages and disadvantages to characterizing the vaginal microbiome with V1V3 (includes V2) and V3V5 (includes V4) primer sets. Species identification of *Lactobacillus* is enabled using a V1V3 primer set; however, V3V5 primers sets are better suited to identify Enterobacteriaceae and Bifidobacteriaceae. Thus, experimental design is essential when examining the pregnant microbiome.

pregnancy with relative altered abundance of multiple taxa (*Romero* et al., 2014; *Aagaard* et al., 2012b). But what impact does this have on either the pregnancy or the developing infant? Although it has long been suggested that intra-uterine infections, such as chorioamnionitis, are the sequelae of ascending microbiota from the upper vaginal tract (*Gonçalves* et al., 2002), the evidence supporting this are relatively sparse (Table 1). Moreover, evidence that the development of highly morbid neonatal conditions, such as neonatal sepsis and necrotizing enterocolitis, are potentially attributable to anatomical displacement of these flora with subsequent inflammation and neonatal acquisition is not actually consistent with respect to representative microbiota (*Claud* and *Walker*, 2001; *Guthrie* et al., 2003; *Yee* et al., 2012). In this dawning era of metagenomic medicine and science we are questioning the notion of anatomically adjacent microbial migration, and are coming to appreciate that many so-called "sterile" niches - notably in and among the female reproductive tract (such as the placenta) - may function as active low biomass ecologic niches which harbour unique microbiomes. These early observations challenge not only our assumed notions of "from whenst and where" our earliest microbiomes are colonized (or seeded), but our concepts of inflammatory mediators, reproductive immunity, and whether microbes in such niches may constitute more friend than foe. This leads to a number of reflections on the rather unexpected beginnings of the neonatal and infant microbiome, which are themselves relatively low biomass microbiomes.

HOW DO WE DETECT LOW BIOMASS MICROBIOMES?

16S-based metagenomics

Sequencing of the 16S rRNA gene using Next-Gen technology has recently been widely exploited to characterize the human microbiome (*Jonasson* et al., 2007; *Liu* et al., 2007). The 16S rRNA gene is an ideal target to classify bacteria due to the nine hypervariable regions in this gene that can be used to distinguish species based on individual nucleotide polymorphisms (Figure 1A; *Klindworth* et al., 2013). However, 16S-amplicon based approaches are limited to a shorter read length as compared to Sanger sequencing, and as a result, only a few hypervariable regions can be contiguously sequenced at a time. Initial work on approach and validation by the HMP Consortium demonstrated that there is variation in the taxonomy profile identified based on sequencing different variable regions. For example, V1V3 amplicons may underestimate *Acinetobacter* and *Escherichia* genera, but V3V5 provides both breadth and depth of communities dominated by these genera. Furthermore, V6V9 may underestimate *Bacteroides* but provides good coverage for *Pseudomonas* and *Escherichia* (*Human Microbiome Project*, 2012b). As a result, the resultant microbiome profile of any given body site will have subtle variations by virtue of the primer set used.

For example, comparison of the vaginal microbiome data from the HMP reveals that V1V3 will distinguish communities primarily by the relative predominant *Lactobacillus* species present, while V3V5 amplicons will reveal either lactobacilli-dominant or lactobacilli-diminished groups. Furthermore, the number of *Lactobacillus* species detected (and thus relative abundance) will vary depending on the 16S region sequenced, with V1V3 revealing more unique *Lactobacillus* operational taxonomic units (OTUs) as compared to V3V5 (*Huse* et al., 2012). However, unlike V3V5, V1V3 does not fully discriminate *Enterobacteriaceae* family projections (including *Escherichia* and *Proteus)*, and some genera (including *Staphylococcus)* (*Fettweis* et al., 2012) (Figure 1B). Few studies have directly compared the vaginal communities described by V6, V7, V8 and V9 to other primer sets, though a number of studies have utilized these regions with good coverage (*Hummelen* et al., 2010; *Ghartey* et al., 2014). Taken together, these data emphasize the need for prudent consideration when choosing primer sets for sequencing with appreciation of both the body site to be characterized, the reference data set to be compared to, and limitations when comparing to other published findings.

Whole genome shotgun (WGS) based metagenomics

Although 16S sequencing is a cost effective approach to perform metagenomics studies, WGS sequencing allows for deep and refined taxonomic classification to the species and strain level, as well as the capacity to capture total gene content and metabolic capacity (*Butler* et al., 2008; *Qin* et al., 2012; *Liu* et al., 2012; *Morgan* et al., 2012; *Aagaard* et al., 2014). However, the sheer volume of data generated by this approach poses significant bioinformatics challenges (*Prakash* and *Taylor*, 2012). Upon receiving sequence reads, quality filtering is performed to remove human contamination, which consists of more than 90% of reads in vaginal or placental samples.(*Human Microbiome Project*, 2012c; *Aagaard* et al., 2014) To get species assignment to provide potential gene expression information, sequence reads are first assembled into

Table 1: Metagenomic studies pertaining to perinatal health

Authors	Year	Site	Technique(s)	Primers utilized	Study Design	Findings
Non-gravid vaginal studies						
NIH HMP Consortium	2012	skin, nares, oral vagina	next-gen sequencing	V1V3 V3V5	longitudinal	characterized healthy reference population
Ravel et al.	2010	mid-vagina (self-collected)	next-gen sequencing	V1V2	cross-sectional	characterized healthy, non- gravid vaginal microbiome
Gajer et al.	2012	mid-vagina (self-collected)	next-gen sequencing	V1V2	longitudinal	demonstrated temporal dynamics of the vaginal microbiome
Macklaim et al.	2013	vagina	meta-transcriptomics		cross-sectional	demonstrated potential or meta-transcriptomics on vaginal swabs
Gravid vaginal studies						
Aagaard et al.	2012	vaginal introitus, posterior fornix, and mid-vagina	next-gen sequencing	V3V5	cross-sectional	characterized healthy, gravid vaginal microbiome
Romero et al.	2014	posterior fornix	next-gen sequencing	V1V2	longitudinal	characterized healthy, gravid vaginal microbiome throughout pregnancy
Walther-António et al.	2014	posterior fornix cervix	next-gen sequencing	V3V5	longitudinal	characterized healthy, gravid vaginal microbiome throughout pregnancy
Beyond the vagina: intestinal microbiome						
Koren et al.	2012	stool	sequencing	V1V2	longitudinal	characterized 1st and 3rd trimester stool
Beyond the vagina: the placenta						
Aagaard et al.	2014	placenta	next-gen sequencing	V1V3 and WGS	population based cross-sectional	the placenta harbours a unique micro-biome profile, most akin to the oral microbiome and varies by virtue of preterm birth and a remote history of antenatal infection

Table 1 (continued): Metagenomic studies pertaining to perinatal health

Authors	Year	Site	Technique(s)	Primers utilized	Study design	Findings
Beyond the vagina: neonatal studies						
Schultz et al.	2004	stool	sequencing	strain specific	longitudinal	vertical transmission from mother to infant
Palmer et al.	2007	stool	sequencing microarray, PCR	universal 16S rRNA	longitudinal	characterized healthy neonatal microbiome
Dominiguez-Bello et al.	2010	oral, vagina skin, rectum	next-gen sequencing	V2	cross-sectional	characterized neonatal microbiome by mode of delivery
Koenig et al.	2011	stool	next-gen sequencing	V1V2	longitudinal	characterized the intestinal microbiome from birth to 2.5 years
Jost et al.	2012	stool	sequencing	Sanger, V5V6	longitudinal	characterized healthy neonatal microbiome
Wang et al.	2013	cord blood amniotic fluid	bacterial culture and sequencing	universal 16S rRNA	cross-sectional	neonates with necrotizing colitis had predominantly one bacteria dominating
Milisavljevic et al.	2013	gastro-esophageal	sequencing	universal 16S rRNA	longitudinal	characterized the microbiome in VLBW infants
Azad et al.	2013	stool	next-gen sequencing	V5, V6, V7	longitudinal	characterized the neonatal microbiome from birth to 4 months while examining mode of delivery and feeding
Rogier et al.	2014	stool	microarray		murine	examined the role of maternal IgA on intestinal microbiome
Ma et al.	2014	colon, anus, stool	next-gen sequencing	V3V5	non-human primate	examined the role of maternal diet on juvenile microbiome

contigs, or genes. This is challenging due to the lack of reference genomes, which results in *de novo* assembly of microbial genomes with the potential to distort the species abundance and generate chimeric genomes (*Pop*, 2009). Following assembly, gene prediction is then possible by analyzing molecular characteristics of existing open reading frames of sequenced genomes (*Zhu* et al., 2010). Given the complexity of assembly and low efficiency, taxonomy classification can also be achieved by alignment of sequence reads to clade-specific markers identified from Integrated Microbial Genomes (IMG) without prior assembly (*Segata* et al., 2012). The reconstruction of functional profile is achieved by mapping reads onto pathway collections such as Kyoto Encyclopedia of Genes and Genomes (KEGG), with additional interference steps for pathway coverage and abundance (*Abubucker* et al., 2012). Further, webtools have recently been developed to perform the tasks described above to facilitate the analysis of WGS data (*Glass* et al., 2010). However, regardless of approach, WGS and 16S sequencing are limited to describing community composition and its potential metabolic or functional capability.

WHAT MICROBES COMMENSALLY COLONIZE THE NEONATE?

Recently, the establishment of the neonatal microbiome has been the subject of debate and investigation. When directly examining the intestinal microbiome of infants born via caesarean or vaginal delivery, culture-based microbiological techniques have demonstrated differences in the colonization of the neonatal intestinal microbiome, particularly of *Bifidobacterium*-like bacteria, *Lactobacillus*-like bacteria, and *Bacteriodes fragilis* (*Grönlund* et al., 1999a). However, a separate study by this group also determined that bacterial enzymes were not altered in the stool of infants based on mode of delivery (*Grönlund* et al., 1999b). Using culture-independent, PCR-based techniques, Penders *et al.,* also showed that *B. fragilis* and *Bifidobacterium* were decreased in caesarean delivered infants in comparison to vaginally delivered infants. However, this study also demonstrated differences in the intestinal microbiome of infants based on formula-feeding or breastfeeding (*Penders* et al., 2006). An Italian study further investigated the intestinal microbiome of infants based on mode of delivery using the V6V8 region of the 16S rRNA gene in PCR-DGGE and PCR-temperature gradient gel electrophoresis (TGGE) assays (*Biasucci* et al., 2008). This group determined that infants born vaginally had increased diversity in their intestinal microbiome when compared to caesarean delivered infants. Again, infants born by caesarean delivery appeared to have an absence of *Bifidobacterium* in their intestinal microbiome (*Biasucci* et al., 2008).

Which microbiota first populate the infant?

Further studies into the establishment of the neonatal microbiome highlight this principle. Jost *et al.,* demonstrated that the intestinal microbiome of infants born vaginally and exclusively breastfed decrease the amount of the Firmicutes phylum, of which *Lactobacillus* belongs, over time while increasing the prevalence of *Bacteroides* species (*Jost* et al., 2012). Thus, although these infants were born

vaginally, a high presence of Firmicutes did not persist. Intriguingly, these vaginally delivered infants could be classified into two cohorts in this study: those that had species of *Bacteriodes* present in their intestinal microbiome and those that did not (*Jost* et al., 2012), and this trend was also seen in a separate study (*Palmer* et al., 2007). This is in contrast to previous studies finding that *Bacteroides* relative abundance differed based on mode of delivery (*Grönlund* et al., 1999a; *Penders* et al., 2006). However, Palmer *et al.* also determined that variations in the *Bacteroides* species seen early in neonatal life were less variant and more consistent in the abundance by one year of age. Additionally, the neonatal intestinal microbiome also appeared more adult-like near the end of the first year of life (*Palmer* et al., 2007). This finding was confirmed in a study by Koenig *et al.,* in which the intestinal microbiome of an infant was monitored for 2.5 years. Here, the authors found that the diversity of the intestinal microbiome increased over time and with the introduction of foods (*Koenig* et al., 2011). Also, *Bacteroides* species were found to increase upon the introduction of vegetables (*Koenig* et al., 2011). Altogether, this data indicate that the neonatal microbiome is highly variable within the first year of life. Therefore, various exposures during this time may have a significant impact on the developing microbiome.

Is it vertical or horizontal transmission of microbes that populate the infant at birth?

An initial study by Schultz and colleagues examined the vertical transmission of a probiotic, *L. rhamnosus* GG, from mother to infant (*Schultz* et al., 2004). Gravid women took the probiotic twice daily from 30 to 36 weeks of gestation. The authors found that all infants born vaginally (4/4) contained this probiotic in their stool while the probiotic was only detected in the stool of half of the caesarean delivered infants (1/2) (*Schultz* et al., 2004). This study demonstrated that vertical transmission did occur between mother and infant. However, this study included a small subject number and did not examine the maternal stool postpartum, which would be relevant to determine the persistence of the probiotic in the maternal microbiome that would provide an opportunity for horizontal transmission. The maternal microbiome during infancy, and not solely delivery mode, may be an important factor in establishing the neonatal microbiome. In fact, a recent study examining the establishment of the intestinal microbiome of healthy, term neonates using qPCR found that bacterial loads between maternal and infant stool were remarkably similar (*Jost* et al., 2012). This study demonstrates that the maternal microbiome at birth and postpartum may be critical in the establishment and development of the neonatal microbiome via horizontal transmission. Although it is unclear if the health and microbiome of the offspring is persistently influenced by mode of delivery, gestational age at delivery appears to be the greater arbiter for the developing microbiome. For instance, differences in the intestinal microbiome of preterm and term neonates has been described (*Schwiertz* et al., 2003). In this study, the authors found that healthy, term neonates that were breast fed had increased diversity in their intestinal microbiome when compared to hospitalized, preterm infants using PCR-DGGE analysis (*Schwiertz* et al., 2003), and additional studies have confirmed that the microbiome of infants with conditions like PTB, very low birth weight, or necrotizing enterocolitis, also have an altered microbiome

(*Schwiertz* et al., 2003; *Hällström* et al., 2004; *Milisavljevic* et al., 2013; *Wang* et al., 2013). However, it is unclear if these alterations are due to early gestational age at delivery or hospitalization since neonatal exposure in early life is pertinent to the establishment of the microbiome.

Does it matter how the infant was delivered?

To date, few studies on this issue regarding mode of delivery have used Next-Gen sequencing techniques. An initial study was performed in Venezuela with nine gravid subjects total, four giving vaginal birth and five having a caesarean delivery (*Dominguez-Bello* et al., 2010). This study used the V2 region of the 16S rRNA gene and demonstrated that the infant microbiome most closely resembled the mothers' vaginal microbiome following vaginal delivery. Similarly, if the infant was born by caesarean delivery, the infant microbiome most closely resembled the skin microbiome. Furthermore, a Canadian study using Next-Gen sequencing techniques for the V5, V6, and V7 regions of the 16S rRNA gene demonstrated that *Escherichia shigella* and *Bacteroides* were significantly diminished in the intestinal microbiome of infants born by caesarean-section (*Azad* et al., 2013). This study also found that infants with the highest species richness and diversity of their intestinal microbiome were born by emergency caesarean-section rather than by an elective caesarean-section or vaginally. However, when followed out to four months postpartum, infants' intestinal microbiome could be differentiated primarily based on mode of feeding. Specifically, infants that were formula-fed had a higher prevalence of Peptostreptococcaceae and Verrucomicrobiaceae when compared to breastfed infants (*Azad* et al., 2013). Thus, mode of feeding may be more crucial than mode of delivery in regards to the long-term establishment of the microbiome. These findings suggest that the establishment of a stable microbiome is not only a question of what bacteria are present at birth, but also what factors, either host-derived or environmental, influence the species that persist.

Furthermore, these exposures at delivery and early life may have lasting effects on the microbiome (*Ma* et al., 2014b; *Ding* and *Schloss*, 2014). For instance, a recent study demonstrated that breastfeeding has an impact in the long-term enterotype of an individual (*Ding* and *Schloss*, 2014). This study is bolstered by murine studies suggesting that maternal antibodies transferred via breast milk may have a persistent impact on the intestinal microbiome (*Rogier* et al., 2014). However, retrospective analysis of the literature determined that mode of delivery may influence obesity in adulthood (*Darmasseelane* et al., 2014). Thus, these aforementioned associations with mode of feeding and delivery may be attributable to differences in the seeding of the neonatal microbiome. Therefore, future studies are needed to entail how and why microbes remain in distinct body sites. In other words, it may not be a question as to who is there and from whenst and where do they arise, but rather why do certain microbes take up and retain residence. As the maternal diet and microbiome has been shown to influence the establishment and development of the infant microbiome, these future studies may reveal early mechanisms of adult metabolic disorders, which may allow for the early treatment and/or prevention of associated diseases.

IS THE VAGINAL MICROBIOME A LIKELY FIRST SOURCE OF SEEDING THE NEXT GENERATIONS MICROBIOME?

As previously overviewed, it has been suggested by many, based on a paucity of data, that the infant microbiome is seeded at the time of vaginal birth (Table 1). Such a supposition would necessitate thorough understanding of the vaginal microbiome during pregnancy, as well as an appreciation for the vaginal microbiome constituent variation across gestational age.

Characterizing the vaginal microbiome

Prior to the advent of Next-Gen sequencing, the characterization of the vaginal microbiome through traditional microbiological techniques (culture-dependent) revealed a predominance of *Lactobacillus* species (*Redondo-Lopez* et al., 1990; *Larsen* and *Monif*, 2001). These early characterizations of the vaginal microbiome resulted in the delineation of "normal" flora (defined as *Lactobacillus* predominant), and "abnormal" or "aberrant" vaginal flora (non-lactobacillus predominant). Early descriptions also attempted to characterize abnormal vaginal flora, most notably in conjunction with bacterial vaginosis (BV). BV is a common and complex alteration of vaginal flora, but the description of the bacteria involved in the vaginal dysbiosis has changed over time. The association between anaerobic cocci and abnormal vaginal discharge was first described by Curtis in 1914, and *Gardnerella vaginalis* was first described as a causative agent for BV in 1955 (*Eschenbach*, 1993; *Ledger*, 1993). However, by the 1990s, multiple other species were found in anaerobic cultures of vaginal discharge from subjects with symptoms consistent with BV (*Faro* et al., 1993; *Hillier* et al., 1993). Despite the multiplicity of causitave agents, one common finding is that women experiencing clinically symptomatic BV tend to be deficient in lactic acid producing species of bacteria that also convert oxygen to H_2O_2 (*Eschenbach* et al., 1989; *Hillier* et al., 1992, 1993). This observation has led to several decades of data demonstrating that adverse reproductive health outcomes accompany "abnormal flora" associated with BV (*Gravett* et al., 1986; *Martius* et al., 1988; *Krohn* et al., 1991; *Hillier* et al., 1995a; *Martin* et al., 1999; *Wiesenfeld* et al., 2003; *Ness* et al., 2005; *Brotman* et al., 2010). For instance, incidence of BV has been associated with preterm birth and with increased risk for acquiring sexually transmitted diseases, such as *Neisseria gonorrhea, Chlamydia trachomatis,* and human immunodeficiency virus (HIV) (*Gravett* et al., 1986; *Martius* et al., 1988; *Krohn* et al., 1991; *Kurki* et al., 1992; *Hillier* et al., 1995b; *Martin* et al., 1999; *Wiesenfeld* et al., 2003; *Ness* et al., 2005; *Brotman* et al., 2010; *Perla* et al., 2012). This increased risk of infection is thought to be due to the deficiency of *Lactobacillus* species that produce lactic acid and H_2O_2 to provide protection from pathogenic bacteria and viruses (*Eschenbach* et al., 1989). Along these lines, previous *in vitro* studies have demonstrated that *L. acidophilus* can protect from infection with *G. vaginalis*, *Bacteroides bivius*, and HIV through a peroxidase dependent mechanism (and *Coombs*, 1991; *Klebanoff* et *Klebanoff* al., 1991). Thus, while BV may indicate vaginal dysbiosis in a clinical setting, there has been historically a lack of data regarding whether vaginal dysbiosis occurred as "normal" flora in women asymptomatic for BV. Currently, Next-Gen sequencing techniques have enabled the vaginal

microbiome to be more thoroughly characterized to determine the bacterial flora of the "normal" versus "abnormal" vagina.

One of the first studies to reveal the complexity of the vaginal microbiome using Next-Gen techniques was performed by Ravel *et. al.* This study recruited nearly 400 women of mixed ethnicities. Samples were prepared for either V1V2 16S sequencing and were scored for BV using Nugent criteria (*Ravel* et al., 2010). Five distinct community state types (CSTs) were revealed, and the majority of these CSTs were dominated by species of lactobacilli. The fourth CST included women with a vaginal microbiome deficient in *Lactobacillus* species, and interestingly, this group had increased incidence of BV. Also, this study demonstrated that the vaginal microbiome could be distinguished by ethnicity. Asian subjects had a higher prevalence of CST III (*L. iners*), non-Hispanic Caucasians had a higher prevalence of CST I (*L. crispatus*), and African-American and Hispanic subjects had a higher prevalence of CST III (*L. iners*) and IV (decreased *Lactobacillus* species) (*Ravel* et al., 2010). However, recent studies have demonstrated the caution that must be utilized when performing analysis such as CSTs (*Koren* et al., 2013). In these investigations, Koren *et al.* demonstrated that these types of cluster analysis are sensitive to the distance metric used for analysis and that multiple distance metrics should be utilized to promote accuracy in data. In fact, this study utilized the data of Ravel *et al.* in their analysis and found varying support for the presence of CSTs based on the analysis used (*Ravel* et al., 2010; *Koren* et al., 2013). Thus, multiple methods of analysis must be used to establish a community state type. An additional method that may be useful to examine microbiome communities across ethnicities may be to utilize single nucleotide polymorphisms (SNPs) in mitochondrial DNA (mtDNA), which provides more precision in analysis (*Ruiz-Pesini* et al., 2007). We have recently used this method in conjunction with analysis of the microbiome through data leveraged from the HMP to examine associations between mtDNA haplotypes and microbiome communities (*Ma* et al., 2014a). While we did see similar microbiome communities as the Ravel group, our analysis has provided a molecular basis in which to describe the structure of microbiome communities.

Variation of the vaginal microbiome during and across pregnancy

With the demonstration that the vaginal microbiome fluctuates based on the menstrual cycle, with intercourse, and (to a much more limited degree) with clinical symptoms of BV, we and others sought to characterize the vaginal microbiome during pregnancy. Nearly every organ system changes during pregnancy to promote pregnancy maintenance or prepare for parturition. In the vagina, increased vascularity and hyperaemia develop in the skin of the vulva and the mucosa of the vagina. Additionally, the vaginal mucosa increases in thickness and cervical secretions increase, which causes the underlying smooth muscle cells to hypertrophy and relax the connective tissue. At the epithelial surface, the vaginal epithelium hypertrophies and causes a crowding of the epithelial cells, which are rich in glycogen (*Nieburgs*, 1947). Oestrogen (namely oestradiol) rises across gestation, and further leads to increases in glycogen levels; glycogen is metabolized into lactic acid resulting in the decrease pH (acidity) of the vagina (*Paavonen*, 1983; *Gregoire* et al., 1971). This

metabolism of glycogen into lactic acid was historically thought to be performed by the vaginal epithelium, since the vaginal lumen is sufficiently distant from the oxygen supply to become anaerobic. However, the primary source of lactic acid became debated when Boskey *et al.* reported that vaginal lactobacilli were capable of producing lactic acid *in vitro* at a rate sufficient to re-acidify the vagina *in vivo* following a neutralizing exposure (*i.e.* ejaculate) (*Boskey* et al., 1999; *Pybus* and *Onderdonk*, 1999). Afterwards, the specific lactate structures in the vagina were explored and it was discovered that the majority of the vaginal lactic acid was of the D-isoform, which cannot be produced by human metabolism (*Boskey* et al., 2001). Thus, vaginal bacteria, namely lactobacilli, appear to be the primary source of lactic acid in the vagina. In pregnancy, the preponderance of *Lactobacillus* species appears to be aided by the oestrogen-induced increase in glycogen that contributes to the vaginal acidic environment, which is not only enhanced by lactobacilli but also fosters *Lactobacillus* growth.

Prior to the advent of metagenomics, the presence of lactobacilli were noted to increase as gestational age advanced during pregnancy (*Nieburgs*, 1947). Further, more recent studies demonstrated a high prevalence of *Lactobacillus* species in the vagina during either the first, second, or third trimester using PCR-DGGE for the V3 region of the 16S rRNA gene, and the species with the highest prevalence were *L. acidophilus* and *L. iners* (*Hernández-Rodríguez* et al., 2011). The utilization of Next-Gen sequencing techniques to study the vaginal microbiome further demonstrated the presence of *Lactobacillus* species in the vagina during pregnancy. Our group initially published this 16S-based metagenomic characterization in a cross-sectional study employing V3V5 16S metagenomics (*Aagaard* et al., 2012b). When compared to the HMP non-pregnant reference subjects, we found that pregnancy was associated with an altered vaginal microbiome and marked by a decrease in alpha diversity at the subgenus level (*Aagaard* et al., 2012b) (Figure 2). When we examined this phenomenon further, we discovered that there was an overall enrichment of the orders Lactobacillus, Clostridiales, Bacteriodales, and Actinomycetales. And upon probing at the species level using supervised machine learning approaches, such as linear discriminate analysis (LDA) effect size (LEfSe) and Boruta feature selection, we discovered that the vaginal microbiome during pregnancy was enriched in *L. iners, L. crispatus, L. jensenii,* and *L. johnsonii* (*Aagaard* et al., 2012b). The increase in lactobacilli may be explained by the increase in oestrogen that occurs during pregnancy. However, direct associations between specific species of *Lactobacillus* and oestrogen levels are lacking and warrant further investigation.

However, the aforementioned studies were cross-sectional, which allows for the characterization of a pregnancy and gestational age-common microbiome signature, but lacks capacity for description of the dynamic changes which may occur in an individual over time. Longitudinal analysis of the vaginal microbiome in a small cohort of women across gestation has been performed using terminal restriction fragment length polymorphism (tRFLP) in 100 gravid women (*Verstraelen* et al., 2009). This study categorized gravidae into two cohorts during the first trimester: lactobacilli-dominant and lactobacilli-diminished. Interestingly, only 16.9% of gravidae that had a lactobacilli-dominant vaginal microbiome in

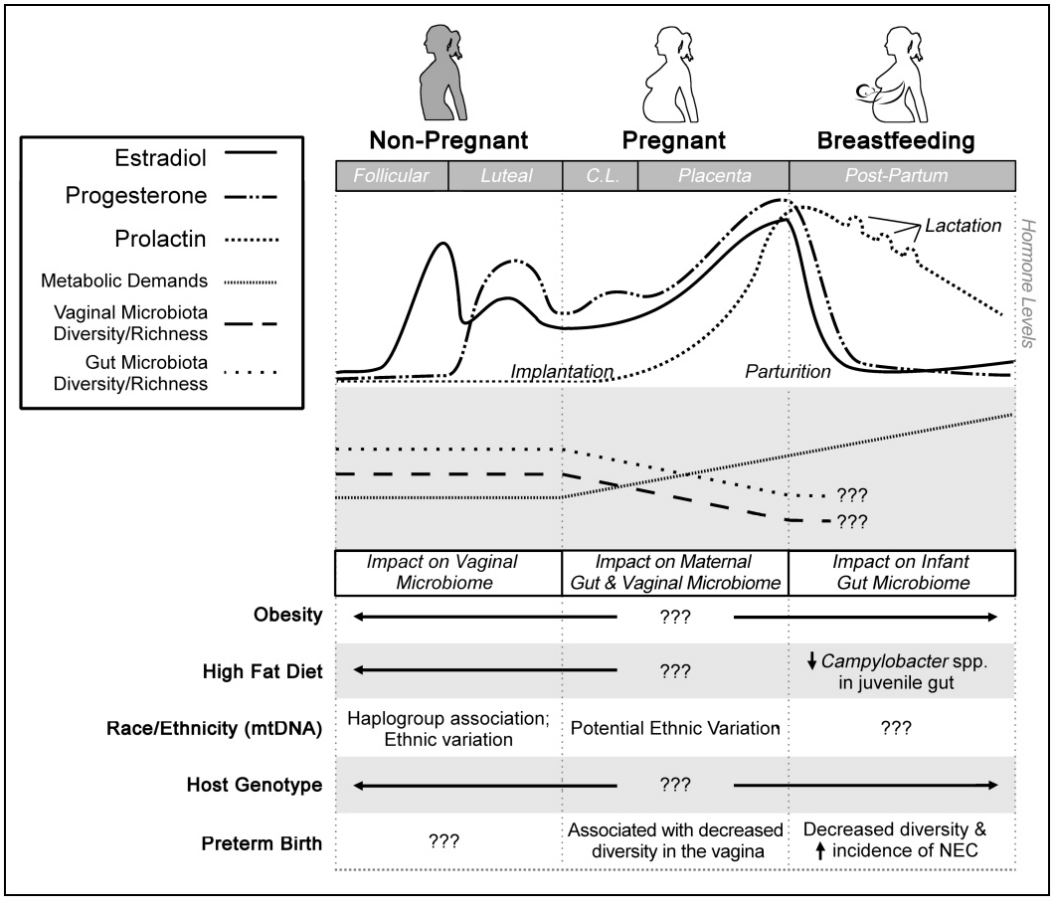


Figure 2: Potential influences on the microbiome during early development.
A number of hormonal changes, environmental exposures and genetic differences may impact the maternal microbiome before and during pregnancy that may alter the developing neonatal microbiome**.** During pregnancy, the maternal intestinal and vaginal microbiome have reduced alpha diversity and species richness. Metabolic demands increase throughout pregnancy and after parturition as the mother is lactating. Oestradiol, progesterone and prolactin levels gradually increase during pregnancy, though it is unclear how these changes affect the maternal microbiome. Increased oestrogen raises glycogen production in the vagina, but how the availability of this substrate structures the vaginal microbiome is unknown. The effect of host genetics on the maternal microbiome throughout pregnancy is relatively unknown. Different ethnicities, which can be inferred by mitochondrial DNA (mtDNA) haplotypes, have been shown to have varied vaginal microbiomes before and after pregnancy. Further studies are needed to understand these differences, and to explore the effect of host genotype on the maternal microbiome. The impact of diet and obesity on the pregnant microbiome is just beginning to be explored. A primate model of maternal high fat diet demonstrated that diet alone can persistently alter the juvenile microbiome at one year of age regardless of juvenile diet. However, how diet alters the maternal environment during pregnancy and how this affects the vertical transmission of bacteria is unknown.

the first trimester decreased the prevalence of lactobacilli during pregnancy. On the other hand, 56.5% of gravidae with a lactobacilli-diminished vaginal microbiome during the first trimester gained prevalence of this genus. In contrast to the cross-sectional study, these longitudinal cohorts demonstrated that *L. crispatus* dominated during pregnancy and that prominence of *L.*

gasseri and/or *L. iners* during the first trimester may be associated with diminished lactobacilli as pregnancy progressed (*Verstraelen* et al., 2009). Recently, Romero *et al.* has performed longitudinal studies of the posterior fornix of the vaginal microbiome during pregnancy using Next-Gen sequencing of the V1V2 region of the 16S rRNA amplicon (*Romero* et al., 2014). These studies utilized the self-collection of vaginal samples over 16 weeks of the non-pregnant cohort and swabbing of the posterior fornix at four prenatal visits from the gravid cohort with 22 subjects. Additionally, the gravid cohort consisted of mainly African-American ethnicity. In their analysis, the authors used the CSTs established by Ravel *et al.* to interrogate the vaginal microbiome during pregnancy (*Ravel* et al., 2010; *Romero* et al., 2014). This study concluded that the vaginal microbiome of gravid women mostly consisted of the CST I or III with odds ratios of 2.986 and 2.136, respectively, and that the vaginal microbiome of gravid women shift toward these two CSTs as their pregnancy progressed (*Romero* et al., 2014). When the stability of the vaginal microbiome was examined during pregnancy, the authors noted that the vaginal microbiome of gravid subjects shifted between CSTs dominated by lactobacilli but rarely shifted to CST IV, which is marked by a diminished abundance of *Lactobacillus* species (*Romero* et al., 2014). However, with a low number and low ethnic diversity of subjects in the study, further studies are needed to confirm these shifts of the vaginal microbiome towards CSTs dominated by lactobacilli as pregnancy progresses. Initial studies of the vaginal microbiome demonstrated that African-American women have a higher prevalence of vaginal CSTs consisting of *Lactobacillus* species (*Ravel* et al., 2010), which was further demonstrated by this recent study (*Romero* et al., 2014). In a separate study, the vaginal microbiome was examined longitudinally during gestation using the V3V5 amplicon of 16S rRNA in 12 subjects that were mostly Caucasian (Walther-António et al., 2014). In agreement with previous studies, these authors demonstrated that alpha diversity decreases as pregnancy progresses and that *L. crispatus* and *L. iners* dominate the vaginal microflora (*Walther-António* et al., 2014). (Figure 2) Intriguingly, these authors suggest that maternal age may be important for the dominance of *L. crispatus* or *L. iners,* with, *L. iners* being dominant in older gravidae (*Walther-António* et al., 2014). While this insight should be kept in mind for future studies, these study had only two subjects with advanced maternal age (34-36) (*Walther-António* et al., 2014). To take these studies further, this group attempted to analyze their data in conjunction with the Romero *et al.* study; however, differences in primer sets and sequencing platforms utilized in these separate studies prevented in depth analysis (*Walther-António et al*., 2014; *Romero* et al., 2014). Despite these challenges, the authors found that while alpha diversity of the vaginal microbiome decreased with gestational age in both African-American and Caucasian subjects, African-American subjects had increased beta diversity between gravid subjects while Caucasian gravidae did not (*Walther-António* et al., 2014). Thus, these studies highlight the need for further longitudinal studies with large subject enrolment and ethnic diversity. Additionally, studies are needed with a high enrolment of BV subjects or women with a dysbiotic vaginal microbiome to lend further insight into vaginal microbiome shifts and stability that are associated with pregnancy.

