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35. MICROBES IMPACTING MAMMALIAN SYSTEMS BIOLOGY

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WOMB WITH A VIEW: THE IMPORTANCE OF MATERNAL-FETAL COMMUNICATION DURING EARLY DEVELOPMENT

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OVERVIEW

What is heritability? Is it merely the vertical transfer of your genomic material from egg and sperm? Or is it a far more complex set of traits which arise from the community of genomic and epigenomic material which is vertically transferred during key intervals of development?

The 'Developmental Origins of Health and Disease [DOHaD] Hypothesis' (*Barker*, 1986; *Fleming* et al., 2018) encompasses a substantial body of evidence which temporally and functionally links maternal exposures to adverse outcomes (largely the non-communicable diseases [NCDs]) in her offspring like obesity, metabolic disorders, cardiovascular disease, and behavioural outcomes (see Figure 1 for a schematic overview).

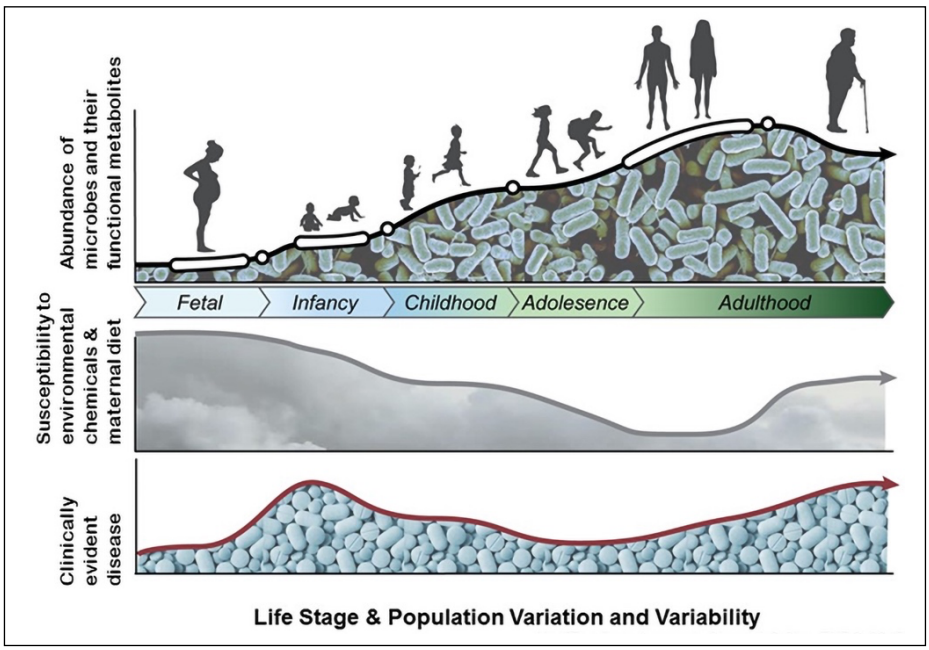

Figure 1: Womb with a view: How do we prepare babies to adapt to the world they are going to be born into? Tolerate commensal microbes and allow colonization? Resist pathogens?
This schematic underlies key questions of our time in microbiome science, and early developmental programming, including foetal development. (Modified from *Aagaard* et al., 2016).

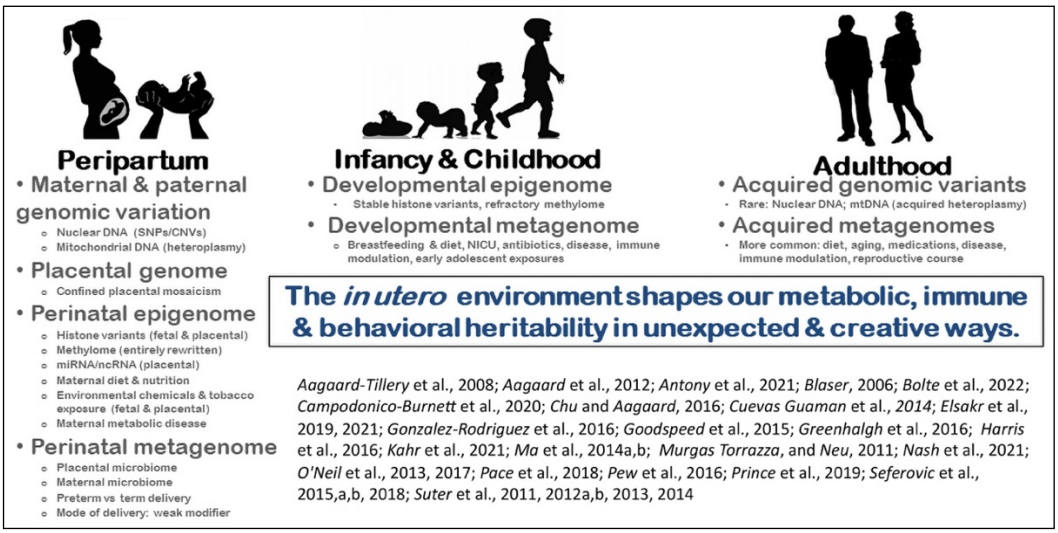

Figure 2: Heritability: What are the molecular mechanisms which facilitate maternal-foetal communication and enable adaptation in a changing world?
This schematic and listed references represent the data from mice to monkeys to humans demonstrating that the majority of our heritable traits are not the product of genomic alleles, but rather epigenomic and metagenomics driven patterning.

Initial mechanistic-minded studies in animal models demonstrated that both maternal nutritional deprivation and a high fat 'Western style' diet feeding brought about meaningful and persistent modulations in postnatal gene expression resulting from epigenomic changes in key metabolic pathways in the offspring (*Aagaard-Tillery* et al., 2008; *Safi-Stibler* and *Gabory*, 2020). More recently, others examined the similar temporal relationships through the lens of microbiome science, leading to the genesis of the 'Hygiene Hypothesis.' The Hygiene Hypothesis suggests that in addition to maternal exposure-driven foetal epigenetic variation, the lack of exposure to microbes early in life predisposes offspring to not only developing these same adverse outcomes, but also atopic and allergic diseases later-in-life (*Blaser*, 2006). However, despite nearly 100 years recognizing these links, we have failed to reveal meaningful mechanistic understandings of 1) how specific maternal elements contribute to functional foetal and early life developmental and 2) how to prevent infant morbidity and mortality. We and others have now spent the last several decades diving into understanding what enables generational adaptation to occur at a far, far more amenable rate than genomic variance and positive selection would allow (*Aagaard-Tillery* et al., 2008; *Aagaard* et al., 2012; *Antony* et al., 2021; *Blaser*, 2006; *Bolte* et al., 2022; *Campodonico-Burnett* et al., 2020; *Chu* and *Aagaard*, 2016; *Cuevas Guaman* et al., *2014*; *Elsakr* et al., 2019, 2021; *Gonzalez-Rodriguez* et al., 2016; *Goodspeed* et al., 2015; *Greenhalgh* et al., 2016; *Harris* et al., 2016; *Kahr* et al., 2021; *Ma* et al., 2014a,b; *Murgas Torrazza*, and *Neu*, 2011; *Nash* et al., 2021; *O'Neil* et al., 2013, 2017; *Pace* et al., 2018; *Pew* et al., 2016; *Prince* et al., 2019; *Seferovic* et al., 2015,a,b, 2018; *Suter* et al., 2011, 2012a,b, 2013, 2014). While Figure 2 depicts a number of mechanisms by which 'heritability' occurs, we focused our discussion at the 2023 Old Herborn University Seminar on the role of the microbiome, its

metagenome, and its accompanying metabolome during key intervals of development (*Aagaard* et al., 2014; *Azad* et al., 2016; *Banerjee* et al., 2020; *Bassols* et al., 2016; *Bolte* et al., 2022; *Butel* et al., 2007; *Chu* and *Aagaard*, 2016; *Chu* et al., 2017; *Claus* et al., 2016; *Collado* et al., 2016; *Gomez-Arango* et al., 2017; *Greenhalgh* et al., 2016; *Doyle* et al., 2014, 2017; *Jasarevic* and *Bale*, 2019; *Liu* et al., 2022; *Ma* et al., 2014a,b; *Nash* et al., 2021; *Pace* et al., 2018, 2021; *Pammi* et al., 2017; *Parnell* et al., 2017; *Prince* et al., 2016, 2019; *Rogier* et al., 2014; *Seferovic* et al., 2015, 2018).

CO-EXISTENCE OF MICROBES AND ANIMALIA SPECIES: HOW ESSENTIAL ARE MICROBES FOR REPRODUCTION AND NORMAL EARLY DEVELOPMENT?

Our laboratory and others have demonstrated that the vaginal, oral and stool microbiota composition and microbiome community function vary during the course of normal pregnancy, thus providing a unique "signature" in pregnancy with relative altered abundance of multiple species and strains (*Aagaard* et al., 2012b; *Butel* et al., 2007; *Chu* et al., 2017; *Liu* et al., 2022; *Ma* et al., 2014a; *Pace* et al., 2021). But what impact does this have on either the pregnancy or the developing infant? Although it has long been suggested that intrauterine infections, such as chorioamnionitis, are the sequelae of ascending microbiota from the upper vagina, we and others have shown that the evidence supporting this notion are relatively sparse and source microbes come from other maternal sites with vertical transfer (both *in* and *ex utero*) or via limited horizontal transfer (*Aagaard* et al., 2014; *Aagaard* and *Hohmann*, 2019; *Azad* et al., 2016; *Banerjee* et al., 2020; *Bassols* et al., 2016; *Bolte* et al., 2022; *Butel* et al., 2007; *Cao* and *Mysorekar*, 2013; *Chen* and *Devaraj*, 2018; *Chen* and *Gur*, 2019; *Chu* et al., 2016, 2017; *Claus* et al., 2016; *Collado* et al., 2016; *Dong* et al., 2015; *Doyle* et al., 2014, 2017; *Goldstein* et al., 2017; *Gomez-Arango* et al., 2017; *Gosalbes* et al., 2013a; *Jasarevic* and *Bale*, 2019; *Kelly* et al., 2021; *Liu* et al., 2022; *Lokugamage* and *Pathberiya*, 2019; *Ma* et al., 2020; *Pace* et al., 2021; *Pammi* et al., 2017; *Parnell* et al., 2017; *Petersen* et al., 2021a, 2021b; *Prince* et al., 2016; *Rogier* et al., 2014; *Roswall* et al., 2021; *Satokari* et al., 2009; *Seferovic* et al., 2019, 2020a,b; *Song* et al., 2021; *Stinson* et al., 2018; *Thaiss* et al., 2016; *Tuominen* et al., 2018; *Zheng* et al., 2015).

Clearly, to exist on Earth is to coexist with microbes. Interactions between microbes and host species within the kingdom Animalia are evident when comparing to their gnotobiotic and/or 'germ free' laboratory derived counterparts. Importantly, germfree derivations of mice, zebrafish, and pigs are capable of survival but are immunologically (*Gensollen* et al., 2016), metabolically (*Cox* et al., 2022), and behaviourally (*Luczynski* et al., 2016) abnormal. Comparing gnotobiotics to naturally-existing Animalia species, which evolved during hundreds of millions of years in a world teeming with microbial communities, it is evident that while survival can occur absent of microbes, normal development is accompanied by immune tolerance to commensal microbes, allowing them to persist while barring highly antigenically and genomically-related pathobionts and pathogens from flourishing.

Indeed, the reliance on microbes for Animalia species fitness is not limited to

immune development. Some animal species, including horses, sheep, and cattle, rely primarily on microbial macromolecules, small molecule intermediates and proteins, and key metabolites as primary sources of dietary energy and micronutrients. Others, like koalas or cuttlefish, have developed highly specific interactions which are hallmark symbiotic characteristics or traits of their species, such as enabling digestion of nutrient-poor food sources (koalas and eucalyptus [*Chong* et al., 2020]) or production of bioluminescence (*McFall-Ngai*, 2014) (cuttlefish). Placental mammals maintain finely tuned relationships with microbial metabolic products, and even seemingly minor disruptions to normal microbial community structure (*e.g*., community dysbiosis) has been associated with the incidence, onset and prevalence of disorders in metabolism and a number of non-communicable diseases in humans and primates (*Girbovan* et al., 2017; *Gosalbes* et al., 2013a; *Huang* et al., 2021; *Moeller* et al., 2018; *van Opstal* and *Bordenstein*, 2015). Although microbial products are capable of influencing foetal development (Figure 1), true vertical transfer requires these microbes be functional, selectable, and capable of colonizing their host during the perinatal window (periconception through early postnatal life). From an ecological perspective, a non-random generational transmission of microbes would provide exclusive opportunities for selection toward host fitness (*Tett* et al., 2019) and limit virulence potential (*Schubbert* et al., 1998). Comprehensive data sources are needed to quantify the contributions of transmission mode toward the acquisition of microbes (*Jiménez* et al., 2008; *Perez* et al., 2007; *Schubbert* et al., 1998; *Tett* et al., 2019) and the mechanisms used to generationally retain them.

WHAT IS THE IMPORTANCE OF MATERNAL-FOETAL COMMUNICATION DURING DEVELOPMENT?

As schematically depicted in Figure 3, there is a fundamental paradox in development: if the womb is sterile and the placenta is a barrier and not a means of communication, how do we tolerate commensal microbes requisite to metabolism, immunity and behaviour and adapt to an ever-changing world? When considering the factors influencing the formation of the foetal microbiome, it is also important to consider how the intrauterine environment itself may influence the microbiota of the developing foetus. Many scientists have challenged the notion of a sterile intrauterine environment in the absence of disease and have purported a distinct placental and amniotic fluid microbiome that is closely similar to the foetal/neonatal microbiome; others argue that any and all representations of microbial content are confounded by contamination (*Aagaard* et al., 2014; *Aagaard* and *Hohmann*, 2019; *Azad* et al., 2016; *Banerjee* et al., 2020; *Bassols* et al., 2016; *Bolte* et al., 2022; *Butel* et al., 2007; *Cao* and *Mysorekar*, 2013; *Chen* and *Devaraj*, 2018; *Chen* and *Gur*, 2019; *Chong* et al., 2020; *Chu* et al., 2016, 2017; *Claus* et al., 2016; *Collado* et al., 2016; *Cox* et al., 2022; *DiGiulio* et al., 2010; *Dong* et al., 2015; *Doyle* et al., 2014, 2017; *Gensollen* et al., 2016; *Girbovan* et al., 2017; *Goldstein* et al., 2017; *Gomez-Arango* et al., 2017; *Gosalbes* et al., 2013a,b; *Hansen* et al., 2015; *Hiltunen* et al., 2021; *Hornef* and *Penders*, 2017; *Huang* et al., 2021; *Jasarevic* and *Bale*, 2019; *Jiménez* et al, 2008; *Kelly* et al., 2021; *Kennedy* et al.,

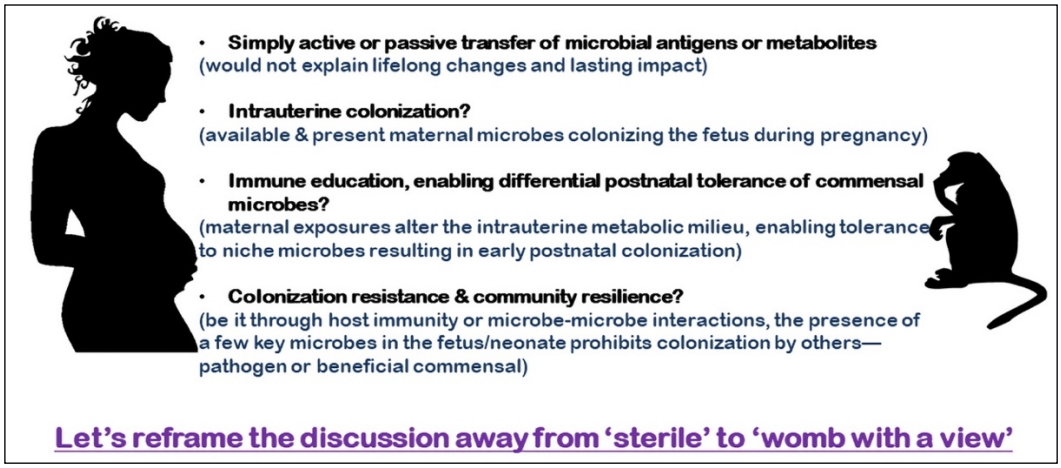

Figure 3: The womb with a view perspective enables us to consider the importance of developing immune tolerance to commensal microbes early in development.
Whether true colonization occurs in utero is yet to be determined, and there is supporting and refuting evidence in both (see the text for references). I remain agnostic as to whether true colonization with live microbes fully occurs in utero. However, what is evident is that microbial products do transfer *in utero* from mother to foetus, inclusive of metagenomic materially, peptidoglycans and other antigens, and microbial metabolites.