How does the vaginal microbiome change among women destined for a preterm birth?

Knowing that term and preterm infants have a variant microbiome, if it were to be solely or even largely seeded by the maternal vagina, then the preterm and term vaginal microbiome would need to vary as well. Prior to the metagenomics era, the Preterm Prediction Study examined the association between BV and PTB (*Meis* et al., 1995). In this study, vaginal specimens were obtained at 24 and 28 weeks gestation, and an association with increased risk for spontaneous PTB at less than 35 weeks gestation was found in 19.8% of women with BV at 28 weeks gestation. However, conclusions could not be drawn regarding whether BV itself was causative of PTB (*Meis* et al., 1995). Additionally, while treatment of BV during pregnancy does eradicate infection, it does not reduce the risk of PTB (*Hillier* et al., 1995b; *Brocklehurst* et al., 2013). Therefore, given the lack of benefit, screening of asymptomatic women in pregnancy is not recommended (*Nygren* et al., 2008). Even more concerning are the findings of two studies that found an increase in preterm delivery (< 34 weeks) among women who tested negative for BV but were treated (*Hauth* et al., 1995; *Vermeulen and Bruinse*, 1999). Thus, the relationship of BV and preterm birth is complicated and the benefit of treatment is questionable. These issues regarding BV and PTB warrant further investigation into these associations, and the examination of the microbiome using Next-Gen sequencing techniques will be of great utility for these studies.

A recent study by Hyman *et al.* has examined the vaginal microbiome in preterm birth. This group used a prospective cohort study with 46 high-risk (previous unexplained PTB) and 42 low-risk (all other gravid) subjects for PTB gravidae enrolled (*Hyman* et al., 2014); however, only 14 subjects were able to be sampled in each trimester. Intriguingly, the investigators found that the presence of lactobacilli did not distinguish term (> 37 weeks) from preterm (< 37 weeks) subjects using Sanger sequencing methods. However, low-risk subjects had a higher prevalence of lactobacilli when compared to high-risk subjects (*Hyman* et al., 2014). Despite the lack of association of lactobacilli with preterm birth, measured alpha diversity was reported as diminished when comparing Caucasian term and preterm subjects (*Hyman* et al., 2014). Among the two longitudinal subjects that ultimately delivered preterm, their vaginal microbiomes were dominated by *L. crispatus* (*Hyman* et al., 2014), which is in contrast to a previous study demonstrating that *L. crispatus* is dominant in healthy term pregnancies (*Verstraelen* et al., 2009; *Aagaard* et al 2012b). Moreover, while two subjects in the Hyman *et al.* study had outgrowths of *Bifidobacterium* and *Ureaplasma* genera, separately, these genera are reported in both normal pregnant subjects as well as non-pregnant (*Ravel* et al., 2010; *Aagaard* et al., 2012b; *Romero* et al., 2014). In sum, while promising, this study underscores the need for broader gestational age-specific, reference-based cohorts in order to define both the effect size and population variance. Prior to the publication of such studies, it would be premature to ascribe such microbiome profiling as indicative or heralding of preterm birth. Moreover, it does not explain the observed variation in the infants microbiome.

VARIANCE OF THE HUMAN MICROBIOME IN PREGNANCY: BEYOND THE VAGINAL COMMUNITY

Maternal intestinal variation

In addition to changes described in the vaginal microbiome during pregnancy, Koren *et al.* investigated the intestinal microbiome during pregnancy using a prospective cohort study and found that as pregnancy progressed, the intestinal microbiome was altered (*Koren* et al., 2012). This study utilized the V1V2 region of the 16S rRNA gene and found that alpha diversity was decreased between the first and third trimesters (Figure 2). Further, stool samples collected from gravidae in the first trimester clustered separately from stool samples collected in the third trimester (*Koren* et al., 2012). These differences in beta diversity were a reflection of increases in Proteobacteria in the stool during the third trimester when compared to stool from the first trimester. When faecal transplants of first and third trimester stool into germ-free mice were performed, mice receiving third trimester stool had increases in inflammatory cytokines and adiposity, similarly to faecal transplants involving obese subjects (*Turnbaugh* et al., 2006; *Koren* et al., 2012; *Ridaura* et al., 2013). These alterations in the intestinal microbiome did not associate with any other co-variates, such as body mass index (BMI), development of gestational diabetes mellitus, or multiparity (*Koren* et al., 2012).

Impact of the maternal diet

Additional alterations in the pregnant microbiome may also be regulated by diet. We have recently shown that diet is the main arbiter of the intestinal microbiome using a non-human primate model (*Ma* et al., 2014b), and a separate study demonstrated that consumption of a high fat diet during pregnancy resulted in impairment of the gut epithelial barrier integrity in a non-obese diabetic (NOD) mouse model (*Xue* et al., 2014). Intriguingly, this group found in two separate models, the NOD mouse model and a sheep model, that inflammation in the intestine of offspring was altered based on maternal diet (*Yan* et al., 2011; *Xue* et al., 2014). In fatal and postnatal life, offspring exposed to a maternal high fat diet had increases in the expression of inflammatory cytokines, Toll-like receptors (TLRs), and their respective signalling pathways (*Yan* et al., 2011; *Xue* et al., 2014). In relation to these studies, we found that maternal diet could persistently alter the intestinal microbiome of the offspring (*Ma* et al., 2014b) (Figure 2). Furthermore, these alterations in the intestinal microbiome resulted in differences in metabolic function using inferred analysis (*Ma* et al., 2014b). Thus, these aforementioned studies illustrate the need to not only expand the number of body sites across populations of women but to incorporate maternal comorbidities, such as diet, into analysis.

THE PLACENTAL MICROBIOME

The placenta has long been considered sterile in normal gestation, where the presence of bacteria in clinical cultures is diagnostic for intrauterine infection and a significant risk for PTB (*Hillier* et al., 1988). The ELGAN studies constituted a large effort that systematically identified bacteria from PTB placentas under this assumption (*Onderdonk* et al., 2008a,b; *Olomu* et al.,

2009). However, there is increasing recognition of a large discordance between the presence of bacteria as per culture-based diagnoses and clinical outcome (*Watts* et al., 1992; *Pettker* et al., 2007; *Buhimschi* et al., 2009; *Han* et al., 2009; *Leviton* et al., 2010; *Stout* et al., 2013; *Combs* et al., 2014; *Fortner* et al., 2014). In fact, the presence of placental or membrane bacteria in the absence of histological infection has been discovered repeatedly over the last few decades (*Hillier* et al., 1988; *Steel* et al., 2005; *Redline*, 2007; *Stout* et al., 2013; *Fortner* et al., 2014). This has led to the recognition of the need to study and redefine our understanding of the role of intrauterine bacteria in gestation.

One of the earliest studies to recognize the absence of pathogenicity of intrauterine bacteria was done using histological analysis. It was shown that membranes of normal pregnancies often contain bacteria, yet show no signs of histological infection (*Steel* et al., 2005). Later studies using similar methodologies discovered intracellular bacteria localized to the trophoblast of the basal plate of the maternal decidua in the absence of chorioamnionitis although there was an association with PTB (*Stout* et al., 2013; *Cao* and *Mysorekar*, 2014). Seemingly in agreement, it was also shown that intra-amnionic invasion is relatively benign in the absence of inflammation with associations between PTB and inflammation alone rather than PTB and bacterial invasion (*Combs* et al., 2014). Intriguing work by the Murtha group showed that high levels of bacteria were strongly associated with premature rupture of membranes (PPROM) and membrane thickness, though there was no histological inflammation in half of the subjects. Although there were bacteria found in subjects of all groups including PPROM (both term and pre-term), PTB, and even normal gestation control, there was no inflammation detected in the majority of subjects (*Fortner* et al., 2014). Thus, these studies suggest that it is not the occurrence of bacteria in the placenta, but the bacterial populations present that may initiate intrauterine infection.

Along these lines, a metagenomic study using a Rhesus macaque model identified over 300 microbial species in the chorioamnion and placenta (*Aagaard* et al., 2013). Interestingly, is was shown that this population was modifiable with a sterile intra-amniotic injection of IL-1ß that induced histological inflammation (*Aagaard* et al., 2013). This suggests that chorioamnionitis may be caused by microbial dysbiosis rather than the presence of bacteria per se. The potential translational implications of this model to human pregnancy was emphasized recently by our description of a vibrant and diverse commensal placental microbiome in normal pregnancies (*Aagaard* et al., 2014). Analysis of over 300 human samples using both 16S and WGS sequencing revealed a low abundance complex community dominated by the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria, found in nearly all samples. Subjects with a remote history of antenatal infection and antibiotic treatment, or who developed PTB, had discrete statistically significant groupings of taxa (*Aagaard* et al., 2014). It was also found that the placental microbiome most closely resembles the oral microbiomes of the supragingival plaque and the dorsum of the tongue; however, the placental microbiome did not closely resemble the stool or the vaginal microbiomes. This implies that the bulk of the low level placental bacteria are likely not ascending nor are contaminants of the stool or the vagina, and

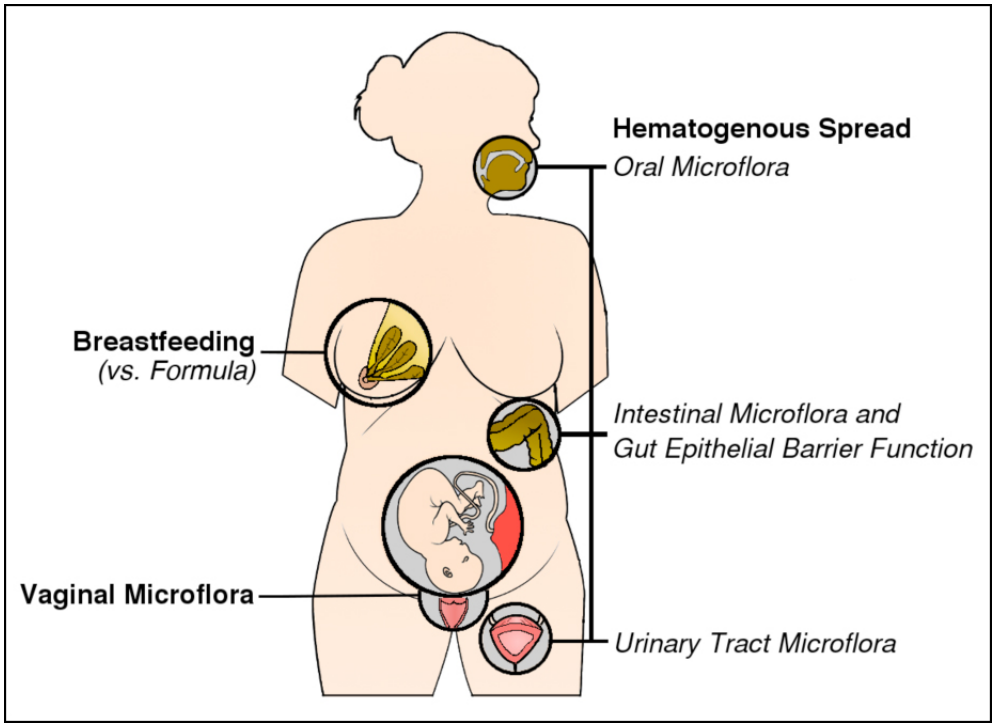


Figure 3: Speculated origins for microbiota colonizing the placenta and seeding the initial neonatal microbiome.
Vaginal microflora likely contribute to the initial seeding of the neonatal microbiome during vaginal deliveries, but the discovery that the uterine environment may not be sterile suggests that colonization of the infant may happen before birth. Recent data demonstrating that the placenta has its own unique microbiome most closely resembling the oral microbiome suggests a potential haematogenous route by which bacteria can seed the placenta and the developing foetus. Microbiota from maternal oral, vaginal, urinary tract and intestine are all potential sources for these colonizing bacteria. Microbiota from breast milk may be an important source of commensal bacteria during early infancy and must be considered when studying the microflora of the neonate.

instead, are quite possibly seeded largely from the oral cavity through haematogenous spread (Figure 3).

The entering of oral microbes into the blood stream as a result of periodontitis or dental procedures is well established (*Han* and *Wang*, 2013), and it has been known for decades that periodontal disease is linked to PTB (*Offenbacher* et al., 1996, 2009; *Goldenberg* et al., 2000; *Michalowicz* et al., 2006; *Macones* et al., 2010). Animal models have demonstrated that bacteria may be spread haematogenously to the placenta (*Han* et al., 2004; *Fardini* et al., 2010), and reports have suggested that oral bacteria may be associated with pregnancy complications (*Katz* et al., 2009; *Han* et al., 2010; *Swati* et al., 2012). *Fusobacterium nucleatum* is an oral pathogen that is frequently found in diagnostic cultures following PTB, PPROM, and stillbirth (*Romero* et al., 1989; *Watts* et al., 1992; *Han* et al., 2004, 2010; *Cahill* et al., 2005). Our finding of Fusobacteria to be a relatively abundant taxon in the placenta supports the hypothesis of haematogenous spread from the oral cavity to the placenta (*Aagaard* et al., 2014), and this theory is further buttressed by the finding of bacteria in

cord blood of normal pregnancies (*Jiménez* et al., 2005). Along these lines, bacteria can spread from the intra-amnion outward to the chorion, which indicates that haematogenous spread from mother to infant may be occurring via cord blood (*Kim* et al., 2009). Thus, haematogenous spread may promote colonization of the placenta and the foetus, but further studies are needed to examine this phenomenon. However, a potential mechanism to compromise the maternal-foetal barrier has been illuminated. In a mechanism akin to *Listeria monocytogenes* (*Bakardjiev* et al., 2006; *Le Monnier* et al., 2007)*, F. nucleatum* expresses adhesin FadA that interacts with E-cadherin, which compromises cell-cell adhesion and membranes (*Lecuit* et al., 2004; *Ikegami* et al., 2009; *Fardini* et al., 2011). However, further studies are necessary to demonstrate definitively that haematogenous spread from the oral cavity is possible for colonization or infection of the placenta.

On-going and future study of the complex origins of neonatal bacteria should consider oral and placental microbiomes in addition to the vaginal microflora. This need is exemplified by studies involving infants born preterm with early-onset neonatal sepsis showing the presence of *Fusobacterium, Ureaplasma,* and *Mycoplasma* in the cord blood, amniotic fluid, and neonatal blood (*Wang* et al., 2013). Additionally, neonates with late onset sepsis or necrotizing enterocolitis have been found to have intestinal microbial dysbiosis which precedes clinical diagnosis (*Mai* et al., 2011, 2013). While the vaginal microbiome may be implicated in these neonatal diseases, significant differences in the abundance of placental bacteria have been found when comparing preterm and term placentas (*Jones* et al., 2009; *Stout* et al., 2013; *Aagaard* et al., 2014). Thus, we speculate that seeding of the neonatal microbiome with bacteria from the placenta, which arose through haematogenous spread, may facilitate early colonization. Thus, this early colonization in combination with variable, modifying host factors (*King* et al., 2007; *Zeldovich* and *Bakardjiev*, 2012) may conceivably provide the initial seeding of a dysbiotic microbiome that may render susceptibility to neonatal disease in a preterm or stressed infant.

CONCLUSIONS AND CLINICAL SIGNIFICANCE

Here we have described the current state of the science on several aspects of the female reproductive microbiome, as well as their current association with perinatal disorders of both the mother and her offspring. By the time this chapter reaches press, we do not doubt that more will be known and what we have described here will quickly be considered out-dated, archaic, and undoubtedly limited in scope and perspective. However, we would consider any such criticisms to be welcomed as they would be testament to the long-needed enthusiasm and interest in human reproductive biology from amongst the broader scientific community.

What we understand today is far more complex and confounded than was appreciated less than a decade ago, and is much simpler than what we will come to realize in coming years. The vaginal microbiome varies from one woman to the next, across the lifespan, and in association with both health and disease states. Simplified views of “less diversity and less rich vaginal microbiomes

are equivalent to disease states" have been challenged and discounted, and concepts of clearly delineated CSTs remain to be fully validated. Previously assumed to be "sterile" reproductive tract tissues have been shown to harbour low biomass microbiomes, and yet we remain unclear as to what, how and when the infant is colonized. What will serve as decisive determinants of community structure is still unknown, and the relative influence of antibiotics, prebiotics, and probiotics (as well as early in life diet and exogenous exposures) has yet to be robustly characterized. A systemic analysis of the microbiome across the reproductive health spectrum (adolescence, pregnancy, postnatal and perimenopausal/menopausal/postmenopausal) will undoubtedly shed light on the most significant and perplexing common disorders of our time. While this is a challenging area of research, the advent of metagenomics combined with integrative multi'omics will enable reproductive scientists and physician scientists to unravel the mysteries plaguing not only our generations' health and disease, but will likely shed light on human and primate co-evolution of host and microbe. We are grateful to be a part of this highly collaborative and interrogative collective of metagenomic physician scientists, and look forward to the many discoveries ahead.

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HUMAN MILK OLIGOSACCHARIDES (HMO) AS PRIMERS OF THE INFANT MICROBIOME

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SUMMARY

Human milk oligosaccharides (HMO) are a group of more than 150 different complex sugars that are highly abundant in human milk, but currently not present in infant formula. HMO composition follows a basic blueprint, but every woman generates a distinct HMO profile. These inter-individual differences are at least in part genetically determined. Whether or not other maternal genetic or environmental factors contribute to HMO biosynthesis remains unknown. HMO prime the infant microbiome through multiple direct and indirect mechanisms. HMO are human milk prebiotics that serve as metabolic substrates for potentially beneficial bacteria in the infant gastrointestinal tract and, thus, help shape the infant gut microbiome. HMO act as soluble decoy receptors that block the attachment of viral, bacterial or protozoan parasite pathogens to epithelial cell surface glycans and have the potential to reduce pathogen colonization and invasion and prevent infectious diseases in the gut and, potentially, also the respiratory and urinary tracts. HMO are antimicrobials that act as bacteriostatic or bactericidal agents. In addition to these direct microbiome-priming effects, HMO also affect host epithelial cell and immune cell responses that indirectly prime the microbiome. While most research currently focuses on potential health benefits for the breast-fed infant, little is known about potential benefits for the breast-feeding mother. In addition, HMO are found in the urine of pregnant women as early as at the end of the first trimester, suggesting that HMO might also affect pregnant women and the growing foetus. Carefully selected preclinical *in vitro* and animal models in combination with mother-infant cohort studies will enable us to investigate the potential benefits of individual structurally defined HMO.

INTRODUCTION

Human Milk Oligosaccharides (HMO) are unconjugated complex sugars (carbohydrates, glycans) that are highly abundant in human milk, but not in infant formula (reviewed in *Bode*, 2012). One litre of mature human milk contains 10-15 g HMO, which often exceeds the total amount of protein and is 100- to 1,000-fold higher than the concentration of oligosaccharides in bovine milk, the basis of most infant formula. HMO concentrations are even higher in human colostrum, the fluid secreted by the mammary gland during the first few days of lactation.

HMO consist of the five

monosaccharide building blocks galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc) and the sialic acid (Sia) derivative N-acetyl-neuramic acid. HMO composition follows a basic blueprint. All HMO carry lactose (Galβ1-4Glc) at the reducing end, which can be elongated in β1-3-linkage by two different disaccharides, either Galβ1-3GlcNAc (type 1 chain) or Galβ1-4GlcNAc (type chain). HMO can be branched if a Gal/GlcNAc disaccharide attaches in β1-6-linkage. HMO with more than 15 disaccharide units have been described, forming complex structural backbones. Structural diversity increase further by the addition of Fuc and Sia.

Fuc can be added to the HMO backbone in α1-2-, α1-3- or α1-4-linkage. HMO fucosylation is highly dependent on the mother's Lewis blood group status (*Kumazaki* and *Yoshida*, 1984; *Johnson* and *Watkins*, 1992; *Chaturvedi* et al., 2001; *Stahl* et al., 2001). Fucosyltransferase 2 (FUT2) catalyses the addition of Fuc in α1-2-linkage on Lewis blood group epitopes as well as on HMO (*Kumazaki* and *Yoshida*, 1984). FUT2 is actively expressed in over 70% of the population (Secretors). Milk of Secretor women contains high concentrations of α1-2-fucosylated HMO. 2'-fucosyllactose (2'FL) is one of the dominant HMO in the milk of secretor women. Non-secretors, however, do not express an active FUT2 and the milk of Non-secretor women lacks α1-2-fucosylated HMO. 2'FL is almost completely absent. Fucosyltransferase 3 (FUT3) on the other hand catalyses the addition of Fuc in α1-3/4-linkage (depending on the type of the underlying HMO backbone), and FUT3 can also be inactive in parts of the population (Lewis negative) (*Johnson* and *Watkins*, 1992). The milk of Lewis negative women has markedly reduced α1-3/4-fucosylated HMO, although they are not completely absent because another fucosyltransferase covers some redundant activity. Depending on the expression of active FUT2 and FUT3 enzymes, women can be separated into four groups: 1. Lewis positive Secretors (FUT2 active, FUT3 active), 2. Lewis negative Secretors (FUT2 active, FUT3 inactive), 3. Lewis positive Non-secretors (FUT2 inactive, FUT3 active), and 4. Lewis negative Non-secretors (FUT2 inactive, FUT3 inactive). Accordingly, the oligosaccharide composition in the milk of women from these four groups varies significantly (*Chaturvedi* et al., 2001; *Stahl* et al., 2001).

Sia can be added to the HMO backbone in α2-3- or α2-6-linkage. Sia contains a carboxyl-group, which, at physiological pH, introduces a negative charge to HMO. Therefore, sialylated or acidic HMO carry one or more negative charges depending on the number of Sia linked to the HMO backbone.

HMO synthesis is in part genetically determined, which is highly apparent with the differential fucosylation between Secretors and Non-secretors described earlier. Genes other than FUT2 and FUT3 that contribute to chain elongation, branching or sialylation might be differentially expressed in different women and lead to a distinct HMO composition. Whether or not environmental factors like nutrition or use of medication and drugs impacts HMO synthesis remains to be elucidated.

In summary, human milk, unlike the milk of most other mammals, contains very high concentrations of a structurally diverse group of more than 150 different complex sugars called human milk oligosaccharides (HMO). HMO composition follows a basic blueprint, but each woman produces a distinct profile of different HMO at different concentrations that can change over the course of lactation. These inter- and

intra-individual differences in HMO composition are in part determined by genetics.

Once ingested by the breast-fed infant, HMO resist the low stomach pH as well as degradation in by pancreatic and brush border enzymes (*Gnoth* et al., 2000; *Engfer* et al., 2000). Approximately, 1% of the ingested HMO are absorbed in the intestine reach the infant's blood, and are excreted intact in the infant's urine (*Rudloff* et al., 1996, 2006, 2011). The majority of HMO is either metabolized by the infant's gut microbes or excreted intact with the infant's faeces (*Albrecht* et al., 2010, 2011). Consequently, HMO are only found in the urine and faeces of breast-fed, but not formula-fed infants. While this article focuses on HMO as primers of the infant microbiome, it is important to emphasize that HMO are not contained in the infant's gut and potentially reach all organs, including the liver and the brain, as well as the respiratory and the urinary tract. Therefore, HMO effects may not be localized to only the gut, but impact the infant on multiple different levels, which may or may not involve microbes.

PREBIOTICS

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota, that confers benefits upon host well-being and health (*Roberfroid* et al., 2007). HMO serve as metabolic substrates for specific bacteria (e.g. *Bifidobacterium longum* subsp. *infantis; B. infantis*, JCM1222). As a consequence, these bacteria have a growth advantage and thrive. Other bacteria that cannot utilize HMO have a disadvantage and do not grow as well or not at all. Thus, HMO are the first prebiotics that humans encounter with their diet. The ability to utilize HMO requires an entire set of enzymes, transporters and other molecules. Certain bacteria have evolved together with HMO and express sialidases to cleave Sia or fucosidases to cleave Fuc to name just a few examples. Only very few bacteria are capable of degrading the entire array of HMO (*LoCascio* et al., 2007; *Marcobal* et al., 2010; *Asakuma* et al., 2011). Other bacteria may only be able to utilize parts of HMO. For example, bacteria with a certain fucosidase may be able to utilize the Fuc, but not Sia or the underlying backbone. Some bacteria may be able to utilize HMO only after other bacteria have removed the Fuc from the backbone, creating a "community feast" where multiple different bacteria may be able to degrade the entire array of HMO, but only when they act together as a community.

While the sequential degradation of HMO by different microbes needs to be further elucidated, it is evident that, based on their structural diversity, different HMO can be metabolized by different bacteria. In other words, not all HMO lead to the same changes in composition and/or activity in the gastrointestinal microbiota and have the same benefits upon host well-being and health. Prebiotic effects are likely structure-specific, raising the question whether or not we can use structurally distinct HMO to elicit a certain response in microbiota composition. Targeted microbiome changes with specific HMO or other glycans might not only be desirable for the neonate, but also for adolescents and adults that suffer from diseases like obesity or inflammatory bowel disease that have been shown to be associated with dysbiosis.

ANTI-ADHESIVES

HMO are more then "food for bugs". Many viral, bacterial or protozoan parasite pathogens need to attach to epithelial surfaces in order to proliferate and in some cases invade and cause disease. In many cases the initial attachment is to epithelial cell surface sugars (glycans) also known as the glycocalyx. While these glycans are often conjugated to proteins or lipids, HMO resemble some of the same glycan structures and serve as soluble decoy receptors that block pathogen binding to epithelial cells. Unbound pathogens can no longer attach and are washed out without causing disease. Norovirus and Rotavirus are examples for viral pathogens that bind to the epithelial glycocalyx; HMO resemble the glycan binding partners and block viral attachment, providing one explanation for the reduced incidence of these viral infections in breast-fed infants compared to formula-fed infants. *Campylobacter jejuni* (*Ruiz-Palacios* et al., 2003) and enteropathogenic *E. coli* (*Manthey* et al., 2014) are examples for bacterial pathogens that follow the same principal and have significant impact on infant health as they are responsible for a majority of bacterial diarrheal episodes. Our lab has recently shown that HMO also prevent the attachment of the protozoan parasite *Entamoeba histolytica* (*Jantscher-Krenn* et al., 2012). While rather uncommon in the US or Europe, *E. histolytica* infects more than 50 million people worldwide and causes the disease amoebiasis, leading to more than 100,000 death annually (*Pritt* and *Clark*, 2008). *E. histolytica* expresses a lectin, a glycan-binding protein, which is a major virulence factor involved in *E. histolytica* attachment to as well as killing and phagocytosis of intestinal epithelial cells (*Cano-Mancera* and *Lopez-Revilla*, 1987; *Saffer* and *Petri*, 1991). HMO block the lectin and prevent attachment, killing and phagocytosis. The effects are structure-specific and require a terminal Gal on the HMO to be effective. Fucosylation of the terminal Gal abolishes the effect.

A growing body of evidence from tissue culture attachment assays and animal infection models support the notion that HMO are structure-specific anti-adhesives. However, well-designed and fully-powered mother-infant cohort studies and, most importantly, intervention studies are required to confirm that individual HMO or a mixture of different HMO reduce the incidence of infectious diseases caused by viral, bacterial and protozoan parasite pathogens. If confirmed, HMO could become urgently needed alternatives or additions to currently existing antibiotics that alone become more and more ineffective as pathogens develop antibiotic resistance.

ANTIMICROBIALS

HMO may protect us from pathogens not only by serving as anti-adhesives at the interface of microbe-host interactions. HMO may have a more direct way of making a pathogen's life difficult. In collaboration with Victor Nizet's lab at the University of California, San Diego, we have recently shown that *Streptococcus agalacticae* (Group B Streptococcus; GBS) is no longer able to proliferate when HMO are present (*Lin* et al., manuscript in preparation). GBS is one of the leading neonatal pathogens affecting about 1 in 2,000 new-borns in the U.S. An estimated 15-40% of all pregnant women

are colonized with GBS in the gastrointestinal or genital tract (*Anthony* et al., 1981; *Campbell* et al., 2000); more than half of them experience miscarriage or stillbirth and 16-53% of colonized mothers may pass the bacteria onto their babies during childbirth (*Phares* et al., 2008; *Muller* et al., 2006). Infected infants can develop sepsis, pneumonia, and life-threatening meningitis. GBS colonization in the genital tract also increases the probability of urinary tract infections (UTIs) in pregnant women (*Muller* et al., 2006). We used multidimensional chromatography and identified specific non-sialylated HMO as having the most pronounced bacteriostatic effect on GBS. We also used a GBS transposon library and identified a GBS mutant that is no longer susceptible to the bacteriostatic effects of HMO. The mutant lacks a gene that encodes a glycosyltransferase and additional *in vitro* studies suggest that GBS employs the glycosyltransferase to incorporate specific HMO into their cell membrane, which then stops GBS proliferation similar to some of the commercially available antibiotics. In fact, HMO synergize with antibiotics like vancomycin or ciproflaxacin and significantly improve their IC_{50} *in vitro* (*Lin* et al., manuscript in preparation). These most recent results are very promising in developing new antibiotics that are based on natural compounds like HMO and synergize with already available antibiotics that, when used alone, start to lose efficacy due to the development of antibiotic resistance.

HMO may not only protect from viral, bacterial or protozoan pathogens. Our most recent work in collaboration with Dr. Cheryl Gale's group at the University of Minnesota suggests that HMO also affect fungal-host interactions (*Gonia* et al., 2015). *Candida albicans*, a prevalent fungal colonizer of the neonatal gut (*Saiman*, 2001; *La Tuga*, 2011; *Heisel*, 2015), causes the overwhelming majority of invasive fungal disease in premature infants and is highly associated with concurrent diagnoses of necrotizing enterocolitis and focal intestinal perforation, life-threatening intestinal disorders (*Coates*, 2005; *Ragouilliaux*, 2007). Treatment with HMO significantly reduced invasion of human premature intestinal epithelial cells (pIECs) by *C. albicans* in a dose dependent manner (*Gonia* et al., 2015). The decreased invasive potential of *C. albicans* correlated with a delay in hyphal growth and morphogenesis as well as a reduction in the ability of *C. albicans* to associate with pIECs, processes important for the initial pathogenesis steps of *C. albicans* infections. Again, HMO appear to directly affect the microbe, here the fungus *C. albicans*, altering hyphal growth and morphogenesis, which then makes it more difficult for the pathogen to attach, invade and cause disease.

ALTERING HOST CELL RESPONSES

HMO may not only impact mircobes directly, but also indirectly by altering host cell responses. HMO have been shown to modulate intestinal epithelial cell apoptosis, proliferation and differentiation (*Kuntz* et al., 2008). HMO have also been shown to alter intestinal epithelial cell gene expression leading to changes in the cell surface glycocalyx (*Angeloni* et al., 2005). Thus, HMO may not only affect microbe-host attachment by serving as soluble decoy receptors as described above, but also by changing the expression of the glycocalyx receptors by reprogramming the epithelial cell. Our own lab recently

set out to investigate whether or not HMO can serve as anti-adhesives not only for bacteria in the infant gut, but also for bacteria in the urinary tract (*Lin* et al., 2014). To our surprise we found that HMO indeed reduce invasion of uropathogenic *E.coli* (UPEC), but not by serving as anti-adhesives that reduce UPEC attachment to epithelial cells. Instead, HMO interact with the epithelial cells and make them more resistant against UPEC attacks. HMO strongly suppress intracellular signalling of apoptotic pathways that renders the epithelial cell irresponsive when UPEC tries to destroy them. The effects are highly structure-dependent and only sialylated HMO like 3'-sialyllactose are effective (*Lin* et al., 2014).

HMO not only alter epithelial cell responses. HMO also affect immune cell responses. For example, we have recently shown that specific sialylated HMO reduce the expression of pro-inflammatory cytokines IL-1β and IL-6 in LPS-stimulated macrophages, both of murine and human origin (*Autran* et al., manuscript in preparation). This and other examples from other immune cell types (*Eiwegger* et al., 2004, 2010) suggest that HMO alter immune responses to pathogens, adding to the repertoire of mechanisms by which HMO interfere with microbe-host interactions and contribute to the protection of the breast-fed infant.

In summary, HMO interfere with microbe-host interactions not only directly by serving as prebiotics, anti-adhesives or antimicrobials, but also indirectly by altering epithelial and immune cell responses that set the stage for microbe-host interactions and help shape microbiota and microbiome compositions.

HMO AS PRIMERS OF THE MOTHER'S MICROBIOME

HMO may not only prime infant mircobiomes. Milk, for example, is not sterile and harbours microbial communities (*Hunt* et al., 2011). When milk is synthesized and temporarily stored in the alveoli and ducts of the mammary gland, HMO are in contact with the milk microbiota and may shape the microbiome by similar mechanism as described for the infant gut microbiome. HMO may serve as metabolic substrates for specific milk microbes and help them thrive. HMO may act as antimicrobials with bacteriostatic or bacteriocidal effects that contain the growth of microbes. HMO may have anti-adhesive effects and prevent bacterial attachment to mammary gland cells surfaces, which may impact bacterial growth. HMO may also have direct effects on epithelial cells or immune cells in the mammary gland, which helps set the environment for certain bacteria to thrive while others are kept in check.

In collaboration with Shelley and Mark McGuire at Washington State University and the University of Idaho we have recently shown strong associations between HMO composition and milk microbiota composition and identified an additional mechanism through which HMO impact microbes (*Hunt* et al., 2012). We found that high concentrations of the HMO 2'FL correlate with high concentrations of *Staphylococcus aureus* in mother's milk. In fact, *S. aureus* grows better in media containing the HMO 2'FL, suggesting the bacteria utilize HMO as a metabolic substrate. To our surprise, HMO concentrations did not decrease when *S. aureus* propagated. Instead, we found that the concentrations of several amino acids decline when *S. aureus* grows on HMO, suggesting that HMO

trigger a metabolic switch that allows the bacteria to utilize specific amino acids more efficiently (*Hunt* et al., 2012).

HMO may not only prime the mother's milk microbiome. Already in the late 1970s, studies revealed that HMO appear in the urine of pregnant women as early as at the end of the first trimester, long before milk is secreted for infant consumption (*Hallgren* et al., 1977; *Hallgren* and *Lundblad*, 1977). These observations are now being confirmed and the data is rapidly expanding. If HMO appear in the urine of pregnant women, do they help shape the urinary tract or vaginal microbiome? Urinary tract infections in pregnant women can lead to ascending infections and cause preterm delivery. Are some women at a higher risk because they lack specific HMO during pregnancy? First reports show that HMO are present in the blood of pregnant women. Do they cross the placental barrier? Do they appear in cord blood and in amniotic fluid? Do HMO impact foetal growth and development long before they are made available to the neonate through mother's milk? Do they shape the women's mircobiome in niches other than urinary and reproductive tract? Do they have systemic effects in pregnant women? Studies are on the way to describe HMO composition, concentration and inter- and intra-individual variations in different fluids and tissues in pregnant women. Results from these studies will lay the foundation for mother-infant-cohort studies that, in combination with suitable preclinical models, will guide us in elucidate the role of HMO as primers of the mother's microbiome and help us understand how HMO benefit the pregnant woman and the growing foetus long before milk is secreted.

THE POWER OF COHORT STUDIES

Most of the data reviewed so far stem from *in vitro* studies in the test tube or in tissue culture or from *in vivo* studies in animal models. The gap between these preclinical studies and clinical intervention studies in humans is immense, carrying the risk that results generated in tissue culture or animal models don't translate to benefit the human neonate. Mother-infant cohort studies are a powerful way to narrow this gap between preclinical and clinical studies. Let's assume preclinical models suggest that HMO reduce a certain disease. Let's further assume that a multidimensional chromatography approach identifies a structurally distinct oligosaccharide within the HMO that is responsible for the beneficial effect. Now, let's assume that mother-infant cohort studies reveal that high concentrations of the oligosaccharide in mother's milk are associated with a lower incidence of the disease in infants. The combination of these results would be very informative in designing an intervention study to confirm that the identified oligosaccharide indeed reduces the disease in question. Preclinical models could continue to help elucidate the underlying mechanisms, and newly developed synthetic approaches could make the specific HMO available to enable clinical studies and application. Due to the immense inter- and intra-individual variation in HMO composition, these cohort studies often require the recruitment of hundreds of mother-infant pairs and the collection and analysis of hundreds and thousands of milk samples. However, until recently HMO analysis was tedious and expensive. For example, in 2012 our lab published results from a study that

investigated associations between HMO composition and mother-to-child HIV transmission via breastfeeding (*Bode* et al., 2012). At that time, we were able to analyse 10-12 samples per week for a total of about 100 milk samples. Conducting cohort studies that analysed the HMO composition of hundreds or thousands of milk samples was simply unfeasible. In the last couple of years, our lab as well as a couple of other labs have developed new rapid high-throughput methods that allow HMO composition analysis from as little as 10 µL milk usually in 96-sample format in parallel in within days (*Autran* et al., manuscript in preparation). In addition, our HPLC-based method uses an internal standard from the very beginning of sample preparation that allows for absolute HMO quantification, which has often been a limitation, especially in most mass spectrometry-based methods. These new analytical methods now enable us to conduct large mother-infant cohort studies with the aim to investigate how specific HMO are associated with health and disease of the infant, but also the mother. For example, HMO composition can now be linked to infant gut microbiota and microbiome composition, risk for infants to develop necrotizing enterocolitis (*Autran* et al., manuscript in preparation) or acquire viral or bacterial infections, or risk for mothers to develop urinary tract infections and deliver their baby prematurely. In addition, the methods now also allow us to study how maternal factors like genetics, health status, nutrition, medication or drugs impact HMO composition.

CONCLUSIONS

Future studies are going to show how individual, structurally defined HMO or mixtures of different HMO benefit the breast-fed infant and the breastfeeding mother, but also pregnant women and the growing foetus. It is important to emphasize that HMO are a group of at least 150 different and structurally distinct oligosaccharides. It is going to be essential to clearly define structure-function relationship in the context of different health states and diseases. Preclinical models have to be carefully selected to generate meaningful data that translates to humans. Multidimensional chromatography is going to be a helpful tool to identify individual and structurally well characterized HMO that are responsible for observed beneficial effects. Mother-infant cohorts are going to provide valuable data to help close the gap between preclinical models and clinical intervention studies. Future research is going to reveal what factors drive HMO composition and how HMO composition impacts health outcomes, in part via priming microbiomes.

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TRYPTOPHAN METABOLITES AND ARYL HYDROCARBON RECEPTOR SIGNALLING BY GUT MICROBES

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SUMMARY

Fungal diseases represent an important paradigm in immunology since they can result from either the lack of recognition or over-activation of the inflammatory response. Current understanding of the pathophysiology underlying fungal infections and diseases highlights the multiple cell populations and cell-signalling pathways involved in these complex conditions. A systems biology approach that integrates investigations of immunity at the systems-level is required to generate novel insights into this complexity and to decipher the dynamics of the host-fungus interaction. It is now clear that a three-way interaction between host, fungi, and microbiota dictates the types of host-fungus relationship. Metagenomics has revealed the complex interactions between fungal and bacterial commensals that, either directly or through the participation of the host immune system, impact on immune homeostasis at mucosal surfaces that, in turn, lead to secondary fungal infections. Metabolomics has captured the dialogue between the mammalian host and its microbiota. The host tryptophan catabolic enzyme, indoleamine 2,3-dioxygenase 1 (IDO1) plays a dominant role in the interplay between tryptophan catabolism by microbial communities, the host's own pathway of metabolite production, and the activation of the aryl hydrocarbon receptor (AhR)/IL-22 axis, eventually impacting on mucosal immune homeostasis and host/fungal symbiosis. Thus, the regulatory loop involving AhR and IDO1 may be exploited for the development of multi-pronged-host- and microbiota-directed therapeutic approaches for mucosal and systemic fungal diseases.

FUNGI ENTER THE METAGENOMIC ERA

Fungi can interact with their hosts (plants, animals or humans) in multiple ways, establishing symbiotic, commensal or pathogenic relationships. Most fungi, such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, and the thermally dimorphic fungi (*Histoplasma capsulatum, Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Penicillium marneffei*, and *Sporothrix schenckii*), are ubiquitous in the environment and humans are exposed by inhaling spores or small yeast cells. As a result, they can interact with humans in multiple ways, establishing symbiotic, commensal, latent or pathogenic relationships

However, among eukaryotes, fungi are particularly prominent residents of the human body (*Huffnagle* and *Noverr*, 2013). More than 400 species of fungi associated with human beings

have been identified (*Cui,* et al., 2013). Very little is known about the biology of the members of the human "mycobiome" (i.e., fungal microbiome) and even less about the interactions that they establish with the human host. Thus, an increased understanding of the importance of mycobiota in shaping the host's immune and metabolic activities will render fungal interactions with their hosts more complex than previously appreciated (*Romani,* et al., 2014). As a corollary, early diagnosis may no longer be a challenge for invasive or chronic fungal diseases, as suggested (*Schelenz,* et al., 2015). Instead, the study of the human mycobiota in the trans-omics era, with a focus on metagenomics and metabonomics, is providing novel insights into the regulation of host/fungus immune homeostasis. Evidence is accumulating to support the exciting concept that the interaction between different biomes and between the host and the mycobiome are critical in the pathogenesis of fungal infections and other human diseases (*Cui,* et al., 2013; *Huffnagle* and *Noverr*, 2013; *Seed*, 2014; *Underhill* and *Iliev*, 2014).

THE HUMAN MYCOBIOME

The development of culture-independent methods has expanded our knowledge of the mycobiomes found in different body sites, their interface with other biomes and their association with human health and diseases (*Cui* et al., 2013). Alterations in the mycobiome are frequently reported to be associated with various diseases such as cystic fibrosis (CF) (*Delhaes* et al., 2012), inflammatory bowel diseases (*Ott* et al., 2008; *Li* et al., 2014;), atopic dermatitis (*Zhang* et al., 2011) or mucocutaneous candidiasis (*Smeekens* et al., 2014). However, it remains to be elucidated whether this variation is primary or secondary to an imbalanced bacterial microbiome. Indeed, interactions of fungi with bacteria *in vitro* have been described (reviewed in: *Wang* et al., 2014) as well as the clinical relevance of these interactions (*Peleg* et al., 2010), such as the occurrence of intractable candidiasis in association with antibiotic-induced dysbiosis (*Krause* et al., 2001) and of mixed fungal-bacterial species in biofilms (*Peleg* et al., 2010). Fungal-bacterial interactions can be antagonistic, synergistic or symbiotic; regardless they influence the physiological characteristics and survival of either one partner and, consequently, impact on host immune reactivity. Variations in the mycobiome can also be secondary to dysregulated host immune reactivity. The traditional view of a single direction by which bacteria stimulate the immune system, leading to inflammation or autoimmune disorders, has been challenged by a more complex view: the gut immune system does not simply protect from pathogens, but is actively involved in the maintenance of a rich and healthy community of gut bacteria (*Kawamoto* et al., 2014). Faults in the immune regulation lead to changes in the bacterial community that in turn feed back into the immune system. Similar to the microbiome, the host/mycobiome interactions also lead to mutual influences. Not only is the host affecting the mycobiome composition and variations by means of genotype, physiology, immune system, and lifestyle but the fungal microbiota may contribute to the balance of inflammation and tolerance at local mucosal surfaces and at distal sites (*Noverr* and *Huffnagle*, 2004).

RESISTANCE AND TOLERANCE MECHANISMS OF ANTIFUNGAL IMMUNITY

As the immune system has evolved to accommodate colonization by symbiotic microbes while retaining the capacity to oppose their infectivity, a fine balance between pro- and anti-inflammatory signals is a prerequisite for a stable host/fungal relationship, the disruption of which may lead to pathological consequences. Indeed, despite the occurrence of severe fungal infections in immunocompromised patients, clinical evidence indicates that fungal diseases also occur in the setting of a heightened inflammatory response, in which immunity occurs at the expense of host damage and pathogen eradication (*Perfect*, 2012). A number of fungal diseases are critical examples of such bidirectional influences between infection and immune-related pathology, a condition that highlights the bipolar nature of the inflammatory process in infection. Early inflammation prevents or limits infection, but an uncontrolled response may eventually oppose disease eradication. This conceptual principle is best exemplified by the occurrence of severe fungal infections in patients with chronic granulomatous disease (*Romani* et al., 2008a), CF (*Iannitti* et al. 2013) or with immune reconstitution inflammatory syndrome (*Singh* and *Perfect*, 2007), an entity characterized by local and systemic inflammatory reactions that can result in quiescent or latent infections manifesting as opportunistic mycoses. Chronic mucocutaneous candidiasis (CMC) and chronic disseminated candidiasis also belongs to the spectrum of fungus-related immune reconstitution inflammatory syndrome (*Legrand* et al., 2008). Thus, an immune response that limits both fungal infectivity and host collateral damage is required to maintain a homeostatic environment (*Casadevall* and *Pirofski*, 2003). This dual role has recently been accommodated within the conceptual framework of a two-component antifungal immune response, i.e., resistance (the ability to limit fungal burden) and tolerance (the ability to limit the host damage caused by either the immune response or other mechanisms). Resistance is meant to reduce pathogen burden through innate and adaptive immune mechanisms whereas a plethora of tolerance mechanisms, despite less known relative to resistance mechanisms, protect the host from immune- or pathogen-induced damage (*Saraiva* and *O'Garra*, 2010).

MICROBIOTA REGULATION OF RESISTANCE AND TOLERANCE TO FUNGI VIA TRYPTOPHAN METABOLISM

The enzyme indoleamine 2,3-dioxygenase 1 (IDO1) and its downstream catabolites sustain the delicate balance between Th1/Th17 pathways and Treg cells, by providing the host with adequate protective immune mechanisms without necessarily eliminating the pathogen or causing undesirable tissue damage (*Zelante* et al., 2009). As a result of their ability to induce differentiation of Treg cells and inhibit Th17 cells, IDO1 is critical to cell lineage commitment in experimental fungal infections and contributes to the overall outcome of inflammation, allergy and Th17-driven inflammation in these infections. Under these circumstances, the Th17 pathway, by inhibiting tryptophan catabolism, may instead favour pathology and provides evidence

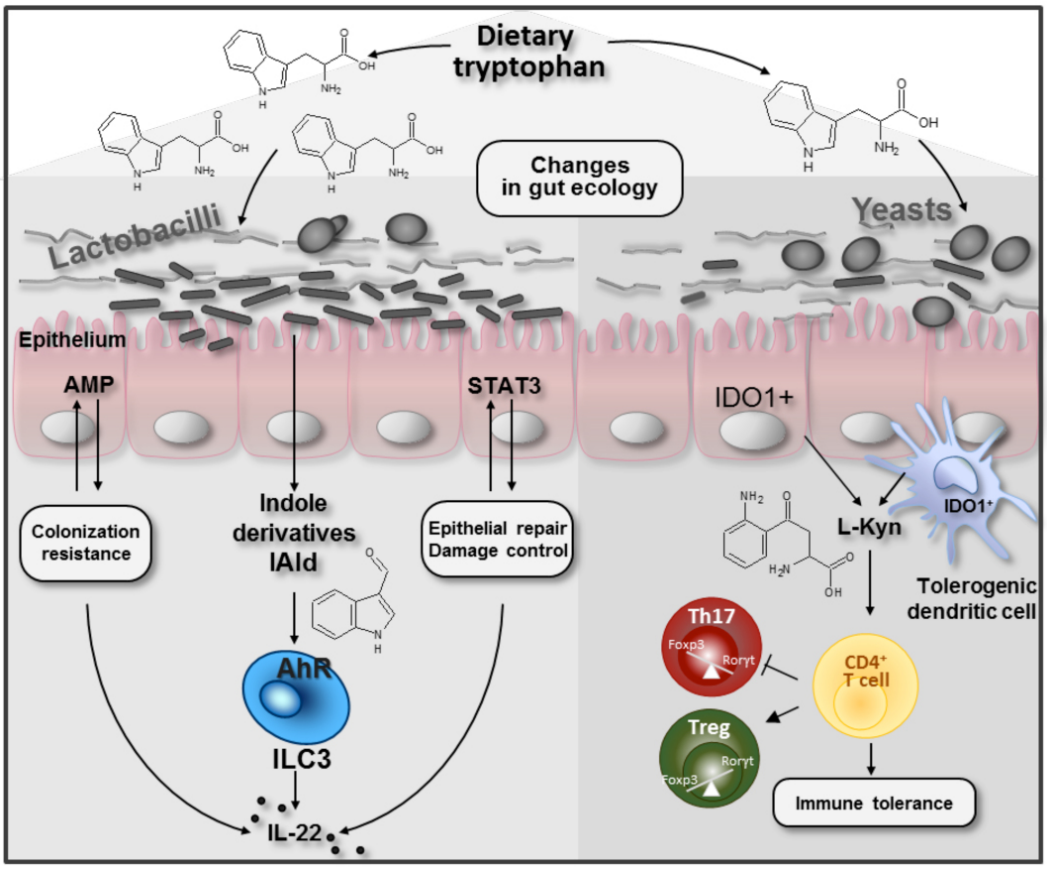

Figure 1: Resistance and tolerance to fungi and its regulation by tryptophan.
The tryptophan metabolism pathway is exploited by the mammalian host and commensals (including fungi) to increase fitness in response to fungi through resistance and tolerance. At mucosal surfaces and skin, the fungal biota promotes the production IL-22, via IL-23 and aryl hydrocarbon receptor (AhR) ligands, by $CD3^{-}NKp46^{+}$retinoic acid-related orphan receptor-γt $(ROR\gamma t)^{+}AhR^{+}$ innate lymphoid cells. By contrast, $NKp46^{-}$ cells produce IL-17A. IL-22 targets epithelial cells, leading to activation of signal transducer and activator of transcription 3 (STAT3) and, together with IL-17A, to production of antimicrobial peptides. Various indole derivatives, which are generated through conversion from dietary tryptophan by commensal intestinal microorganisms, act as endogenous ligands for AhR, and thereby contribute to IL-22 production. Fungus-induced activation of tryptophan catabolism by indoleamine 2,3–dioxygenase (IDO1) expressed by dendritic cells and epithelial cells leads to the production of immunologically active compounds that induce the transcription of FOXP3 and suppress the transcription of RORγt. These findings support a model in which the AhR/IL-22/IL-17A axis control initial fungal growth (i.e., resistance) and epithelial cell homeostasis. By contrast, the exploitation of the IFN-γ/IDO1 axis for functional specialization of antifungal regulatory mechanisms (i.e. tolerance) may have allowed the fungal microbiota to evolve with the mammalian immune system, survive in conditions of inflammation and prevent dysregulated immunity. The balance between resistance and tolerance to fungi may accommodate the spectrum of host/fungus relationships, ranging from protection and immunopathology to fungal persistence and immunosuppression.

accommodating the apparently paradoxical association of chronic inflammation with fungal disease (*Romani* et al., 2008a). IDO1 is a 'metabolic' enzyme conserved through the past 600 million years of evolution. Initially

recognized in infection because of antimicrobial activity ('tryptophan starvation' of intracellular parasites), IDO1 is now widely recognized as suppressor of acute inflammatory responses and regulator of mammalian immune homeostasis (*Zelante* et al., 2009). Not surprising, IDO1 may represent an evasion mechanism for microbes that establish commensalism or chronic infection (*Zelante* et al., 2009). In their capacity to induce Tregs and inhibit Th17, IDO1-expressing DCs and epithelial cells and kynurenines revealed an unexpected potential in the control of inflammation, allergy and Th17-driven inflammation in these infections (*Grohmann* et al., 2007; *Romani* et al., 2008b).

Commensal-driven mucosal responses are up-regulated in IDO1 deficiency (*Harrington* et al., 2008) and IL-22 responses are up-regulated in conditions of defective adaptive immunity (*De Luca* et al., 2010) and IDO deficiency (*Zelante* et al., 2013). AhR is a ligand-activated transcription factor that mediates IL-22 production (*Trifari* et al., 2009). A variety of indole derivatives act as endogenous ligands for AhR (*Heath-Pagliuso* et al., 1998) and are generated through conversion from dietary tryptophan by commensal intestinal microbes (*Bjeldanes* et al., 1991). Recent evidence has shown that AhR is involved in the (patho)physiology of skin including the regulation of skin pigmentation, photocarcinogenesis, and skin inflammation (*Esser* et al., 2013; *Di Meglio* et al., 2014). Of interest is the ability of *Malassezia*-derived indoles to activate AhR correlated with local immunoregulation (*Vlachos* et al., 2012) and pathogenicity in seborrhoeic dermatitis (*Gaitanis* et al., 2008). Similarly, metabolomics has revealed that bioactive indoles with AhR agonist activity are also present in mice with candidiasis (*Zelante* et al., 2013). Thus, the trpyptophan metabolism pathway is exploited by commensals and the mammalian host to increase fitness in response to fungi via induction of resistance and tolerance at the skin and mucosal surface. The new findings support a mode (Figure 1) in which the IL-22 axis controls the initial fungal growth (i.e., resistance) and epithelial cells homeostasis likely exploiting primitive anti-fungal effector defence mechanisms. In contrast, the exploitation of the IFN-γ/IDO1 axis for functional specialization of antifungal regulatory mechanisms (i.e. protective tolerance) may have allowed the fungal microbiota to co-evolute with the mammalian immune system, to survive in conditions of high-threat inflammation and to prevent dysregulated immunity (*Zelante* et al., 2009). The two pathways, although non-redundant, are reciprocally regulated and compensate each other in the relative absence of either one (*De Luca* et al., 2010), consistent with the theme that adaptive immunity depends on innate immunity but innate immunity requires adaptive regulation. This finding not only helps to explain the association of fungal infections with dysbiosis but also points to the essential help the microbiota may provide in fungal colonization and pathogenicity in immunodeficient patients.

ADVANCING HOST- AND MICROBIOTA-DIRECTED THERAPY FOR FUNGAL DISEASES ALONG THE AHR/IDO1 PATHWAY

A plethora of preclinical models suggests that the tryptophan to kynurenine immune tolerance pathway is active in cancer immunity, autoimmunity, infection, transplant rejection, and allergy. Drugs targeting this pathway are

already in clinical trials with the aim at reverting cancer-induced immunosuppression (*Platten* et al., 2014). However, the tryptophan to kynurenine pathway via IDO1 also plays a dominant role in the interplay between tryptophan catabolism by microbial communities, the host's own pathway of metabolite production, and the orchestration of AhR-dependent T-cell immune homeostasis. As AhR stimulation may lead in turn to IDO1 activation via an autocrine AhR-IL6-STAT3 signalling loop (*Litzenburger* et al., 2014), such a positive feed-forward loop between IDO1 and AhR may have allowed the mycobiota to co-evolve with the mammalian immune system, to survive under conditions of high-threat inflammation and to prevent dysregulated immunity in response to environmental cues. This implicates that the AhR/IDO1 loop could be exploited for rational host- and microbial-directed therapies in high-risk patients and suggests that antifungal therapy should consider inter-individual variations in the active human microbiome. Thus, challenging existing paradigms with new perspectives from the cross-talk between fungi, the immune system and the microbiota will eventually lead toward the development of multi-pronged therapeutic approaches for mucosal and systemic fungal diseases.

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ROLE OF INTESTINAL BACTERIA IN GUT INFLAMMATION AND OBESITY

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SUMMARY

It has increasingly been recognized that the gut microbiota has a major impact on host physiology. It is therefore not surprising that various diseases have been linked to perturbations in the gut microbiome. Obesity, which is often accompanied by metabolic disorders, and inflammatory bowel disease (IBD) are associated with shifts in the microbial communities inhabiting the digestive tract. It is not completely clear whether differences in the microbiome observed between healthy and diseased subjects are consequences or causes of these disorders. The transferability of the obese phenotype by microbiota transplantation suggests a role of the intestinal microbiome in disease development. Similarly, the observation that germfree mice in contrast to conventional mice are protected from colitis in certain animal models also supports a role of the gut microbiota in the development of IBD. However, the mechanisms underlying disease promotion by intestinal bacteria are far from being understood. This review summarizes both human and animal studies that aimed to more precisely define the role of intestinal bacteria in obesity development. A number of explanations for the role of intestinal bacteria have been proposed but which bacteria-derived molecules are involved and what their targets are in the host has only been uncovered in a few cases. Available evidence indicates that there is more than one way in which the intestinal microbiota may contribute to disease development and that it is necessary to use complementary approaches to unravel the underlying molecular mechanisms.

INTRODUCTION

The microbial communities inhabiting the gastrointestinal tract support important physiological host functions and thereby contribute to disease prevention. Disruption of the microbiota has been associated with various diseases including inflammatory bowel disease (IBD) and obesity (*Ley* et al., 2005, 2006b; *Qin* et al., 2010). Effects of the intestinal microbiome on the host can be attributed to its immense catalytic potential and to its interactions with the host immune system. Conversely, genetic traits, initial colonization as well as lifestyle and environmental factors influence the composition and activity of the gut microbiota. Diet is a key factor in the development of the gut microbiota because it is the main substrate source for the microbial community (*Blaut*, 2013). Therefore, dietary changes may lead to shifts in microbiota composition and in the spectrum of microbial fermentation

products (*David* et al., 2014). Obesity and associated diseases are clearly connected to dietary habits, but it is also clear that the gut microbiota affects host energy metabolism (*Backhed* et al., 2004). The microbiome of obese subjects has recently been reported to be less rich in bacterial organisms and genes compared with non-obese individuals (*Le Chatelier* et al., 2013). However, even though various bacterial species were found to be associated with the obese phenotype the exact role of these organisms is still obscure.

IBD is a complex disease involving genetic and environmental factors. Intestinal bacteria are thought to play a major role in the development of IBD because certain mouse models for IBD do not develop disease symptoms as long as they are germfree (*Sellon* et al., 1998). IBD patients and healthy subjects can be distinguished based on their microbiota composition (*Qin* et al., 2010). However, so far it has not been possible to identify a single bacterial species or groups of bacteria that definitely account for the disease. Moreover, the microbial patterns observed in IBD patients may be consequence rather than cause of the disease. A number of host gene variants have been identified as possible contributors to IBD development, but they only explain a small proportion of cases. Various bacterial species have positively or negatively been associated with IBD. For example, IBD patients harbour reduced cell numbers of *Faecalibacterium prausnitzii* (*Frank* et al., 2007; *Sokol* et al., 2009; *Joossens* et al., 2011). In contrast, adherent and invasive *E. coli* strains (*Darfeuille-Michaud* et al., 2004), *Salmonella, Campylobacter* (*Gradel* et al., 2009) *Mycobacterium avium* subspecies *paratuberculosis*, *Helicobacter* spp. and *Fusobacterium varium* have been proposed to contribute to IBD (*Hold* et al., 2014).