2021, 2023; *Liu* et al., 2022; *Lokugamage* and *Pathberiya*, 2019; *Luczynski* et al., 2016; *Ma* et al., 2020; *McFall-Ngai*, 2014; *Mishra* et al., 2021; *Moeller* et al., 2018; *Onderdonk* et al., 2008a; *Pace* et al., 2021; *Pammi* et al., 2017; *Parnell* et al., 2017a,b; *Perez* et al., 2007; *Perez-Muñoz* et al., 2017; *Petersen* et al., 2021a, 2021b; *Prince* et al., 2016; *Rackaityte* et al., 2020; *Rogier* et al., 2014; *Roswall* et al., 2021; *Satokari* et al., 2009; *Schubbert* et al., 1998; *Seferovic* et al., 2019, 2020a,b; *Song* et al., 2021; *Stinson* et al., 2017, 2018; *Tett* et al., 2019; *Thaiss* et al., 2016; *Theis* et al., 2020; *Tuominen* et al., 2018; *van Opstal* and *Bordenstein*, 2015; *Zheng* et al., 2015). Evidence for a unique placental microbiome by virtue of metagenomic characterization stems from a metagenomics study of 320 placentae which demonstrated a low-biomass microbial community of the placental parenchyma and chorionic villi (*Aagaard* et al., 2014). Our study demonstrated a unique placental microbiome niche composed of non-pathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla that was most akin to the human oral microbiome by composition. Other researchers have demonstrated a similarity between the placental microbiota and the neonatal meconium, suggesting that the microbiota may be transferred across the placenta and into the foetus before being excreted into the amniotic fluid as foetal urine; others again argue that these metagenomics signals are largely the result of contamination (*Aagaard* et al., 2014; *Aagaard* and *Hohmann*, 2019; *Bassols* et al., 2016; *Cao* and *Mysorekar*, 2013; *Chen* and *Devaraj*, 2018; *Chen* and *Gur*, 2019; *Chong* et al., 2020; *Collado* et al., 2016; *Cox* et al., 2022; *DiGiulio* et al., 2010; *Dong* et al., 2015; *Doyle* et al., 2014, 2017; *Gensollen* et al., 2016; *Girbovan* et al., 2017; *Goldstein* et al., 2017; *Gomez-Arango* et al., 2017; *Gosalbes* et al., 2013a,b; *Hansen* et al., 2015; *Hiltunen* et al., 2021; *Hornef* and *Penders*, 2017; *Huang* et al., 2021;

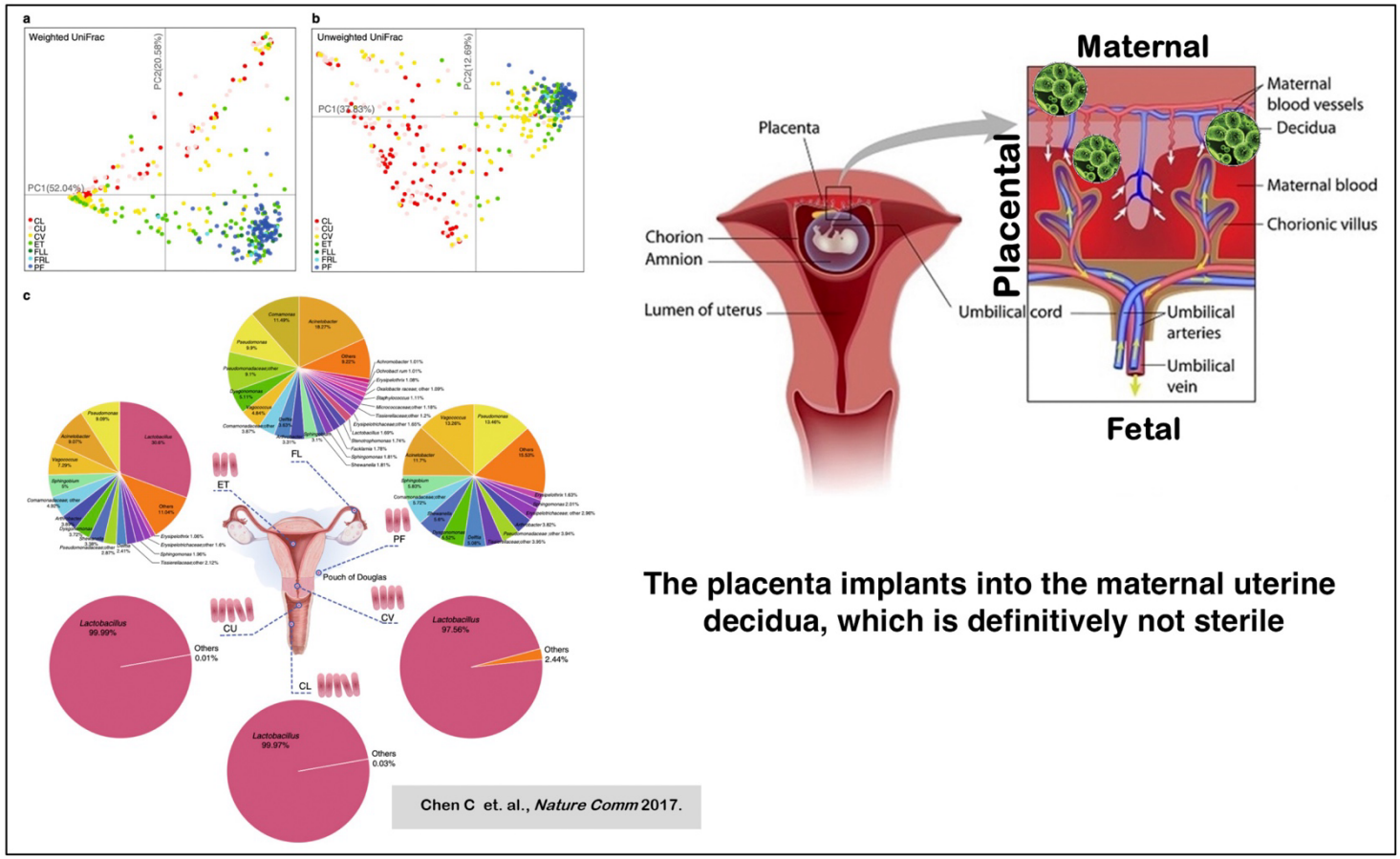

Figure 4: Differing interpretations of highly consistent data: there is a metagenomic signal in amniotic fluid and placenta and the fetus; whether it represents live and persistent microbial colonization *in utero* is yet unclear (see the text for references). The importance of these signals for maternal-fetal communication and fetal adaptation is evident.

Jiménez et al, 2008; *Kelly* et al., 2021; *Kennedy* et al., 2021, 2023; *Lokugamage* and *Pathberiya*, 2019; *Luczynski* et al., 2016; *Ma* et al., 2020; *McFall-Ngai*, 2014; *Mishra* et al., 2021; *Moeller* et al., 2018; *Onderdonk* et al., 2008b; *Parnell* et al., 2017a,b; *Perez* et al., 2007; *Perez-Muñoz* et al., 2017; *Petersen* et al., 2021a, 2021b; *Prince* et al., 2016; *Rackaityte* et al., 2020; *Roswall* et al., 2021; *Satokari* et al., 2009; *Schubbert* et al., 1998; *Seferovic* et al., 2019, 2020a,b; *Song* et al., 2021; *Stinson* et al., 2017, 2018; *Tett* et al., 2019; *Thaiss* et al., 2016; *Theis* et al., 2020; *Tuominen* et al., 2018; *van Opstal* and *Bordenstein*, 2015; *Zheng* et al., 2015). Our publications and those of Gosalbes and colleagues (postulate that because the neonatal meconium microbiota differs from the dominant bacterial groups found in the maternal skin, faecal, and vaginal niches, the neonatal microbiota is unlikely to originate in those maternal locations. Instead, they argue that because meconium is formed starting at mid-gestation in foetal life (17 weeks and beyond), the microbiota detected in meconium is likely not simply due to contact with maternal habitus at time of delivery (*Gosalbes* et al., 2013a). Indeed, it is well established that the meconium expressed within minutes to days of birth has been present in the small bowel since at least 20 weeks of gestation. Chen and Gur hypothesize that maternal comorbidities (including diabetes and hypertension) results in changes in the maternal intestinal, oral, and vaginal microbiomes that facilitate the translocation of bacteria to the intrauterine environment either haematogenously or through direct ascension, suggesting that the placenta is more conduit than barrier (*Chen* and *Gur*, 2019). Recent work from Peterson and colleagues demonstrated that newborns who developed immunoglobulin E (IgE)-mediated allergic sensitization (atopy) by one year of age have a less diverse gut metabolome at birth as measured in the meconium, which begins forming in the foetal gut during the second trimester (Peterson et al., 2021b). They argue that deficiency in microbiota maturation and immune development likely begins in utero rather than at time of delivery.

It is important to acknowledge, however, that there is a debate regarding the existence of a placental/amniotic fluid or foetal microbiome (Figure 4). In our own work, while we have consistently distinguished a metagenomic signal in the placenta from that of contaminant controls, we have also been explicit in noting it to be of low biomass, low abundance, and sparse (*Aagaard* et al., 2014; *Aagaard* and *Hohmann*, 2019; *Azad* et al., 2016; *Banerjee* et al., 2020; *Bolte* et al., 2022; *Butel* et al., 2007; *Chu* et al., 2017; *Claus* et al., 2016; *Goldstein* et al., 2017; *Jasarevic* and *Bale*, 2019; *Liu* et al., 2022; *Ma* et al., 2020, 2014b; *Nash* et al., 2021; *Pace* et al., 2018, 2021; *Pammi* et al., 2017; *Prince* et al., 2019; *Rogier* et al., 2014; *Seferovic* et al., 2015b, 2018, 202b). We have also remained consistently agnostic as to whether the placental or intrauterine microbiome is truly alive and colonizing, with a yet unclear functional role. With further scientific advancement and continued curiosity, we are confident that investigators will determine whether these consistently observed low-biomass communities are alive and colonize the foetus or alternatively enable later colonization through processes of immune tolerance or colonization resistance.

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. 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 that suggest "less diverse and less rich vaginal microbiomes are equivalent to disease states" have been challenged and discounted.

Previously assumed to be "sterile" reproductive tract tissues have been shown to harbour low biomass metagenomes, and yet we remain unclear as to what, how and when the infant is colonized. When we consider species outside humans and mice, the dogma that all foetuses develop in a microbiologically and immunologically naïve environment is not supported by the current published literature. Although the presence of pioneering prenatal microbiota is often considered to be a novel concept, experimental and observational evidence of its existence dates back almost 100 years for many species, including humans. The advent of highly selective cultivation media and concerns regarding contamination-prone nucleic acid sequencing has led to appropriate and necessary questioning of the evidence supporting prenatal exposure to maternal microbiota. Upon comprehensive review of the literature inclusive of heterogenous and orthogonal rigorous methodologies among multiple Animalia species, the state of the science is most consistent with the notion that *in utero* exposure to microbes is sufficient, although not necessary for all species, for normal development when considering heterogeneous cohorts and species on nearly every continent. This robust set of findings may facilitate the transition into a new era pursuing a deeper functional understanding in order to improve the health of humans and animals worldwide.

While we and others have clearly shown that there is, at most, transient differences in the microbiota of neonates born via Caesarean when compared to those born by vaginal delivery, these changes are limited to a few taxa, are not durable, and appear to resolve during infancy. As such, it is not surprising that meaningful lifelong impacts on the development of the metabolic and immune system in mammalian offspring have yet to be causally linked. However, the potential for reduction of harm readily resides within our reach if we can reduce the disparities that contribute to increased Caesarean delivery risk. For example, with a lens on our diabetic or obese population, based on multiple lines of evidence, we can confidently anticipate three benefits of widened availability of optimized nutrition with relief of food scarcity and unrestricted access to medical care both preconception and prenatally. We would (1) improve glycaemic control, optimizing foetal growth, (2) lower the Caesarean delivery rate and increase the duration of human milk feeding, and (3) potentially mitigate the longer-term risks from exposure to these conditions *in utero*. This heightened attention to maternal perinatal health - like improved glucose control and appropriate weight gain - can be attained with widened access to clinical and public health interventions and patient counselling without incurring any additional risk to mom or foetus. While there are many outstanding questions regarding

the neonatal and infant microbiome, we caution that societal focus needs to shift away from a narrow focus on reducing Caesarean delivery rates to meet a certain quota - which as we have demonstrated may be difficult to achieve in today's society - to optimizing maternal healthcare before, during, and after pregnancy. Additionally, efforts to restore the neonatal and infant microbiomes with vaginal or faecal seeding may fail to yield beneficial outcomes and may be harmful at both individual and broader societal levels. As physicians, our first responsibility is to do no harm. Despite growing popularity of neonatal seeding in the general press, health care practitioners and patients should only perform seeding practices within the confounds of strict scientific protocols to ensure safety. To ultimately improve maternal and neonatal outcomes in association with presumptively beneficial alterations in the microbiome, attention should be directed to established beneficial realms of improved access and availability of preconception and prenatal care, nutritional counselling, lactation services, and limiting food scarcity and other health disparities.

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LITERATURE

Aagaard-Tillery, K.M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R., Lane, R.H.: Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J. Mol. Endocrinol. 41, 91-102 (2008).

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T.-A., Coarfa, C., Raza, S., Rosenbaum, S., Van den Veyver, I., Milosavljevic, A., Gevers, D., Huttenhower, C., Petrosino, J., and Versalovic, J.:. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PLoS One **7**, e36466 (2012b).

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., and Versalovic, J.: The placenta harbors a unique microbiome. Sci. Transl. Med. 6, 237ra265 (2014).

Aagaard, K., Stewart, C.J., and Chu, D.: Una destinatio, viae diversae: Does exposure to the vaginal microbiota confer health benefits to the infant, and does lack of exposure confer disease risk? EMBO Rep. 17, 1679-1684 (2016).

Aagaard, K. and Hohmann, E.: Regulating microbiome manipulation. Nat. Med. 25, 874-876 (2019).

Antony, K.M., Romezi, M., Lindgren, K., Mitchell, K.B., Venable, S.F., Racusin, D.A., Suter, M.A., and Aagaard, K.M.: Maternal Metabolic Biomarkers are Associated with Obesity and Excess Gestational Weight Gain. Am. J. Perinatol. 38(S 01), e173-e181 (2021).

Azad, M.B., Konya, T., Persaud, R.R., Guttman, D.S., Chari, R.S., Field, C.J., Sears, M.R., Mandhane, P.J., Turvey, S.E., Subbarao, P., Becker, A.B., Scott, J.A., and Kozyrskyj, A.L.; CHILD Study Investigators: Impact of maternal intrapartum antibiotics, method of birth and breastfeeding on gut microbiota during the first year of life: a prospective cohort study. BJOG 123, 983-993 (2016).

Banerjee, S., Suter, M.A., and Aagaard, K.M.: Interactions between Environmental Exposures and the Microbiome: Implications for Fetal Programming. Curr. Opin. Endocr. Metab. Res. 13, 39-48 (2020).

Barker, D.: Infant mortality, childhood nutrition, and ischaemic heart disease in england and wales. Lancet 327, 1077-1081 (1986).

Bassols, J., Serino, M., Carreras-Badosa, G., Burcelin, R., Blasco-Baque, V., Lopez-Bermejo, A., and Fernandez-Real, J.-M.: Gestational diabetes is associated with changes in placental microbiota and microbiome. Pediatr. Res. 80, 777-784 (2016).

Blaser, M.J.: Who are we? Indigenous microbes

and the ecology of human diseases. EMBO Rep. 7, 956-960 (2006).
Bolte, E.E., Moorshead, D., and Aagaard, K.M.: Maternal and early life exposures and their potential to influence development of the microbiome. Genome Med. 14, 4 (2022).
Butel, M.-J., Suau, A., Campeotto, F., Magne, F., Aires, J., Ferraris, L., Kalach, N., Leroux, B., and Dupont, C.: Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. J. Pediatr. Gastroenterol. Nutr. 44, 577-582 (2007).
Campodonico-Burnett, W., Hetrick, B., Wesolowski, S.R., Schenk, S., Takahashi, D.L., Dean, T.A., Sullivan, E.L., Kievit, P., Gannon, M., Aagaard, K., Friedman, J.E., and McCurdy, C.E.: Maternal Obesity and Western-Style Diet Impair Fetal and Juvenile Offspring Skeletal Muscle Insulin-Stimulated Glucose Transport in Nonhuman Primates. Diabetes 69, 1389-1400 (2020).
Cao, B. and Mysorekar, I.U.: Intracellular bacteria in placental basal plate localize to extravillous trophoblasts. Placenta 35, 139-142 (2013).
Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., Li, F., Yu, X., Feng, Q., Wang, Z., Xie, H., Chen, X., Zeng, C., Wen, B., Zeng, L., Du, H., Tang, H., Xu, C., Xia, Y., Xia, H., Yang, H., Wang, J., Wang, J., Madsen, L., Brix, S., Kristiansen, K., Xu, X., Li, J., Wu, R., and Jia, HJ.: The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. Nat. Commun. 8, 875 (2017).
Chen, H.J. and Gur, T.L.: Intrauterine Microbiota: Missing, or the Missing Link? Trends Neurosci. 42, 402-413 (2019)
Chen, X. and Devaraj, S.: Gut Microbiome in Obesity, Metabolic Syndrome, and Diabetes. Curr. Diab. Rep. 18, 129 (2018).
Chong, R., Cheng, Y., Hogg, C.J., and Belov, K.: Marsupial gut microbiome. Front. Microbiol. 11, 1058 (2020).
Chu, D.M. and Aagaard, K.M.: Microbiome: Eating for trillions. Nature 532 316-317 (2016).
Chu, D.M., Ma, J., Prince , A.L., Antony, K.M., Seferovic, M.D., and Aagaard, K.M.: Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. Nat. Med. 23, 314-326 (2017).
Claus, S.P., Guillou, H., and Ellero-Simatos, S.: The gut microbiota: a major player in the toxicity of environmental pollutants? NPJ Biofilms Microbiomes 2, 16003 (2016).
Collado, M.C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S.: Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci. Rep. 6, 23129 (2016).
Cox, T.O., Lundgren, P., Nath, K., and Thaiss, C.A.: Metabolic control by the microbiome. Genome Med. 14, 80 (2022).
Cuevas Guaman, M., Sbrana, E., Shope, C., Showalter, L., Hu, M., Meloche, S., and Aagaard, K.: Administration of antenatal glucocorticoids and postnatal surfactant ameliorates respiratory distress syndrome–associated neonatal lethality in Erk3−/− mouse pups. Pediatr. Res. 76, 24-32 (2014).
DiGiulio, D.B., Romero, R., Amogen, H.P., Kusanovic, J.P., Bik, E.M., Gotsch, F., Kim, C.J., Erez, O., Edwin, S., and Relman, D.A.: Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: A molecular and culture-based investigation. PLoS One 3, e3056 (2008).
DiGiulio, D.B., Romero, R., Kusanovic, J.P., Gómez, R., Kim, C.J., Seok, K.S., Gotsch, F., Mazaki-Tovi, S., Vaisbuch, E., Sanders, K., Bik, E.M., Chaiworapongsa, T., Oyarzún, E., and Relman, D.A.: Prevalence and diversity of microbes in the amniotic fluid, the fetal immune response, and pregnancy outcome in women with preterm prelabor rupture of membranes. Am. J. Reprod. Immunol. 64, 38-57 (2010).
Dong, X.-D., Li, X.-R., Luan, J.-J. Liu, X.-F., Peng, J., Luo, Y.-Y., and Liu, C.-J.: Bacterial communities in neonatal feces are similar to mothers' placentae. Can. J. Infect. Dis. Med. Microbiol. 26, 90-94 (2015).
Doyle, R.M., Alber, D.G., Jones, H.E., Harris, K., Fitzgerald, F., Peebles, D., and Klein, N.: Term and preterm labour are associated with distinct microbial community structures in placental membranes which are independent of mode of delivery. Placenta 35, 1099-1101 (2014).
Doyle, R.M., Harris, K., Kamiza, S., Harjunmaa, U., Ashorn, U., Nkhoma, M., Dewey, K.G., Maleta, K., Ashorn, P., and Klein, N.:

Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. PLoS One 12, e0180167 (2017).