This indicates that in both obesity and IBD different bacteria may cause similar disease symptoms even though the underlying mechanisms are different. There is an urgent need for a more precise understanding of how bacteria contribute to disease development, in particular for the identification of the bacterial molecules involved as well as their targets in the host organism.

REVIEW AND DISCUSSION

Role of intestinal bacteria in obesity and metabolic disease

Obesity may be considered a lifestyle disease because it is caused by an excessive caloric intake in conjunction with a lack of physical activity. Owing to its increasing incidence in many countries obesity is considered an epidemic, in particular because it is mostly associated with many other disorders such as hypertension, diabetes, dyslipidaemia, and non-alcoholic fatty acid liver disease. This development causes considerable costs for public health systems and is therefore an important research topic in medical research.

Jeffrey Gordon and colleagues were the first scientists to propose that obesity affects microbiota composition. Both genetically obese mice homozygous for a mutation in the leptin gene (*ob/ob*) (*Ley* et al., 2005) and obese human subjects (*Ley* et al., 2006a) displayed a shift in the gut microbiota compared to respective controls. Obesity was associated with an increase in the proportion of the Firmicutes at the expense of the Bacteriodetes. Even though this pattern could not be observed by other investigators (*Duncan* et al., 2008;

Schwiertz et al., 2010) (possibly due to the use of different methods for microbiota analysis) differences in gut microbiota composition between lean and obese human subjects or mice have been observed repeatedly (*Turnbaugh* et al., 2006, 2008, 2009a; *Jumpertz* et al., 2011). There is also experimental evidence that diet rather than the obese phenotype affects microbiota composition. This conclusion was drawn from an experiment, in which two genetically distinct mouse strains, which were fed the same high-fat diet, displayed essentially the same microbiota composition even though one of the strains was obese while the other one was not (*Hildebrandt* et al., 2009). This experiment suggests that diet is critically involved in microbiota composition. From a microbiological point of view this is not really surprising because consumption of a high-fat diet for example causes an increased excretion of bile acids into the digestive tract which, in turn, lowers the cell counts of bacteria that are sensitive to bile acids while bacteria that are resistant to bile acids are not affected or even proliferate in such conditions. Administration of the bile acid cholate to mice leads to increased cell numbers of Firmicutes at the expense of Bacteroidetes, in particular to an increase in Clostridia and Erysipelotrichi (*Islam* et al., 2011).

The most intriguing observations in this field relate to the transferability of the obese phenotype by transplantation of the microbiota from an obese donor mouse or human subject to germfree mice (*Turnbaugh* et al., 2006, 2009b; *Ridaura* et al., 2013). It is not quite clear which features render a gut microbiota obesogenic. What are the bacterial factors that cause this effect in the recipient mice and what are the targets in the host organism? It has been proposed that an obesogenic microbiota improves energy harvest from the diet. However, how this is brought about is not yet clear. Several possible explanations have been proposed:

1. The gut microbiota leads to an increased formation of short chain fatty acids (SCFA) which can be utilized for energy generation, lipogenesis and/or gluconeogenesis. In agreement with a role of SCFA in obesity development, obese subjects were reported to have higher faecal SCFA concentrations compared to normal-weight subjects (*Schwiertz* et al., 2010). On the other hand, high faecal SCFA concentrations indicate that the diet consumed was rich in fermentable fibre. Fibre-rich diets usually have a lower energy density compared to highly digestible diets and the intake of diets rich in dietary fibre correlates with a lower incidence of obesity and symptoms of the metabolic syndrome (*Slavin*, 2005). This can be explained by the regulatory function of SCFA, which are ligands of the G-protein coupled receptors FFAR2 (Free Fatty Acid Receptor 2) and FFAR3 (formerly GPR43 and GPR41) found in ileal and colonic entero-endocrine L cells, adipocytes and immune cells (*Brown* et al., 2003). Following the activation of these FFARs by SCFA, leptin is secreted by adipocytes (*Xiong* et al., 2004) and peptide YY (PYY) and glucacon like peptide 1 (GLP-1) are secreted by entero-endocrine cells (*Tazoe* et al., 2008; *Tolhurst* et al., 2012). Leptin and PYY reduce appetite (*Wren and Bloom*, 2007) and GLP-1 stimulates insulin production, improves insulin sensitivity and promotes satiety.
2. High-fat diets lead to low-grade inflammation caused by increased permeability of the intestinal epithelium for bacterial lipopolysaccharides (LPS) and increased concentration of LPS in blood; this is

referred to as metabolic endotoxaemia (*Cani* et al., 2007). In agreement with this proposed mechanism association of germfree mice with an LPS-containing *Enterobacter cloacae* strain, which had been isolated from an obese Chinese patient, developed low-grade inflammation and obesity to greater extent than germfree mice in response to a high-fat diet (*Fei and Zhao*, 2013). However, the authors of a very recent study did not find any indications for an impaired gut barrier in response to high-fat diet feeding even though glucose tolerance was impaired and signs of low-grade inflammation in adipose tissue were observed (*Kless* et al., 2015).

3. Intestinal bacteria promote the formation of Angiopoietin-like protein 4 (ANGPTL4) also called Fasting-induced adipose factor (FIAF) in the intestine (*Backhed* et al., 2004). ANGPTL4 is a downstream target of the nuclear peroxisome proliferator-activated receptor family and an inhibitor of lipoprotein lipase (LPL). Inhibition of LPL by ANGPTL4 results in reduced plasma triglyceride levels and decreased deposition of released fatty acids as triglycerides in adipose tissue (*Lichtenstein* and *Kersten*, 2010). Germfree mice display lower intestinal ANGPTL4 mRNA levels than conventional mice and in accordance with this explanation less body fat (*Backhed* et al., 2004). Increased ANGPTL4 mRNA levels in intestinal mucosa of germfree versus conventional mice were confirmed in another study, but the plasma protein levels of ANGPTL4 in germfree mice were not higher than those in the conventional mice (*Fleissner* et al., 2010). This finding is in conflict with a critical role of ANGPTL4 in obesity development. Mice colonized with *Lactobacillus paracasei* strain F19 displayed higher serum ANGPTL4 levels and reduced fat accumulation (*Aronsson* et al., 2010), suggesting that intestinal bacteria do not necessarily reduce circulating ANGPTL4 levels as proposed (*Backhed* et al., 2004).
4. The gut microbiome contributes to obesity development by enhancing nutrient uptake. In agreement with this explanation, Hooper *et al*. (2001) observed that mice monoassociated with *Bacteroides thetaiotaomicron* had higher mRNA levels of genes involved in nutrient uptake such as Na^+/glucose co-transporter (SGLT1), co-lipase and liver fatty acid-binding protein (L-FABP) compared to germfree mice. A recent study in gnotobiotic mice suggests that *C. ramosum*, a member of the Erysipelotrichi, promotes obesity development in mice fed a high-fat diet by enhancing nutrient uptake (*Woting* et al., 2014). This is in accordance with a recent human study, which reported an association of obesity with low bacterial gene content and presence of *C. ramosum* (*Le Chatelier* et al., 2013). Furthermore, symptoms of the metabolic syndrome in diabetic women were reported to correlate with faecal *C. ramosum* (*Karlsson* et al., 2013). We compared mouse groups that differed in their microbiological status and that were fed a high-fat diet (HFD) or a low-fat diet (LFD) (*Woting* et al., 2014). The first group of mice was associated with a simplified human intestinal (SIHUMI) microbiota of eight bacterial species, including *C. ramosum*, the second group with SIHUMI except *C. ramosum* (SIHUMIw/oCra), and the third group with *C. ramosum* only (Cra). After feeding the mice the HFD for 4 weeks, there was no

difference between the mouse groups in energy intake, diet digestibility, gut permeability, and parameters of low-grade inflammation but the SIHUMI and Cra mice fed the HFD gained significantly more body weight and body fat and displayed higher food efficiency than the SIHUMIw/oCra mice and the germfree mice, respectively, also fed the HFD. Glucose transporter 2 (*Glut2*) and fatty acid translocase (*CD36*) mRNA levels in small intestinal mucosa were significantly higher in the obese SIHUMI and Cra mice than in the less obese SIHUMIw/oCra mice. The data suggest that in this animal model upregulation of small intestinal glucose and fat transporters contribute to their increased body fat deposition (*Woting* et al., 2014). It remains to be shown whether this is a general mechanism and to which extent it may contribute to obesity compared to the other mechanisms that have been proposed.

Role of intestinal bacteria in inflammatory bowel disease (IBD)

IBD encompasses two major forms of inflammatory conditions of the digestive tract: Crohn's disease and ulcerative colitis. It is now generally accepted that IBD arises as a result of a disturbed interaction of genetic and environmental factors. Various gene variants have been identified that predispose the host organism to IBD. Examples include Nucleotide-binding oligomerization domain (NOD) 1 and 2, Toll-like receptor (TLR) 1 and 2 or Autophagy-related protein 16-1 (ATG16L1). These gene variants may lead to a disturbed barrier function of the gut epithelium and in consequence to a loss of homeostasis between intestinal microbiota and immune system. The intestinal microbiota and nutrition are considered the most important environmental factors in the development of IBD (*Hold* et al., 2014). As mentioned above for metabolic disease the exact role of intestinal bacteria in IBD development is not really clear, in particular whether specific bacteria are involved or whether there is a disturbance in the microbiome, which is not exactly defined. The latter is referred to as dysbiosis. The microbiomes of patients suffering from Crohn's disease or ulcerative colitis differ from those of healthy subjects (*Qin* et al., 2010). However, first of all it is not clear whether the microbiota patterns observed in patients are cause or consequence of the disease and second it has so far not been possible to deduce the mechanism(s) underlying IBD development. IBD is associated with a reduction in diversity and shifts in certain microbial populations (*Manichanh* et al., 2006; *Frank* et al., 2007; *Wohlgemuth* et al., 2009). Several studies concurrently report reduced cell numbers of *Faecalibacterium prausnitzii* in IBD patients (*Frank* et al., 2007; *Sokol* et al., 2009; *Joossens* et al., 2011). *F. prausnitzii* is considered to be protective against IBD because oral administration of this organism or of spent *F. prausnitzii* growth media improved symptoms of a colitis induced by trinitrobenzosulphonate (TNBS); it blocks Nuclear Factor kappa B (NFκB) and the formation of Interleukin (IL)-8 (*Sokol* et al., 2008). In contrast to *F. prausnitzii*, cell numbers of *E. coli* are increased in both Crohn patients and interleukin 10-deficient (IL-$10^{-/-}$) mice (*Darfeuille-Michaud* et al., 1998; *Wohlgemuth* et al., 2009). The *E. coli* strains detected in epithelial lesions of Crohn patients turned out to preferentially belong to the group of adherent entero-invasive *E. coli* (AIEC) (*Darfeuille-Michaud* et al., 2004). There are also indications

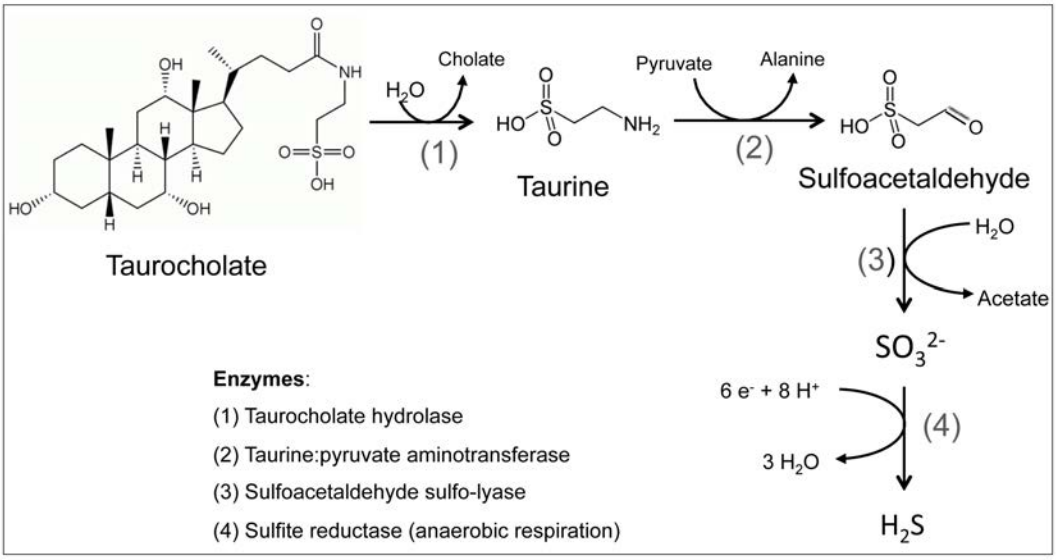

Figure 1: Pathway of taurocholate conversion by *Bilophila wadsworthia*.
This pathway enables the organism to generate sulphite, which is used as an external electron acceptor to generate ATP by anaerobic respiration (*Devkota* et al., 2012).

for a role of *Mycobacterium avium* subspecies *paratuberculosis*, *Helicobacter* spp. and *Fusobacterium varium* in IBD development (*Hold* et al., 2014). However, these and other differences in gut microbiota are not generally observed which may be due to the high inter-individual variability of the gut microbiota.

In the recent decades there has been an increase in the incidence of IBD. This development was accompanied by changes in lifestyle. As nutrition is a very important lifestyle factor, which also affects the gut microbiome, various studies investigated possible correlations between diet and the incidence of IBD. In particular Japan in the last decades underwent considerable changes in nutrition, from a traditional Japanese diet to a more Western-oriented diet. The latter is characterized by an increased consumption of fat, animal protein, milk, and milk products as well as a reduced intake of rice. Interestingly, these changes coincide with an increase in the incidence of IBD in the Japanese population (*Asakura* et al., 2008). Even though a direct effect of diet cannot be excluded it appears reasonable to assume that possible dietary effects are mediated by the gut microbiome. One animal study provides an interesting example on how diet could promote the onset of IBD. Two groups of IL-$10^{-/-}$ mice, which are prone to developing colitis, were fed two isocaloric high-fat diets that did not differ in macronutrient composition but just in the type of dietary fat. One diet contained milk fat (MF), i.e. saturated fatty acids, whereas the other one contained poly-unsaturated fatty acids (PUFAs). Colitis incidence, inflammatory score, and pro-inflammatory markers were considerably increased in the MF diet-fed mice compared to the PUFA diet-fed mice (*Devkota* et al., 2012). The authors demonstrated that this effect was caused by changes in the spectrum of bile acids, specifically by increased concentrations of taurocholate (Figure 1). The latter stimulates the growth of

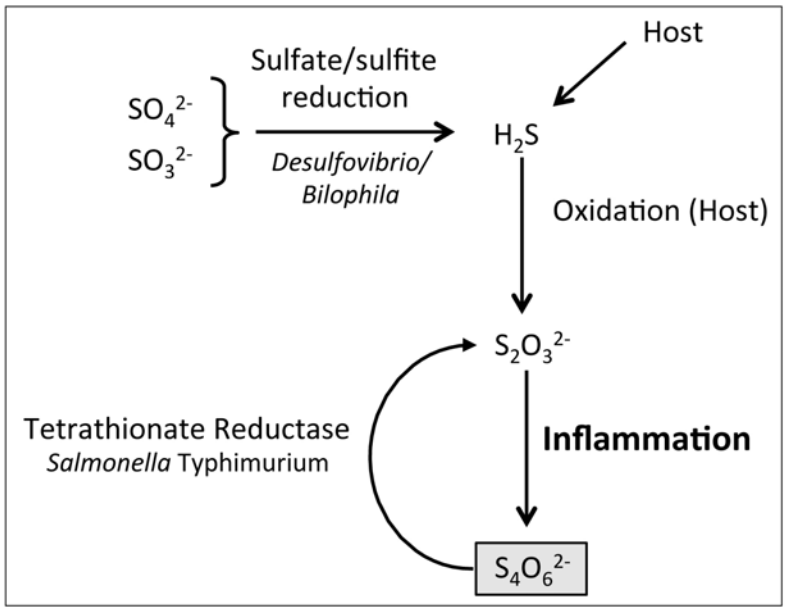

Figure 2: Formation of tetrathionate ($S_4O_6^{2-}$) which *Salmonella enterica* Typhimurium utilizes as an electron acceptor.
Tetrathionate is only formed under inflammatory conditions in the gut. It gives *S.* Typhimurium a growth advantage leading to an outgrowth of the organism in the inflamed intestine (*Winter* et al., 2010).

the pro-inflammatory *Bilophila wadsworthia*, a common isolate from infected organs or tissues such appendix, biliary tract and liver (*Finegold* and *Jousimies-Somer*, 1997; *Summanen* et al., 1995). *B. wadsworthia* belongs phylogenetically to the Desulfovibrionaceae within the δ-Proteobacteria, but unlike other genera of this family, *B. wadsworthia* is not capable of sulphate reduction. This organism rather converts the sulphonic group of taurine to sulphite, which in turn stimulates the growth of *B. wadsworthia* in the intestinal tract owing to the organism's ability to utilize sulphite as an electron acceptor (Figure 1). These experiments showed for the first time a causal relationship between diet and IBD development. However, it has to be emphasized that it is unclear whether these observations are of any relevance to the human situation.

Tetrathionate has been identified as a molecule that stimulates the growth of *Salmonella enterica* serovar Typhimurium (*Winter* et al., 2010). As a response to infection by this organism host neutrophils produce reactive oxygen species. They react with thiosulfate ($S_2O_3^{2-}$) giving rise to tetrathionate ($S_4O_6^{2-}$). Thiosulfate is a detoxification product formed by epithelial cells through the oxidation of hydrogen sulphide, which can be produced by both intestinal bacteria and the host (Figure 2). However, tetrathionate formation is only observed under inflammatory conditions suggesting that the pathogen, by inducing an inflammatory response, creates favourable conditions that promote its own growth. *S.* Typhimurium is capable of utilizing tetrathionate as an external electron acceptor enabling the organism to gain energy from substrates that without an external electron acceptor cannot be utilized. *S.* Typhimurium's ability to utilize tetrathionate as an electron acceptor confers a substantial selective growth advantage on

this pathogen whose proliferation causes an aggravation of intestinal inflammation.

Whereas *B. wadsworthia* and *S.* Typhimurium have pro-inflammatory properties, *Akkermansia muciniphila,* a member of the phylum Verrucomicrobia, is considered a beneficial organism (*Derrien* et al., 2008). However, in a recent gnotobiotic animal study we showed that mice associated with a background microbiota of eight bacterial species (SIHUMI) plus *A. muciniphila* (SIHUMI-A) developed a more severe gut inflammation when challenged with *S.* Typhimurium (SIHUMI-AS) as compared to SIHUMI mice without *Akkermansia* but also challenged with *S.* Typhimurium (SIHUMI-S) or as compared to unchallenged mice (SIHUMI or SIHUMI-A) (*Ganesh* et al., 2013). Pro-inflammatory cytokines and *S.* Typhimurium cell numbers in mesenteric lymph nodes of SIHUMI-AS mice were considerably higher than in SIHUMI-S mice. Interestingly, the number of mucin filled goblet cells in caecal tissue of mice was much lower than in SIHUMI-S, SIHUMI-A or SIHUMI mice. The proportions of the microbial community members in SIHUMI-AS mice differed very much from those of the other three communities: *S.* Typhimurium accounted for 94% of total bacteria in the SIHUMI-AS mice but for only 2.2% in the SIHUMI-S mice suggesting that the comcomitant presence of *A. muciniphila* and *S.* Typhimurium led to a severe disturbance of the microbial community. We proposed that *A. muciniphila* exacerbates *S.* Typhimurium-induced intestinal inflammation by its ability to disturb host mucus homeostasis (*Ganesh* et al., 2013). However, this has not yet been demonstrated experimentally and requires clarification.

CONCLUSION

The intestinal microbiome has been linked to various diseases including obesity, metabolic disease and IBD. However, the underlying molecular mechanisms are mostly not understood or unproven. Gnotobiotic and knockout animal models are valuable tools for testing hypotheses derived from correlations between metagenomics/ metabolomic data and disease symptoms. In a few instances it has been possible to identify dietary components or host-derived molecules that enhance the growth of certain intestinal bacteria and thereby directly promote or aggravate intestinal inflammation. In a complex disease such as the metabolic syndrome intestinal bacteria probably play more than one role in disease development. It will be necessary to scrutinize proposed hypotheses to come to a more precise understanding of how bacteria contribute to disease development.

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THE GUT MICROBIOTA, SHORT CHAIN FATTY ACIDS AND APPETITE

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INTRODUCTION

Modern science has left little doubt that in terms of evolutionary success, bacteria must be at its pinnacle. Every single corner of this planet, and its inhabitants, has a substantial community of bacteria perfectly adapted to their environment. From the depth of the sea to the inners of our own guts, bacteria have learnt to adapt and survive and in many cases have made themselves essential to the overall capacity of its host. Indeed, given the intrinsic role of mitochondria, with its ancestral bacterial endosymbiotic origin, we can safely say that bacteria have not only survived for billions of years, but have been fundamental to the evolution of complex life (*Lane*, 2014). Moreover, and leaving aside mitochondria, bacteria themselves have evolved to symbiotically co-exist with most organisms, conferring them with unique traits and possibilities. To humans, as with all living animals, bacteria have become a unique set of "friends with benefits".

Currently, it is estimated that there are at least 70 different groups of bacteria in the world, however only four appears to inhabit the human gut, forming what has become known as the microbiome (*Macfarlane* et al., 2004). Most of these bacteria are selected at birth, or soon thereafter (*Palmer* et al., 2007), and their overall population appear to be highly dependent on their ability to interact with intestinal derived IgA, which in turn allows them to penetrate the mucous layer protecting this tissue. Here, the microbiota flourishes and is in continuous interaction with our bodies. In ideal conditions, this interaction leads to clear benefits to both parties; however the mechanisms associated with this interaction, both positive and in some cases negative, are not fully understood. What is clear is that more and more research is showing that the synergetic balance between the microbiota and our bodies can be readily disrupted, in many cases in unforeseen ways, by medicines and diet. This arises mainly from the fact that we currently have not proper handle on the extent and depth at which our gut microbiota interact and modulate our own physiology. Indeed, there is increasing evidence that alteration in the microbiome may lead to many chronic disorders, including obesity, type II diabetes, the metabolic syndrome, autoimmune diseases (*Hansen* et al., 2015) and even some neurological and cognitive conditions (*Wang* and *Kasper*, 2014). It is this close correlation between the microbiome disruption and disease development that is driving current research, all of it aimed at understanding the microbiome-host interaction and how we can use this knowledge to prevent and/or treat many modern non-inheritable diseases.

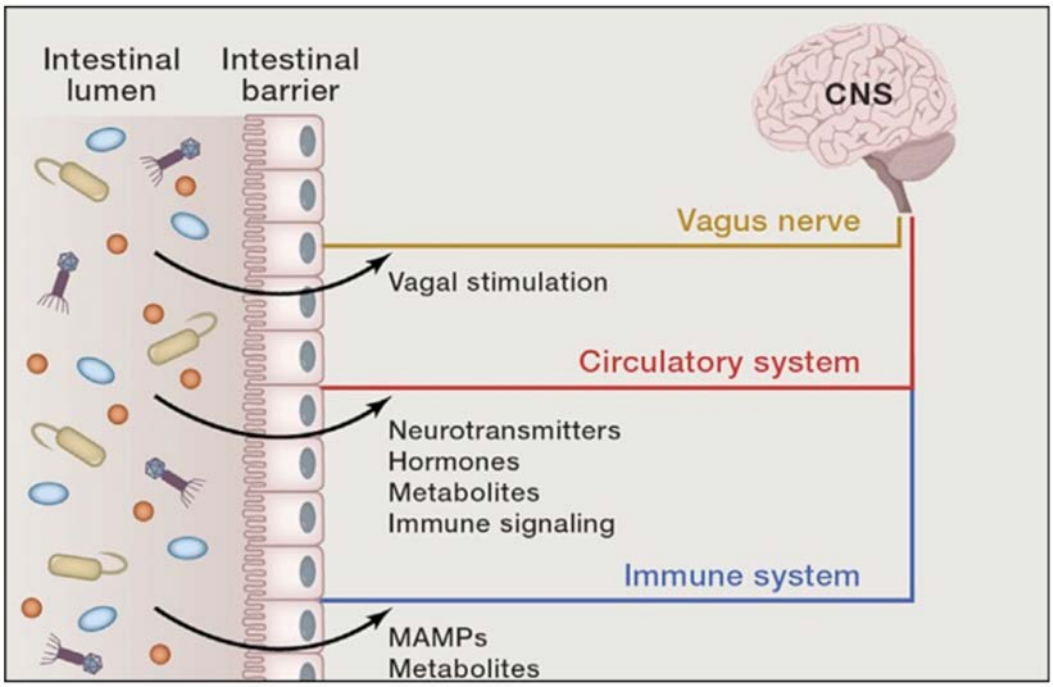

Figure 1: Interaction between the gut microbiota and the brain (Adapted from *Sampson* et al., 2015).

MICROBIOTA-HOST INTERACTION

Broadly speaking, the microbiome interacts with its host in three different, yet linked, manners. Firstly, and at its most basic, the symbiotic relationship between gut microbiota and our bodies leads the former to increase energy availability to our system by digesting food components that would otherwise be lost through the faeces. It is now well accepted that under some dietary conditions the gut microbiota can increase energy supply by well over 20% (*Krajmalnik-Brown* et al., 2012). This energy is principally in the form of short chain fatty acids (SCFAs - mainly acetate, propionate and butyrate), and monosaccharides, together with gases such as CO_2, CH_4, and H_2. Gut microbes have also been shown to produce vitamins, detoxify plant components as well as metabolise medicine in unexpected ways. Secondly, the microbiome can directly interact with our bodies through its modulation of the immune system and the production of neuroactive compounds (*Sekirow* et al., 2010). Indirectly it also modifies levels of many gut peptides which in turn modulate a myriad of anatomical, physiological and metabolic systems, including affecting the blood-brain barrier permeability (*Braniste* et al., 2014). Finally, the gut microbiota derived products e.g. SCFA interact directly with different organs including the brain (*Frost* et al., 2014). Interestingly, yet less understood is the interaction between the gut microbiota and our central nervous system (*Sampson* et al., 2015). This interaction again appears to take three distinctive, yet interconnected paths: directly through the Vagus nerve and indirectly through the circulatory system and the immune system (Figure 1).

In recent years, a number of groups

around the world, including our own, have turned their attention to the modulation of brain function by the gut microbiota through the circulatory system, with especial focus on the production and role of gut-microbiota derived SCFAs in brain function.

SCFA PRODUCTION AND BRAIN METABOLISM

Our gut microbiota is believed to be made up of over 500 bacterial species, with Bacteroidetes and Firmicutes accounting for >90% of the phylotypes (*Eckburg* et al., 2005). A key action of this microbiota population is the production of large amounts of SCFAs. Colonic levels of SCFAs can reach well >100 mM concentration, derived principally from the fermentation of indigestible carbohydrates, unabsorbed sugars, cellulosic/non-cellulosic polysaccharides and some proteins normally found in our diets (*Cummings* et al., 1987). A number of studies with preclinical models and humans have shown that increasing the level of non-digestible carbohydrates in the diet, including inulin, has significant effects on both the colonic microbiota population and the amount of SCFAs produced (*Arora* et al., 2012). As previously mentioned, acetate, propionate and butyrate are the main SCFAs produced in our colon, in a ratio that varies according to our diet of around 40:40:20 to 75:15:10, respectively (*Rubinstein* et al., 1969; *Macfarlane* et al., 2003). In the case of butyrate, this SCFA is mainly utilised by colonocytes, with little or no butyrate being detected in circulation (*Roediger*, 1980). Propionate on the other hand is both utilised by colonocytes, as well as reaching the liver where it serves as substrate for gluconeogenesis and also appears to modulate cholesterol synthesis (*Mortensen* and *Clausen*, 1996). Acetate, the most abundant of SCFAs, is mostly spared by colonocytes and is found in high concentration in plasma (*Cummings* et al., 1987). Indeed, and again depending on the diet, acetate can reach mmol concentration in plasma. Yet, despite its abundance, the overall function of acetate is still unknown, with recent work suggesting that this SCFA may be metabolised in many organs, is associated with energy metabolism (*Kondo* et al., 2009) and appears to be closely linked to histone and protein acetylation (*Soliman* and *Rosenberger*, 2011).

Until recently, SCFAs were believed to play no direct role on brain function, exerting their effects through modulation of inflammatory processes and/or behaved as signalling molecules that interact with the G-protein-coupled receptors, leading to changes in hormones production from L-cells, including the satietogenic hormones glucagon-like-peptide-1 (GLP-1) and peptide-YY (PYY) (*Tolhurst* et al., 2012; *Psichas* et al., 2014). Moreover, propionate and acetate have been shown to affect adipogenesis and through this, leptin production. Thus, through modulation of gut and adipocyte derived hormones, SCFAs was thought to affect brain function, including appetite and satiety (*Xiong* et al., 2004; *Samuel* et al., 2008). However, more recently and through the use of advanced imaging techniques, it has been possible to demonstrate that the SCFA acetate appears to reach many organs in the body, besides the liver. This points towards the fact that this acetate may not only affect brain metabolism indirectly, but may play an important role in the overall function of the central nervous

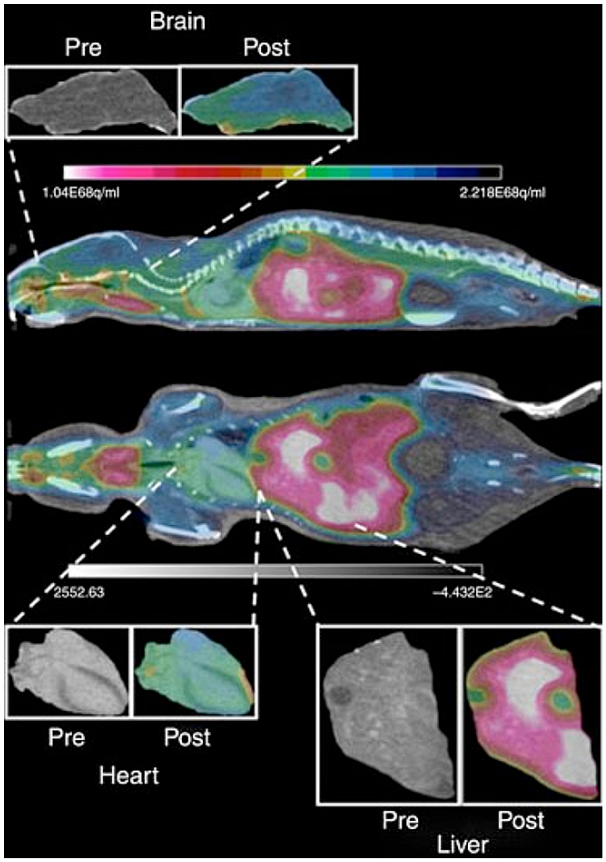

Figure 2: Biodistribution of ^{11}C-acetate in a mouse by PET imaging.
Note that acetate can be detected in most organs, including the brain (Adapted from *Frost* et al., 2014).

system. In the brain, acetate is able to directly affect neuronal function in a number of key brain regions, especially those associated with appetite and satiety control (*Anastasovska* et al., 2012).

ACETATE IMAGING AND BIODISTRIBUTION

The advent of *in vivo* imaging techniques, including magnetic resonance imaging (MRI) and positron emission tomography (PET), has made it possible for researchers to obtain quantitative data on biodistribution, *in vivo*, of many compounds, including SCFAs (*Song* et al., 2009). Carbon-11 labelled acetate was initially produced as a potential PET marker for clinical studies of tumour metabolism (*Vavere* et al., 2008); however it quickly became clear that here was a unique opportunity to get a better understanding of the overall biodistribution of this SCFA. From these studies it became clear that acetate is actively taken up by many organs, including the liver, skeletal muscle, spleen, heart and adipose tissue (Figure 2). More importantly it has been shown to reach the brain in small, yet significant amounts,

independent of route of administration (ip/rectal) or whether it arose directly from dietary components or given in purified form (*Frost* et al., 2014).

However, due to the nature of the PET technique it was not possible to assess the overall metabolism of acetate in different organs (*Grassi* et al., 2012). Carbon-11 has a rather short half-life (c.a. 20 minutes) making longitudinal studies extremely difficult. Moreover, due to the inability of PET to determine the chemical nature of the compound-giving rise to the positron-signal, it is not possible to identify acetate-derived metabolites. These issues could in theory be overcome by the use of hyperpolarised ^{13}C-acetate, in combination with MRI (*Bastiaansen* et al., 2013), yet no such experiments have been carried out so far. This is principally due to the fact that this technique is not widely available to the scientific community, especially those involved in nutritional studies, and also due to the overall cost associated with this methodology.

ACETATE AND APPETITE

Brain plays an important role in the control of appetite and it was first the lesions or surgical transections of a nuclei of the hypothalamus such as the arcuate nucleus (ARC), ventromedial nucleus (VMH) and paraventricular nucleus (PVN) resulting in changes in daily food intake that supported this link (*Dube* et al., 1999; *Penicaud* et al., 1983). More recent work has shown that these nuclei express a number of key neuropeptides receptors that are activated by gut peptides or adipokines and regulate food intake (*Van Den Top* et al., 2004; *Satoh* et al., 1997). Two sets of neurons exists in the ARC; orexigenic neuropeptides Neuropeptide Y (NPY) and Agouti Related Peptide (AgRP) increase food intake and induce obesity, whereas Pro-opiomelanocortin (POMC) and Cocaine and Amphetamine Regulated Transcript (CART) inhibit food intake and these signals from the ARC projects to other nuclei (*Parkinson* et al., 2009).

Manganese-enhanced MRI (MEMRI) is used for functional MRI in animals due to the unique T1 contrast generated by the paramagnetic analogue Mn^{2+} (*Kuo* et al., 2005; *Lin* and *Koretsky*, 1997). Due its ability to mimic Ca^{2+} ions, Mn^{2+} ions can permeate presynaptic voltage-gated calcium channels (VGCC) and induce neurotransmitter release from depolarised nerve terminals (*Narita* et al., 1990). The technique has been implemented successfully to monitor brain activation in the regions controlling appetite (*Kuo* et al., 2007; *Anastasovksa* et al., 2012).

Acetate has been shown to be actively metabolised in neuronal and astrocyte cell culture *in vitro* (*Brand* et al., 1997). Supplementation of fermentable carbohydrates such as inulin has long been shown to reduce appetite in animal studies and this effect has been thought to be through the effect of SCFA on gut peptides (*Cani* et al., 2004) but recently appetite suppressing effect of acetate was shown to be through hypothalamic neuronal activations (*Frost* et al., 2014). Intraperitoneal administration of acetate resulted in reduced food intake. Interestingly when acetate was encapsulated in liposomes limiting its distribution to the brain, no reduction in food intake was observed which would suggest that acetate has a direct neuronal function. Using ^{13}C HR-MAS it was shown that acetate, administered or produced in

the colon, in the hypothalamus incorporates into the glutamate-glutamine transcellular cycle, increasing lactate and GABA labelling, thus supporting hypothalamic glutamatergic or gabaergic neurotransmissions. This increased gabaergic neurotransmission together with increased lactate oxidation will result in increased ATP production in turn inhibiting AMPK activity and activating Acetyl-CoA Carboxylase. The increased ACC activity induces Malonyl-CoA expression which activates POMC neurons and therefore reduces food intake. By using MEMRI hypothalamic activation was also monitored which showed increased activation with acetate administration in hypothalamic brain regions such as arcuate nucleus, which is populated by POMC neurons among others.

SUMMARY

Short chain fatty acids derived from the gut microbiota fermentation of non-digestible carbohydrate, principally, acetate have now been shown to reach the brain where it can affect important function including appetite and satiety. The extent of this effect can be readily observed in the significant decrease in food intake in preclinical models. However, acetate is also metabolised in other organs which themselves may indirectly affect appetite. It is therefore imperative that central and peripheral effects are studied in isolation to determine the extent by which acetate directly affects brain function and behaviour.

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MIMICKING THE HUMAN GUT MICROBIOME COMPOSITION AND FUNCTIONALITY

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SUMMARY

This paper highlights the current *in vitro* technological advances in simulating the gut microbiome composition and functionality with enabling technologies related to the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). While *in vitro* models offer the standard advantages over *in vivo* studies (low costs, no ethical constraints, multi-parametric testing), their core value lies in their ability to simulate regional and micro-environmental differences that occur along the longitudinal and radial axis of the gut. We will provide evidence that these models can be used:

1. to simulate microbiome differences between proximal and distal colon regions,
2. to establish distinct luminal and mucus-associated microbial communities, and
3. to enable intimate host-microbe interactions near the gut epithelial surface.

More specifically, we will present published and unpublished data obtained with the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) and the Host-Microbe Interaction (HMI-) module. Finally, our insights into the specific micro-environmental behaviour of gut microbes will be used to propose some future perspectives for (*in vitro*) gut microbiome research.

INTRODUCTION

Human health is influenced by a multitude of determinants: genetics, way of delivery, diet, lifestyle, medical practices, hygiene, the exposome… Also the human microbiome is considered a major factor in the health equation, either directly or through interaction with any of the aforementioned factors. In the last decade of scientific research, different -omics approaches have revolutionized the field by shedding light on correlations between health status and microbiome composition, specific expression of genes, translation into proteins or production of specific metabolites. The number of these studies has exponentially grown in recent years, yet scientists struggle to find preventive or therapeutic measures that tackle any of the microbial processes (*de Vrieze*, 2015). This is mainly caused by the lack of mechanistic insight in host-microbe interaction processes and the lack of appropriate model systems that allow dynamic sampling of specific environments.

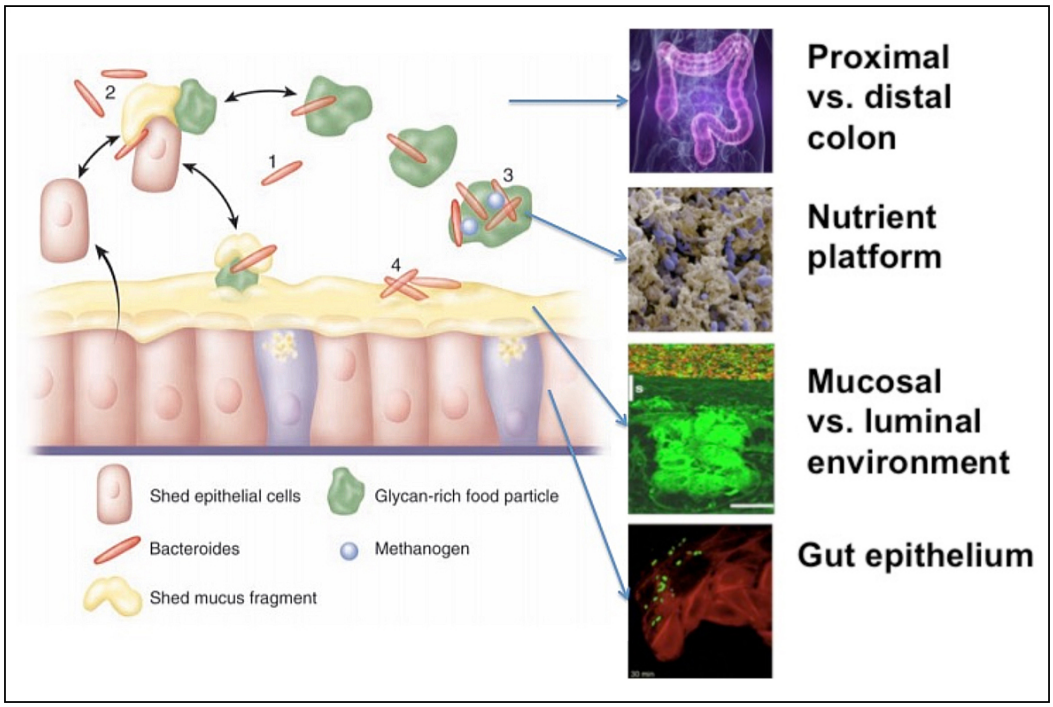

Figure 1: Depiction of different gut micro-environments and altered microbial colonization profiles (picture adapted from *Bäckhed* et al., 2005).

GUT MICROENVIRONMENTS

"The" gut microbiome is composed of different microbial consortia that are composition-wise and functionally highly diverse because of their association with different gut micro-environments (Figure 1) (*Bäckhed* et al., 2005). Because of differences in residence time, physicochemical or enzymatic stressors in the upper digestive tract, diverse presence of M-cells or variable thickness of the mucus layer along the gastrointestinal tract, the microbial colonization is distinct and highly specific. Already in the colon alone, there are significant differences between the proximal and distal region when it comes down to microbiome composition and the fermentative metabolism of dietary substances or the metabolic potency towards secondary plant metabolites, pharmaceuticals or pollutants. The suspension in the gut lumen cannot just be considered as a homogeneous mixture of non-digestible dietary components, microbiota and host secretions. It is rather a heterogeneous suspension where nutrient platforms offer a scaffold for microorganisms to adhere to and where they can interact with one another, for example to cross-feed on dietary fibre resulting in the production of a diverse short chain fatty acid profile. When focusing on the mucus layer overlying the epithelium, one must appreciate the existence of specific physicochemical and immunological gradients that dictate what microorganisms have a higher preference (or success rate) to become part of the mucosal microbiome. The existence of an oxygen gradient over the epithelium and mucus

layer can result in altered colonization and even modulated gene expression, for example by *Shigella flexnerii* pathotypes (*Marteyn* et al., 2010). The mucosal surface and intervillus region is also known for their altered fluid shear forces, with low fluid shear prevailing near the mucosa and for example directing virulence gene expression by pathogenic *Salmonella* strains (*Höner zu Bentrup* et al., 2006). These mucosal microbes are interesting from a health point of view, because of their close proximity to the gut epithelium and, hence, higher potency to interact with the human host. Specific microorganisms can even further migrate through the mucus layer and actually reach epithelial cells where they can more profoundly interact with the host, sometimes leading to actual pathogenesis. Finally, because of the gut being an open ecosystem, changes in the aforementioned determinants (diet, medical practices, lifestyle…) add an additional layer of complexity to the microbiome composition. This necessitates a dynamic monitoring of the gut microbiota and a correlation with changing environmental parameters.

NEED FOR MODEL SYSTEMS

For obvious reasons, human microbiome research is physiologically most representative when conducted on biological samples from human subjects. However, only relying on the analysis of faecal microbiota does not give an accurate view on the colonization ability and dynamics of the microbial consortia in the different gut microenvironments. While biopsy samples may give a closer view, the sampling procedure is far from straightforward and it does not allow to dynamically monitoring the microbiome upon dietary shifts or during disease progress. While animal models - even the gnotobiotic models that are humanized with human microbiota - give some more flexibility in the analysis of different gut regions, also these samples are restricted to endpoint measurements. Moreover, interpretation of the scientific data must always take into account to what extent these models are representative for human biological processes.

To address the issues of gut microenvironment differences and microbiome dynamics, specific lab-scale model systems have been developed over recent years. While the biggest limitation of these model systems is obviously the lack of a physiological environment, they do provide certain advantages over *in vivo* observations. Firstly, specific microenvironments can be simulated, both in a longitudinal direction (proximal vs. distal gut regions) as in a cross-sectional direction (luminal vs. mucosal regions). Secondly, these environments can be sampled in a dynamic way, allowing the study of microbial adaptations to a changing environment. Finally, and this is probably the biggest *in vitro* asset, due to the control over several digestive, enzymatic and physicochemical parameters, scientists are able to conduct mechanistic research. These benefits can however not be overestimated. *In vitro* models always need a proper validation against human *in vivo* data and the generated scientific insights need to be used to support *in vivo* observations or to direct research prior to entering clinical research.

One of the models that has tried to encompass most of the digestive processes going from the upper digestive

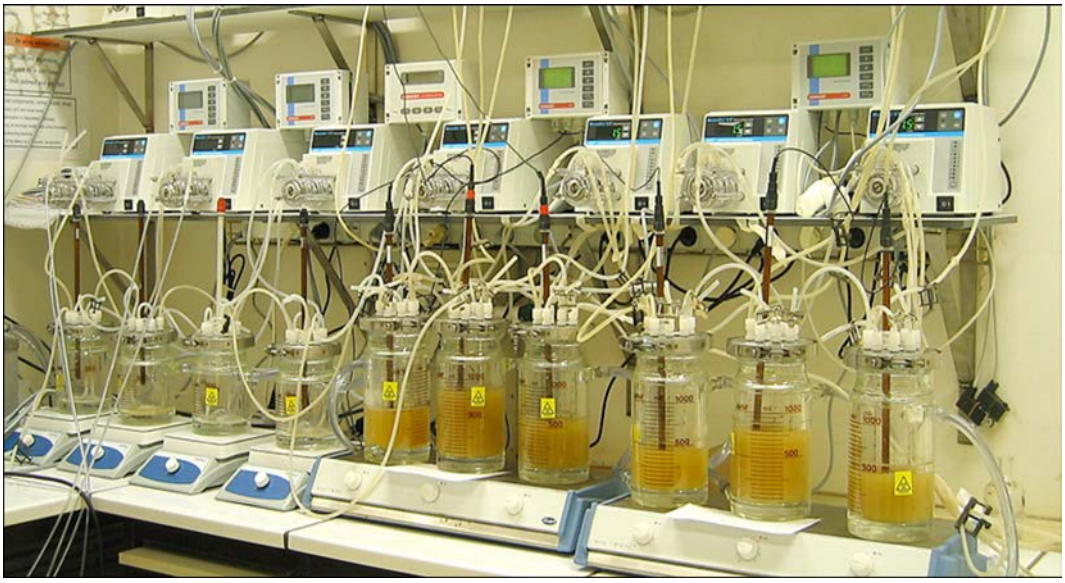

Figure 2: Experimental setup of a TWIN-SHIME system, composed of 2 parallel units of stomach, small intestine, ascending colon, transverse colon and descending colon compartments.

tract (stomach, small intestine) to the lower gut (proximal and distal colon) is the simulator of the human microbial ecosystem, or SHIME® (registered name by Ghent University and ProDigest) (Figure 2) (*Molly* et al., 1993). The model system has been validated against human *in vivo* data both for microbiome composition and fermentative activity (*Molly* et al. 1994) as for very specific metabolic conversions that are distinguishing individuals from one another (*Possemiers* et al., 2006). While at first, SHIME research primarily focused on metabolic interactions in the lumen, the last 5 years have seen a significant improvement of the model by including a mucosal environment by incorporation of mucus coated microcosms (*Van den Abbeele* et al., 2013). This so-called M-SHIME (mucosal SHIME) (Figure 3) allows not only the luminal microbes to settle in the system, but also the colonization of mucosal microbes on surfaces that are representative of the *in vivo* situation, at least from the glycoprotein perspective.

Another improvement of the SHIME model is the host-microbe interaction (HMI) module (Figure 4) (*Marzorati* et al., 2014). This bicompartmental module separates gut microbes from epithelial cells through a mucin-covered semi-permeable polyamide membrane. Unlike Transwell® systems, the HMI module has in- and outlets at both the microbial and host side of the membrane. This enables connecting the microbial compartment to the M-SHIME and seeding the host compartment with 3D organotypic colon epithelium. In a proof-of-concept, this module was successfully used to study host responses upon prebiotic modulation of the intestinal microbiota (*Possemiers* et al., 2013).

Interestingly, two specific physicochemical conditions prevailing near the mucosa can also be simulated with the HMI device. Firstly, by providing oxygen from the host compartment and maintaining anaerobiosis in the microbial compartment it is possible to

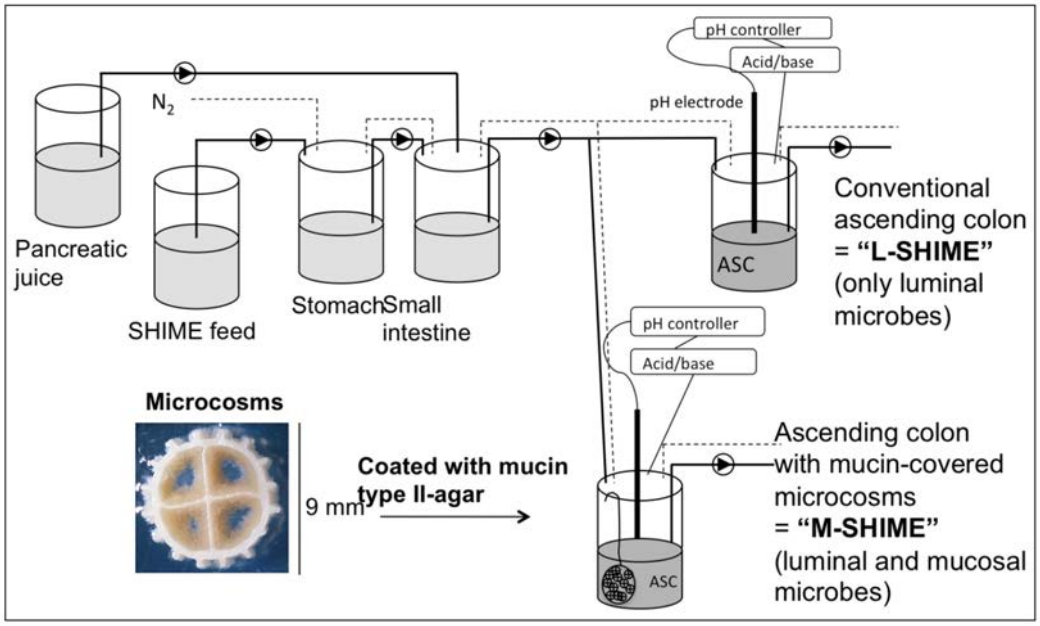

Figure 3: Experimental setup of the M-SHIME system. Mucin coated microcosms or mucus beads are brought into the respective colon compartments thereby creating a mucosal contact surface next to the luminal suspension (*Van den Abbeele* et al., 2013).

establish an oxygen gradient over the mucus layer and thereby mimic the mucosal environment more closely. This is for example interesting to study what impact hypoxic stress may have towards microbiome colonization in a scenario of insufficient mucosal oxygenation with IBD patients. Secondly, the design of the HMI module is such that it allows control over fluid shear conditions over the mucus layer, another important parameter that not only affects morphology and gene expression of epithelial cells, but also the gene expression of microorganisms in that environment.

MIMICKING THE MUCOSAL MICROBIOME AND MICROBIAL BEHAVIOUR IN THE MUCOSA

In this paper, we primarily focus on the mucosal microbiome and the role that specific microorganisms may play in health and disease. *In vivo* data have demonstrated the microbiome from biopsy samples to have a distinct composition as opposed to faecal microbiota (*Zoetendal* et al., 2002; *Swidsinski* et al., 2002). Moreover, there seems to be specific correlations between specific mucosal microorganisms and health status: for example, typical mucosal colonizers such as the butyrate producing microorganisms such as *Faecalibacterium prausnitzii,* downregulated in patients with Crohn's disease (*Willing* et al., 2009) and metabolic syndrome. More detailed *in vivo* analysis on murine samples also indicates the higher preference of butyrate producing Clostridia to colonize the mucosal environment (*Nava* et al., 2011). In that respect, it is interesting to note that the M-SHIME system

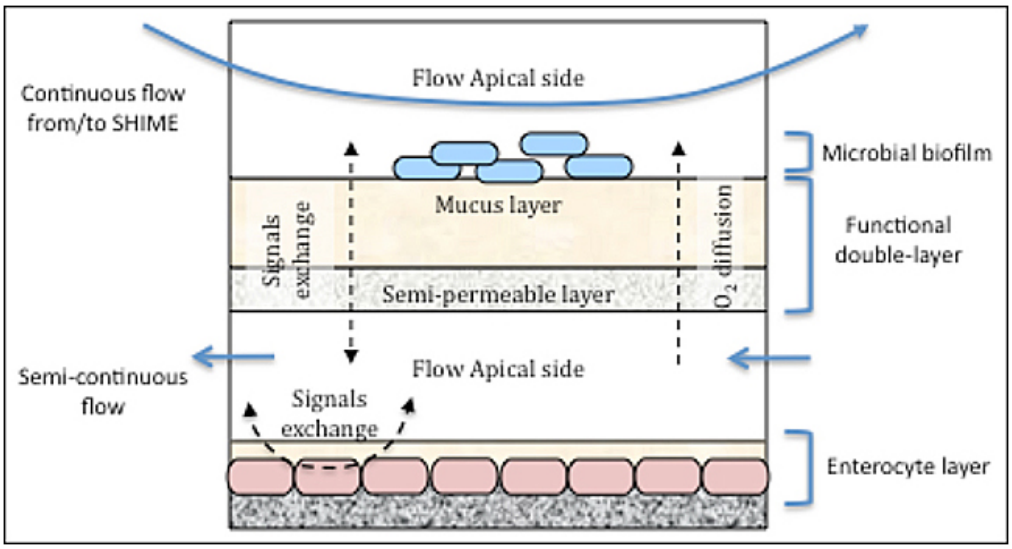

Figure 4: Host microbe interaction module comprising flow cell with low fluid shear conditions representing the mucosal microbial compartment and a host compartment (*Marzorati* et al., 2014).

displays similar colonization profiles with much higher abundances of *Clostridium* clusters IV and XIVa in the mucosal environment as opposed to the luminal suspension (*Van den Abbeele* et al., 2013). In addition, micro-array analysis for 1100 microbiome phylotypes also demonstrates M-SHIME to preserve unique microbiome features that distinguish individuals from one another.

The finding of a more pronounced colonization of the mucosal environment by butyrate producers requires some further attention. Whole genome shotgun data from the human microbiome project demonstrate these butyrate producing *Clostridium* cluster IV and XIVa to reach abundances up to 30% of the entire microbiome (*Karlsson* et al., 2012). It thus plays a dominant role in the gut ecosystem. Moreover, monoassociation of gnotobiotic mice with several of these butyrate producing Clostridia was found to result in an upregulation of Foxp3+ in CD4 cells, indicating the role of these microorganisms in regulatory T cell function (*Atarashi* et al., 2011). This can either occur through bacterial cell-associated antigens or through secreted compounds. To exemplify, *Faecalibacterium prausnitzii* was previously found to be an anti-inflammatory commensal following microbiome analysis of Crohn's disease patients (*Sokol* et al., 2009). Only recently, the compound thought to elicit the anti-inflammatory effects in epithelial cell culture models and an animal model of chemical-induced colitis, was revealed to be a 15 kDa protein, termed MAM (microbial anti-inflammatory molecule) (*Quévrain* et al., 2015).

The example of *F. prausnitzii*'s anti-inflammatory properties is particularly interesting. As mentioned above, Clostridium cluster IV butyrate producers, from which *F. prausnitzii* is the most abundant, are strong colonizers of the mucosal environment (*Willing* et al., 2009). Butyrate producing clostridia are typically anaerobic and given the fact that oxygen gradients may exist over the mucus layer, it is intriguing to find out what mechanism lies behind

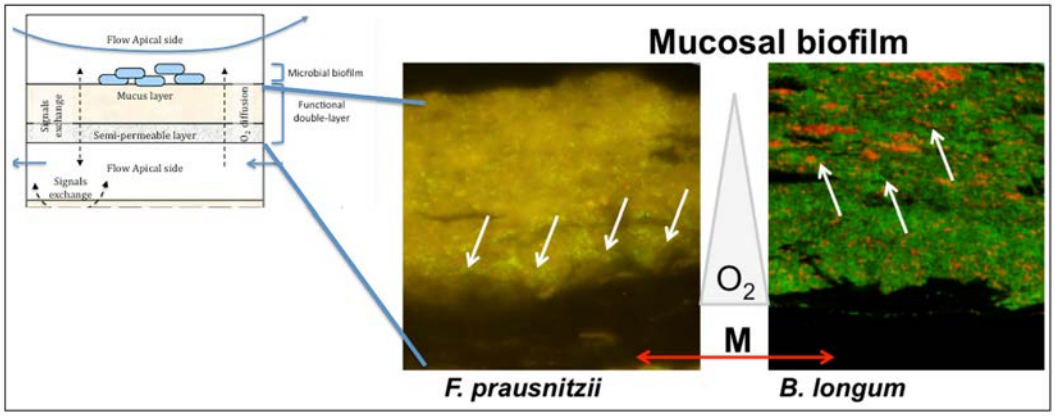

Figure 5. FISH analyses a) positioning of F. prausnitzii (left panel - fluorescent microscopy) and bifidobacteria (right panel - Confocal Laser Scanning Microscopy) in the microbial biofilm with respect to the membrane and mucus layer (M), as indicated by the white arrows. Oxygen concentration (O_2) is assumed to decrease from the bottom to the top of the biofilm. The green background is auto-fluorescence of the matrix: EPS, and non-responding bacteria in the left panel, while in the right panel it corresponds to bacteria stained with the EUB338 probe FITC labeled, and also some auto-fluorescent EPS (adapted from *Marzorati* et al., 2014).

their successful colonization. To monitor this mucosal colonization process more in-depth, the HMI module is particularly useful. Coupling the HMI to SHIME enables *in vitro* cultured gut microbiota to colonize the mucus layer on top of the semi-permeable membrane in the HMI module. This results in the establishment of an early stage biofilm where the positioning of different microorganisms of interest can be determined. Using FISH probes, *Marzorati* et al., (2014) monitored the mucosal colonization of Bifidobacteria and *Faecalibacterium prausnitzii* with the HMI module and came to interesting findings. While the strictly anaerobic bifidobacteria tended to colonize the upper side of the mucus layer, the anaerobic *F. prausnitzii was* mainly found back in the lower part of the mucus, i.e. at the anoxic/oxic interphase (Figure 5). *Khan* et al., (2012) demonstrated that *F. prausnitzi*i can grow in the oxic-anoxic interphase due to the fact that this microorganism, despite being oxygen sensitive, copes with O2 because of a special extracellular electron shuttle of flavins and thiols. Similar to the *in vivo* situation - where small amounts of oxygen permeate from blood vessels towards the gut lumen - in the HMI module, oxygen diffusion from the aerobic lower chamber to the anaerobic upper chamber (Figure 4) probably results in more oxidative conditions at the base of the biofilm. Availability of the flavin/thiol electron shuttle gives *F. prausnitzii* a selective advantage over other gut microbes enabling it specific mucosal colonization. These findings were supported by the dynamic monitoring of *F. prausnitzii* with qPCR. Over a 48h experiment, a decreasing concentration of *F. prausnitzii* was noted in the luminal compartment and an increasing one in the mucus layer, as opposed to an unchanging bifidobacteria concentration in the lumen and decreasing bifidobacteria concentrations in the mucus layer. This demonstrates the potency of the HMI module to maintain the preferential mucosal colonization of specific gut microorganisms within the mucus layer and the possibility to complement

in vivo observations with mechanistic explanatory data.

This mucosa-specific functional behaviour is not solely confined to *F. prausnitzii*. Using hydrodynamic chronoamperometry with a rotating disk electrode *Prévoteau* et al., (2015) recently demonstrated that also *Butyricicoccus pullicaecorum* - another butyrate producing Clostridia cluster IV member with anti-inflammatory potential (*Eeckhaut* et al., 2012) - has the ability to use riboflavins as electron shuttle for coping with oxidative stress. In a scenario of inflammation, a status of epithelial hypoxia is often encountered due to impaired perfusion or the metabolic demands of localized inflammatory cells (*Marteyn* et al., 2011). This may be one of the reasons where flavin-using electron shuttling butyrate producing Clostridia may loose their selective colonization advantage over other strict anaerobes that cannot make use of electron shuttles to cope with oxidative stress. It is clear that such specific micro-environment functions of gut microorganisms cannot be studied *in vivo,* and that *in vitro* model systems are required to elucidate these mechanisms.

The abundance of butyrate producing Clostridia is not merely correlative with health status. Mucosal butyrate producers may also confer stability to the gut microbiome when challenged by stress factors. We provide one example from dietary related stress and one example from chronic gut inflammation.

One of the most recent insights has come from Western diets that have become increasingly rich in linoleic acid (LA). Just like other poly-unsaturated fatty acids linoleic acid has a strong antimicrobial activity, thereby potentially compromising the gut microbiome. In addition, rumen microbiology has learned us that certain bacteria can convert linoleic acid to more saturated products. This process is called biohydrogenation, in which hydrogen gas is combined with linoleic acid to gradually saturate the double bounds to subsequently vaccenic acid and the completely saturated end-product stearic acid. With each biohydrogenation step the antimicrobial activity is decreased. Interestingly, *Devillard* et al. (2007) have previously described that among a wide range of human gut bacteria, the most important biohydrogenating species are *Roseburia* and *Butyrivibrio* species, which are specialists in converting linoleic acid towards vaccenic acid. Both genera belong to the butyrate producing Clostrida cluster XIVa. It was thus hypothesized that these biohydrogenating and butyrate producing species may confer stability upon challenging an *in vitro* cultured gut microbiome with linoleic acid.

Using M-SHIME (containing both a mucosal as luminal environment) as opposed to L-SHIME (which only has a luminal environment), De Weirdt et al. (unpublished) investigated the importance of the presence of a mucosal environment. Exposing either M-SHIME or L-SHIME to 1 g/L of linoleic acid (which corresponds to the theoretical concentration colon bacteria would experience upon consumption of a Western diet) revealed that the biohydrogenating potential in the presence of a mucus layer (M-SHIME) was 6 times higher than in the absence of a mucus layer (L-SHIME). This indicates a higher biohydrogenating potential of the gut microbiome when mucosal bacteria are able to thrive.