Elsakr, J.M., Dunn, J.C., Tennant, K., Zhao, S.K., Kroeten, K., Pasek, R.C., Takahashi, D.L., Dean, T.A., Velez Edwards, D.R., McCurdy, C.E., Aagaard, K.M., Powers, A.C., Friedman, J.E., Kievit, P., and Gannon, M: Maternal Western-style diet affects offspring islet composition and function in a non-human primate model of maternal over-nutrition. Mol. Metab. 25, 73-82 (2019).

Elsakr, J.M., Zhao, S.K., Ricciardi, V., Dean, T.A., Takahashi, D.L., Sullivan, E., Wesolowski, S.R., McCurdy, C.E., Kievit, P., Friedman, J.E., Aagaard, K.M., Edwards, D.R.V., Gannon, and M.: Western-style diet consumption impairs maternal insulin sensitivity and glucose metabolism during pregnancy in a Japanese macaque model. Sci. Rep. 11, 12977 (2021).

Fleming, T.P., Watkins, A.J., Velazquez, M.A., Mathers, J.C., Prentice, A.M., Stephenson, J., Barker, M., Saffery, R., Yajnik, C.S., Eckert, J.J., Hanson, M.A., Forrester, T., Gluckman, P.D., and Godfrey, K.M.: Origins of lifetime health around the time of conception: causes and consequences. Lancet 391, 1842-1852 (2018).

Gensollen, T., Iyer, S.S., Kasper, D.L., and Blumberg, R.S.: How colonization by microbiota in early life shapes the immune system. Science 352, 539-544 (2016).

Girbovan, A., Sur, G., Samasca, G., and Lupan, I.: Dysbiosis a risk factor for celiac disease. Med. Microbiol. Immunol. 206, 83-91 (2017).

Goldstein, R.F., Abell, S.K., Ranasinha, S., Misso, M., Boyle, J.A., Black, M.H., Li, N., Hu, G., Corrado, F., Rode, L., Kim, Y.J., Haugen, M., Song, W.O., Kim, M.H., Bogaerts, A., Devlieger, R., Chung, J.H., and Teede, H.J.: Association of Gestational Weight Gain With Maternal and Infant Outcomes: A Systematic Review and Meta-analysis. JAMA 317, 2207-2225 (2017).

Gomez-Arango, L.F., Barrett, H.L., McIntyre, H.D., Callaway, L.K., Morrison, M., and Dekker Nitert, M.: Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. Sci. Rep. 7, 2860 (2017).

Gonzalez-Rodriguez, P., Cantu, J., O'Neil, D., Seferovic, M.D., Goodspeed, D.M., Suter, M.A., and Aagaard, K.M.: Alterations in expression of imprinted genes from the H19/IGF2 loci in a multigenerational model of intrauterine growth restriction (IUGR). Am. J. Obstet. Gynecol. 214, 625.e1-625.e11 (2016).

Goodspeed, D., Seferovic, M.D., Holland, W., Mcknight, R.A., Summers, S.A., Branch, D.W., Lane, R.H., and Aagaard, K.M.: Essential nutrient supplementation prevents heritable metabolic disease in multigenerational intrauterine growth-restricted rats. FASEB J. 29, 807-819 2015.

Gosalbes, M.J., Llop, S., Vallès, Y., Moya, A., Ballester, F., and Francino, M.P.: Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. Clin. Exp. Allergy 43, 198-211 (2013a).

Gosalbes, M.J., Llop, S., Vallès, Y., Moya, A., Ballester, F., and Francino, M.P.: Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. Clin. Exp. Allergy 43, 198-211 (2013b).

Greenhalgh, K., Meyer, K.M., Aagaard, K.M., and Wilmes, P.: The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. Environ. Microbiol. 18, 2103-2116 (2016).

Hansen, R., Scott, .KP., Kha,n S., Martin, J.C., Berry, S.H., Stevenson, M., Okpapi, A., Munro, M.J., and Hold, G.L.: First-pass meconium samples from healthy term vaginally-delivered neonates: an analysis of the microbiota. PLoS One 10, e0133320 (2015).

Harris, R.A., Alcott, C.E., Sullivan, E.L., Takahashi, D., McCurdy, C.E., Comstock, S., Baquero, K., Blundell, P., Frias, A.E., Kahr, M., Suter, M., Wesolowski, S., Friedman, J.E., Grove, K.L., and Aagaard, K.M.: Genomic Variants Associated with Resistance to High Fat Diet Induced Obesity in a Primate Model. Sci. Re. 6, 36123 (2016).

Hiltunen, H., Hanani, H., Luoto, R., Turjeman, S., Ziv, O., Isolauri, E., Salminen, S., Koren, O., and Rautava, S.: Preterm infant meconium microbiota transplant induces growth failure, inflammatory activation, and metabolic disturbances in germ-free mice. Cell Rep. Med. 2, 100447 (2021).

Hornef, M. and Penders, J.: Does a prenatal bacterial microbiota exist? Mucosal Immunol. 10, 598-601 (2017).

Huang, Y., Liao, J., Liu, X., Zhong, Y., Cai, X., and Long, L.: Review: The role of intestinal dysbiosis in Parkinson's disease. Front. Cell. Infect. Microbiol. 11, 615075 (2021).

Jašarević, E. and Bale, T.L.: Prenatal and postnatal contributions of the maternal microbiome on offspring programming. Front. Neuroendocrinol. 55, 100797 (2019).

Jiménez, E., Marín, M.L., Martín, R., Odriozola, J.M., Olivares, M., Xaus, J., Fernández, L., and Rodríguez, J.M.: Is meconium from healthy newborns actually sterile? Res. Microbiol. 159, 187-193 (2008).

Kahr, M.K., Antony, K.M., Galindo, M., Whitham, M., Hu, M., Aagaard, K.M., and Suter, M.A.: SERUM GLP-2 is Increased in Association with Excess Gestational Weight Gain. Am. J. Perinatol. 40, 400-406 (2021).

Kelly, J.C., Nolan, L.S., and Good, M.: Vaginal seeding after cesarean birth: Can we build a better infant microbiome? Med 2, 889-891(2021).

Kennedy, K.M., Gerlach, M.J., Adam, T., Heimesaat, M.M., Rossi, L., Surette, M.G., Sloboda, D.M., and Braun, T.: Fetal meconium does not have a detectable microbiota before birth. Nat. Microbiol. 6, 865-873 (2021).

Kennedy, K.M., de Goffau, M.C., Perez-Muñoz, M.E., Arrieta, M.-C., Bäckhed, F., Bork, P., Braun, T., Bushman, F.D., Dore, J., de Vos, W.M., Earl, A.M., Eisen, J.A., Elovitz, M.A., Ganal-Vonarburg, S.C., Gänzle, M.G., Garrett, W.S., Hall, L.J., Hornef, M.W., Huttenhower, C., Konnikova, L., Lebeer, S., Macpherson, A.J., Massey, R.C., McHardy, A.C., Koren, O., Lawley, T.D., Ley, R.E., O'Mahony, L., O'Toole, P.W., Pamer, E.G., Parkhill, J., Raes, J., Tarrei, T., Salonen, A., Segal, E., Segata, N., Shanahan, F., Sloboda, D.M., Smith, G.C.S,. Sokol, J., Spector, T.D., Surette, M.G., Tannock, G.W., Walker, A.W., Yassour, M., and Walter, J.: Questioning the fetal microbiome illustrates pitfalls of low-biomass microbial studies. Nature 613, 639-649 (2023).

Liu, Y., Elworth, R.L., Jochum, M.D., Aagaard, K.M., and Treangen, T.J.: De novo identification of microbial contaminants in low microbial biomass microbiomes with Squeegee. Nat. Commun. 13, 6799 (2022).

Lokugamage, A.U. and Pathberiya, S.D.C.: The microbiome seeding debate - let's frame it around women-centred care. Reprod. Health 16, 91 (2019).

Luczynski, P., McVey Neufeld, K.-A., Oriach, C.S., Clarke, G., Dinan, T.G., and Cryan, J.F.: Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. Int. J. Neuropsychopharmacol. 19, pyw020 (2016).

Ma, J., Coarfa, C., Qin, X., Bonnen, P.E., Milosavljevic, A., Versalovic, J., and Aagaard, K.: mtDNA haplogroup and single nucleotide polymorphisms structure human microbiome communities. BMC Genomics 15, 257 (2014a).

Ma, J., Prince, A.L., Bader, D., Hu, M., Ganu, R., Baquero, K., Blundell, P., Alan Harris, R., Frias, A.E., Grove, K.L., and Aagaard, K.M.: High-fat maternal diet during pregnancy persistently alters the offspring microbiome in a primate model. Nat. Commun. 5, 3889 (2014b).

Ma, J., Qiao, Y., Zhao, P., Li, W., Katzmarzyk, P.T., Chaput, J.-P., Fogelholm, M., Kuriyan, R., Lambert, E.V., Maher, C., Maia, J., Matsudo, V., Olds, T., Onywera, V., Sarmiento, O.L., Standage, M., Tremblay, M.S., Tudor-Locke, C., and Hu, G.; ISCOLE Research Group: Breastfeeding and childhood obesity: A 12-country study. Matern. Child Nutr. 16, e12984 (2020).

McFall-Ngai, M.J.: The importance of microbes in animal development: Lessons from the squid-vibrio symbiosis. Annu. Rev. Microbiol. 68, 177-194 (2014).

Mishra, A., Lai, G.C., Yao, L.J., Aung, T.T., Shentel, N., Rotter-Maskowitz, A., Sheperdson, E., Singh, G.S.N., Pai, R., Shanti, A., Wong, R.M.M., Lee, A., Khyriem, C., Dutertre, C.A., Chakarov, S., Srinivasan, K.G., Shadan, N.B., Zhang, X.-

M., Khalilnezhad, S., Cottier, F., Tan, A.S.M., Low, G., Chen, P., Fan, Y., Hor, P.X., Lee, A.K.M., Choolani, M., Vermijlen, D., Sharma, A., Fuks, F., Straussman, R., Pavelka, N., Malleret, B., McGovern, N., Albani, S., Chan, J.K.Y., and Ginhoux, F.: Microbial exposure during early human development primes fetal immune cells. Cell 184:3394-3409.e20 (2021).

Moeller, A.H., Suzuki, T.A., Phifer-Rixey, M., and Nachman, M.W.: Transmission modes of the mammalian gut microbiota. Science 362, 453-457 (2018).

Murgas Torrazza, R. and Neu, J.: The developing intestinal microbiome and its relationship to health and disease in the neonate. J. Perinatol. 31, Suppl. 1., S29-S34 (2011)

Nash, M.J., Dobrinskikh, E., Newsom, S.A., Messaoudi, I., Janssen, R.C., Aagaard, K.M., McCurdy, C.E., Gannon, M., Kievit, P., Friedman, J.E., and Wesolowski, S.R.: Maternal Western diet exposure increases periportal fibrosis beginning in utero in non-human primate offspring. JCI Insight 6, e154093 (2021).

Onderdonk, A.B., Delaney, M..L, DuBois, A.M., Allred, N., and Leviton, A.: Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. Am. J. Obstet. Gynecol. 198, 110.e1-110.e7 (2008a).

Onderdonk, A.B., Hect, J.L., McElrath, T.F., Delaney, M.L., Allred, E.N., and Leviton, A.: Colonization of second-trimester placenta parenchyma. Am. J. Obstet. Gynecol. 199, 52.e1-52.e10 (2008b).

O'Neil, D., Mendez-Figueroa, H., Mistretta, T.A., Su, C., Lane, R.H., and Aagaard, K.M.: Dysregulation of Npas2 leads to altered metabolic pathways in a murine knockout model. Mol. Genet. Metab. 110, 378-387 (2013).

O'Neil, D.S., Stewart, C.J., Chu, D.M., Goodspeed, D.M., Gonzalez-Rodriguez, P.J., Shope, C.D., and Aagaard, K.M.: Conditional postnatal deletion of the neonatal murine hepatic circadian gene, Npas2, alters the gut microbiome following restricted feeding. Am. J. Obstet. Gynecol. 217, 218.e1-218.e15 (2017).

Pace, R.M., Prince, A.L., Ma, J., Belfort, B.D.W., Harvey, A.S., Hu, M., Baquero, K., Blundell, P., Takahashi, D., Dean, T., Kievit, P., Sullivan, E.L., Friedman, J.E., Grove, K., and Aagaard, K.M.: Modulations in the offspring gut microbiome are refractory to postnatal synbiotic supplementation among juvenile primates. BMC Microbiol. 18, 28 (2018).

Pace, R.M., Chu, D.M., Prince, A.L., Ma, J., Seferovic, M.D., and Aagaard, K.M.: Complex species and strain ecology of the vaginal microbiome from pregnancy to postpartum and association with preterm birth. Med. 2, 1027-1049 (2021).

Pammi, M., Cope, J., Tarr, P.I., Warner, B.B., Morrow, A.L., Mai, V., Gregory, K.E., Kroll, J.S., McMurtry, V., Ferris, M.J., Engstrand, L., Engstrand Lilja , H., Hollister, E.B., Versalovic, J., and Neu, J.: Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. Microbiome 5, 31 (2017).

Parnell, L.A., Briggs, C.M., Cao, B., Delannoy-Bruno, O., Schrieffer, A.E., and Mysorekar, I.U.: Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. Sci. Rep. 7, 11200 (2017a).

Parnell, L.A., Brigs, C.M., Cao, B., Belannoy-Bruno, O., Schrieffer, A.E., and Mysorekar, I.U.: Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. Sci. Rep. 7, 11200 (2017b).

Perez, P.F., Doré, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E.J., and Donnet-Hughes, A.: Bacterial imprinting of the neonatal immune system: lessons from maternal cells? Pediatrics 119, e724-e732 (2007).

Perez-Muñoz, M.E., Arrieta, M.-C., Ramer-Tai,t, A.E., and Walter, J.: A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: implications for research on the pioneer infant microbiome. Microbiome 5, 48 (2017).

Petersen, C., Dai, D.L.Y., Boutin, R.C.T., Sbihi, H., Sears, M.R., Moraes, T.J., Becker, A.B., Azad, M.B., Mandhane, P.J., Subbarao, P., Turvey, S.E., and Finlay, B.B.: A rich meconium metabolome in human infants is associated with early-life gut microbiota compo-

sition and reduced allergic sensitization. Cell. Rep. Med. 2, 100260 (2021a).

Petersen, C., Dai, D.L.Y., Boutin, R.C.T., Sbihi, H., Sears, M.R., Moraes, T.J., Becker, A.B., Azad, M.B., Mandhane, P.J., Subbarao, P., Turvey, S.RE., and Finlay, B.B.: A rich meconium metabolome in human infants is associated with early-life gut microbiota composition and reduced allergic sensitization. Cell. Rep. Med. 2, 100260 (2021b).

Pew, B.K., Harris, R.A., Sbrana, E., Guaman, M.C., Shope, C., Chen, R., Meloche, S., and Aagaard, K.: Structural and transcriptomic response to antenatal corticosteroids in an Erk3-null mouse model of respiratory distress. Am. J. Obstet. Gynecol. 215, 384.e1-384.e89 (2016).

Prince, A.L., Ma, J., Kannan, P.S., Alvarez, M., Gisslen, T., Harris, R.A., Sweeney, E.L., Knox, C.L., Lambers, D.S., Jobe, A.H., Chougnet, C.A., Kallapur, S.G., and Aagaard, K.M.: The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. Am. J. Obstet. Gynecol. 214, 627.e1-627.e16 (2016).

Prince, A.L., Pace, R.M., Dean, T., Takahashi, D., Kievit, P., Friedman, J.E., and Aagaard, K.M. :The development and ecology of the Japanese macaque gut microbiome from weaning to early adolescence in association with diet. Am. J. Primatol. 81, e22980 (2019).

Rackaityte, E., Halkias, J., Fukui, E.M., Mendoza, V.F., Hayzelden, C., Crawford, E.D., Fujimura, K.E., Burt, T.D., and Lynch, S.V.: Viable bacterial colonization is highly limited in the human intestine in utero. Nat. Med. 26, 599-607 (2020).

Rogier, E.W., Frantz, A.L., Bruno, M.E.C., Wedlund, L., Cohen, D.A., Stromberg, A.J., and Kaetzel, C.S.: Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. Proc. Natl. Acad. Sci. USA 111, 3074-3079 (2014).

Roswall, J., Olsson, L.M., Kovatcheva-Datchary, P., Nilsson, S., Tremaroli, V., Simon, M.-C., Kiilerich, P., Akrami, R., Krämer, M., Uhlén, M., Gummesson, A., Kristiansen, K., Dahlgren, J., and Bäckhed, F.: Developmental trajectory of the healthy human gut microbiota during the first 5 years of life. Cell Host Microbe 29, 765-776 (2021).

Safi-Stibler, S. and Gabory, A.: Epigenetics and the Developmental Origins of Health and Disease: Parental environment signalling to the epigenome, critical time windows and sculpting the adult phenotype. Semin. Cell Dev. Biol. 97, 172-180 (2020).

Satokari, R., Grönroos, T., Laitinen, K., Salminen, S., and Isolauri, E.: Bifidobacterium and Lactobacillus DNA in the human placenta. Lett. Appl. Microbiol. 48, 8-12 (2009).

Schubbert R, Hohlweg U, Renz D, Doerfler W. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. Mol. Gen. Genet. 1998;259:569-76.

Seferovic, M.D., Goodspeed, D.M. Chu, D.M., Krannich, L.A., Gonzalez-Rodriguez, P.J., Cox, J.E. and Aagaard, K.M.: Heritable IUGR and adult metabolic syndrome are reversible and associated with alterations in the metabolome following dietary supplementation of 1-carbon intermediates. FASEB J. 2640-4652 (2015a).

Seferovic, M.D., Goodspeed, D.M., Chu, D.M., Krannich, L.A., Gonzalez-Rodriguez, P.J., Cox, J.E., and Aagaard, K.M.: Heritable IUGR and adult metabolic syndrome are reversible and associated with alterations in the metabolome following dietary supplementation of 1-carbon intermediates. FASEB J. 29, 2640-2652 (2015b).

Seferovic, M.D., Beamish, C.A., Mosser, R.E., Townsend, S.E., Pappan, K., Poitout, V., Aagaard, K.M., and Gannon, M.: Increases in bioactive lipids accompany early metabolic changes associated with beta-cell expansion in response to short-term high-fat diet. Am. J. Physiol. Endocrinol. Metab. 315, E1251-E1263 (2018).

Seferovic, M.D., Pace, R.M., Carroll, M., Belfort, B., Major, A.M., Chu, D.M., Racusin, D.A., Castro, E.C.C., Muldrew, K.L., Versalovic, J., and Aagaard, K.M.: Visualization of microbes by 16S in situ hybridization in term and preterm placentas without intraamniotic infection. Am. J. Obstet. Gynecol. 221, 146.e1-146.e23 (2019).

Seferovic, M.D., Mohammad, M., Pace, R.M., Engevik, M., Versalovic, J., Bode, L., Haymond, M., and Aagaard, K.M.: Maternal diet alters human milk oligosaccharide composition with implications for the milk metagenome. Sci. Rep. 10, 22092 (2020a).

Seferovic, M.D., Mohammad, M., Pace, R.M., Engevik, M., Versalovic, J., Bode, L., Haymond, M., and Aagaard, K.M.: Maternal diet alters human milk oligosaccharide composition with implications for the milk metagenome. Sci. Rep. 10, 22092 (2020b).

Song, S.J., Wang, J., Martino, C., Jiang, L., Thompson, W.K., Shenhav, L., McDonald, D., Marotz, C., Harris, P.R., Hernandez, C.D., Henderson, N., Ackley, E., Nardella, D., Gillihan, C., Montacuti, V., Schweizer, W., Jay, M., Combellick, J., Sun, H., Garcia-Mantrana, I., Raga, F.G., Collado, M.C., Rivera-Viñas, J.I., Campos-Rivera, M., Ruiz-Calderon, J.F., Knight, R., and Dominguez-Bello, M.G.: Naturalization of the microbiota developmental trajectory of Cesarean-born neonates after vaginal seeding. Med. 2, 951-964 (2021).

Stinson, L.F., Payne, M.S., and Keelan, J.A.: Planting the seed: Origins, composition, and postnatal health significance of the fetal gastrointestinal microbiota. Crit. Rev. Microbiol. 43, 352-369 (2017).

Stinson, L.F., Payne, M.S., and Keelan, J.A.: A Critical Review of the Bacterial Baptism Hypothesis and the Impact of Cesarean Delivery on the Infant Microbiome. Front. Med. (Lausanne) 5, 135 (2018).

Suter, M., Bocock, P., Showalter, L., Hu, M., Shope, C., McKnight, R., Grove, K., Lane, R., and Aagaard-Tillery, K.: Epigenomics: maternal high-fat diet exposure in utero disrupts peripheral circadian gene expression in nonhuman primates. FASEB J. 25, 714-726 (2011).

Suter, M.A., Chen, A., Burdine, M.S., Choudhury, M., Harris, R.A., Lane, R.H., Friedman, J.E., Grove, K.L., Tackett, A.J., and Aagaard, K.M.: A maternal high-fat diet modulates fetal SIRT1 histone and protein deacetylase activity in nonhuman primates. FASEB J. 26, 5106-5114 (2012a).

Suter, M.A., Sangi-Haghpeykar, H., Showalter, L., Shope, C., Hu, M., Brown, K., Williams, S., Harris, R.A., Grove, K.L., Lane, R.H., and Aagaard, K.M.: Maternal high-fat diet modulates the fetal thyroid axis and thyroid gene expression in a nonhuman primate model. Mol. Endocrinol. 26, 2071-2080 (2012b).

Suter, M.A., Takahashi, D., Grove, K.L., and Aagaard, K.M.: Postweaning exposure to a high-fat diet is associated with alterations to the hepatic histone code in Japanese macaques. Pediatr. Res. 74, 252-258 (2013).

Suter, M.A., Ma, J., Vuguin, P.M., Hartil, K., Fiallo, A., Harris, R.A., Charron, M.J., and Aagaard, K.M.: In utero exposure to a maternal high-fat diet alters the epigenetic histone code in a murine model. Am. J. Obstet. Gynecol. 210, 463.e1-463.e11 (2014).

Tett, A,, Huang, K.D., Asnicar, F., Fehlner-Peach, H., Pasolli, E., Karcher, N., Armanini, F., Manghi, P., Bonham, K., Zolfo, M., De Filippis, F., Magnabosco, C., Bonneau, R., Lusingu, J., Amuasi, J., Reinhard, K., Rattei, T., Boulund, F., Engstrand, L., Zink, A., Collado, M.C., Littman, D.R., Eibach, D., Ercolini, D., Rota-Stabelli, O., Huttenhower, C., Maixner, F., and Segata, N.. The Prevotella copri complex comprises four distinct clades underrepresented in westernized populations. Cell Host Microbe 26, 666–679 (2019).

Thaiss, C.A., Zmora, N., Levy, M., and Elinav, E.: The microbiome and innate immunity. Nature 535, 65-74 (2016).

Theis, K.R., Romero, R., Winters, A.D., Jobe, A.H., Gomez-Lopez, N., and Young, V.B.: Lack of evidence for microbiota in the placental and fetal tissues of Rhesus macaques. mSphere 5, e00210-e00220 (2020).

Tuominen, H., Rautava, S., Syrjänen, S., Collado, M.C., and Rautava, J.: HPV infection and bacterial microbiota in the placenta, uterine cervix and oral mucosa. Sci. Rep. 8, 9787 (2018).

van Opstal, E.J. and Bordenstein, S.R.: Rethinking heritability of the microbiome. Science 349, 1172-1173 (2015).

Zheng, J., Xiao, X., Zhang, Q., Mao, L., Yu, M., and Xu, J.: The Placental Microbiome Varies in Association with Low Birth Weight in Full-Term Neonates. Nutrients 7, 6924-6937 (2015).

THE ROLE OF THE GUT MICROBIOTA AND *LACTIPLANTIBACILLUS PLANTARUM* WJL STRAIN IN JUVENILE GROWTH DURING CHRONIC UNDERNUTRITION

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SUMMARY

The World Health Organization defines malnutrition as a deficiency, excess, or imbalance in a person's energy and/or nutrient intake. However, it is becoming increasingly clear that not only diet per se, but also the intestinal microbiota plays a fundamental role in the development of diseases associated with malnutrition. Next-generation sequencing, the renewed interest in bacterial culturing techniques, and the use of gnotobiotic models have enabled major advances in causally linking the role of the microbiota and specific bacterial strains in the aetiology/prevention of diseases associated with undernutrition, including wasting, stunting, and underweight. We have shown that the *Lactiplantibacillus plantarum* strain, selected in the gnotobiotic invertebrate Drosophila model for its growth-promoting properties, is able to increase the growth of juvenile undernourished mice in both gnotobiotic and specific pathogen-free animals, and that this effect is dependent on the expression of the NOD2 receptor by intestinal epithelial cells. These results suggest that administration of selected bacterial strains may represent a novel strategy for alleviating the persistent stunting that is one of the long-term consequences of undernutrition, which still affects millions of children under five years of age in low- and middle-income countries.

ACUTE MALNUTRITION

When a developing young organism faces specific nutritional challenges such as undernutrition (low-calorie or low-protein diet), it becomes stunted, that is, it is too small for its age. Because stunting affects both physical and cognitive development, it is not just a problem in childhood; the effects often persist throughout someone's life. In addition to being too small for age, stunting is associated with a dysbiotic gut microbiota that is immature for age (*Subramanian* et al., 2014). The causal evidence for this observation comes from germ-free animal models. Smith and colleagues transplanted into germ-free mice the faecal microbiota of monozygotic Malawian twins, who had become discordant for kwashiorkor, a form of severe acute malnutrition. After feeding these mice with a suboptimal diet they observed more severe weight loss in mice colonized with kwashiorkor-associated microbiota compared to the mice colonized by microbiota of healthy twin. This phenomenon was

associated with different metabolic profile and more labile responses to the renutrition regime of the kwashiorkor-associated microbiota (*Smith* et al., 2013). In a subsequent study, the same group showed that microbiota from undernourished children is also able to transmit impaired growth, altered bone morphology, and metabolic abnormalities in the muscle, liver, and brain to recipient gnotobiotic mice (*Blanton* et al., 2016). Moreover, they found that dysbiotic microbiota of these undernourished children is permissive for the invasion of bacterial taxa from the healthy microbiota and these invading bacterial taxa could improve growth impairments. Supplementing the microbiota of undernourished donors with cultured representatives of these invasive species again ameliorated growth and metabolic abnormalities of recipient mice (*Blanton* et al., 2016). Along these lines, Tidjani Alou and colleagues combined culturomics and metagenomics methods to identify potential probiotics candidates that were present in stool samples of healthy children and absent in malnourished patients with kwashiorkor. As a result, a complex of 12 species was identified in healthy children that was missing in kwashiorkor patients. They concluded that microbiotherapy based on selected strains has the potential to improve the current treatment of severe acute malnutrition and prevent relapse and death by re-establishing a healthy gut microbiota (*Tidjani Alou* et al., 2017). In addition to administering specific bacterial strains, another way to alter the intestinal microbiome is to provide substrates that promote the growth of specific bacterial taxa. Gehrig and colleagues showed that improving therapeutic diets by supplementing them with ingredients that increase the representation of growth-promoting bacterial taxa in the microbiota of undernourished donors promoted healthy growth in both preclinical mouse and piglet models and in a subsequent human study (*Gehrig* et al., 2019). These results not only confirmed the transferability of findings from preclinical models, but also simultaneously illustrated a possible new approach for treating malnutrition in children.

CHRONIC UNDERNUTRITION

Contrary to the acute malnutrition, chronic malnutrition is a result of inadequate nutrition over a long period of time leading to the failure of linear growth and, as a result, to short and thin individuals. Its causative factors are still poorly understood (*Gordon* et al., 2012) and until recently, nothing was known about the role of the microbiota in this condition in mammals (*Schwarzer* et al., 2016). In the invertebrate gnotobiotic Drosophila model of chronic protein undernutrition, microbiota in general and specific commensal strains of *Acetobacter* and *Lactobacillus* in particular have been shown to promote host systemic growth during the juvenile phase (*Shin* et al., 2011; *Storelli* et al., 2011). Specifically, Storelli and colleagues studied different strains of one of the most abundant Drosophila commensal species, *Lactiplantibacillus plantarum* (Lp). We were able to show that a single strain LpWJL can recapitulate the growth benefit effect of the full microbiota. This effect was strictly strain-specific and relied on endocrine tissue-specific activity of the TOR kinase, whose activity is regulated by circulating amino acid levels and controls the production of systemic hormonal growth signals, such as the

insulinlike peptides, including the Drosophila analogues of mammalian insulin-like growth factor (IGF-1) (*Storelli* et al., 2011).

In mammals, the juvenile growth is governed both by the nutritional input and the activity of somatotropic axis (*Breier*, 1999). Growth hormone (GH) is released from the anterior lobe of the pituitary gland in a pulsatile pattern and acts by binding to its receptor in the membranes of the target cells in the liver or peripheral organs (*Hartman* et al., 1993). The binding leads to the initiation of a signalling cascade, culminating in the phosphorylation of transducer and activator of transcription (STAT) proteins and induction of transcription of GH-regulated genes, in particular insulin-like growth factor-1 (IGF-1), IGF-1 binding proteins (IGFBPs) and suppressors of cytokine signalling (SOCSs) (*Bartke* et al., 2013). IGF-1 is the main mediator of GH actions and it also inhibits GH release by classical negative feedback loop. Upon protein malnutrition or starvation, the GH signalling pathway is inhibited, the levels of IGF-1 decrease, and the negative feedback loop on GH production is compromised. This leads to the increased levels of GH in the circulation and individuals enter maintenance (survival) mode. The GH resistance state seems to be an adaptive response to undernutrition in order to maintain euglycemia and preserve energy (*Fazeli* and *Klibanski*, 2014).

To investigate the role of the intestinal microbiota in mammals' juvenile growth, we studied the role of the microbiota on growth kinetics of conventional mice compared to germ-free animals on standard breeding diet or under chronic undernutrition (*Schwarzer* et al., 2016). We found that under normal nutritional conditions, the microbiota of infant mice was necessary to maximize systemic weight gain and linear growth. At the same time, the microbiota improved bone growth parameters, including femur length, cortical thickness, and cortical and trabecular bone fraction. On the molecular level higher growth kinetics of CV mice was accompanied by higher level of circulating IGF-1 and IGFBP-3, its major binding protein, despite GH levels similar to GF animals. The peak of circulating IGF-1 levels at day 28 after birth, which corresponded to a spurt growth period in CV mice, was not observed in GF animals. These data suggested higher sensitivity of conventional animals to GH actions and highlighted the major role of IGF-1 in postnatal growth. Further, the importance of the intestinal microbiota and its composition for juvenile growth on both normal breeding and low-protein diets was recently shown by Darnaud and colleagues (*Darnaud* et al., 2021). Two different defined mouse minimal microbiota, Oligo-MM^{12} (*Eberl* et al., 2019) and GM15, showed an enhanced ability to buffer the deleterious effects of a low-protein diet on systemic growth in post-weaning mice compared to an SPF microbiota, with concomitant improvement in circulating levels of insulin-like growth factor-1 (IGF-1). Given the modularity, stability, and easy tractability of the minimal microbiota, this model offers new opportunities for research focused on how the microbiota affects host physiology in general and juvenile growth in particular (*Darnaud* et al., 2021). In contrast to CV or gnotobiotic consortium-colonized mice, the growth of GF animals was completely arrested when challenged by chronic undernutrition. Yet, when we monocolonized GF mice with lactobacilli strains selected in Drosophila for their different growth promoting abilities, we observed a strain-dependent increase in the magnitude of linear and ponderal growth. The growth-promoting strain LpWJL was able to recapitulate the effect of the

entire microbiota on growth. The improved mouse growth associated with the complete microbiota and a selected LpWJL strain was accompanied by restored GH sensitivity and IGF-1 production in liver and in the peripheral tissues (*Schwarzer* et al., 2016).

Our findings on the conservation of the growth-promoting properties of the LpWJL strain in both the invertebrate Drosophila and the mammalian mouse model were limited to the gnotobiotic animals. Therefore, we asked the obvious question: Is LpWJL capable of alleviating stunting in conventional juvenile mammals as well? To this end, we developed a new preclinical mouse model for nutritional stunting in conventional mice with reduced circulating IGF-1 and insulin levels (*Schwarzer* et al., 2023). Previously, consumption of similar diet low in proteins and fats during the early-stage of life, in combination with exposure to certain commensal bacterial species (*e.g*. Bacteroidales species and *Escherichia coli*) was shown to contribute to the development of environmental enteropathy (EE). EE is characterized by increased gut inflammation, intestinal permeability and villous blunting, which as a result leads to growth failure and stunting (*Brown* et al., 2015). Contrary to the study of Brown and colleagues, our model of stunting was not associated with small intestinal inflammation but with altered small intestine crypt cell proliferation. Using this model, we have shown that repeated administration of LpWJL sustains the postnatal growth of malnourished conventional animals by orchestrating metabolic and hormonal changes in the juvenile host manifested as improved circulating levels and activity of both IGF-1 and insulin. The improved IGF-1 and insulin levels and activity were in accordance with a recent clinical study in which the authors reported a significant increase in the levels of both of these hormones in severe acute malnourished Bangladeshi children, who received microbiota-directed therapeutic food treatment (*Gehrig* et al., 2019). Further, we identified administration of heat-killed LpWJL bacteria as well as its isolated cell walls as being sufficient bacterial signals stimulating animal growth. This prompted us to search for a possible receptor on the host side enabling the sensing of bacterial cell-wall motifs. In *in vitro* assays, the LpWJL purified cell-walls were able to engage TLR2 and NOD2 receptors. We therefore administered LpWJL to undernourished NOD2 KO and MyD88 KO mice. While we observed increased growth in the MyD88 KO mice upon LpWJL administration, this effect was lost in the NOD2 KO mice. Further, by using the tissue specific NOD2 KO mice, we identified that NOD2 receptor expression is necessary in intestinal epithelial cells in order for LpWJL to mediate increased intestinal crypt cell proliferation, type I interferon–regulated gene induction, IGF-1 production, and postnatal growth promotion in malnourished conventional animals (*Schwarzer* et al., 2023) (Figure 1).