De Weirdt et al. (unpublished data) then investigated to what extent this difference in biohydrogenating functionality was also reflected in the microbiome composition. Co-occurrence analysis on Illumina based NGS of the microbiome's 16S rRNA genes

revealed two distinct microbial populations under control conditions: one large population affiliates with the lumen and has many positive correlations between mainly fermentative bacteria, while a smaller mucosal population contains several of the aforementioned butyrate producing Clostridia clusters including the biohydrogenating Roseburia and Pseudobutyrivibrio. Construction of co-occurrence networks for samples that had been exposed to LA, reveals an interesting shift. It is no longer possible to distinguish the 2 populations with region-specific affinity. Instead, the two specialist biohydrogenating genera move to the centre of a new co-occurrence network where they interact with a lot more genera than was the case for the control situation. These data show that members of the mucosal microbiome with specific functional behaviour (i.e. biohydrogenation) may bring stability to the entire gut microbiome upon dietary stressors such as linoleic acid.

The possibility of using butyrate-producing Clostridia to protect the gut microbiome may also extend towards disease scenarios. Particularly with respect to chronic gut inflammation, clear correlations with microbial dysbiosis and abundance of butyrate producing Clostridia have been observed before. *Willing* et al. (2009) showed that butyrate producing clostridia were lower in abundance in patients with IBD. In addition, *Faecalibacterium prausnitzii* and *Butyricoccus pullicaecorum*, both members from the Clostridium cluster IV, have been shown to elicit a protective effect towards gut barrier function both *in vitro* as *in vivo* (*Sokol* et al., 2009; *Eeckhaut* et al., 2012). Again, model systems of the gut mucosa can reveal more in depth what role any of these microorganisms may fulfil with respect to protection of gut barrier function.

Geirnaert et al. (unpublished) recently inoculated colon compartments from M-SHIME model systems with microbiota from either Crohn's patients during active disease or in remission. While microbiome analysis showed significantly different colonization in the lumen and mucus, metabolic analysis revealed that the cultured colon microbiota originating from active disease had a significantly reduced (30%) butyrate production. M-SHIME colon compartments were then treated with a cocktail of 6 butyrate producing Clostridia from both cluster IV as XIVa. Intestinal water was prepared from the different colon compartments and subjected to Caco-2 epithelial cell cultures during their differentiation process. Trans-epithelial electrical resistance measurements and trans-epithelial lucifer yellow transport experiments were then conducted to find out to the impact on epithelial barrier function.

Intestinal water generated from untreated SHIME colon samples resulted in a 50% drop of the TEER value compared to the control situation. This indicates that secreted metabolites from gut bacterial origin pose a certain stress towards tight junction proteins. These findings were supported by a drop in mitochondrial activity and a sharp increase in paracellular transport of lucifer yellow. As a positive control, addition of 2 mM butyrate (a physiological concentration expected *in vivo)* restored TEER values indicating the protective effect from butyrate towards epithelial barrier functioning. Interestingly, intestinal water derived from colon vessels treated with the cocktail of butyrate producing Clostridia also resulted in normalized TEER values and lucifer yellow transport, independent of butyrate levels. These recent findings with M-SHIME systems connected to Caco-2 epithelial cell cultures can help to support previous findings on the

ability of butyrate producing bacteria to improve gut barrier functioning with bioactive peptides (*Quévrain* et al., 2015) or to stimulate regulatory T-cells (*Atarashi* et al., 2011).

CONCLUSION

To conclude, the importance of micro-environment differences for the preferential colonization of gut microbiota and their functionality cannot be underestimated. While *in vivo* samples cannot always grasp these differences, several *in vitro* model systems have been developed that can mimic these different micro-environments. While very useful in providing mechanistic information to support *in vivo* observations, *in vitro* data can neither be overestimated. Each model system comes with its boundaries, and needs a proper validation with human *in vivo* data.

Another advantage of model systems of the gut micro-environment is the ability to study the micro-environment behaviour of specific microorganisms. This not only contributes to our understanding of their role in host-microbe interactions, but it also provides opportunities for exploring these environments with the purpose of isolating microorganisms with novel metabolic traits etc… (Van den Abbeele et al., 2013).

The increasing insight in the putative health-promoting role of functionally important groups such as butyrate producing bacteria indicate that next generation probiotics will no longer belong solely to the classic lactic acid producing strain, but will also include strains from other phyla. Such efforts obviously need to come with the necessary risk assessment steps and depending on the domain of application (pharmaceutical vs. nutraceutical) and the type of application (preventive vs. therapeutic), selection criteria will be more stringent. In addition, current legislation is not yet adapted for the inclusion of these novel strains and a debate between scientists, regulators and industry is highly warranted.

Finally, the last aspect where *in vitro* model systems can become very useful is the exploration of and preclinical testing of novel biotherapeutics. The last couple of years have brought a revolution in this field, especially with respect to ecosystem restoration. The best-known example is the highly successful application of faecal microbial transplants to cure *Clostridium difficile* associated diarrhoea. While the success of FMT for CDAD primarily lies in the sudden diversification of the microbiome, thereby tackling microbial dysbiosis (the primary reason why *Clostridium difficile* is so successful), this success cannot be simply extrapolated to other pathologies. Especially when considering FMT for disease where inherent gut barrier function may be compromised (Crohn's disease, ulcerative colitis, metabolic syndrome…), extreme caution is necessary given the badly characterized nature of faecal transplants and the risk of disease or allergen transmission from the donor to the receiving patient. Already now, research groups have started to tackle these FMT disadvantages by making cocktails of microorganisms of diverse phylogeny and functionality. This has already resulted in the successful treatment of 2 CDAD patients with a defined consortium of 33 microorganisms, isolated from a healthy individual's faecal microbiome (Petrof et al., 2013).

Knowledge about microbial composition and functionality in specific gut

micro-environments may result in new candidate strains that can be taken up in such defined microbial cocktails. *In vitro* model systems will become useful tools to help in the identification and isolation of such microorganisms and in the preclinical testing of the resulting biotherapeutic products.

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MICROBIAL COMMUNITY DYNAMICS IN *CLOSTRIDIUM DIFFICILE* INFECTION: CONNECTING THE DOTS

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SUMMARY

The Human Microbiome Project established a deep, molecular understanding of interactions between microbial communities and their hosts, and provided insight into how the host and epigenetic factors can influence intestinal ecosystems. In this context, we give a brief overview of the capacity for metagenomics and metabolomics to explore host-pathogen interactions over multiple life stages. Young children are naturally resistant to toxigenic *Clostridium difficile,* which forms part of the normal developing microbiome. By studying both paediatric and adult cases, we hope to discover why susceptibility to *C. difficile* changes as we age. Our mini-review considers current risk factors in *Clostridium difficile* infection and emerging treatment options, including faecal microbiota transplantation as promising therapy when antibiotics fail. Manipulation of the intestinal microbiota offers a relatively unexplored niche for targeted therapy, but incomplete genome coverage, bioinformatics discrepancies and inferred biological function highlight the need for a multi-'omic approach to complement next generation sequencing and provide a direct measure of the altered intestinal phenome.

INTRODUCTION

Antibiotic resistance among pathogenic bacteria is a major emerging public health threat with estimates of worldwide deaths reaching 10 million annually by 2050 (*Review on Antimicrobial Resistance*, 2014). Overuse of antibiotics and adaptability of bacteria have created a growing number of "super bugs" that are resistant to multiple antibiotics. *Clostridium difficile* is one such organism and is rapidly becoming one of the major public health threats of the 21st century. *C. difficile* is a spore-forming anaerobe that is the single leading cause of nosocomial infections in some hospitals (*Pant* et al., 2013; *Magill* et al., 2014). In the western world, it is by far the most deadly enteric pathogen triggering colonic disease due to the secretion of two potent exotoxins (Figure 1) (*Taylor* et al., 1981; *Savidge* et al., 2003; *Genth* et al., 2008; *Kuehne* et al., 2010). The incidence and severity of *C. difficile* infection (CDI) has risen dramatically in the United States since 2000 with almost half a million annual cases including 30,000 deaths in 2011(*Lessa* et al., 2015). Because of this perceived threat to patients, the Centers for

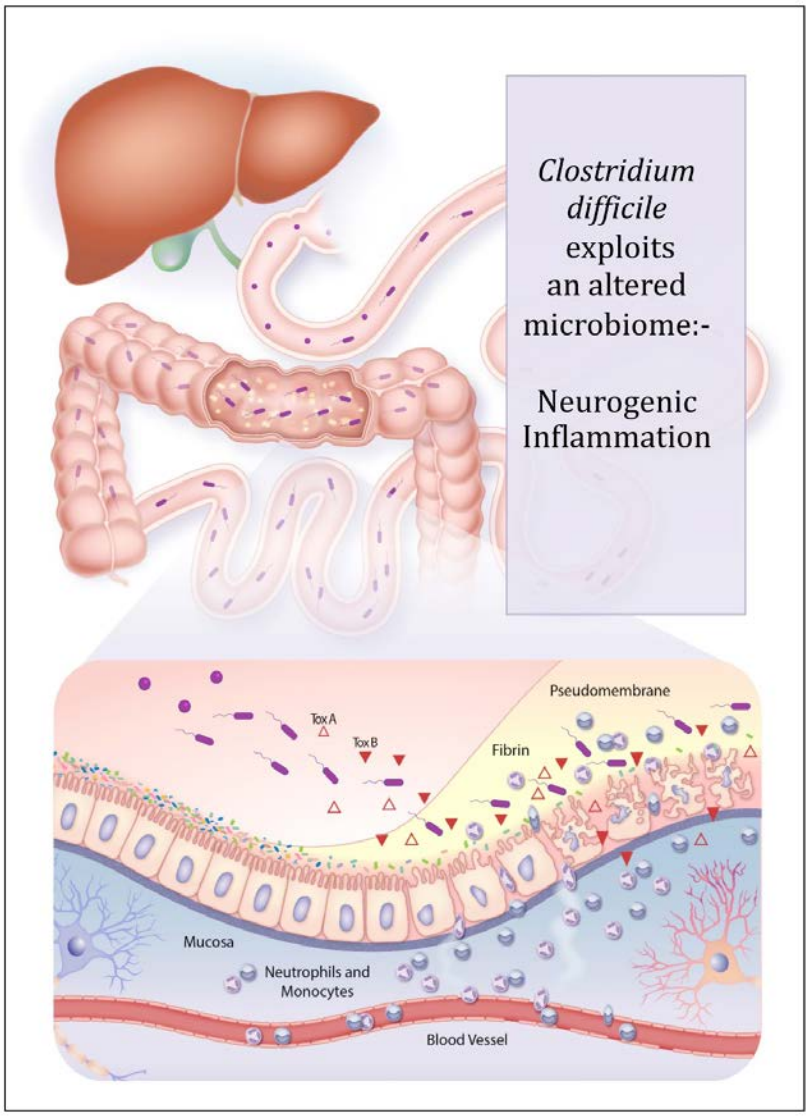

Figure 1: Schematic of *C. difficile* infection in a patient.

Disease Control and Prevention assigned an urgent hazard level (*Centers for Disease Control and Prevention*, 2013) to this pathogen and urged the scientific community to identify risk factors to better manage new, but expensive treatment (e.g. Dificid®).

Due to the growing international concern regarding both clinical management and dissemination of *C. difficile*, the United States and Europe now require hospitals to report symptomatic CDI cases. Because *C. difficile* is resistant to most antibiotics, first line therapy includes off-label use of metronidazole or oral vancomycin in clinically severe and recurrent cases (*Cohen* et al., 2010; *Goldberg* et al., 2015). However, up to 35% of CDI patients will experience a clinical recurrence following cessation of antibiotic use despite a favourable response to treat-

ment (*McFarland* et al., 1999; *Garey* et al., 2008; *Johnson*, 2009; *Kelly*, 2012; *Goldberg* et al., 2015). Of these patients, up to 50% will experience subsequent infective episodes, adding to patient morbidity (*Fekety* et al., 1997; *McFarland* et al., 1999, 2002). Notably, half of the recurrent episodes involve a new *C. difficile* strain, strongly suggesting that epigenetics and the intestinal ecosystem modulate host susceptibility to this pathogen (*Young* and *Schmidt*, 2004; *Garey* et al., 2008; *Antonopoulos* et al., 2009; *Rupnik* et al., 2009; *Centers for Disease Control and Prevention*, 2012; *Britton* and *Young*, 2012; *Peery* et al., 2012; *Theriot* et al., 2014). The heralded clinical success of faecal microbiota transplantation (FMT) in recurrent CDI cases (>90% efficacy) (*Burke* and *Lamont*, 2013; *McKinney*, 2013) is strongly supportive of host-microbe interactions being important in preventing CDI onset. The procedure involves single to multiple orogastric or intracolonic infusions of faecal bacteria originating from healthy donors. Typically, only a single treatment is necessary to eradicate disease in patients that previously experienced multiple recurrent CDI episodes. Although FMT is considered a medical triumph against recurrent CDI, it is also regarded as a treatment of last resort because of safety and social concerns, especially in children. After an initial 2013 FDA regulatory ruling (*McKinney*, 2013) FMT is now only available for recurrent CDI in a limited number of health centres. Due to limited access, self-administered FMT is becoming a common practice in the community, raising additional ethical and safety concerns.

CURRENT TREATMENTS FOR CDI

Current herapeutic options for *C. difficile* include metronidazole (currently the most common treatment), oral vancomycin and the newly approved fidaxomicin (Dificid®). Access to vancomycin and fidaxomicin is somewhat restricted due to high cost. However, vancomicin has increased market share with significant compounding occurring in hospital pharmacies, allowing capitalization of cheaper, generic formulations. Fidaxomicin is an important new antibiotic that reduces recurrent episodes with some strains of *C. difficile*. However, a critical liability may be a reduced effect on recurrence for the clinically important epidemic strains (24.4% vs. 23.6% for FXD and VA for ribotype 027) demonstrating a significant market opportunity for alternative approaches, notably microbial therapeutics with either defined bacterial communities or single non-toxigenic *C. difficile*.

It is proposed that toxin inactivation by the Merck antitoxin IgG monoclonal antibodies provide protection against recurrence. Merck recently advanced an antibody combination into Phase III clinical trials. Reported Phase II trial results demonstrated that single injections, when used with standard antibiotics, reduced disease recurrence to as low as 7%. A cogent rationalization for the reduction is lacking but Merck's investment provides evidence of substantial interest in a therapy directed toward reducing CDI recurrence. Other experimental therapies currently in development include vaccines, toxin-absorbing polymers, bile acid analogues and probiotics. Notably, toxin-binding resins also target bile acids. In support of a vaccine development program, antibodies against both toxins are protective in hamsters, and serum

anti-toxin antibodies in patients correlate with protection against symptomatic disease and recurrence. However, the scope of antitoxin vaccination remains uncertain since patients with severe CDI are usually elderly and critically ill. While effective in hamster CDI models, toxoid-based vaccines currently in Phase III trials have not proven as protective in patients and are associated with several inherent shortcomings, including batch-to-batch variations and potential residual toxicity. Given this critical need to generate predictors of clinical outcome, there is currently much interest in understanding the intestinal ecosystem that is vulnerable to *C. difficile* and epigenetic factors that promote antibiotic resistance in this pathogen.

ALTERED MICROBIAL COMMUNITY DYNAMICS IN CDI

Antibiotic exposure is a major risk factor for CDI development due to disruption of the indigenous microbiota (*Young* and *Schmidt*, 2004; *Jernberg* et al., 2007; *Dethlefsen* et al., 2008). However, the specific changes in microbiome structure that lead to increased risk are poorly defined. In an effort to increase our understanding of colonization resistance and identify microbial communities that are strongly associated with CDI risk, several investigators have characterized differences in the microbiomes of subjects with and without CDI (*Manges* et al., 2010; *Antharam* et al., 2013; *Vincent* et al., 2013; *Schubert* et al., 2014), as well as in patients before and after FMT (*Song* et al., 2013; *Dutta* et al., 2014; *Seekatz* et al., 2014). These studies primarily utilized three methods to assess the differences between the intestinal communities from groups of individuals: (1) alpha-diversity, which describes the microbiota community in terms of richness or diversity, (2) beta-diversity, a comparison of communities between samples, and (3) comparisons of the relative abundance of microbial taxa between individuals or clinical groups (*Schubert* et al., 2014). Results from these studies consistently illustrate that microbial communities in patients with CDI and antibiotic-associated diarrhoea (AAD) are less diverse and structurally different than those isolated from healthy subjects. These studies found that healthy stools were dominated by Bacteroidiaceae, Lachnospiraceae and Ruminococcaceae, while CDI and AAD patients typically exhibited an enrichment of Enterobacteriaceae, Enterococcaceae and Lactobacilliaceae (*Manges* et al., 2010; *Antharam* et al., 2013; *Song* et al., 2013; *Dutta* et al., 2014; *Schubert* et al., 2014; *Seekatz* et al., 2014).

In children, the clinical significance and outcomes associated with CDI remain poorly defined, even though the incidence is rising (*Nylund* et al., 2011). It is well established that young children are more refractory to CDI than adults despite the fact that microbiome studies clearly demonstrate that these populations exhibit similar low diversity (*Larson* et al., 1982; *Al-Jumaili* et al., 1984; *Jangi* and *Lamont*, 2010). Although colonization with toxigenic *C. difficile* is prevalent during the first two years of life, clinical disease is rare (*Larson* et al., 1982; *Sandora* et al., 2011; *Rousseau* et al., 2012). One study found that asymptomatic carriage rates increase in the first year of life and then drops to 6% by 24-36 months (*Rousseau* et al., 2012). While there is speculation regarding passive transfer of maternal antibodies (*Rolfe* and *Song*, 1995; *Dallas* and

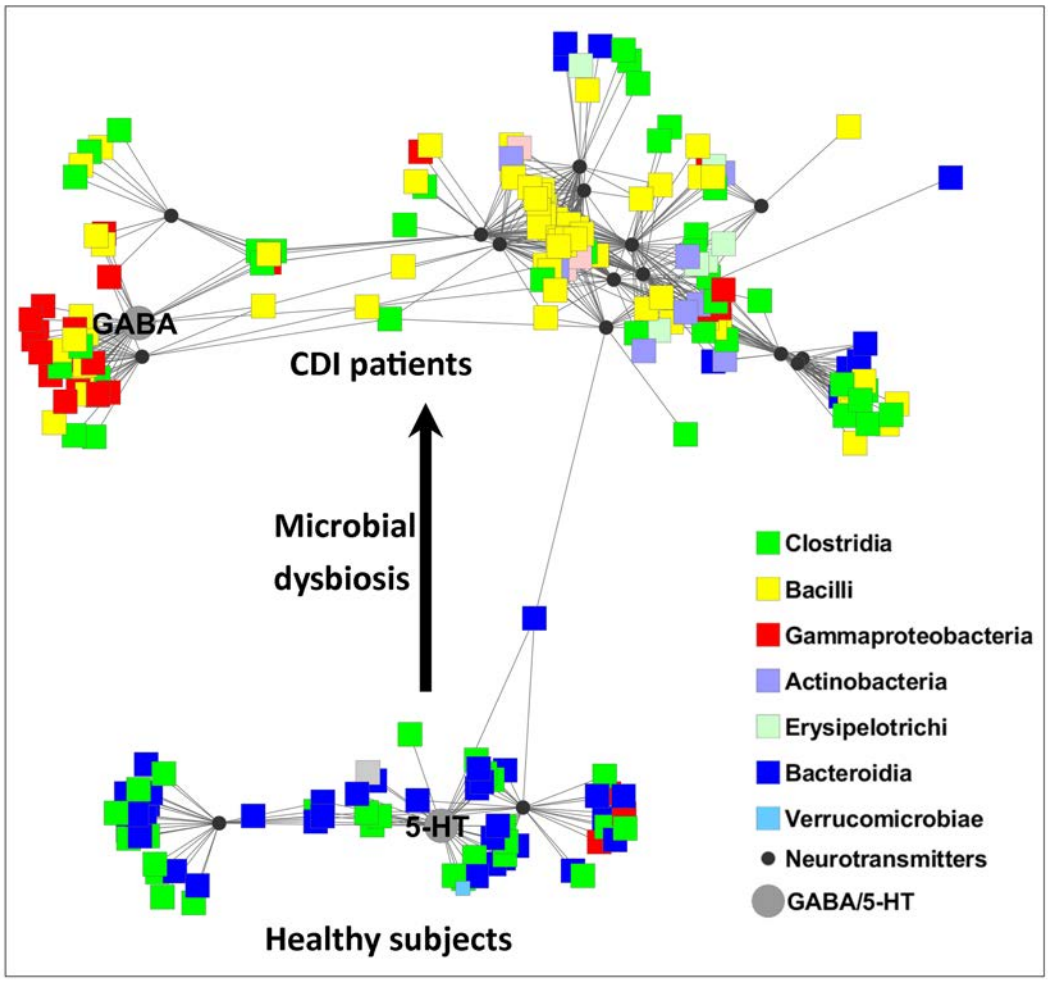

Figure 2: Operational taxonomical units (OTU)-Neurotransmitter correlation sub-networks: OTU's are shown as squares and coloured according to their assigned taxonomic class. The neurotransmitters are shown as black circles. The specific neurotransmitters, GABA and 5-HT, which are associated with disease and healthy subjects, respectively, are highlighted as larger gray circles.

Rolfe, 1998), a protective gut microbiome during infancy and a lack of toxin receptors (*Eglow* et al., 1992) have been proposed, the mechanism for asymptomatic *C. difficile* carriage in this population remains unknown. Not surprisingly, molecular diagnostics, which are more sensitive and specific, are problematic in this population. Aside from the >50% increase in CDI incidence, concerns about detection of colonization rather than true disease have been raised (*Gould* et al., 2013; *Longtin* et al., 2013; *Moehring* et al., 2013). A recent study by *Leibowitz* et al. (2015) found that hospitalized children aged 1-18 years (19% with diarrhoea and 24% without diarrhoea), tested positive for *C. difficile* by *tcdB*-specific PCR. Furthermore, high rates of *C. difficile* colonization have been reported in paediatric populations with additional co-morbidities, such as cancer (*Dominguez* et al., 2014) and IBD (*Hourigan* et al., 2013; *Pant* et al., 2013). As a result, the American

Academy of Pediatrics guidelines caution that testing for CDI should be exclusively performed on children who meet the clinical criteria (*Schutze* et al., 2013). They also note that test results for infants may be difficult to interpret due to high asymptomatic colonization rates and suggest that in children between 1 and 3 years of age, other causes of diarrhoea should be considered and tested for, even if the *C. difficile* test is positive. Notably, children with co-morbid conditions, such as IBD, are more susceptible to CDI, are more likely to recur and are prone to treatment failure. Moreover, CDI is known to affect IBD severity and is associated with higher rates of hospitalization (*Pant* et al., 2013; Kellermayer, 2015; *Sandberg* et al., 2015). The ability to identify symptomatic CDI in a population with high rates of asymptomatic *C. difficile* carriage would improve diagnostics and treatment for at-risk children. Furthermore, a better understanding of disease resistance in the paediatric population is likely to identify key host and microbial metabolic pathways, along with microbial species that may protect young children from developing clinical disease despite a lack of microbial diversity.

Through a re-examination of published microbiome data and our own metagenomics analyses, we found that in addition to the altered community dynamics described above, expansion of the Peptostreptococcaceae and Clostridiaceae families occur in adult and paediatric CDI populations, especially in recurrent cases. Bipartite correlation networks of stool operational taxonomical units (OTU) with neurotransmitter levels show that many OTUs associated with CDI development share identity with apparent γ-aminobutyric acid (GABA) producing Clostridiales, whereas OTUs associated with mucosal serotonin are lost (Figure 2). Both GABA and serotonin are potent neurotransmitters with important regulatory function in the intestine, including homeostasis of motility, mucosal barrier, blood flow and secretion, and modulating immune function. Preliminary global metabolomics profiling of CDI patient stool specimens demonstrate inverse correlations between GABA and serotonin that predict treatment failure in CDI patients (Figure 2). Intracellular recording of sensory neurons in the intestine confirm luminal GABA alone can induce action potentials that are likely enhanced by the *C. difficile* toxins. These findings are supported by potent GABA-producing Clostridiales yielding increased morbidity and mortality in animal models of CDI.

MICROBIAL NEUROTRANSMISSION IN THE INTESTINE

Our preliminary multi-'omics data are of interest because recent reports link serotonin receptor uptake inhibitors (SERT) and zolpidem (a $GABA_A$ receptor agonist) use with CDI development in at-risk patients. Therefore, microbial-derived neurotransmitters may represent novel druggable disease targets for therapeutic intervention in CDI although not all microbial-derived neurotransmitters potentiate CDI pathogenesis (Figure 3). Our laboratory recently demonstrated that luminal nitric oxide intermediary signals are capable of ameliorating CDI pathogenesis via a mechanism involving S-nitrosylation and inactivation of the *C. difficile* toxins (*Savidge* et al., 2011). The intestine is a rich source of nitric oxide and hydrogen sulphide neurotransmitters

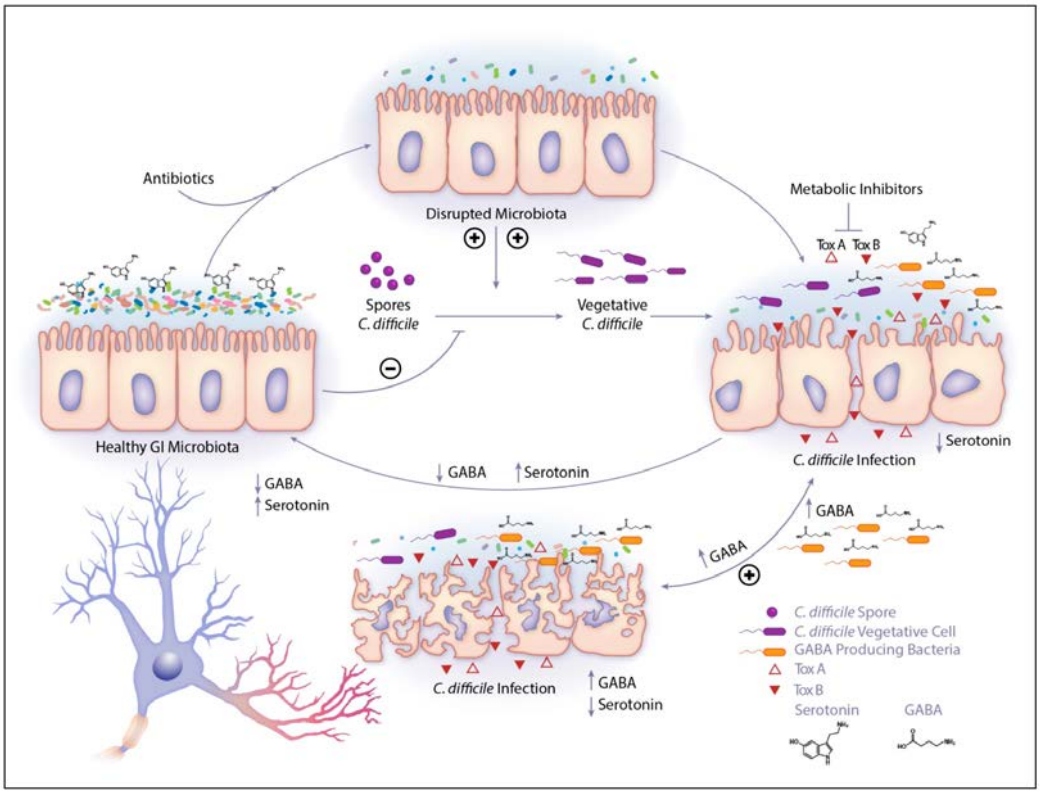

Figure 3: Antibiotic administration alters the intestinal microbiota, creating an environment that favours spore germination through reduced bile acid conjugation, and exacerbates *C. difficile* toxin activity by production of GABA and reduction of mucosal serotonin.

generated from bacterial conversion of dietary substrates such as nitrites. For example, *E. coli* is a prominent species that generates bioactive nitric oxide in both the small and large intestine using different oxygen dependent mechanisms. Because antibiotic-induced dysbiosis in CDI is associated with expansion of Enterobacteriaceae (*Song* et al., 2013; *Schubert* et al., 2014; *Seekatz* et al., 2014), this has the likely consequence of shifting the balance of nitric oxide generating bacteria in the colon. Our inability to culture the majority of microbial species in the intestine limits characterization of other neurotransmitter regulators in CDI. For example, expansion of antibiotic resistant Lactobacilliaceae in CDI patients may be associated with elevated histamine signalling, an important regulator of intestinal permeability, immune function and motility. Similarly, depletion of short chain fatty acid producing bacteria in CDI patients may exert epigenetic effects on neuronal signalling in the intestine resulting in altered immune responses, intestinal motility and luminal pH. At present, it is not clear whether alterations in microbial-derived neurotransmitters represent cause or consequence in CDI disease pathogenesis, but emerging data suggest further studies are needed to establish their role.

In conclusion, treatment options for patients experiencing recurrent CDI are limited and often involve long-term antibiotic administration, which poses a serious threat to development of

anti-bacterial resistance and reinfection by *C. difficile*. Because new antibiotic treatments are currently regarded as cost prohibitive as frontline treatment for CDI and efficacy decreases significantly with each recurrence, identifying at-risk patients for treatment failure is imperative. We expect that multi-'omics biomarkers may offer some candidate leads and provide insight into an alternative clinical cure that is not dependent on new antibiotics.

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UNCOVERING THE COMMUNICATIONS THAT MODULATE HOST PHYSIOLOGY IN HOST-MICROBE RELATIONSHIPS

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SUMMARY

Alterations to the microbiome have been associated with numerous aspects of health and disease. Application of microbiome studies to the improvement of human health will require identifying under which contexts the components of the microbiome have a causal influence on health and disease, identifying the smallest consortia of taxa capable of recapitulating the phenotypic variation observed by the whole community, and identifying the molecular communication signals between the consortia and the host to enable targeted interventions to improve health. Here we summarize research progress on experimental platforms and designs to move from causation, to causative organism, to molecular communications.

INTRODUCTION

Human microbiome studies suggest the microbiota can serve as a biomarker of disease progression, severity, and treatment efficacy (*Faith*, et al., 2013; *Karlsson* et al., 2013; *Scher* et al., 2013; *Gevers* et al., 2014; *Subramanian* et al., 2014; *Taur* et al., 2014). Further research and refinement will determine if microbiome-based biomarker assays are sufficiently precise, sensitive, and cost effective for clinical practice. In parallel, there is a need to determine in which diseases the microbiome influences the onset and progression of pathogenesis in a causative manner and to identify the microbial members or community structures with a causative role in disease susceptibility as well as any environmental or host factors that operate in parallel or in concert with the microbiota to modulate disease risk and severity (*de Vos* and *de Vos*, 2012; *Ahern* et al., 2014). The ultimate goal is to determine through what molecular language microbes communicate these beneficial and deleterious effects and the mechanisms by which these communications influence health and disease so that we may intervene to promote health.