CONCLUSIONS

Eukaryotic cells and multicellular organisms have evolved in the world of bacteria. Therefore, during their evolution, they constantly interacted with bacteria and now form symbiotic relationships. In recent years, with advances in sequencing and -omics techniques, we have begun to see the various facets of this symbiotic relationship between host and microbiota.

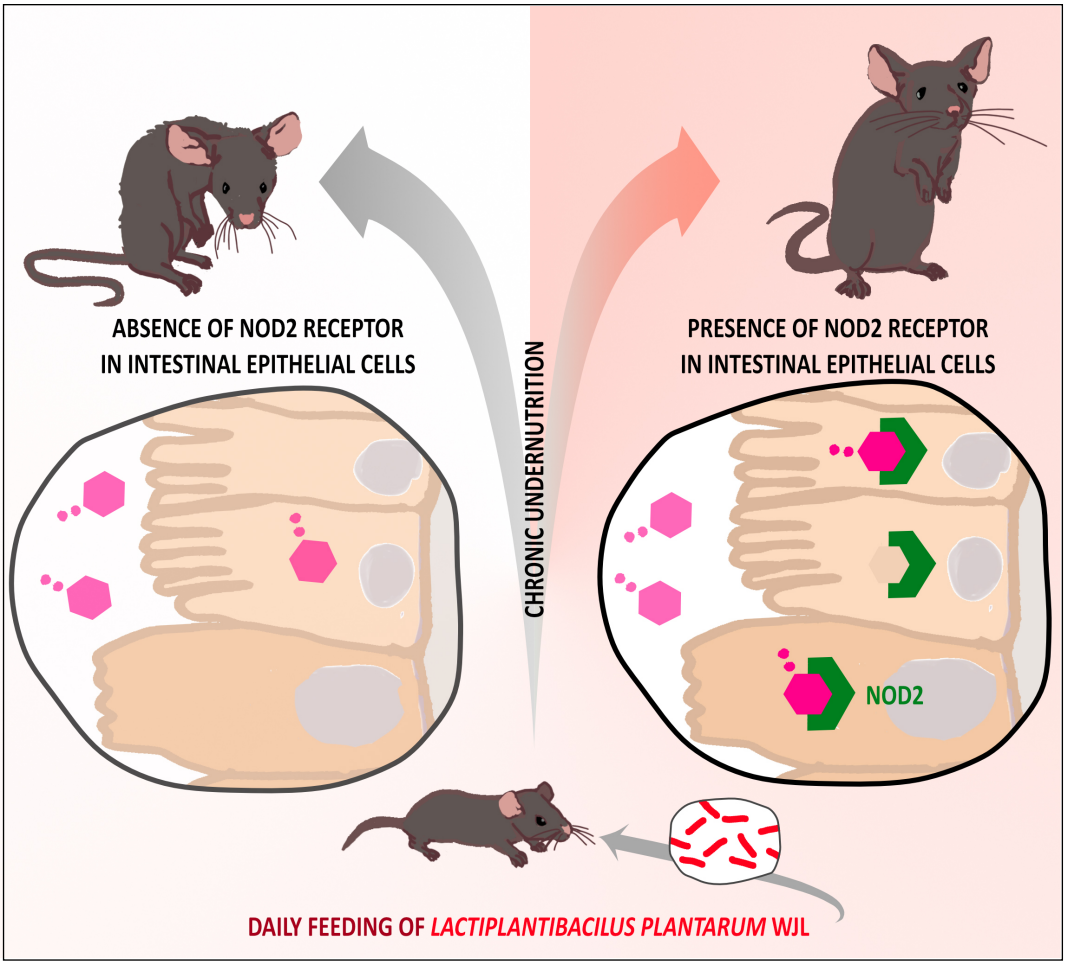

Figure 1: Feeding of *Lactiplantibacillus plantarum* WJL (LpWJL) to chronically undernourished juvenile mice enhance their longitudinal and ponderal growth. LpWJL cell wall motifs are sensed by the pattern recognition receptor NOD2 in the intestinal epithelial cells, enhancing the proliferation of crypt stem cells and sustaining postnatal juvenile growth despite chronic undernutrition. (Illustration created by Petra Schwarzer.)

Ample evidence from both human studies and experimental data from animal models shows that the gut microbiota plays a critical role in the growth of the juvenile host (*Schwarzer* et al., 2018). In a mouse model of chronic undernutrition, we showed that not only microbiota but also a selected strain of *Lactiplantibacillus plantarum* (LpWJL) is able to alleviate the complete stunting observed in germ-free mice. Further, the same LpWJL strain improved linear and ponderal growth in conventional undernourished mice upon daily intervention. The isolated bacterial cell-walls and the NOD2 receptor in the intestinal epithelial cells were both necessary for the observed beneficial effects. In a broad perspective, our findings suggest that certain validated bacterial strains or defined components of their cell wall, together with nutritional therapy, may represent a novel and complementary strategy to buffer the adverse effects of chronic undernutrition on human postnatal growth, which still affects more

than 160 millions of children below 5 years of age in low- and middle-income countries.

Outstanding questions
1) *Lactiplantibacillus plantarum* WJL or NOD2 agonist supplementation supports growth of chronically undernourished mouse model with no small intestinal enteropathy. Would the supplementation be effective also in undernourished models with environmental enteropathy (*i.e*. small intestinal inflammation)?
2) Would *Lactiplantibacillus plantarum* WJL or NOD2 agonist supplementation enhance the efficacy of nutritional interventions in undernourished mice and children?
3) Are other bacteria outside of *Lactobacillaceae* family also able to promote growth of undernourished juvenile animals? And if yes, how?

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LITERATURE

Bartke, A., Sun, L.Y., and Longo, V.: Somatotropic signaling: trade-offs between growth, reproductive development, and longevity. Physiol. Rev. 93, 571-598 (2013).

Blanton, L.V., Charbonneau, M.R., Salih, T., Barratt, M.J., Venkatesh, S., Ilkaveya, O., Subramanian, S., Manary, M.J., Trehan, I., Jorgensen, J.M., Fan, Y-M, Henrissat, B., Leyn, S.A., Rodionov, D.A., Osterman, A.L., Maleta, K.M., Newgard, C.B., Ashorn, P., Dewey, K.G., and Gordon, J.I.: Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. Science 351, 10.1126 (2016).

Breier, B.H.: Regulation of protein and energy metabolism by the somatotropic axis. Domest. Anim. Endocrinol. 17, 209-218 (1999).

Brown, E.M., Wlodarska, M., Willing, B.P., Vonaesch, P., Han, J., Reynolds, L.A., Arrieta, M.C., Uhrig, M., Scholz, R., Partida, O., Borchers, C.H., Sansonetti, P.J., and Finlay, B.B.: Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. Nat. Commun. 6, 7806 (2015).

Darnaud, M., Vadder, F.D., Bogeat, P., Boucinha, L., Bulteau, A.-L., Bunescu, A., Couturier, C., Delgado, A., Dugua, H., Elie, C., Mathieu, A., Novotná, T., Ouattara, D.A., Planel, S., Saliou, A., Šrůtková, D., Yansouni, J., Stecher, B., Schwarzer, M., Leulier, F., and Tamellini, A: A standardized gnotobiotic mouse model harboring a minimal 15-member mouse gut microbiota recapitulates SOPF/SPF phenotypes. Nat. Commun. 12, 6686 (2021).

Eberl, C., Ring, D., Munch, P.C., Beutler, M., Basic, M., Slack, E.C., Schwarzer, M., Srutkova, D., Lange, A., Frick, J.S., Bleich, A., and Stecher, B.: Reproducible Colonization of Germ-Free Mice With the Oligo-Mouse-Microbiota in Different Animal Facilities. Front. Microbiol. 10, 2999 (2019).

Fazeli, P.K., and Klibanski, A.: Determinants of GH resistance in malnutrition. J. Endocrinol. 220, R57-65 (2014).

Gehrig, J.L., Venkatesh, S., Chang, H.W., Hibberd, M.C., Kung, V.L., Cheng, J., Chen, R.Y., Subramanian, S., Cowardin, C.A.,

Meier, M.F., O'Donnell, D., Talcott, M., Spears, L.D., Semenkovich, C.F., Henrissat, B., Giannone, R.J., Hettich, R.L., Ilkayeva, O., Muehlbauer, M., Newgard, C.B., Sawyer, C., Head, R.D., Rodionov, D.A., Arzamasov, A.A., Leyn, S.A., Osterman, A.L., Hossain, M.I., Islam, M., Choudhury, N., Sarker, S.A., Huq, S., Mahmud, I., Mostafa, I., Mahfuz, M., Barratt, M.J., Ahmed, T., and Gordon, J.I.: Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. Science 365, eaau4732 (2019).

Gordon, J.I., Dewey, K.G., Mills, D.A., and Medzhitov, R.M.: The human gut microbiota and undernutrition. Sci. Transl. Med. 4, 137ps12 (2012).

Hartman, M.L., Veldhuis, J.D., and Thorner, M.O.: Normal control of growth hormone secretion. Horm. Res. 40, 37-47 (1993).

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J.: Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334, 670-674 (2011).

Schwarzer, M., Gautam, U.K., Makki, K., Lambert, A., Brabec, T., Joly, A., Srutkova, D., Poinsot, P., Novotna, T., Geoffroy, S., Courtin, P., Hermanová, P.P., Matos, R.C., Landry, J.J.M., Gérard, C., Bulteau, A.-L., Hudcovic, T., Kozáková, H., Filipp, D., Chapot-Chartier, M.-P., Šinkora, M., Peretti, N., Gomperts Boneca, I., Chamaillard, M., Vidal, H., De Vadder, F., and Leulier, F.: Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice. Science 379, 826-833 (2023).

Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova, P., Martino, M.E., Balmand, S., Hudcovic, T., Heddi, A., Rieusset, J., Kozakova, H., Vidal, H., and Leulier, F.: Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. Science 351, 854-857 (2016).

Schwarzer, M., Strigini, M., and Leulier, F.: Gut Microbiota and Host Juvenile Growth. Calcif. Tissue Int. 102, 387-405 (2018).

Smith, M.I., Yatsunenko, T., Manary, M.J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A.L., Rich, S.S., Concannon, P., Mychaleckyj, J.C., Liu, J., Houpt, E., Li, J.V., Holmes, E., Nicholson, J., Knights, D., Ursell, L.K., Rob Knight, R., and Gordon, J.I.: Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. Science 339, 548-554 (2013).

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F.: Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab. 14, 403-414 (2011).

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., Benezra, A., DeStefano, J., Meier, M.F., Muegge, B.D., Barratt, M.J., VanArendonk, L.G., Zhang, Q., Province, M.A., Petri Jr., W.A., Ahmed, T., and Gordon, J.I.: Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature 510, 417-421 (2014).

Tidjani Alou, M., Million, M., Traore, S.I., Mouelhi, D., Khelaifia, S., Bachar, D., Caputo, A., Delerce, J., Brah, S., Alhousseini, D., Sokhna, C., Robert, C., Diallo, B.A., Diallo, A., Parola, P., Golden, M., Lagier, J.-C., and Raoult, D.: Gut Bacteria Missing in Severe Acute Malnutrition, Can We Identify Potential Probiotics by Culturomics? Front. Microbiol. 8, 899 (2017).

TRIPARTITE INTERACTIONS AMONG FUNGI, BACTERIA AND THE MAMMALIAN HOST

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SUMMARY

Fungal infections are difficult diseases to manage in humans. Developing antifungal drugs is an exceptionally challenging task due to the close evolutionary relationship between fungi and humans, making it hard to find fungal-specific inhibitors without toxicity to humans. Optimal implementation of antifungal treatments will require improved understanding of not only antifungal resistance mechanisms, but also of the tripartite interactions between fungi, bacteria and the mammalian host. We emphasized in this review how the definition of the mechanisms behind this tripartite interaction may provide us with an understanding of multi-kingdom community processes that allows for the development of novel therapeutic approaches for human fungal diseases.

INTRODUCTION

Some fungi cause diseases in healthy people, but most fungal infections occur in individuals already experiencing serious illness, such as cancer, solid organ and hematopoietic stem cell transplantation, intensive care and recently COVID-19 (*Brown* et al., 2012). Among the estimated 1.5–5.0 million fungal species on planet Earth (*O'Brien* et al*.,* 2005) only several hundred cause disease in humans, and very few are able to affect healthy people. Animals co-evolved with fungi, and the sophisticated and potent human immune system arose from the constant challenge posed by the microbial world. Fungal pathogens likely adapted their pathogenic repertoire to other animal prey - mammals, insects, and even unicellular amoebae - before encountering and attacking humans (*Casadevall*, 2012). For a fungus, parasitizing a human is a demanding strategy that demands, among others, to withstand the human immune system. Although not unique among infectious agents, fungi possess complex and unusual relationships with the vertebrate immune system, partly due to some prominent features (see below).

Developing antifungal drugs is an exceptionally challenging task due to the close evolutionary relationship between fungi and humans, making it hard to find fungal-specific inhibitors without toxicity to humans. Only a few antifungal drugs have been approved in the past few decades (*McCarty and Pappas*, 2021). Although these drugs are effectively used for current treatments, there are some drawbacks to prolonged usage, including the emergence of multi-drug resistant species of *Candida auris* (*Forsberg* et al*.,* 2019) and the increased incidence of voriconazole-resistant *Aspergillus fumigatus*

isolates (*van der Linden* et al., 2013). This underscores the need for new approaches in the management of fungal diseases. A renewed focus on repurposing of drugs used for other diseases provides a promising approach for antifungal drug discovery because their pharmacodynamic, pharmacokinetic, and toxicity profiles are well established (*Bouz and Dolezal*, 2021). In addition, alternative therapies, such as immune cell therapies, vaccines, and monoclonal antibodies, have shown potential in animal models, although these are not yet available for clinical use (*Bernardes and Hohl*, 2020). Optimal implementation of antifungal treatments will require improved understanding of not only target and drug efflux-based antifungal resistance mechanisms, but of the tripartite interactions between fungi, bacteria and the mammalian host. Despite the abundance of bacterial–fungal interactions in nature and the clinical environment, very little is known about the molecular mechanisms underlying these interactions and their importance to human health. Unravelling the mechanisms that microorganisms use in a competitive, polymicrobial environment would not only deepen our understanding of microbial pathogenesis but may also provide important insights into novel pathways that are amenable for the development of new antimicrobial drugs. History has demonstrated the power of understanding such interactions, with the identification of penicillin being the consequence of a bacterial–fungal interactions on a contaminated agar plate.

BACTERIAL-FUNGAL INTERACTIONS IN THE GASTROINTESTINAL TRACT

Fungi and bacteria coexist creating complex communities that are important in agriculture and human health. Bacteria comprise more than 90% of the human microbiome, most belonging to four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (*Qin* et al., 2010). The human microbiome also harbours other microbes such as fungi, accounting for ~ 0.1% of the human gut microbiome by shotgun sequencing, viruses (bacteriophages), archaea, and protozoans, collectively making a median 1.5×10^{11} cell counts per gram of faecal material. The study of the gut mycobiota from healthy stool identified Ascomycota and Basidiomycota as the most abundant taxa, and *Saccharomyces, Candida, Malassezia* and *Cladosporium* as the dominant genera (*Nash* et al., 2017). Of interest, the most abundant genera of fungi in the guts of mice were also those present in humans (*Dollive* et al., 2013). A number of studies have indicated that the gut mycobiome (*Wang* et al., 2023) and its interactions with bacterial species (*Santus* et al., 2021) influence gut homeostasis and are relevant to human health. Indeed, beneficial interactions between bacteria and fungi are continuously being explored as potential probiotic interventions for intestinal disease. *Candida albicans* is a member of the intestinal microbiota in the majority of the human population. This underscores *C. albicans'* adaptation to life in the intestine without inducing competitive interactions with other microbes or immune responses detrimental to its survival. Specific conditions such as a dysbalanced microbiome, a suppression of the immune system, and an impaired intestinal barrier can predispose for invasive, mostly nosocomial *C. albicans* infections. Colonization of the intestine and translocation through the intestinal barrier are fundamental

aspects of the processes preceding life-threatening systemic candidiasis. However, protective effects have also been described, including the ability of this commensal to orchestrate the usage of multiple receptor-signalling pathways in dendritic cells, ultimately affecting antifungal resistance and tolerance (see below) (*Romani* et al., 2002; *Romani*, 2011). Interestingly, *C. albicans* also demonstrates probiotic properties by enhancing the growth of two strictly anaerobic commensal bacteria, *Bacteroides fragilis* and *Bacteroides vulgatus*, likely via aerobic respiration and/or antioxidant production (*Valentine* et al., 2019). As a matter of fact, the administration of antifungal drugs exacerbated colitis (*Wheeler* et al., 2016) while *C. albicans* affects the recolonization of the cecum by the microbiota in mice treated with antibiotics (*Mason* et al., 2012; *Erb Downward* et al., 2013). Although antibiotic-treated *C. albicans*-colonized mice showed reduced expression of specific immune response genes it is also likely that fungal yeasts directly interact with bacteria. The yeast *Saccharomyces boulardii* has indeed been extensively studied as a potential probiotic due to its protective effect against various bacterial gastrointestinal pathogens, including *Clostridiodes difficile, Helicobacter pylori, Vibrio cholerae, Salmonella enterica serovar Typhimurium, Shigella flexneri, and Escherichia coli (Ansari* et al., *2023)*.

Fungus-bacterium interactions are however bidirectional with a variety of reciprocal interactions encompassing antagonistic and pathogenetic interactions in addition to beneficial ones (*Nogueira* et al., 2019; *Santus* et al., 2021; *Zhang* et al., 2022). For instance, the abundance of fungi is regulated by gut bacteria. Firmicutes and Bacteroidetes restrict colonization of *C. albicans* in the mouse gut by activating transcription factor HIF-1α in intestinal cells, which causes an increase in the antimicrobial peptide LL-37 (*Fan* et al., 2015). *C. albicans* and lactic acid bacteria (LAB) have the same metabolic niches throughout the gastrointestinal tract. Dysbiosis of *C. albicans* causes altered levels of LAB, especially *Lactobacillus* spp. and *Enterococcus* spp. Lactobacilli inhibit *C. albicans*, while *Enterococcus faecalis* and *C. albicans* are mutualistic (*Zeise* et al., 2021). In an in vitro gut model, *L. rhamnosus* modified the metabolic environment, altering the expression of virulence-related genes and reducing *C. albicans* induced epithelial damage (*Graf* et al., 2019; *Alonso-Roman* et al*.,* 2022). Moreover, probiotics strains, such as *L. acidophilus*, *L. reuteri, L. casei GG*, and *Bifidobacterium* spp., have shown efficacy in limiting the severity of *C. albicans* infections in both immunocompromised and germ-free mice (*Wagner* et al., 1997) and inhibiting the in vitro formation of polymicrobial biofilms (*Hager* et al., 2019). Furthermore, metabolites produced by a consortium of bacterial species derived from healthy human faecal samples effectively inhibited the growth of *C. albicans* in liquid culture. Species of *Roseburia* and *Bacteroides ovatus* were directly responsible for these antifungal effects (*Garcia* et al., 2017).

Alternatively, interactions between fungi and bacteria have the potential to enhance microbial pathogenesis. For instance, both *C. albicans* and *S. cerevisiae* enhance the pathogenicity of the opportunistic pathogen *Acinetobacter baumannii* by producing ethanol (*Smith* et al., 2004) or the quorum-sensing molecule farnesol (*Peleg* et al., 2008). *C. albicans* allows the growth of strict anaerobes, including *C. difficile*, both in vivo (*Panpetch* et al., 2019) and in vitro (*van Leeuwen* et al., 2016), under aerobic conditions due to the rapid reduction of dissolved oxygen in the vicinity of

the yeast (*Lambooij* et al*.,* 2017) and *C. difficile* inhibited *C. albicans* hyphal growth through the secretion of the small molecule p-cresol (*van Leeuwen* et al*.,* 2016). However, another study found that prior colonization of mice with *C. albicans* protected mice from *C. difficile* infection (*Markey* et al., 2018), a finding implicating the effects of *Candida* colonization on mucosal immune homeostasis. Finally, while Enterobacteriaceae were required for *C. albicans*-mediated enhancement of colitis (*Sovran* et al., 2018), likely due to the ability of enterohemorrhagic *E. coli* to enhance *C. albicans* epithelial invasion vitro (*Yang* et al., 2016), *S. marcescens* employed a type VI secretion system to deliver antifungal toxins to kill *C. albicans* (*Trunk* et al., 2018) and S. *typhimurium* employed a type III secretion system to block hyphal formation (*Kim and Mylonakis*, 2011). Thus, these few examples highlight the complex nature of fungal-bacterial interactions in the mammalian gut, and the broad impact of fungi on bacterial species within microbiomes (*Pierce* et al., 2021). The spectrum of findings points to new possibilities and challenges in addressing the global spread of drug-resistant fungal pathogens and the diminishing pipelines of effective antifungal drugs (*Chow* et al., 2023). Ultimately, the crucial role of the fungal-bacterial interactions across habitats and ecosystems is well established (*Deveau* et al., 2018; *Steffan* et al., 2020).

TRIPARTITE INTERACTIONS: FUNGI, BACTERIA AND THE MAMMALIAN HOST

Like other microorganisms, fungi interact with the immune system at mucosal surfaces in ways that are important both for host defence and for regulating the immune system (*Underhill and Iliev*, 2014). As said, fungi possess complex and unusual relationships with the vertebrate immune system, including their ability to exist in different forms and to reversibly switch from one to the other in infection. Examples are the dimorphic fungi (*H. capsulatum, P. brasiliensis, C. immitis, and B. dermatitidis*) which transform from saprobic filamentous molds to unicellular yeasts in the host, the filamentous fungi (such as *Aspergillus* spp.) that, inhaled as unicellular conidia, may transform into a multicellular mycelium, and some species of *Candida*, capable of growing in different forms such as yeasts, blastospores, pseudohyphae and hyphae. This implicates the existence of a multitude of recognition and effector mechanisms to oppose fungal infectivity at the different body sites. For commensals, two prominent features are important. The highly effective strategies of immune evasion have evolved to enable survival in the host environment and the prolonged antigenic stimulation of the host can have profound immunoregulatory consequences. Thus, in the context of the antagonistic relationships that characterize the host-pathogen interactions, the strategies used by the host to limit fungal infectivity are necessarily disparate and, in retaliation, fungi have developed their own elaborate tactics to evade or overcome these defences (*Romani*, 2004, 2011). This may have resulted in an expanded repertoire of cross-regulatory and overlapping antifungal host responses. Indeed, through the involvement of different pattern recognition receptors, cells of the innate immune system not only discriminate between the different forms of fungi, but also contribute to resistance and tolerance to fungi at the level of the adaptive T helper immunity (*Romani*, 2004, 2011; *Underhill and Iliev*, 2014). Resistance is

the ability of the host to reduce the success of infection or to increase the rate of clearance of the pathogens. Tolerance is the ability to reduce the detrimental effects of the pathogens on the performances of the hosts, either directly or by limiting immunopathological mechanisms. Infectivity diminishes naturally among resistant hosts but not necessarily among tolerant ones, as these harbour the pathogen with no or moderate loss in performance. Resistance and tolerance are associated with fitness costs, which arise from the diversion of limiting resources away from biological processes related to performance. The host organism is a complex mosaic of cell populations that requires adequate supplies of nutrients for maintenance, growth and proliferation. Because many nutrient requirements may be shared by host cells, pathogens and indigenous microbiota, all these cells may potentially compete for growth-limiting resources.

THE SHARING OF TRYPTOPHAN METABOLISM

Amino acid metabolic pathways are crucial regulators of immunity from plants (*Zeier*, 2013) to mammals (*Grohmann and Bronte*, 2010). Amino acids serve as the building blocks of proteins, so their importance in immune activity requiring cell division, cytokine and chemokine production and other de novo protein synthesis requirements is self-evident. In addition to this process, some amino acids, or their downstream metabolites, have been implicated as anti-microbial agents. Tryptophan (Trp) is a central hub for host/microbial information processing. Trp is an essential amino acid for humans and must be obtained from the diet. Besides being involved in protein synthesis, Trp is a versatile precursor and can be metabolized by both host and microbial enzymes to generate a variety of molecules involved in different fundamental processes (*Borghi et al.,* 2020; *Grifka-Walk et al.,* 2021; *Li et al.,* 2022; *Seo and Kwon*, 2023). Three pathways have gained considerable interest for their role at the interface between the host, the microbiome and pathogens. These pathways include the host kynurenine and serotonin pathways and the microbial indole pathway (*Seo and Kwon*, 2023). The host kynurenine pathway and the microbial indole pathway, converge on a central xenobiotic receptor, the Aryl Hydrocarbon Receptor (AhR), a critical regulator at the host/microbe interface (*Zelante et al.,* 2013; *Metidji et al.,* 2018; *Dong and Perdew*, 2020; *Stockinger et al.,* 2021). The kynurenine pathway accounts for ~95% of overall Trp degradation and the first and rate-limiting step is mediated by indoleamine 2, 3–dioxygenase (IDO)1, along with IDO2 (a paralogue of IDO1) and the tryptophan 2,3-dioxygenase, TDO2, resulting in the formation of kynurenine. The kynurenine pathway, and IDO1 in particular, has been associated with the promotion of tolerance in different inflammatory conditions (*Cervenka et al.,* 2017). For instance, the Trp metabolic pathway crucially provides immune homeostasis in fungal infections by taming heightened inflammatory responses and inducing immune and tissue tolerance, an activity to which the host, fungi and the microbiota cooperatively contributed (*Romani et al.,* 2015). These results provide proof-of concept demonstration of the druggability of host metabolic pathways for antifungal tolerance defences. The serotonin pathway also influences the interactions between host and microbes. For instance, commensal bacteria regulate the synthesis of serotonin by the host

(*Yano et al.,* 2015), and serotonin may modulate the composition of the gut microbiome (*Stasi et al.,* 2019). The third important pathway is represented by the indole pathway whereby different species of bacteria produce indole compounds via the major metabolic pathways involving the activity of: i) tryptophanase generating indole, ii) aromatic amino acid aminotransferase generating indole-3-acetaldehyde and indole-3-aldehyde (3-IAld) and iii) tryptophan deaminase generating indole-3-pyruvic acid (*Morgan et al.,* 2023). Indoles are very attractive molecules as they have been shown to augment health span across a broad range of evolutionarily diverse species from different phyla, to control bacterial fitness, including antibiotic resistance and strengthening of the epithelial barrier function (*Wikoff et al.,* 2009; *Venkatesh et al.,* 2014; *Lee et al.,* 2015; *Dodd et al.,* 2017; *Roager and Licht* 2018; *Hendrikx and Schnabl* 2019; *Ornelas et al.,* 2022). Microbiota-derived indoles are ligands of AhR, thus suggesting that the host AhR has evolved to sense and respond to the presence of the microbiota resulting in maintenance of homeostasis. In addition to its function as xenobiotic receptor, AhR has been implicated in a wide range of physiological activities, including the bidirectional communication with the microbiome for modulating host immunity, tolerance and metabolism (*Zelante et al.,* 2013; *Metidji et al.,* 2018; *Stockinger et al.,* 2021; *Nieves et al.,* 2022).

Our group has previously identified a microbial pathway of Trp utilization that regulates *Candida* commensalism and mucosal homeostasis in the gut (*Zelante et al.,* 2013; *Roager and Licht* 2018; *Puccetti et al.,* 2023). Indeed, the commensal *L. reuteri*, by switching to Trp as energy source, produces 3-IAld via the aromatic amino acid aminotransferase pathway. 3-IAld, in turn, by working as a ligand of AhR, stimulated innate lymphoid cells to release IL-22 that efficiently controlled *C. albicans* colonization by promoting epithelial integrity and the release of antimicrobial peptides. The scenario emerging from these findings appear to trace the dichotomy of resistance versus tolerance back to the different pathways of Trp utilization. Indeed, the microbial AhR-IL-22 axis appear to promote resistance by means of primitive antifungal defence mechanisms that include the homeostatic maintenance of the triad microbiota, epithelium and immune system. In contrast, the host IDO1 pathway emerges as a functional specialization of antifungal tolerance mechanisms that evolved to facilitate the establishment of a fungal microbiome. It should also be noted that although the IDO1 product kynurenine and the microbial product 3-IAld are both ligands of AhR, the outcome may be different because AhR activation results in a variety of effects that depend, among others, on the ligand itself. Therefore, the IDO1 pathway and the microbial Trp metabolic pathway, although intersecting at a common node, may underlie distinct functional activities in the resistance vs tolerance antifungal response.

CONCLUSIONS

Fungi serve as a biological scaffold for bacterial attachment. Consequences of these interactions are not just limited to the respective microorganisms, but also have major impacts in the immune system resulting in a network of bidirectional interactions that operates across the health and disease spectrum (Figure 1). It is clear that individual microbes have important effects on the host, and

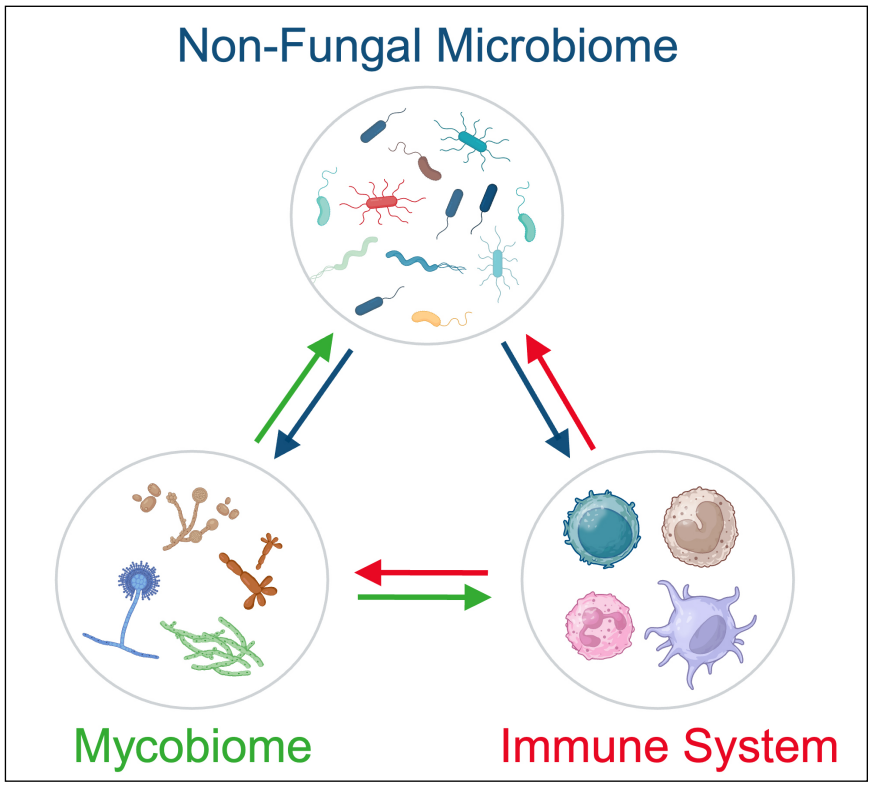

Figure 1: Schematic representation of the bidirectional network among the microbiome, the mycobiome and the immune system. The figure was made on BioRender.

that a balance of the microbiota is necessary for homeostasis. Examining the mechanisms behind the fungi/bacteria balance will provide us with an understanding of multi-kingdom community processes that allows for the development of disease-specific therapeutic approaches in different ecological settings. In so doing, we have discovered that harnessing of the AhR/IDO1 pathway may represent a much-needed strategy for improving and preventing the burden of human fungal diseases. By shifting the current view of pathogenesis from pathogen- to host-oriented views, we provided proof-of-concept evidence of the feasibility of therapeutic approaches to reduce infectious disease burden by targeting host and microbial-derived immunometabolic checkpoints leading to tolerance. The druggability of this pathway with microbial metabolites to promote homeostasis and microbial symbiosis at mucosal surfaces is becoming a reality.

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LITERATURE

Alonso-Roman, R., Last, A., Mirhakkak, M.H., Sprague, J.L., Moller, L., Grossmann, P., Graf, K., Gratz, R., Mogavero, S., Vylkova, S., Panagiotou, G., Schauble, S., Hube, B., and Gresnigt, M.S.: Lactobacillus rhamnosus colonisation antagonizes Candida albicans by forcing metabolic adaptations that compromise pathogenicity. Nat. Commun. 13, 3192 (2022).

Ansari, F., Alian Samakkhah, S., Bahadori, A., Jafari, S.M., Ziaee, M., Khodayari, M.T., and Pourjafar, H.: Health-promoting properties of Saccharomyces cerevisiae var. boulardii as a probiotic; characteristics, isolation, and applications in dairy products. Crit. Rev. Food Sci. Nutr. 63, 457-485 (2023).

Bernardes, M. and Hohl, T.M.: Fungal Infections Associated With the Use of Novel Immunotherapeutic Agents. Curr. Clin. Microbiol. Rep. 7, 142-149 (2020).

Borghi, M., Puccetti, M., Pariano, M., Renga, G., Stincardini, C., Ricci, M., Giovagnoli, S., Costantini, C., and Romani, L.: Tryptophan as a Central Hub for Host/Microbial Symbiosis. Int. J. Tryptophan Res. 13, 1178646920919755 (2020).

Bouz, G. and Dolezal, M.: Advances in Antifungal Drug Development: An Up-To-Date Mini Review. Pharmaceuticals (Basel) 14, 1312 (2021).

Brown, G.D., Denning, D.W., Gow, N.A., Levitz, S.M., Netea, M.G., and White, T.C.: Hidden killers: human fungal infections. Sci. Transl. Med. 4, 165rv13 (2012).

Casadevall, A.: Fungi and the rise of mammals. PLoS Pathog. 8, e1002808 (2012).

Cervenka, I., Agudelo, L.Z., and Ruas, J.L.: Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health. Science 357, eaaf9794 (2017).

Chow, E.W.L., Pang, L.M., and Wang, Y.: Impact of the host microbiota on fungal infections: New possibilities for intervention? Adv. Drug Deliv. Rev. 198, 114896 (2023).

Deveau, A., Bonito, G., Uehling, J., Paoletti, M., Becker, M., Bindschedler, S., Hacquard, S., Herve, V., Labbe, J., Lastovetsky, O.A., Mieszkin, S., Millet, L.J., Vajna, B., Junier, P., Bonfante, P., Krom, B.P., Olsson, S., van Elsas, J.D., and Wick, L.Y.: Bacterial-fungal interactions: ecology, mechanisms and challenges. FEMS Microbiol. Rev. 42, 335-352 (2018).

Dodd, D., Spitzer, M.H., Van Treuren, W., Merrill, B.D., Hryckowian, A.J., Higginbottom, S.K., Le, A., Cowan, T.M., Nolan, G.P., Fischbach, M.A., and Sonnenburg, J.L.: A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature 551, 648-652 (2017).

Dollive, S., Chen, Y.Y., Grunberg, S., Bittinger, K., Hoffmann, C., Vandivier, L., Cuff, C., Lewis, J.D., Wu, G.D., and Bushman, F.D.: Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. PLoS One 8, e71806 (2013).

Dong, F. and Perdew, G.H.: The aryl hydrocarbon receptor as a mediator of host-microbiota interplay. Gut Microbes 12, 1859812 (2020).

Erb Downward, J.R., Falkowski, N.R., Mason, K.L., Muraglia, R., and Huffnagle, G.B.: Modulation of post-antibiotic bacterial community reassembly and host response by Candida albicans. Sci. Rep. 3, 2191 (2013).