One common source of host-microbe communication is the rich intestinal metabolic diversity derived from the diet, the host, and the gut microbiota. These metabolites are in constant flux from the metabolic processing and modifications by both the host and the microbiota. Given that each person has a unique collection of roughly one-hundred organisms harboured in their gut microbiota, unique dietary preferences driven by their personal tastes and cultural influences, and their own genome's unique potential to absorb and utilize metabolic input, interpersonal metabolite diversity represents a large potential influence on disease susceptibility with rich

opportunities for synergism. Advances in microbiome and gnotobiotic methods have uncovered a few examples of these synergisms in model systems (*Hansen* and *Sartor*, 2007; *Ivanov* et al., 2009; *Faith* et al., 2014; *Woting* et al., 2014; *Chassaing* et al., 2015). As we move forward, advances in metabolomics, microbiome tools, microbial culturing, and human microbiome study design will foster greater complexity in model systems and improved identification of key microbial components and metabolites influencing health.

THE INFLUENCE OF INTERPERSONAL MICROBIOME VARIABILITY ON HEALTH

Quantifying the influence of interpersonal microbiome variability on health remains a challenge. Given the interacting components of diet, host genotype, and microbiota, an essential factor in quantifying the microbiome's influence is to design experiments that control for diet and host genotype. Gnotobiotic animals colonized with the gut microbiota sampled from individual humans represent one such system (*Turnbaugh* et al., 2009). These "humanized" microbiota mice have the benefit of allowing different human microbiota to be tested for their differential influences in the context of a diverse array of gnotobiotic animal models (mostly rat and mouse) where both diet and genotype can be controlled. Sixty to seventy percent of the genus-level taxa in a human microbiota colonize a gnotobiotic animal, and both 16S rRNA amplicon sequencing and metagenomics methods indicate that mice colonized with a human microbiota exhibit significantly greater similarity to their human donor's microbiota than to mice colonized with the microbiota of the human donor's sibling or unrelated human donors (*Turnbaugh* et al., 2009; *Goodman* et al., 2011; *Ridaura* et al., 2013).

Colonization of germ-free animals with human gut microbes leads to numerous alterations in host physiology including increases in colonic lamina propria regulatory T cells, increases in adiposity, and alterations in intestinal metabolite profiles (*Atarashi* et al., 2013; *Faith* et al., 2014). Importantly, in the context of understanding human disease, mouse models can display differential phenotypic responses when colonized with the microbiota of different human donors. For example in *Ridaura* et al. (2013), gnotobiotic mice were colonized with the gut microbiota of one of four different twin pairs discordant for obesity. Two weeks later, the animals colonized with the gut microbiota from one of the four obese human donors had significantly increased total body and fat mass relative to mice colonized with their discordant lean sibling. In addition, co-housing animals harbouring an obese donor's microbiota with animals harbouring a lean donor's microbiota prevented the adiposity phenotypes. Interestingly, these observations where diet dependent and did not occur under the metabolic conditions fostered by consumption of a high fat, low fruit and vegetable diet. Differential responses to interpersonal variability in human gut microbiota composition have also been observed in the context of irritable bowel syndrome (IBS) where the transfer of faecal microbiota from IBS patients, characterized by hypersensitivity to colorectal distension, to gnotobiotic rats led to an increase in abdominal

contractions in response to colorectal distensions relative to rats colonized with non-hypersensitive healthy volunteers (*Crouzet* et al., 2013). In addition, early evidence suggest that germ-free mice after colonization with the faecal microbiota of humans with diarrhoea dominant IBS (IBS-D) phenocopy features of their microbiota donor, with animals receiving the IBS-D donor having significantly faster transit time than those receiving the microbiota of healthy controls (*Bercik* et al., 2012).

Although the use of humanized microbiota gnotobiotic models is still in its infancy, early evidence across a range of models suggest these studies will enable a broader understanding of the potential for interpersonal variation in microbiota composition to generate phenotypic variation in gnotobiotic animals receiving the donor microbiotas. It is also critical to understand the role of interpersonal variation in microbiota composition directly in humans. While safety and practical concerns prevent identical experimental paradigms in humans, faecal microbiota transplantation (FMT) whereby an individual with a particular disease has the faecal microbiota of a healthy donor infused into their intestine, provides the unique opportunity to gain a better understanding of the role of specific donor microbiota in humans. FMT has repeatedly proven to be a highly effective treatment for recurrent *Clostridium difficile* infections with a cure rate of >90%. Given this efficacy and the high rate of association of alterations in the microbiota to different diseases, there are numerous clinical trials being designed and underway for other clinical indications. The second largest current application of FMT is in clinical trials of Ulcerative Colitis (UC) where a few case reports and clinical trials have already been published (*Angelberger* et al., 2013; *Moayyedi* et al., 2015; *Rossen* et al., 2015). From these early results, it is clear that if FMT significantly improves UC, the magnitude and efficacy of the intervention is far less than in recurrent *C. difficile*. Nonetheless there are early hints that donor selection may be essential to efficacy (*Moayyedi* et al., 2015), which suggests that simply taking a "healthy" individual's microbiota as a donor to a patient with a particular disease is likely overly simplistic. If this variability in donor microbiota efficacy holds true in UC and other indications it provides the rare opportunity to understand overlapping microbiotas (the donor and the subset of the donor that engrafts in the recipient) in the context of humans with different genotypes and environmental histories and enables the quantification of interpersonal microbiota features such as the prevalence of effective donors for different indications.

IDENTIFYING MICROBIAL EFFECTOR STRAINS

Given the diversity of responses of different rodent models and phenotypes to interpersonal variation in gut microbiota composition, a subsequent challenge is to identify the microbial consortia or individual taxa within each community that are responsible for the observed differences. Gnotobiotic animals colonized with defined consortia provide a means to identify such taxa (*Faith* et al., 2010). These models systems have enabled the identification of bacterial strains and consortia that modulate regulatory T cells (*Round* and *Mazmanian*, 2010; *Geuking* et al., 2011; *Atarashi* et al., 2013; *Faith* et al., 2014), Th17 cells (*Ivanov* et al., 2009), tumorigenesis (*Arthur* et al., 2012), and

colitis (*Hansen* and *Sartor*, 2007; *Eun* et al., 2014). More recently, advances in high throughput microbial culturing systems (*Goodman* et al., 2011; *Lagier* et al., 2012) have enabled the isolation, archiving, and reuse of personalized culture collections, from individual human donors, whose membership contains the majority of genus level taxa from the original community (*Goodman* et al., 2011). Coupling such systems with high throughput combinatorial gnotobiotic screening experiments (*Ahern* et al., 2014; *Faith* et al., 2014) or targeted in vitro assays (*Romano* et al., 2015) provides the opportunity to identify the specific strains driving variation in phenotypic diversity between animals colonized with the microbiota of different humans. Such experiments and follow-up mechanistic studies will determine if variability in pathogenesis is driven by the same taxa or mechanisms in different donors, the extent to which the whole community context is necessary for full pathology manifestation, and could identify key beneficial organisms for addition in future FMT clinical trials and pathogenic organisms to target for removal.

Although animal models provide a valuable resource to understand host-microbe interactions and move towards mechanistic underpinnings, at this still early stage in our knowledge of the human microbiome, it is unclear if the transfer of phenotypic variation from humans to mice is mediated by the same strains and mechanisms in rodents as in the original human community (assuming the microbiota contributes in a causative way to a given phenotype in humans). It is clearly a possibility, even in cases where the microbiota contributes to phenotypic variation or disease pathogenesis in a casual way, that the community members responsible for these changes only do so in the context of a specific host where their molecular communications with the host are interpreted in a host-dependent manner. Therefore it is essential that, in addition to advances in gnotobiotic models, we push forwards technologies and study designs to move human microbiota studies towards more finely grained quantitation of the microbial consortia that explain disease risk. One such method to quantify the risk associated certain taxa harnesses the unique data of FMT. For indications where FMT is effective, but only in a subset of individuals, analysing the strain-specific differences in microbiota engraftment in responders and non-responders provides the possibility to identify key taxa that must be removed or added for a successful therapy. The drastic heterogeneity between individuals' microbiota creates a formidable challenge to this type or research, as the recipient microbiota will vary drastically - and likely completely at the strain-level (*Faith* et al., 2013, 2015) - from person to person. However, FMT studies that use one donor microbiota to treat several individuals provide at least a consistent donor microbiota to generate safety profiles and to begin quantifying the molecular details of faecal microbiota transplantation and how strain-specific differences in engraftment modulate FMT efficacy.

Improved design of human microbiota studies is also critical outside the realm of FMT. Current human studies often feature 16S rRNA amplicon sequencing. Although this method is effective in identifying high abundance organisms that are uniquely associated with a community or that are highly enriched in a pathogenic site (*Relman* et al., 1990, 1992), it lacks the resolution to identify unique strain variants of common species and genera. Disease associated changes in the microbiota to date have been dominated by descriptions of broad

community characteristics such as reductions in diversity and alterations in the abundance of different phyla. Although such descriptions can serve as weak biomarkers of disease, they also are sufficiently broad to encompass the microbiota of healthy individuals and lack sufficient detail to provide insight into disease aetiology or potential clinical interventions. Strain-level resolution techniques would provide the ability to identify unique strains from common species that are enriched or absent from individuals with disease. Early evidence suggests unrelated individuals do not share microbes at the strain level, thus strain level techniques would best be employed in family studies where strains are known to be shared between individuals (*Faith* et al., 2015) and perhaps in the context of a geographically isolated population where the total set of microbes in the community are perhaps more limited and freely shared between individuals. Such studies are currently limited by the precision, resolution and depth attainable by current microbiome methods, although improvements in metagenomics algorithms or experimentally-driven advances (*Beitel* et al., 2014) could enable studies such as using microbial inheritance patterns to quantify the risk associated with all of the taxa harboured in an individual's gut microbiota in the context of disease (*Faith* et al., 2015).

IDENTIFYING COMMUNICATION MECHANISMS OF MICROBIAL EFFECTOR STRAINS

To fully realize the potential of microbiome inspired health interventions, we must not only identify the organisms that modulate health but also the communication signals used to do so. Although this large challenge is virtually unexplored in the context of human health, animal studies have begun to identify the specific proteins or metabolites that mediate cross talk between the host immune system and microbes. For example, Peterson et al. characterized the influence of both strain-specific (*Peterson* et al., 2007) and species-specific IgA (*Peterson* et al., 2015) on the colonization and transcriptional expression of the abundant commensal organism *Bacteroides thetaiotaomicron*. *Yang* et al. (2014) characterized the T-cell antigen receptor repertoire of intestinal Th17 cells in animals colonized by segmented filamentous bacteria (SFB), a known potent inducer of Th17 cells in mice, showing both the localization of SFB specific T-cells and the potential of the immune system to maintain the specific Th17 response even in the context of co-colonization with a strong Th1 cell inducer (*Listeria monocytogenes*). Several groups have shown the potential for abundant microbially generated compounds like short chain fatty acids to increase regulatory T-cells (*Furusawa* et al., 2013; *Smith* et al., 2013). Importantly, metabolic output can vary between strains of the same species leading to strain-specific phenotypic outcomes such as the observation by *Fukuda* et al. (2011), that one of three tested strains of species *Bifidobacterium longum* were capable of preventing lethal infection with enterohaemorrhagic *Escherichia coli* O157:H7 in gnotobiotic mice. Similarly, *Romano* et al. (2015) found strain-specific variability in the potential of *Edwardsiella tarda* species to generate trimethylamine (a precursor of potential atherosclerotic compound trimethylamine-N-

oxide) from choline. Again these results highlight the importance of strain-specific variation in functional capacity to interact with and manipulate the host. While numerous individuals harbour *B. longum* and *E. tarda* species in their microbiota, a smaller fraction harbour specific strains with a given functional potential.

The above examples of microbe-host communication are mostly focused on targeted examples where some idea of the molecular signal is known by the nature of the interaction (T-cell or B-cell) or the abundance of the molecule and its use in prior clinical interventions (short-chain fatty acids). Nonetheless, platforms designed to identify effector strains, such as the combinatorial gnotobiotics strategies described above, can serve to narrow the search from the communications from a community of organisms with the host down to the smallest microbial consortium sufficient for phenotype modulation. In addition, we have previously shown these combinatorial gnotobiotic search strategies can be used to identify the specific microbial strains whose presence/absence best explains the observed variation in metabolite diversity (*Faith* et al., 2014). Identifying microbial effector strains in culture-dependent assays provides the potential to use *in vitro* cultures to perform gnotobiotic experiments where animals are fed a supply of crude extracts of one or more specific microbes, microbial supernatants from culture media processed by the target organisms, or some fractionated form of these proteins and metabolites to further refine the potential microbial effector signals that interact with the host to modulate health. Similar crude extract and fractionation studies could be performed in a culture independent manner in the context of animal experiments where some aspect of host health can be transmitted via the microbiota and also in the context of humanized microbiota experiments where different human microbial communities differentially modulate health in gnotobiotic animals.

CONCLUSION

Although much progress has been made in the characterization of the microbes in and on our body surfaces, we are still far from the day where we understand the molecular communication signals driving the phenotypic variation associated with interpersonal differences in microbiome composition. However, gnotobiotic animals combined with defined collections of specific microbial strains are aiding in the dissection of these communications and will be critical for understanding and designing the next generation of clinical studies to understand host-microbe communication systems in humans.

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THERAPIES AIMED AT MANIPULATING THE GUT MICROBIOME

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SUMMARY

It is now recognized that disturbances in host microbial populations may be linked to acute infections such as *Clostridium difficile* and to chronic diseases including inflammatory bowel disease, cardiovascular disease, cancer, obesity, diabetes, and metabolic disorders. The gut microbiome is influenced through dietary exposures, drugs, and environmental factors. Features generally associated with health include a high level of diversity, stability and resilience of the gut microbiome over time and a predominance of obligate anaerobic bacteria that greatly outnumber facultative anaerobic species. A dysbiosis or reduction in health promoting metabolites can have a significant impact on host barrier, immune function, and physiology and may, if not cause disease, certainly exacerbate or prolong the disease process. It follows therefore, that strategies such as diet modifications, pre-and probiotics, and faecal microbial transplantation (FMT) may be beneficial in both the prevention and treatment of disease by modifying either microbial composition or function. Probiotics, defined as live microbes that, when ingested, have health-promoting effects have been examined for both preventative and treatment roles in a number of diseases. Despite a multitude of studies demonstrating numerous molecular mechanisms of action, benefits of probiotics in clinical studies remain modest. Recent metagenomic research is identifying shifts in other dominant commensals that are associated with human disease and future probiotic therapy may focus on other possibly more relevant bacterial strains such as *Faecalibacterium prausnitzii* or *Akkermansia muciniphila.* Faecal microbial transplantation delivers a complete microbial ecosystem consisting of a wide array of microbes. FMT has been shown to be highly effective with >90% cure rate in recurrent *Clostridium difficile* infection (RCDI) and is currently being investigated for treatment of numerous diseases. However, in that there is extensive variability between individuals in their microbiota composition, personalized approaches may be required in order to effectively utilize therapies aimed at manipulating the balance of the gut microbiota.

INTRODUCTION

Our gut microbiome changes throughout life and can be influenced by diet, drugs, and environmental exposures. Numerous studies to date suggest that alterations or "dysbiosis" in the gut microbiome are linked with chronic diseases as well as being associated with acute infectious states. This "dysbiosis", defined as a disruption of the normal balance between the gut microbiota and host along with a decrease in overall diversity has been

associated with obesity, type 2 diabetes, irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease, autoimmune arthritis, chronic kidney disease, multiple sclerosis, autism, and cancer (*Backhed* et al., 2004; *Turnbaugh* et al., 2006; *Berer* et al., 2011; *Wang* et al., 2011; *Morgan* et al., 2012; *Qin* et al., 2012; *Kang* et al., 2013; *Ng* et al., 2013; *Wu* et al., 2013; *Brusca* et al., 2014; *Ramezani* and *Raj*, 2014; *Louis* et al., 2014). It is not clear however, whether it is the overall low diversity or the increase or decrease of specific microbial taxa that are most important and whether these changes are causative or associative. However, although many questions remain to be answered, research to date in animal models and human studies supports the concept of developing strategies aimed at targeting the gut microbiota to restore homeostasis through increasing diversity and shifting the balance of gut commensals.

MICROBIAL COMPOSITION AND FUNCTION

Studies showing that microbial composition and gene richness were able to distinguish healthy obese individuals from those with metabolic disease (*Le Chatelier*, 2013) suggests that profiling the microbial genome on a functional basis may be useful in predicting disease course in some human diseases (*Fang*, 2013). In addition, animal studies showing that disease phenotypes such as obesity, metabolic syndrome and colitis can be transferred to healthy recipients through faecal transplantation along with studies in humans that faecal transplantation with donor faecal material can cure *C. difficile* colitis (*Turnbaugh* et al., 2006; *Garrett* et al., 2007; *Turnbaugh* et al., 2008; *Aroniadis* and *Brandt*, 2013) argues strongly for a major role of gut microbes in the development and modulation of various human diseases although underlying mechanisms have not as of yet been completely defined. A greater understanding of factors that influence the gut microbiome as well as which components of the microbiota are most important in a particular condition is necessary in order to effectively develop therapeutic interventions based on manipulating the gut microbiome to promote health or treat disease (*Hollister* et al., 2014). If the concept that all healthy humans have a "core" microbiome was true, then achieving this "core" group would represent a clear therapeutic target. However, results from metagenomic studies to date have shown that there are extreme levels of inter-individual variability even among closely related individuals, and there does not appear to be a core microbiome, at least in terms of species (*Qin* et al., 2010; *Shafquat* et al., 2014). Alternatively, the idea exists that a core healthy microbiome may be defined by metabolic and functional aspects, suggesting that identification of these metabolic pathways and specific metabolites may lead to the identification of specific metabolic pathways to target (*Shafquat* et al., 2014). Features generally associated with health include stability and resilience over time and a predominance of obligate anaerobic bacteria that greatly outnumber facultative anaerobic species. Often a reduction in the obligate anaerobes is accompanied by an increase in facultative anaerobes in disease states, including members of the Enterobateriaceae family of the Proteobacteria phylum. This family includes several pathogens including *Salmonella*, *Shigella*,

*Klebsiell*a, *Proteus* and *E. coli*. However, currently it is not known if specific species, a metabolic functional profile, or other factors are most important in the maintenance of health and/or induction of disease, and which should be targeted for therapeutics. How viruses, archaea and eukaryotes interact with bacteria to maintain gut homeostasis also remains to be clearly determined.

BENEFICIAL ACTIVITIES OF GUT MICROBIOTA

One of the key mechanisms by which gut microbes are thought to exert health benefits is through the production of short chain fatty acids (SCFA) by the breakdown and fermentation of polysaccharides. SCFAs include acetate, propionate, and butyrate, with the overall abundance produced dependent upon the diet of the host and microbial composition of the colon. Bifidobacteria and lactobacilli produce mainly lactate and acetate, which can contribute to health benefits through reduction of pH and immune modulation (*Fukuda* et al., 2011), but they do not produce butyrate or propionate, two SCFAs which have been identified to exert highly beneficial local and systemic immunological effects (*Louis* et al., 2014; *Flint* et al., 2015). Butryate and propionate are produced primarily by bacteria belonging to the Clostridium clusters XIVa and IV, and to the Bacteroidetes phylum (*Louis* et al., 2010; *Reichardt* et al., 2014). Complex carbohydrate fermentation by bacteria leads to the production of short-chain fatty acids including acetate, butyrate and proprionate (*Flint* et al., 2008). Acetate maintains gut barrier function and can prevent pathogen translocation (*Fukuda* et al., 2011). Butyrate is the primary energy source for colonocytes and also has numerous anti-inflammatory effects (*Zimmerman* et al., 2012). A lack of butyrate results in colonocyte cell death and autophagy (*Donohoe* et al., 2011). In addition to SCFAs, numerous metabolites and structural components of gut commensals interact with host epithelial and immune cells to influence barrier function and immunoregulatory activity. Gut microbes also provide protection against infection by pathogenic organisms through colonization resistance. This protection may include competition for nutrients or attachment sites on the mucosa, production of antimicrobial compounds, or stimulation of host defences. Colonization resistance may also help keep potentially pathogenic commensals from multiplying and inducing disease. Thus, a dysbiosis or reduction in health promoting metabolites can have a significant impact on host barrier, immune function, and physiology and may, if not cause disease, certainly exacerbate or prolong the disease process. It follows therefore, that strategies such as diet modifications, pre-and probiotics, and faecal microbial transplantation may be beneficial in both the prevention and treatment of disease by modifying either microbial composition or function.

PROBIOTICS

Probiotics, defined as live microbes that, when ingested, have health promoting effects have been examined for both preventative and treatment

roles in a number of diseases (*Ghouri* et al., 2014; *Ferolla* et al., 2015). A multitude of studies have delineated molecular mechanisms of probiotic strains in modulating host physiology and immune function through interactions between the host and various effector molecules, including cell surface proteins, release of bioactive molecules, lipoteichoic acid, peptidoglycan, and exopolysaccharides (*Bron* et al., 2012; *Lee* et al., 2013). Oral intake of probiotics has been shown to significantly alter host gene expression both in a strain-selective manner (*van Baarlen* et al., 2011) and in a host-dependent manner (*Mariman* et al., 2015). There is clinical evidence that probiotics have some efficacy in the prevention of necrotizing enterocolitis in infants, in relieving symptoms of irritable bowel syndrome, and also in the prevention of antibiotic-associated diarrhoea. However, whether probiotics are able to reverse dysbiosis and restore gut homeostasis has not yet been demonstrated. Some studies have demonstrated that intake of probiotics can alter both the composition and metabolic activity of existing microbes in the gut (*McNulty* et al., 2011) while others have shown no effect on composition but significant effects on microbial gene expression (*Lahti* et al., 2013; *Eloe-Fadrosh* et al., 2015). Further, the existing gut microbiota can also have effects on gene expression of the probiotic (*Lahti* et al., 2013) suggesting that the host microbiota may have a significant influence on the individual response to probiotic. Other host factors that change response to probiotics include diet (*Ohland* et al., 2013; *Yadav* et al., 2013; *Degirolamo* et al., 2014; *Tachon* et al., 2014) and the existing microbiome (*Ferrario* et al., 2014). Further, different probiotic strains taken together can have competitive or inhibitory effects on each other (*Ringel-Kulka* et al., 2014) and the host may become adapted to continual ingestion of probiotics and prebiotics or bacterial products (*Dykstra* et al., 2011; *Chambers* et al., 2014; *Komura* et al., 2014). These studies clearly demonstrate that the use of probiotics to mediate human health or treat disease involves a complex reciprocal interaction of the probiotic, host immune function, and commensal microbiota encountered by the probiotic. Further studies are required to truly understand how to properly use these individual strains for beneficial purposes.

FUTURE OF PROBIOTIC THERAPY

While clinical trials have shown modest benefits from probiotic therapy, overall the results have been relatively modest. Most of the strains used as commercial probiotics include lactobacilli and bifidobacteria, even though neither of these are major colonizers of the adult human gut and defects in these have not yet been linked with any human disease in adults. Further, metagenomic research is clearly identifying shifts in other dominant commensals that are associated with human disease (*Backhed* et al., 2004; *Turnbaugh* et al., 2006; *Frank* et al., 2007; *Morgan* et al., 2012; *Qin* et al., 2012; *Kang* et al., 2013; *Ng* et al., 2013; *Wu* et al., 2013). The identification of specific organisms, such as *Faecalibacterium prausnitzii* (*Varela* et al., 2013; *Cao* et al., 2014), which has been shown to be reduced in patients with inflammatory bowel disease, as well as other butyrate producing microbes such as *Roseburia* spp., has led to the suggestion that these organisms should be used as

probiotic preparations in patients with IBD to help manage the disease. In addition, *Akkermansia muciniphila* (*Everard* et al., 2013; *Cani* and *Van Hul*, 2015), which is reduced in patients with metabolic syndrome and diabetes, and specific microbial-produced metabolites in autism spectrum disorders (*Siniscalco* and *Antonucci*, 2013; *Frye* et al., 2015) may be of more relevance for treating specific conditions in adults.

FAECAL MICROBIAL TRANSPLANTATION

Faecal microbiota transplantation (FMT), a process of transferring stool from a healthy individual to a sick person, has been shown to be highly effective with >90% cure rate in recurrent *Clostridium difficile* infection (RCDI) (*Cammarota* et al., 2014). Unlike probiotic therapy which involves only a few species of microorganisms, faecal microbial transplantation delivers a complete microbial ecosystem consisting of a wide array of microbes. RCDI is one of the most common hospital acquired infections. There has been a large increase in the number of infections along with increased severity and mortality over the past decade, associated with significant health care costs. Following a course of antibiotic therapy, approximately 20-30% of patients will experience a recurrence. Unfortunately, the risk of recurrence continues to increase with each subsequent episode, and no conventional treatment has been proven effective. Recent research suggests that development of RCDI involves alterations in bile acid metabolism. In particular, germination of *C. difficile* spores can be either inhibited or stimulated by a complex mixture of bile salts. Cholate and chenodeoxycholate are metabolized into the secondary bile acids deoxycholate and lithocholate. Deoxycholate stimulates germination while lithocholate inhibits germination (*Sorg* and *Sonenshein*, 2008, 2009). Administration of antibiotics shifts the bile acid pool and allows for spore germination (*Giel* et al., 2010). Antibiotics also reduce the diversity of microbiota and thus decrease competition for available nutrients. In that FMT is so effective at treating RCDI, this procedure is rapidly gaining acceptance throughout the world although questions still remain about the optimal route of administration, quality control, durability of response and long-term outcomes.

Studies have shown that several defined communities of microbes are equally as effective in the treatment of RCDI as is FMT. A combination of 10 facultative aerobes and anaerobes was effective against RCDI (*Tvede* and *Rask-Madsen*, 1989) as was a 33-strain combination (*Petrof* et al., 2013). Recent reports of success of freeze-dried (*Tian* et al., 2015) and encapsulated forms (*Hirsch* et al., 2015; *Stollman* et al., 2015), as well as the use of selected strains of *Clostridium* either in live form or as spores (*Gerding* et al., 2015) (Seres Health Ecobiotic®) suggests that in the very near future a much more targeted approach will be undertaken to cure recurrent *C. difficile* infection. Indeed, it may be possible to use a single strain of *Clostridium* based upon an ability of the particular strain to modulate bile acid metabolism to restore gut homeostasis in patients who are colonized with *C. difficile* (*Buffie* et al., 2015). This is a clear indication of how an approach based on an understanding of the underlying mechanism of disease can lead to effective therapy focusing on manipulating the gut microbiome.

FAECAL MICROBIAL TRANSPLANTATION AND INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, is a chronic, relapsing and remitting set of conditions characterized by an excessive inflammatory response leading to the destruction of the gastrointestinal tract. While the exact aetiology of inflammatory bowel disease remains unclear, increasing evidence suggests that the human gastrointestinal microbiome plays a critical role in disease pathogenesis. Manipulation of the gut microbiome has therefore emerged as an attractive alternative for both prophylactic and therapeutic intervention against inflammation. Probiotics have had limited benefit in ulcerative colitis, and have demonstrated no benefit in the treatment of Crohn's disease (*Wasilewski* et al., 2015). *Borody* and *Campbell* (2012) reported using FMT as an induction and maintenance therapy in patients with IBD, with as many as 69 rectal infusions, to successfully treat 3 patients. However, in most cases, the results are not as consistent or as durable as in the setting of CDI. Most of the published reports consist of small case series, which suffer from significant heterogeneity with regards to disease activity, mode and frequency of delivery, and duration of follow up. A systematic review published in 2012 included 9 case series/reports of FMT to treat IBD (N=26); it found that 19/25 patients experienced symptomatic improvement, 13/17 ceased taking IBD medications within 6 weeks, and 15/24 had no active disease 3-36 months after FMT (*Anderson* et al., 2012). A recent case series also found that 7/9 paediatric patients with mild-to-moderate UC disease activity experienced clinical improvement, and 3/9 achieved clinical remission within 1 week after a 5-day course of daily FMT enema (*Kunde* et al., 2013). On the other hand, another pilot study by *Vermeire* et al. (2012) examined the role of FMT in the management of 4 patients with refractory Crohn's disease who had failed corticosteroids, immunomodulators and anti-TNF therapy. These 4 patients received 3 doses of FMT by naso-jejunal infusion over 2 days but none experienced clinical, biological or endoscopic benefit 8 weeks later. More importantly, the faecal bacterial composition of these 4 patients did not show clustering with their donors after FMT, unlike the cases in recurrent CDI. Intense FMT treatment of 3 paediatric UC patients using a combination of colonoscopy and enemas during a 6-12 week period showed significant clinical benefit and also an expansion of rare taxa and significant changes in colonic mucosal gene expression (*Kellermayer* et al., 2015). Two randomized control trials published recently demonstrated a possible potential for the use of FMT in treating patients with ulcerative colitis, although neither demonstrated a large response (*Moayyedi* et al., 2015; *Rossen* et al., 2015).

Thus, while manipulating the gut microbiome still remains a potential therapeutic target in patients with IBD, the question remains as to why some individuals have dramatic improvements while others do not and also why continual therapy appears to be necessary. In IBD patients with active inflammation, the luminal environment that the transplanted microbiota enters contains factors (e.g. nitrate, reactive oxygen species, viruses, phages) that may prevent successful colonization of some species and allow for the bloom of microbes received from the donor that are adapted to living in

inflammatory environments (*Winter* et al., 2013). In the colon, bacterial species are primarily anaerobes which lack the ability to respire oxygen and rely on fermentation of complex polysaccharides for growth. Dysbiosis in patients with intestinal inflammation is characterized by a marked decrease in obligate anaerobes and an increased relative abundance of facultative anaerobes such as Gammaproteobacteria and Bacilli (*Frank* et al., 2007). Members of the Enterobacteriaceae family are adapted to survival in the presence of inflammatory mediators such as reactive oxygen and nitrogen species (*Winter* et al., 2013). In the inflamed gut, increased growth of these pathogenic organisms can act to reduce gut barrier function and stimulate the immune system, thus propagating inflammatory responses. At the same time, a reduction in anaerobic bacteria that produce either butyrate or other anti-inflammatory molecules would also contribute to an increased inflammatory state. Thus, under these conditions, the newly transplanted microbes may initially exert an anti-inflammatory effect due both to the production of immunoregulatory molecules (e.g. short-chain fatty acids) and generation of signalling molecules (e.g. secondary bile acids), but over time a susceptible individual that has a genetically-determined defect in handling bacteria or in barrier function may develop an inflammatory response directed towards the newly transplanted microbes. Thus, under these conditions, while targeting the microbiota may initially be effective in reducing gut inflammation at induction FMT, due to patient genetic susceptibility, continual maintenance FMT would be necessary.