Fan, D., Coughlin, L.A., Neubauer, M.M., Kim, J., Kim, M.S., Zhan, X., Simms-Waldrip, T.R., Xie, Y., Hooper, L.V., and Koh, A.Y.: Activation of HIF-1alpha and LL-37 by commensal bacteria inhibits Candida albicans colonization. Nat. Med. 21, 808-814 (2015).

Forsberg, K., Woodworth, K., Walters, M., Berkow, E.L., Jackson, B., Chiller, T., and Vallabhaneni, S.: Candida auris: The recent emergence of a multidrug-resistant fungal pathogen. Med. Mycol. 57, 1-12 (2019).

Garcia, C., Tebbji, F., Daigneault, M., Liu, N.N., Kohler, J.R.. Allen-Vercoe, E., and Sellam, A.: The Human Gut Microbial Metabolome Modulates Fungal Growth via the TOR Signaling Pathway. mSphere 2, e00555-17 (2017).

Graf, K., Last, A., Gratz, R., Allert, S., Linde,

S., Westermann, M., Groger, M., Mosig, A.S., Gresnigt, M.S., and Hube, B.: Keeping Candida commensal: how lactobacilli antagonize pathogenicity of Candida albicans in an in vitro gut model. Dis. Model. Mech. 12, dmm039719 (2019).

Grifka-Walk, H.M., Jenkins, B.R., and Kominsky, D.J.: Amino Acid Trp: The Far Out Impacts of Host and Commensal Tryptophan Metabolism. Front. Immunol. 12, 653208 (2021).

Grohmann, U. and Bronte, V.: Control of immune response by amino acid metabolism. Immunol. Rev. 236, 243-264 (2010).

Hager, C.L., Isham, N., Schrom, K.P., Chandra, J., McCormick, T., Miyagi, M., and Ghannoum, M.A.: Effects of a Novel Probiotic Combination on Pathogenic Bacterial-Fungal Polymicrobial Biofilms. mBio 10, e00338-19 (2019).

Hendrikx, T. and Schnabl, B.: Indoles: metabolites produced by intestinal bacteria capable of controlling liver disease manifestation. J. Intern. Med. 286, 32-40 (2019).

Kim, Y. and Mylonakis, E.: Killing of Candida albicans filaments by Salmonella enterica serovar Typhimurium is mediated by sopB effectors, parts of a type III secretion system. Eukaryot. Cell 10, 782-790 (2011).

Lambooij, J.M., Hoogenkamp, M.A., Brandt, B.W., Janus, M.M., and Krom, B.P.: Fungal mitochondrial oxygen consumption induces the growth of strict anaerobic bacteria. Fungal Genet. Biol. 109, 1-6 (2017).

Lee, J.H., Wood, T.K., and Lee, J.P.: Roles of indole as an interspecies and interkingdom signaling molecule. Trends Microbiol. 23, 707-718 (2015).

Li, Y., Liu, N., Ge, Y., Yang, Y., Ren, F., and Wu, Z.: Tryptophan and the innate intestinal immunity: Crosstalk between metabolites, host innate immune cells, and microbiota. Eur. J. Immunol. 52, 856-868(2022).

Markey, L., Shaban, L., Green, E.R., Lemon, K.P., Mecsas, J., and Kumamoto, C.A.: Precolonization with the commensal fungus Candida albicans reduces murine susceptibility to Clostridium difficile infection. Gut Microbes 9, 497-509 (2018).

Mason, K.L., Erb Downward, J.R., Mason, K.D., Falkowski, N.R., Eaton, K.A., Kao, J.Y., Young, V.B., and Huffnagle, G.B.: Candida albicans and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. Infect Immun 80, 3371-3380 (2012).

McCarty, T.P. and Pappas, P.G.: Antifungal Pipeline. Front. Cell. Infect. Microbiol. 11, 732223 (2021).

Metidji, A., Omenetti, S., Crotta, S., Li, Y., Nye, E., Ross, E., Li, V., Maradana, M.R., Schiering, C., and Stockinger, B.: The Environmental Sensor AHR Protects from Inflammatory Damage by Maintaining Intestinal Stem Cell Homeostasis and Barrier Integrity. Immunity 49, 353-362.e35 (2018).

Morgan, E.W., Dong, F., Annalora, A.J., Murray, I.A., Wolfe, T., Erickson, R., Gowda, K., Amin, S.G., Petersen, K.S., Kris-Etherton, P.M., Marcus, C.B., Walk, S.T., Patterson, A.D., and Perdew, G.H.: Contribution of Circulating Host and Microbial Tryptophan Metabolites Toward Ah Receptor Activation. Int. J. Tryptophan Res. 16, 11786469231182510 (2023).

Nash, A.K., Auchtung, T.A., Wong, M.C., Smith, D.P., Gesell, J.R., Ross, M.C., Stewart, C.J., Metcalf, G.A., Muzny, D.M., Gibbs, R.A., Ajami, N.J., and Petrosino, J.F.: The gut mycobiome of the Human Microbiome Project healthy cohort. Microbiome 5, 153 (2017).

Nieves, K.M., Hirota, S.A., and Flannigan, K.L.: Xenobiotic receptors and the regulation of intestinal homeostasis: harnessing the chemical output of the intestinal microbiota. Am. J. Physiol. Gastrointest. Liver Physiol. 322, G268-G281 (2022).

Nogueira, F., Sharghi, S., Kuchler, K., and Lion, T.: Pathogenetic Impact of Bacterial-Fungal Interactions. Microorganisms 7, 459 (2019).

O'Brien, H.E., Parrent, J.L., Jackson, J.A., Moncalvo, J.M., and Vilgalys, R.: Fungal community analysis by large-scale sequencing of environmental samples. Appl. Environ. Microbiol. 71, 5544-5550 (2005).

Ornelas, A., Dowdell, A.S., Lee, J.S. and Colgan, S.P.: Microbial Metabolite Regulation of Epithelial Cell-Cell Interactions and Barrier Function. Cells 11, 944 (2022).

Panpetch, W., Somboonna, N., Palasuk, M., Hiengrach, P., Finkelman, M., Tumwasorn, S., and Leelahavanichkul, A.: Oral Candida administration in a Clostridium difficile mouse model worsens disease severity but is attenuated by Bifidobacterium. PLoS One 14, e0210798 (2019).

Peleg, A.Y., Tampakakis, E., Fuchs, B.B., Eliopoulos, G.M., Moellering Jr., R.C., and Mylonakis, E.: Prokaryote-eukaryote interactions identified by using Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 105, 14585-14590 (2008).

Pierce, E.C., Morin, M., Little, J.C., Liu, R.B., Tannous, J., Keller, N.P., Pogliano, K., Wolfe, B.E., Sanchez, L.M., and Dutton, R.J.: Bacterial-fungal interactions revealed by genome-wide analysis of bacterial mutant fitness. Nat. Microbiol. 6, 87-102 (2021).

Puccetti, M., Pariano, M., Wojtylo, P., Schoubben, A., Giovagnoli, S., and Ricci, M.: Turning Microbial AhR Agonists into Therapeutic Agents via Drug Delivery Systems. Pharmaceutics 15, 506 (2023).

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H, Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta, H.I.T.C., Bork, P., Ehrlich, S.D., and Wang, J.: A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59-65 (2010).

Roager, H.M. and Licht, T.R.: Microbial tryptophan catabolites in health and disease. Nat. Commun. 9, 3294 (2018).

Romani, L.: Immunity to fungal infections. Nat. Rev. Immunol. 4, 1-23 (2004).

Romani, L.: Immunity to fungal infections. Nat. Rev. Immunol. 11, 275-288 (2011).

Romani, L., Bistoni, F., and Puccetti, P.: Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol. 10, 508-514 (2002).

Romani, L., Zelante, T., Palmieri, M., Napolioni, V., Picciolini, M., Velardi, A., Aversa, F., and Puccetti, P.: The cross-talk between opportunistic fungi and the mammalian host via microbiota's metabolism. Semin. Immunopathol. 37, 163-171 (2015).

Santus, W., Devlin, J.R., and Behnsen, J.: Crossing Kingdoms: How the Mycobiota and Fungal-Bacterial Interactions Impact Host Health and Disease. Infect Immun 89, e00648-20 (2021).

Seo, S.K. and Kwon, B.: Immune regulation through tryptophan metabolism. Exp. Mol. Med. 55, 1371-1379 (2023).

Smith, M.G., Des Etages, S.G., and Snyder, M.: Microbial synergy via an ethanol-triggered pathway. Mol. Cell. Biol. 24, 3874-3884 (2004).

Sovran, B., Planchais, J., Jegou, S., Straube, M., Lamas, B., Natividad, J.M., Agus, A., Dupraz, L., Glodt, J., Da Costa, G., Michel, M.L., Langella, P., Richard, M.L., and Sokol, H.: Enterobacteriaceae are essential for the modulation of colitis severity by fungi. Microbiome 6, 152 (2018).

Stasi, C., Sadalla, S., and Milani, S.: The Relationship Between the Serotonin Metabolism, Gut-Microbiota and the Gut-Brain Axis. Curr. Drug Metab. 20, 646-655 (2019).

Steffan, B.N., Venkatesh, N., and Keller, N.P.: Let's Get Physical: Bacterial-Fungal Interactions and Their Consequences in Agriculture and Health. J. Fungi (Basel) 6, 243 (2020).

Stockinger, B., Shah, K., and Wincent, E.: AHR in the intestinal microenvironment: safeguarding barrier function. Nat. Rev. Gastroenterol. Hepatol. 18, 559-570 (2021).

Trunk, K., Peltier, J., Liu, Y.C., Dill, B.D., Walker, L., Gow, N.A.R., Stark, M.J.R., Quinn, J., Strahl, H., Trost, M., and Coulthurst, S.J.: The type VI secretion sys-

tem deploys antifungal effectors against microbial competitors. Nat. Microbiol. 3, 920-931 (2018).

Underhill, D.M. and Iliev, I.D.: The mycobiota: interactions between commensal fungi and the host immune system. Nat. Rev. Immunol. 14, 405-416 (2014).

Valentine, M., Benade, E., Mouton, M., Khan, W., and Botha, A.: Binary interactions between the yeast Candida albicans and two gut-associated Bacteroides species. Microb. Pathog. 135, 103619 (2019).

van der Linden, J.W., Camps, S.M., Kampinga, G.A., Arends, J.P., Debets-Ossenkopp, Y.J., Haas, P.J., Rijnders, B.J., Kuijper, E.J., van Tiel, F.H., Varga, J., Karawajczyk, A., Zoll, J., Melchers, W.J., and Verweij, P.E.: Aspergillosis due to voriconazole highly resistant Aspergillus fumigatus and recovery of genetically related resistant isolates from domiciles. Clin. Infect. Dis. 57, 513-520 (2013).

van Leeuwen, P.T., van der Peet, J.M., Bikker, F.J., Hoogenkamp, M.A., Oliveira Paiva, A.M., Kostidis, S., Mayboroda, O.A., Smits, W.K., and Krom, B.P.: Interspecies Interactions between Clostridium difficile and Candida albicans. mSphere 1, e00187-16 (2016).

Venkatesh, M., Mukherjee, S., Wang, H., Li, H., Sun, K., Benechet, A.P., Qiu, Z., Maher, L., Redinbo, M.R., Phillips, R.S., Fleet, J.C., Kortagere, S., Mukherjee, P., Fasano, A., Le Ven, J., Nicholson, J.K., Dumas, M.E., Khanna, K.M., and Mani, S.: Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. Immunity 41, 296-310 (2014).

Wagner, R.D., Pierson, C., Warner, T., Dohnalek, M., Farmer, J., Roberts, L., Hilty, M., and Balish, E.: Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. Infect. Immun. 65, 4165-4172 (1997).

Wang, L., Zhang, K., Zeng, Y., Luo, Y., Peng, J., Zhang, J., Kuang, T., and Fan, G.: Gut mycobiome and metabolic diseases: The known, the unknown, and the future. Pharmacol. Res. 193, 106807 (2023).

Wheeler, M.L., Limon, J.J., Bar, A.S., Leal, C.A., Gargus, M., Tang, J., Brown, J., Funari, V.A., Wang, H.L., Crother, T.R., Arditi, M., Underhill, D.M., and Iliev, I.D.: Immunological Consequences of Intestinal Fungal Dysbiosis. Cell Host Microbe 19, 865-873 (2016).

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., and Siuzdak, G.: Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc. Natl. Acad. Sci. USA 106, 3698-3703 (2009).

Yang, W., Zhou, Y., Wu, C., and Tang, J.: Enterohemorrhagic Escherichia coli promotes the invasion and tissue damage of enterocytes infected with Candida albicans in vitro. Sci Rep 6, 37485 (2016).

Yano, J.M., Yu, K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L., Nagler, C.R., Ismagilov, R.F., Mazmanian, S.K., and Hsiao, E.Y.: Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 161, 264-276 (2015).

Zeier, J.: New insights into the regulation of plant immunity by amino acid metabolic pathways. Plant Cell Environ. 36, 2085-2103 (2013).

Zeise, K.D., Woods, R.J., and Huffnagle, G.B.: Interplay between Candida albicans and Lactic Acid Bacteria in the Gastrointestinal Tract: Impact on Colonization Resistance, Microbial Carriage, Opportunistic Infection, and Host Immunity. Clin. Microbiol. Rev. 34, e0032320 (2021).

Zelante, T., Iannitti, R.G., Cunha, C., De Luca, A., Giovannini, G., Pieraccini, G., Zecchi, R., D'Angelo, C., Massi-Benedetti, C., Fallarino, F., Carvalho, A., Puccetti, P., and Romani, L.: Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 39, 372-385 (2013).

Zhang, F., Aschenbrenner, D., Yoo, J.Y., and Zuo, T.: The gut mycobiome in health, disease, and clinical applications in association with the gut bacterial microbiome assembly. Lancet Microbe 3, e969-e983 (2022).

EARLY-LIFE GUT MICROBIOTA AND NEURODEVELOPMENTAL OUTCOMES

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SUMMARY

Over the last decades, studies have revealed that the gut microbiota has much wider effects on host physiology and development than originally believed, including the modulation of brain development and behaviour. Recent insights into the complexity and magnitude of the human microbiota and its wide-ranging impact on health and disease have given way to a paradigm shift in our conceptualization of the origin of human brain disorders. Evidence from preclinical research, cross-sectional clinical studies, and preliminary microbiota-targeted intervention studies implicates the gut microbiota as a potential key susceptibility factor in neurodevelopmental and psychiatric disorders, including autism spectrum disorder (ASD). However, the microbiota-gut-brain axis field is nascent, and further investigations are needed to unravel the precise mechanisms mediating the intricate microbiota-host interactions and the potential of microbiota-based therapeutic strategies for these conditions. This article briefly highlights recent findings implicating the microbiota-gut-brain axis in the neurobiology of ASD, and novel metabolite-based therapeutic approaches to manage ASD. The emerging roles of the bacterial peptidoglycan signalling pathway in early life gut-brain communication are also presented.

INTRODUCTION

It is now well recognized that environmental influences during early life can profoundly affect the formation and later function of neural brain circuits, a phenonomen termed developmental programming. One such external environmental factor is the gut microbiota – the trillions of microorganisms inhabiting the gastrointestinal (GI) tract, including bacteria, archaea, fungi, and viruses – that through evolution has adapted to coexist in a mutualistic relationship with mammals (*Davenport* et al., 2017). During birth and rapidly thereafter, the new-born is massively colonized with microbes via vertical transmission from mother to infant (*Dominguez-Bello* et al., 2019; *House* et al., 2016). This postnatal microbial colonization process contributes to the developmental programming of epithelial barrier function, gut homeostasis, and angiogenesis, as well as promoting the development of the immune system (*Hooper* et al., 2012). A rapidly expanding body of research has revealed that the gut microbiota exerts a broader range of effects on host physiology and development beyond the GI tract, including the early life programming of brain circuits involved in the control of the stress response, motor activity, and cognitive functions (for a review, see: *Cryan* et al., 2019). Preclinical studies

using germ-free (GF) animals have shown that gut microbiota influences a wide-range of neurodevelopmental processes, including the maturation and function of microglia, primary resident immune cells of the brain (*Erny* et al., 2015; *Thion* et al., 2018), synaptogenesis (*Diaz Heijtz* et al., 2011), blood-brain barrier (BBB) formation and integrity (*Braniste* et al., 2014) and myelination (*Hoban* et al., 2016), as well as complex behaviours (*e.g.*, social and anxiety-like behaviours) (*Clarke* et al., 2013; *Diaz Heijtz* et al., 2011; *Neufeld* et al., 2011). Other studies have shown that gut microbiota influences behavioural abnormalities and brain pathologies observed in numerous animal models of neurodevelopmental and psychiatric disorders (*Lum* et al., 2020; *Needham* et al., 2018; *Sgritta* et al., 2019; *Tabouy* et al., 2018). One important lesson that has emerged from these preclinical studies is that many effects of the gut microbiota on the brain are sex-dependent (*Jaggar* et al., 2020). These studies also indicate that there are sensitive periods in early life during which the gut microbiota plays a critical role in shaping neural circuit formation and function.

In humans, the maturation of the gut microbiota occurs during the first years of postnatal life, occurring in parallel with critical neurodevelopmental processes (*e.g.*, synaptogenesis, myelination, and synaptic pruning) and the onset of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (for a review, see: *Borre* et al., 2014; *Diaz Heijtz*, 2016). It is increasingly acknowledged that neurodevelopmental and psychiatric disorders are often co-morbid with GI problems and an altered gut microbiota (*Cryan* et al., 2020). In addition, a growing number of preclinical and epidemiological studies suggest that early-life antibiotic exposure, that reduces microbiota diversity, could have detrimental effects on neurodevelopmental trajectories, and subsequently be a contributing factor to behavioural and psychological disturbances emerging in childhood (*Neuman* et al., 2018; *Otten* et al., 2022). The current challenge is to translate findings from animal models into humans, and to untangle the complex and dynamic interactions between the microbiota and the developing brain.