FUTURE OF FAECAL MICROBIAL TRANSPLANTS

Many questions remain to be answered before FMT becomes a feasible treatment option for diseases other than *C. difficile* infection. Optimal dosage, frequency of treatment, preparation of donor material, and route of administration needs to be determined. In that each donor is different, this represents a clear challenge to implementation of this type of therapy. In addition, potential long-term effects and the risk of transferring either infectious material or susceptibility to disease needs to be evaluated.

CONCLUSION

In order to develop new therapies aimed at returning our microbiome to a healthy state, future research should seek to understand why and how our gut microbiome changes and to understand the functional consequences of those changes. It is clear that interactions between the gut microbiome and the host have a major role in health and disease; therefore, manipulation of gut microbiota represents a therapeutic target. However, in that there is extensive variability between individuals in their microbiota composition, personalized approaches may be required in order to effectively utilize therapies aimed at manipulating the balance of the gut microbiota. In the future, a detailed analysis of an individual's gut microbes may indeed be part of their health care and biomarkers identified that can be followed for changes that may herald imminent onset of disease.

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MICROBIAL METABOLISM AND MAMMALIAN PHYSIOLOGY: SUMMARY OF THE 29TH OLD HERBORN UNIVERSITY SEMINAR

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OVERVIEW AND KEY CONCEPTS

In this 29th edition of the Old Herborn University Seminar, microbial metabolism and mammalian physiology were discussed as one integrated biological system. The lectures, discussions, and contributions to this monograph emphasize the web of interactions between diet, the microbiome (placental, vaginal, oral, and intestinal), microbial metabolites, and mammalian cells (epithelial and immune). Central concepts included various holistic perspectives on microbial and mammalian (rodent, primate, and human) biology. Prenatal and postnatal nutrition may have a lasting impact on mammalian growth and development. In addition to the ingestion of active dietary components, microbes may generate bioactive metabolites by luminal conversion of dietary substrates or de novo biosynthesis in bacterial cells. These metabolites may impact mammalian physiology via the microbiome or directly on mammalian cell signalling pathways. Host-microbe metabolic axes must be considered in terms of metabolite flow across organ systems and across time in man's microbial ages (Figure 1). In this Seminar, leading scientists considered the contributions of microbial cells (species) and their metabolites on mammalian cell function, signalling pathways, and bidirectional communication networks. In this way, we are considering the microbial-mammalian interface of human biology, and how microbes can impact human health and susceptibility to disease.

MICROBES AND HUMAN DEVELOPMENT

New concepts to consider include the prenatal impact of the microbiome during foetal development and compositional relationships between microbiomes at different body sites. Microbial communities may impact human biology even during foetal development. During pregnancy the placenta grows and develops with its own microbiome forming a distinct collection of human-associated bacterial taxa. The placenta has long been considered sterile during gestation, and, similar to other previously "sterile" body sites, microbial communities have been characterized in the placenta (*Aagaard* et al., 2014).

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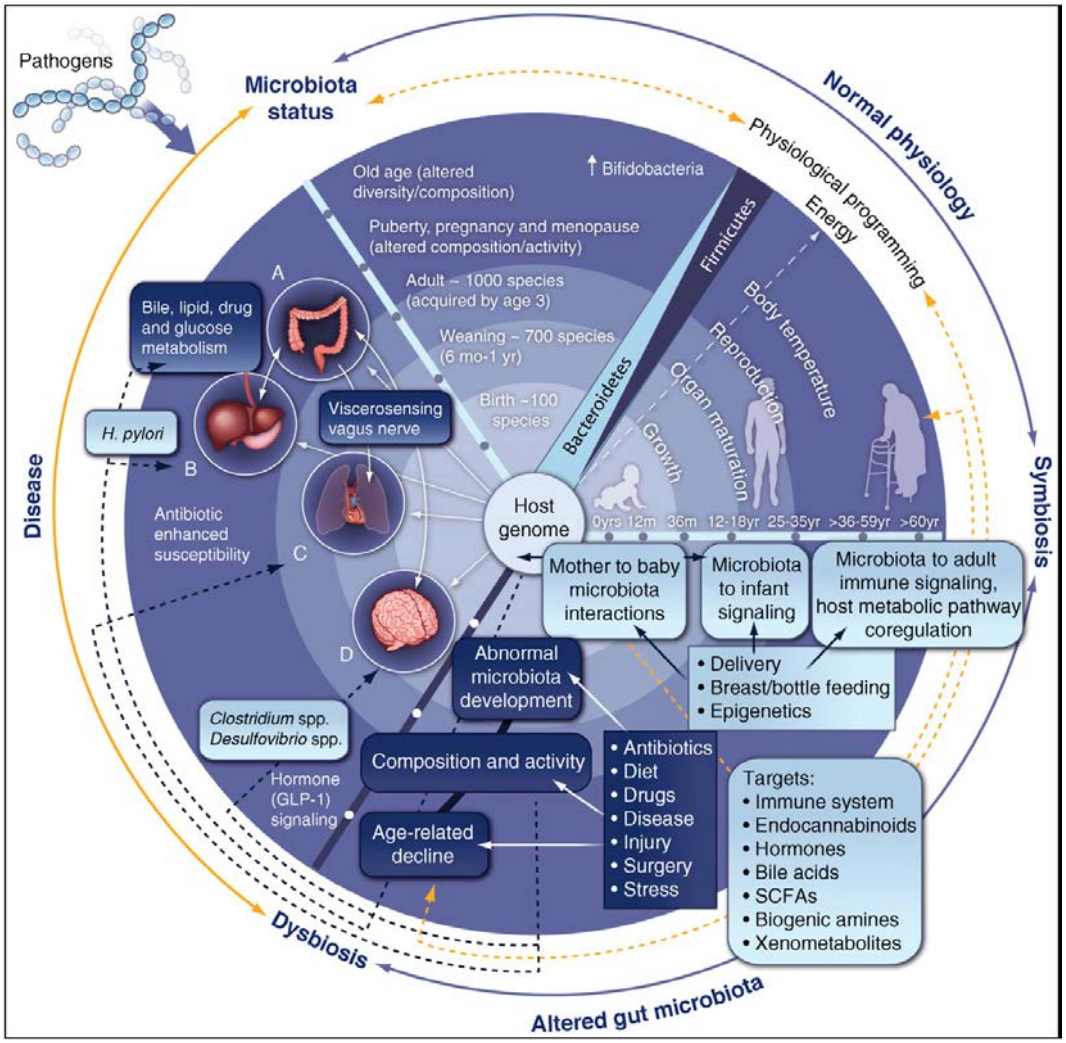

Figure 1: The human microbiome, microbial metabolism and mammalian physiology.
The gut microbiota affects human health from birth to elderly life stages and is important for maintaining human physiology and energy production. The composition of the gut microbiome in early life has an effect on the risk of developing disease in later life. (Reprinted with permission from *Nicholson* et al., 2012.)

The presence of pathogenic bacteria in placental cultures is considered diagnostic for intrauterine infection and a significant risk factor for preterm birth (*Hillier* et al., 1988). Findings in diagnostic microbiology must now be placed in the context of the placental microbiome and a broader array of commensal microbes in pregnancy. Term and preterm infants have discordant microbiomes, and future diagnostic tests may detect differences in placental microbial biomarkers that may be useful clinically. Taxa in the placental microbiome that may discriminate spontaneous preterm birth include members of the phyla Tenericutes and Fusobacteria. Could placental microbes or deficiencies in microbial features contribute to preterm birth along with maternal and environmental factors? In a practical sense, perhaps

microbial metabolites generated by placental bacterial taxa may be used to monitor women at risk of preterm birth.

Tantalizing evidence suggests that one microbiome may influence the composition and function of a distinct microbiome at a different body site. In other words, does the microbial community at a superficial body site potentially influence the microbiome established in a separate body compartment? In this case, the placental microbiome most closely resembles the oral microbiomes of the supragingival plaque and the dorsum of the tongue by bacterial composition (*Aagaard* et al., 2014). Seeding of the neonatal microbiome with bacteria from the placenta, which arises through hematogenous spread, may facilitate prenatal colonization of the foetus and *in utero* exposure to microbial metabolites. The oral-to-placental microbiome connection may help drive physiological and immunologic development of the foetus and help dictate susceptibility to preterm birth or neonatal disease (*King* et al., 2007; *Zeldovich* and *Bakardjiev*, 2012). We are beginning to make connections between microbial communities at different body sites and how microbial and human cells are linked together as one biological system.

This Seminar highlighted the importance of diet before and after birth with respect to microbial composition and function in the developing infant and child. Maternal diet during pregnancy may have a lasting impact on the composition of the gut microbiome postnatally. In foetal and postnatal life, offspring exposed to a maternal high fat diet yielded increased expression of inflammatory cytokines, Toll-like receptors, and their respective signalling pathways (*Yan* et al., 2011, *Xue* et al., 2014). Results of a study by *Aagaard* et al. (2014) on maternal diet modification of the primate foetal epigenome suggest that obesity caused by a high-caloric, high-fat maternal diet yielded changes in the juvenile intestinal microbiome of non-human primates. The quality of the diet during pregnancy may yield persistent effects on the gut microbiome during childhood several years after the initial dietary exposure. This phenomenon of diet's persistent effects on mammalian metabolism via changes in the human microbiome demands further attention.

After birth, neonatal nutrition may have a lasting impact on microbiome composition, function, and disease susceptibility. The intestinal microbiome of infants born vaginally and exclusively breastfed diminished the relative amounts of members of the phylum Firmicutes, while increasing the relative abundance of *Bacteroides* species (*Jost* et al., 2012). The maternal microbiome at birth and postpartum may be critical in the establishment and development of the neonatal microbiome via horizontal transmission. Human milk oligosaccharides (HMO) are the third most abundant component in human milk, and breastfeeding may serve as an important mechanism for dictating how the gut microbiome is "seeded" early in life. More than 150 different HMOs have been identified, and were discussed by Lars Bode in this Seminar (*Bode*, 2012). Only humans have such a high quantity and structural diversity of HMOs with inter- and intrapersonal differences in composition. These differences are determined partly by human genetics or maternal diet during lactation. HMOs are maternal compounds contributing to the development of the postnatal gut microbiome following childbirth. HMOs may function as prebiotics from human milk, and should be added to the list of plant-derived oligosaccharides considered as prebiotics (*Gibson* and *Roberfroid*, 1995; *Roberfroid*, 2007). HMOs also

have antimicrobial features partly due to anti-adhesive properties that may inhibit bacterial pathogens and alter the ability of commensal species to colonize the intestine. By altering colonization, HMOs may help shape the nature of bacterial communities. For example, HMOs reduced the ability of enteropathogenic *E. coli* (EPEC) to attach in a tissue culture adhesion assay and mouse EPEC infection (*Manthey* et al., 2014). HMOs may have indirect effects on the infant microbiome, namely as epithelial and immune cell modulators. Specific HMO structures such as disialyl-lacto-N-tetraose (DSLNT) improved survival and reduced pathology scores in a rat necrotizing enterocolitis (NEC) model (*Jantscher-Krenn* et al., 2012). The effects of DSLNT on intestinal pathology are highly structure-specific. Perhaps specific HMOs can prevent or suppress the pathology of severe NEC in infants, directly by acting on the intestinal mucosa, and raise the possibility that microbial metabolites may act directly as immunomodulators as well. The biology of HMOs serves as a reminder that key dietary components early in life may affect mammalian biology via changes in the composition and function of the microbiome, or directly on mammalian cells by modulating immune cell signalling via pattern recognition receptors (TLRs), production of antimicrobial peptides, mucin composition, and cytokine production in the intestinal mucosa.

THE BACTERIAL COMMUNITY: GUT MUCOSAL BIOLOGY INTERFACE

Dietary components such as carbohydrates and lipids clearly affect microbial composition in the intestine. Microbes that respond to elements of the diet, in turn, may influence whole body metabolism and affect the ability of mammals to absorb and process nutrients. This interplay of diet, the microbiome, and mammalian metabolism represents a central theme of this OHUS Monograph. In rodent models, diets differing in polysaccharide and fat content yielded major differences in gut microbial composition and correspondingly microbial metabolism. The caecal microbiota of genetically obese ob/ob mice, lean ob/+ and wild-type siblings, and their ob/+ mothers were compared after ingesting the same polysaccharide-rich diet (*Ley* et al., 2005). Regardless of kinship, ob/ob (obese) mice had approximately half the number of Bacteroidetes than lean mice and a proportional increase in Firmicutes taxa. Colonization of germ-free mice with obesity-associated microbiota resulted in increased total body fat, when compared to mice colonized by microbiota associated with leanness. The gut microbiota may produce metabolites, and directly or indirectly affect patterns of mammalian metabolism and weight gain (*Turnbaugh* et al., 2006).

Specific bacterial species have been identified that are associated with alterations in mammalian body metabolism and weight gain (*Le Chatelier* et al., 2013). Studies demonstrated that humans afflicted with diabetes and/or obesity harbour increased cell numbers of *Clostridium ramosum* (Erysipelotrichi) (*Qin* et al., 2012; *Karlsson* et al., 2013; *Le Chatelier* et al., 2013). The researchers investigated the role of *C. ramosum* in the pathophysiology of obesity in gnotobiotic mice. The results showed that Simplified Intestinal Human Microbiota (SIHUMI) mice with

C. ramosum had enhanced diet-induced obesity compared with SIHUMI mice without *C. ramosum*. This species of bacteria proliferated in SIHUMI mice fed a high-fat diet (HFD). Small intestinal glucose and fat transporters in these animals may be upregulated, leading to increase in body fat deposition. This upregulation in HFD-fed SIHUMI mice may be caused by *C. ramosum* increasing gene expression of the fatty acid transport proteins ileal FA-transport protein-4 (FATP4) and *CD36*. In addition, enterocytes of obese mice contained increased gene expression of perilipin, which is a fat storage protein (*Woting* et al., 2014). So, gut microbes may proliferate when exposed to dietary fat and may enhance the body's ability to absorb and metabolize simple sugars and triglycerides. Diet alters the microbiome, and the microbiome, in turn, changes the ability of mammals to absorb and metabolize nutrients.

The availability of nutrients and degree of flexibility of resident bacteria in finding nutrients determine the diversity and mutualistic cooperation among the component species in human gut microbiota (*Backhed* et al., 2005). Nutrient availability and microbial composition are complicated by the presence of different bacterial layers in the intestinal lumen (luminal) and adjacent to the mucosa (mucosal). These "layers" of microbes and luminal content are affected by hydrodynamics (fluid shear), oxygen content, and the presence of mucus (*Van den Abbeele* et al., 2011). The composition and function of the mucosal microbiome may directly affect the biology of the intestinal mucosa, but this area adjacent to the mucosa is difficult to access and dynamics of microbiome colonization are challenging to study. Butyrate-producing organisms of the groups *Clostridium* cluster IV and XIVa are enriched in the mucosal microbiome (discussed in this monograph by Tom van de Wiele) (*Nava* et al., 2011). Promotion of regulatory T cell accumulation in mice is caused by the spore-forming components of native intestinal microbiota, especially *Clostridium* clusters IV and XIVa (*Atarashi* et al., 2011). *In vitro* models, consistent with *in vivo* data, showed that strictly anaerobic bifidobacteria tend to reside in the upper part of the mucus layer, but the Firmicute, *F. prausnitzii*, mainly colonized the lower part of the mucus, at the anoxic/oxic interphase and adjacent to the intestinal epithelium. Intestinal bacteria and their metabolites are stratified in layers spanning the lumen and mucosa-associated spaces.

The metabolism of carbohydrates by specific microbes generates precursors of short chain fatty acids (SCFAs) such as butyrate. Generation of butyrate provides a fuel source for the intestinal epithelium, enhances tight junction formation and promotes intestinal "barrier" function. Deficiencies of butyrate-producing microbes such as *F. prausnitzii* (*Sokol* et al., 2008) have been demonstrated in disease states such as inflammatory bowel disease (e.g. Crohn's disease) (*Willing* et al., 2009). Models such as M-SHIME detected clear differences in healthy versus Crohn's disease-associated microbiomes, and data showed that human participants with active disease yielded lower butyrate production. In summary, deleterious effects on microbial composition of the mucosal microbiome caused by shifts in the intestinal environment may culminate in ineffective functioning of the gut mucosa and increased disease susceptibility. Conversely, abundance of microbial metabolites such as SCFAs may promote healthy immune and epithelial cell function, and disease resistance. The compositional and functional

stratification of the intestinal microbiome in "layers" can be studied with new model systems and provide insights into microbial-mucosal interactions.

MICROBIAL METABOLITES AND MAMMALIAN PHYSIOLOGY

Microbial metabolites represent the biochemical currency of communication between microbial and mammalian cells in the gut and the gut-brain axis. As we consider the links between diet and the microbiome, and the microbiome and mammalian physiology before and after birth, the roles of microbial metabolites in different body compartments must be considered. SCFAs are well-known examples of microbial metabolites affecting the intestinal epithelium. Three main SCFAs produced in the human colon are acetate, propionate, and butyrate. Propionate and acetate affect adipogenesis and leptin production. By modulating gut and adipocyte-derived hormones, SCFAs may affect the gut-brain axis and brain function, including appetite and satiety (*Xiong* et al., 2004; *Samuel* et al., 2008). Acetate is able to directly affect neuronal function in the brain, especially regions associated with appetite and satiety control (*Anastasovska* et al., 2012). Using carbon-11 labelled acetate as a potential PET marker, acetate is actively assimilated by many organs including the liver, skeletal muscle, spleen, heart, and adipose tissue. Acetate may reach the brain in small, yet significant amounts, regardless of the route of administration (*Frost* et al., 2014). Supplementation of fermentable carbohydrates has long been known to reduce appetite in animal studies, and the appetite suppressing effect of acetate was recently shown to occur via hypothalamic neuronal activation (*Frost* et al., 2014). Whether adminis
tered or produced in the colon, acetate has a direct effect on hypothalamic neurons by its incorporation into the glutamate-glutamine transcellular cycle, increasing GABAergic transmission and activating acetyl-CoA carboxylase (ACC). The increased ACC activity induces malonyl-CoA expression, which activates pro-opiomelanocortin neurons and therefore reduces food intake (see contribution by J. Bell in this monograph). The SCFA, acetate, provides a clear example of how one microbial metabolite mediates communication between different organs such as the gut and the brain.

In addition to appetite suppression and body metabolism, microbial metabolites may modulate the immune system by activating or suppressing immune responses in the gut mucosa. Fungi within the microbiome may promote resistance to pathogenic microbes and tolerance to commensal microbes. The mammalian tryptophan catabolic enzyme, indoleamine 2,3-dioxygenase 1 (IDO1), plays a key role in the conversion of dietary or microbial tryptophan into kynurenines and promotion of tolerance to commensal microbes. The capacity of IDO1-expressing dendritic cells, epithelial cells, and kynurenines to induce Tregs and inhibit Th17 has revealed their unexpected potential to control inflammation, allergy, and Th17-driven inflammation in fungal infections (*Grohmann* et al., 2007; *Romani* et al., 2008). Conversely, the exploitation of the IFN-γ/IDO1 axis for functional specialization of antifungal regulatory mechanisms may have enabled fungal microbiota to co-evolve with the mammalian immune system,

and to prevent dysregulated immunity (*Zelante* et al., 2009). In contrast, beneficial microbes may convert tryptophan into bioactive indole compounds. Indole derivatives act as endogenous ligands for the arylhydrocarbon receptor (AhR) (*Heath-Pagliuso* et al., 1998) and are generated through conversion from dietary tryptophan by commensal intestinal microbes (*Bjeldanes* et al., 1991). By binding AhR, the microbiome may stimulate mammalian immune cells to produce the cytokine lL-22 and thereby enhancing protective mucus production, suppressing IL-17 production, and stimulating production of antimicrobial peptides via STAT3 activation. The lesson in *Lactobacillus*-mediated indole-3-aldehyde (one example of indole derivative) production from tryptophan is that bacterial metabolites generated by the microbiome may have a profound impact on immunity. The metabiotic concept refers to metabolites produced by the microbiome or probiotic species that may confer specific benefits on mammalian physiology or immunity. Metabiotics such as indole derivatives may suppress inflammation and promote immunologic resistance to different pathogens.

Gamma-aminobutyric acid (GABA) is another important co-metabolite produced by glutamate decarboxylases (GADs) of mammals and bacteria. Amino acid decarboxylation systems, such as the GAD system, enable bacterial species to cope with acid stress, and GABA is enriched in the intestines of patients with recurrent *C. difficile*-associated disease (CDAD). Most intestinal GABA measured in stool is microbial in origin, and this metabolite (GABA) appears to potentiate the risk of *C. difficile* infection. Individuals consuming the $GABA_A$ receptor agonist, zolpidem (Ambien®), have increased risk of *C. difficile* infection, adding support to the pathogenic role of GABA in CDAD. Data from mouse models also support the ability of GABA receptor signalling to exacerbate intestinal inflammation. In contrast to elevated amounts of microbial GABA in recurrent CDAD, the bile acid metabolite lithocholate is associated with healthy controls and patients cured post-FMT. The scientific community is beginning to identify specific microbes and microbial metabolites that contribute to human health and alter disease susceptibility. New classes of microbial biomarkers are being characterized, and GABA is a good example. So we are beginning to identify specific microbial metabolites and how their receptors/signalling pathways may protect patients or enhance disease susceptibility.

SYSTEMS BIOLOGY AND THERAPEUTIC MICROBIOLOGY

Many published studies have explored associations between differences in microbial composition and human health and disease states, including but not limited to inflammatory bowel disease, necrotizing enterocolitis, and obesity. However, few microbiome studies could verify causation. Scientists should attempt to verify causation, try to identify microbial effector strains and metabiotics, and modify effector strains to improve mammalian health. To identify microbial effector strains, one must isolate individual microbes or rely on differences in metabolite profiles. *Faith* et al. (2014) approached the challenge of identifying microbial effector strains. One of several human faecal samples transmitted a metabolic/immunologic phenotype to

recipient germ-free mice and resulted in efforts to identify the key microbe(s) involved by metagenomic and microbial sequencing. Ninety-four bacterial consortia were taken from the culture collection and were introduced as humanized gut microbiota to germ-free mice. Feature selection algorithms and follow-up mono-colonization studies may identify effector bacterial strains. An unexpected range of bacterial strains was found to promote accumulation of colonic regulatory T cells (Tregs) and expand Nrp1 (lo/-) peripheral Tregs. Other strains modulated adiposity in mice and caecal metabolite concentrations (*Faith* et al., 2014). The greatest numbers of Tregs in the colonic lamina propria were associated with *B. intestinalis* colonization (*Faith* et al., 2014). Specific metabolites such as quinic acid, a cyclic polyol, were detected and correlated with effector bacterial strains. As effector strains are identified, their specific metabolic features may be useful in determining which effector strains (and metabolites) may be ideally suited for particular research and clinical applications. These studies point to the utility of combining intestinal microbiology with well-defined gnotobiotic mouse models so that specific functions of the microbiome can be linked to specific microbes.

Another key challenge in microbiome research is to understand the role of interpersonal microbiome variation in disease. Researchers investigated gut microbiota from twins with discordance for obesity that modulated metabolism in mice. They found that co-housing mice containing an obese twin's microbiota (Ob) with mice containing the lean co-twin's microbiota (Ln) led to suppression of body mass and adiposity increases in Ob cage mates. As mice are coprophagic, rescue is correlated with invasion of specific members of Bacteroidetes from the Ln microbiota into the Ob microbiota (*Ridaura* et al., 2013). Another study of interpersonal microbiome variation in disease showed that faecal microbiota transplantation induced 7 of 9 remissions in patients with active ulcerative colitis from one donor (*Moayyedi* et al., 2015). Thus, even if a donor does not have disease, this donor may not be an appropriate donor for someone that does have the disease. Thus, microbial composition is important in terms of determining whole body metabolism and careful selection of stool donors for faecal microbiota transplantation (FMT). Determining the causative drivers of diverse responses from diverse microbiota is a key challenge in microbiome research.

The relative diversity and abundance of each body habitat's microbes varie between individuals (*Human Microbiome Project Consortium*, 2012). On the other hand, metabolic pathways are relatively conserved between individuals in a healthy population (*Human Microbiome Project Consortium*, 2012). Diet has a large role in modulating microbiota, and it is a primary determinant of gut microbial composition. Although short-term dietary interventions might rapidly change community structures within the gut microbiome, long-term shifts in steady-state microbial communities require longer term and substantial dietary changes (*Voreades* et al., 2014). Antibiotics reduce the relative diversity within gut microbiomes. Researchers found substantial variations in microbial communities of subjects given a single antimicrobial agent (ciprofloxacin). These changes occurred between two courses of antibiotics administered to subjects. When the study ended, the composition of the gut microbiota in every subject had stabilized, although it was changed from its initial state. Thus, alternative stable states may shift depending on the

frequency, duration and nature of antimicrobial exposure (*Dethlefsen* and *Relman*, 2011).

Several options for microbial manipulation provide opportunities for changing microbial composition and function. Studies showed that probiotics were effective for infectious diarrhoea (*Francavilla* et al., 2012; *Dinleyici* and *Vandenplas*, 2014). A systemic review and meta-analysis showed significant beneficial effects of probiotics that reduced global symptoms or abdominal pain (*Ford* et al., 2014). A systematic review of probiotics, prebiotics, and synbiotics in inflammatory bowel disease demonstrated that some studies yielded improvement trends, but definitive treatments had been evasive (*Ghouri* et al., 2014). Diet of the host can have a large effect on responses to probiotics. Also, probiotics can alter microbial composition quite differently depending upon the diet (*Degirolamo* et al., 2014). The effects of probiotic treatment are strongly influenced by the existing intestinal microbial ecosystem. Confounding factors in human studies of probiotics include the individual's diet, established microbial communities in a particular individual, and the luminal environment.

Faecal microbiota transplantation (FMT) is a method to radically change microbial composition by introducing faecal microbiota from a healthy person to a person with an intestinal disorder. Researchers found that infusion of donor faeces was significantly superior to vancomycin in treatment of recurrent *C. difficile* infection (*van Nood* et al., 2013). FMT caused bacterial composition to normalize quickly from a state of dysbiosis. Although the microbiome in the subject reflected the donor implant material at 1 day post-FMT, the composition was highly varied at later time points (*Weingarden* et al., 2015). It is likely that mechanisms contributing to FMT's effectiveness as a treatment strategy in patients with recurrent *C. difficile* infection stem from the ability to interfere with pathogen spore germination and competition by *C. difficile* (*Britton* and *Young*, 2014). Importantly, FMT can be considered as the simultaneous administration of hundreds of bacterial taxa with different functional features. For example, functional classes of bacterial strains may include lactate producers, methanogens, mucin degraders, and short chain fatty acid producers. Restoration of many microbiome-related functional features after one or several procedures (FMT) may be necessary for restitution of a severely depleted microbiome. Disorders of microbial ecology (e.g. CDAD) secondary to antibiotic consumption in susceptible individuals emphasize the need for replacement strategies with consortia and pools of microbes harbouring diverse microbiologic functions.

Future perspectives on FMT include customization of bacterial combinations with defined and known specific biological properties to produce predictable responses. Additionally, development of standardized conditions for transplantation (inoculation) and maintenance of faecal-derived microbial communities, and analyses of donor-recipient microbiome compatibilities represent next steps in advancing FMT and other microbial consortia-based replacement strategies. In addition to global and multifunctional considerations, precision microbiome reconstitution has been shown to impact bile acid-mediated resistance to CDAD (*Buffie* et al., 2015). In this example, one species, *Clostridium scindens*, has 7-dehydroxylase activity resulting in production of bile acid metabolites that mediate resistance to *C. difficile* infection. One of these bile acid metabolites,

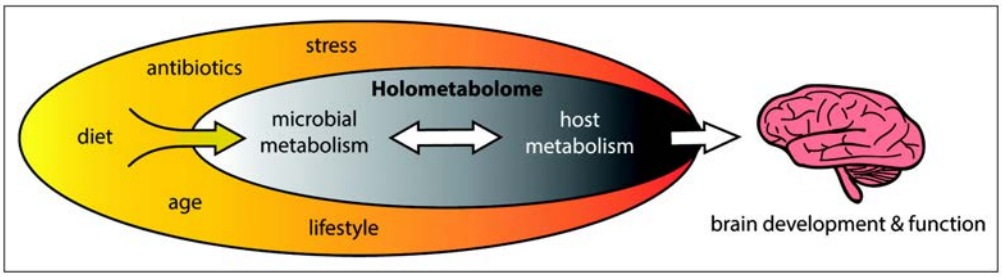

Figure 2: The human-microbiome holometabolome. Environmental factors including diet and medications influence microbial and human metabolism together as one physiological system. Changes in the holometabolome likely affect gut biology, the brain-gut axis and brain development and function. (Reprinted with permission from *Selkrig* et al., 2014.)

lithocholate, was found enriched in healthy controls and FMT-responsive patients by Tor Savidge and colleagues. So, we come full circle by considering microbiome replacement therapy (FMT), identifying individual effector bacterial strains (*C. scindens*), and detecting individual bile acid metabolites conferring disease resistance.

SUMMARY AND FUTURE DIRECTIONS

This 29th edition of the Old Herborn University Symposium (June 2015) placed the spotlight on the overlapping contact points between diet, the gut microbiome, microbial metabolism, and human metabolism/physiology. The "Holometabolome" concept as proposed in 2014 provides a conceptual framework by linking environmental factors with microbial and mammalian metabolism, and considering how metabolites from microbes and mammals impact the biology of the intestine and remote organ systems (e.g. brain) (Figure 2). The importance of the microbiome *in utero* and the impact of breast milk drive home the point that humans rely on microbes and microbial metabolites during the earliest weeks to months of human life. The exciting reality is that microbial composition and function can be manipulated by diet and that effects of the microbiome on early human development cannot be underestimated.

Beyond early human life, microbial taxa communicate effectively with mammalian cells at the mucosal interface, resulting in different metabolic and immunologic profiles later in life. Several important concepts in microbiome science have emerged. First, one microbiome (oral) may seed a different microbiome (placenta) and enable it to function with the foetus. Dietary impact of the microbiome may persist for many years. Compositional and functional stratification of the gut microbiome reminds us that the microbiome is a differentiated organ with different functional aspects. By describing various microbial metabolites and their potential roles, we must think in terms of microbiome-related contributions to the entire host. The gut microbiome will be tapped to understand the needs/identity/skills of individual microbes so that functions can be attached. The relative contribution of microbiomes to heredity is an interesting question to ponder. Finally, intentional manipulation of the gut

microbiome by introducing intact microbial communities (FMT), single or selected beneficial microbes (probiotics), or dietary shifts (including prebiotics) will hopefully yield opportunities to improve human health and prevent or cure diseases. Insights into microbial and human metabolism may help us identify new biomarkers, diagnostic tools, and therapeutic targets in the future.

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