THE GUT MICROBIOTA AND AUTISM SPECTRUM DISORDER

Autism spectrum disorder (ASD) is an early-onset neurodevelopmental disorder, defined by the presence of social communication and interaction challenges in conjunction with restricted, repetitive behaviours and atypical sensory processing (*Lord* et al., 2018). The worldwide prevalence rate of ASD is currently estimated to be 1% (*Zeidan* et al., 2022). Manifestations of ASD are heterogenous, reflecting a spectrum of symptoms that may include individuals with intellectual disability and limited language ability, as well as individuals with average- or above-average intellectual abilities and typical language development. Moreover, many children with ASD experience GI and immune dysfunction, as well as a range of co-occurring somatic and psychiatric conditions including sleep disorders, epilepsy, and anxiety (*Hsiao*, 2014; *Leader* et al., 2022; *Madra* et al., 2020; *Zeidan* et al., 2022). For instance, several studies have shown that GI symptoms, such as abdominal pain, diarrhoea, constipation, and flatulence, are more common in children with ASD than their

neurotypically developing peers (*Chaidez* et al., 2014) and are positively associated with the severity of behavioural problems such as irritability, aggression, and repetitive behaviours (*Adams* et al., 2011; *Chakraborty* et al., 2021; *Wang* et al., 2011). Almost two decades ago, a small open-label study observed that treatment with oral vancomycin (a non-absorbable antibiotic that is active against Gram-positive bacteria) resulted in short-term benefit in a small group of children with regressive-onset autism (*Sandler* et al., 2000). Although antibiotics are not a suitable intervention strategy for the management of ASD, this study was among the first to suggest a link between the gut microbiota and ASD. Since then, multi cross-sectional studies have also reported an altered gut microbiota composition in children and adolescents with ASD, with lower gut bacterial diversity and an underrepresentation of beneficial bacteria (*e.g.*, Bifidobacterium species), along with an increased abundance of potentially pathogenic bacteria *Desulfovibrio* and *Clostridium* genera (*Andreo-Martinez* et al., 2022; *Bezawada* et al., 2020; *Iglesias-Vazquez* et al., 2020; *Xu* et al., 2019). There is, however, little consensus regarding the magnitude of changes or the existence of a specific microbial signature associated with ASD. It is worth mentioning that most of these studies were performed several years after children received the diagnosis of ASD and did not adequately record detailed dietary information or potential medical use. A recent study by Yap and colleagues provided evidence that ASD-associated gut microbiota changes can be attributed largely to a lack of dietary diversity (*Yap* et al., 2021). It is well-known that children with ASD often exhibit selective eating patterns (*Bourne* et al., 2022), and that they are more frequently exposed to antibiotics during the first years of life (*House* et al., 2016), which can adversely impact the gut microbiota (*Otten* et al., 2022). Nevertheless, the causal potential of the gut microbiota in ASD has been supported by preclinical studies including genetic (*e.g.*, Shank3B mutant mice) environmental (*e.g.*, valproic acid, GF, and maternal high-fat diet) and idiopathic rodent models of ASD (BTBR T+ Itpr3tf/J mice) (for a review, see: Alamoudi et al., 2022). Further support has been gathered from studies transferring the faecal gut microbiota from autistic children into GF mice, resulting in behavioural and molecular changes relevant to this condition (*Sharon* et al., 2019). However, it remains unclear whether the gut microbiota contributes to, or modifies the likelihood of ASD onset before any dietary changes occur. Indeed, a recent multi-omics analysis has highlighted the limitations of cross-sectional cohort studies (*Morton* et al., 2023), thus advocating for prospective, longitudinal multi-omics studies in combination with comprehensive patient metadata to elucidate the role of the gut microbiota in ASD.

EARLY-LIFE MICROBIOME DIFFERENCES IN INFANTS AT HIGH FAMILIAL RISK FOR ASD

ASD is now understood to be a multifactorial neurodevelopmental condition, involving genetic susceptibility, environmental risk factors, and gene-environmental interactions. One such risk factor is having a sibling with ASD, with studies consistently demonstrating a higher prevalence among siblings and in families with a history of ASD (*Lord* et al., 2018). Moreover, previous

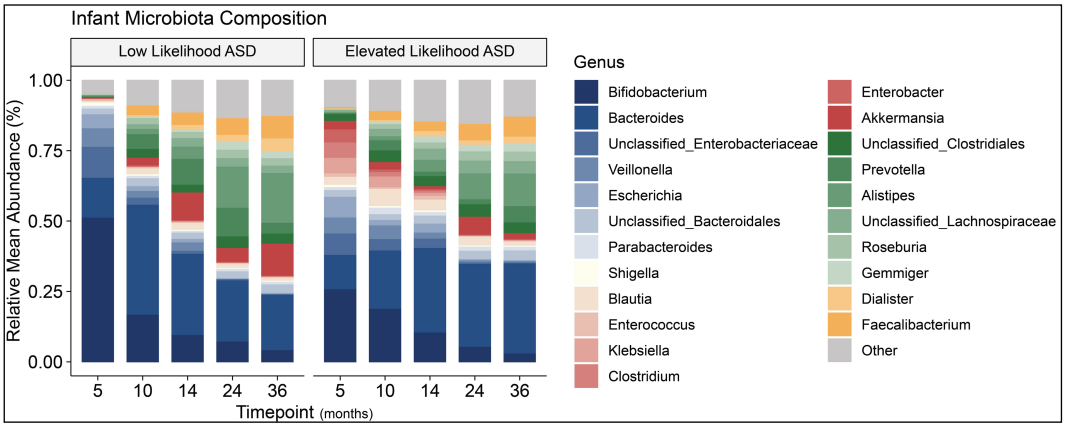

Figure 1: Gut microbiota composition level of infants at low likelihood (LL) and elevated likelihood (EL) of ASD from 5 to 36 months of age.
The relative mean abundance of the top taxa was calculated using the aggregated OTUs at the genus level. The largest differences between the two groups were observed at 5 months of age. The LL group harboured more Bifidobacteria (51% vs. 26%), while the EL group had more Clostridium (5% vs. 0.005%) compared to the LL group (Reprinted with permission from *Zuffa* et al., 2023)

investigations have shown that about 50% of younger siblings of children with ASD develop in an atypical manner, approximately 20% develop ASD, and another 30% show delays or deficits in other areas of development or behaviour (*Ozonoff* et al., 2014). Using a prospective longitudinal study design, Zuffa and colleagues recently studied the developmental profile of the faecal microbiota and metabolome in infants with and without a family history of ASD (in first- or second-degree relatives) across the first 3 years of life (*Zuffa* et al., 2023), a critical period when the gut microbiota and brain are both undergoing rapid development (*Borre* et al., 2014).

At 5 months of age, this study found pronounced alterations in the gut microbiota composition of infants at elevated likelihood (EL) of developing ASD (*i.e.*, siblings of children with ASD) compared to low-likelihood (LL) infants (*i.e.*, infants without a family history of ASD) (see Figure 1). Specifically, infants in the EL group harboured less beneficial *Bifidobacterium* species (*B. bifidum, B. longum* and *B. kashiwanohense*) and more *Clostridium* (*C. bolteae*, *C. difficile*, *C. clostridioforme*, *C. neonatale)* species compared to infants in the LL group. Members of the genus *Bifidobacterium* are among the first colonizers of the neonatal human gut and have been associated with various health benefits (*e.g.*, folate production, protection against pathogens and development of the immune system). On the other hand, *Clostridium* species, which have previously been linked to ASD, are considered pathobionts and responsible for inflammation when homeostasis is disrupted (*Lees* et al., 2016).

Untargeted metabolic profiling highlighted that infants in the LL group excreted greater amounts of faecal γ-aminobutyric acid (GABA) GABA, which progressively declined with age (*Zuffa* et al., 2023). In contrast, infants in the EL group did not display similar developmental changes, with GABA being consistently low across the first 36 months of life (*Zuffa* et al., 2023). Furthermore, integrated microbiome-metabolome analysis revealed a strong positive correlation between the

identified *Bifidobacterium* species and GABA, as well as negative associations with *Clostridium* species (*Zuffa* et al., 2023). These observations raised the possibility that competition between *Clostridium* and *Bifidobacterium* species may exist and lead to reduced availability of GABA in the infant gut. To test this hypothesis, Zuffa and colleagues used a simplified *in vitro* model with different ratios of the identified *Bifidobacterium* and *Clostridium* species in the presence of GABA and its metabolic precursors (*Zuffa* et al., 2023). Fascinatingly, *in vitro* experiments revealed that *Bifidobacterium species* can produce GABA while *Clostridium* species can consume it, indicating that a delicate balance exists between these bacteria genera in the infant gut, with consequences for the bioavailability of GABA and its modulatory effects on the infant host.

The general development level including gross motor functions, visual perception, fine motor skills, receptive language, and expressive language of infants in the EL and LL groups was evaluated using the Mullen Scales of Early Learning (MSEL) test at 5 and 36 months of age and with Autism Diagnostic Observation Schedule Second Edition (ADOS-2; an assessment tool for the diagnosis and evaluation of ASD) at 36 months of age. Consistent with prior literature, no significant developmental changes were observed between infants in the EL and LL groups at 5 months (*Gamliel* et al., 2007). At 36 months, however infants in the EL group had lower MSEL and ADOS-2 compared to infants in the LL group. Although none of the infants were diagnosed with ASD at 36 months of age, more subtle presentations of this condition might still be diagnosed later in development, when daily functional demands increase (*e.g.*, in the school setting). Moreover, other co-occurring somatic and psychiatric conditions may not be evident at this early age. Despite this limitation, this study clearly demonstrated that alterations in gut microbiota composition and functionality of infants at EL of ASD occur before any atypical development could be detected at 36 months of age (*Zuffa* et al., 2023), supporting a possible role of the gut microbiota in emerging behavioural variability later in life.

Emerging evidence suggests that the development of the infant gut microbiota is influenced by host genetics, but that this effect is subtle and affects only certain bacteria species (*Palmeira* et al., 2022). The absence of major confounding perinatal risk factors such as preterm birth and antibiotic exposure in the above cohort of infants suggest a role for host genetic risk factors in the observed early-life differences in gut microbiota composition and functionality of infants in the EL group. Interestingly, recent studies have shown that many high-confidence risk genes for ASD are expressed not only in the developing brain, but also in the developing gut (*Niesler* and *Rappold*, 2021). Moreover, recent preclinical studies have shown that mutations in some of these genes (*e.g.*, Shank3B mutant) alter the enteric nervous system, leading to abnormal GI motility and structural changes in the GI tract (for a review, see: *Alamoudi* et al., 2022). These findings suggest that genetic variants previously linked to behavioural symptoms in ASD could contribute to the development of an atypical gut microbiota due to alterations in the host gut environment.

POTENTIAL ROLE OF BACTERIAL-DERIVED GABA IN NEURODEVELOPMENT

GABA acts as the main inhibitory neurotransmitter in the central nervous system (CNS) and is significantly involved in a wide range of physiological and psychological processes. Alterations in the central GABAergic system have been implicated in the neurobiology of several neurodevelopmental and psychiatric disorders, including ASD (for a review, see: *Zhao* et al., 2021). Early in life, however, GABAergic synaptic transmission is excitatory, and plays an important role in brain development (*Ganguly* et al., 2001). GABAergic dysfunction disturbs the optimal excitatory/inhibitory (E/I) balance in the brain, altering the integrity and function of neural circuits. Indeed, an imbalance in E/I neurotransmission ratio in cortical networks has been suggested as a plausible neurobiological pathway underlying cognitive symptomatology in ASD (*Marenco* et al., 2018). Over the past decades, studies have shown that several commensal gut bacteria produce neurotransmitters such as dopamine, histamine, serotonin, and GABA. Several members of the genus *Bifidobacterium*, such as *Bifidobacterium dentium*, *Bifidobacterium longum* subsp. infantis and *Bifidobacterium adolescentis* have been shown to produce GABA (*Hidalgo-Cantabrana* et al., 2017). It remains unclear whether bacterial-derived GABA present in systemic circulation enters the brain. For instance, Luck and colleagues showed that GF mice mono-colonized with *Bifidobacterium dentium,* which possesses the enzymatic machinery to produce GABA from glutamate, glutamine, and succinate, displayed increases in faecal GABA concentrations, but no changes within the brain (*Luck* et al., 2021). Nonetheless, there is evidence that the central GABA system can be modulated via the vagus nerve and enteric nervous system (ENS). In a landmark study by Bravo and colleagues, it was demonstrated that ingestion of *Limosilactobacillus reuteri,* a probiotic with anti-inflammatory properties, can regulate emotional behaviour and central GABA receptor expression in mice via the vagus nerve (*Bravo* et al., 2011). Other animal studies have implicated GABA producing species such as *Bifidobacterium infantis* in the early-life programming of the immune system (*Henrick* et al., 2021), raising the possibility that bacterial-derived GABA may indirectly modulate brain development and subsequent behaviour via multiple pathways.

NEW MICROBIOME-BASED THERAPEUTIC APPROACHES TO MANAGE ASD

There is growing evidence from animal studies that direct metabolites of the gut microbiota or products of the combinatorial metabolism between the microbiota and host (so called "neuroactive microbial metabolites") can cross the BBB (*Swann* et al., 2020), and directly modulate neural networks involved in the control of affective, social, and cognitive processes (*Needham* et al., 2022). In humans, several neuroactive gut-bacterial-derived metabolites such as 4-ethylphenyl sulphate (4EPS), p-cresyl sulphate (pCS), and other structurally related phenolic molecules, were elevated in faeces and plasma of individuals with ASD (*Needham* et al., 2021; *Zheng* et al., 2021). Moreover, preclinical studies using mouse models of ASD indicate that 4EPS is likely to contribute

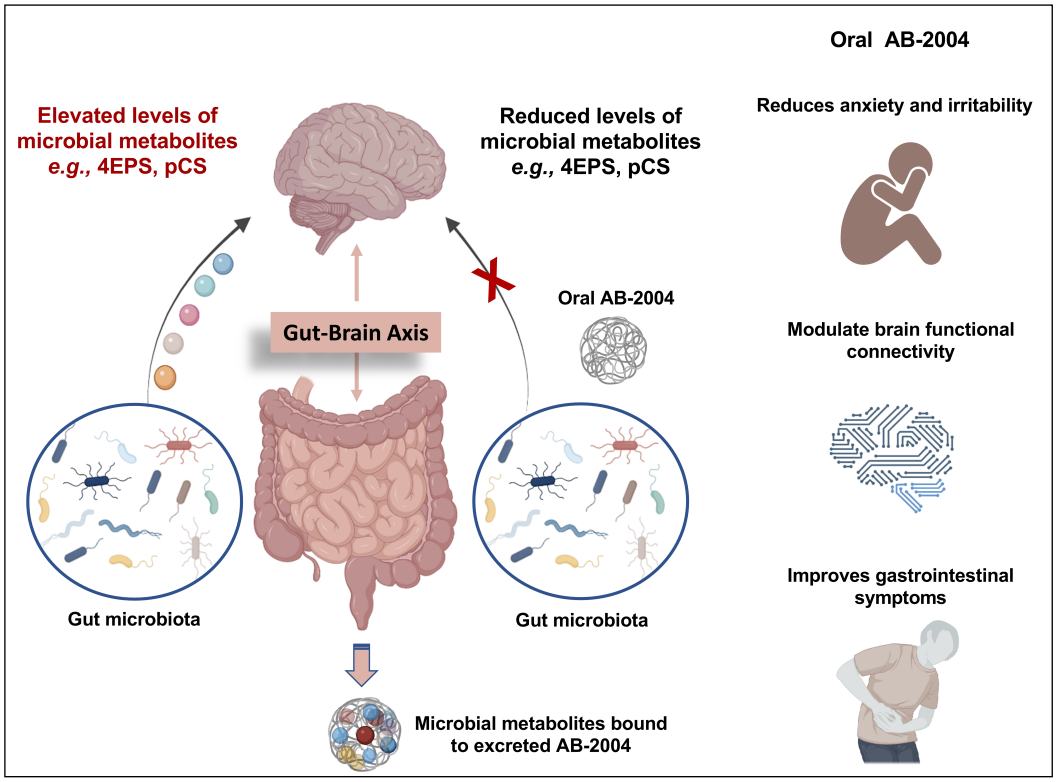

Figure 2: Treatment with AB-2004 improves gastrointestinal problems, brain functional connectivity and non-behavioural symptoms of ASD. **(**Modified from *Diaz Heijtz* et al., 2022).
Several biological pathways of communication along the gut-brain axis have been implicated, including the production of gut-bacterial-derived metabolites (so called neuroactive microbial metabolites) that can cross the blood brain barrier, and directly modulate the brain. Individuals with autism spectrum disorder (ASD) show elevated amounts of various neuroactive microbial metabolites such as 4-ethylphenyl sulphate (4EPS) and p-cresyl (pCS) in faeces and plasma. AB-2004 directly targets these metabolites in the gut, diminishing systemic exposure and limiting their impact on the brain.

to atypical neurodevelopment and GI dysfunction in mammals (*Hsiao* et al., 2013). Consequently, metabolite-based therapeutics targeting metabolites of the gut microbiota have emerged as an attractive new therapeutic approach to manage ASD. As such, rather than targeting the altered gut microbiota composition in ASD, this approach directly targets dysregulated neuroactive microbial metabolites in the gut, diminishing systemic exposure and limiting their impact on the brain. In a recent study, Campbell and colleagues provided the first preliminary clinical evidence that AB-2004, a first-in-class therapeutic that targets neuroactive microbial metabolites in the gut, can help reduce anxiety and irritability in adolescents with ASD, as well as GI problems (*Campbell* et al., 2022). AB-2004 is a spherical carbon absorbent that has high affinity for uremic toxins and related aromatic metabolites such as 4EPS, pCS, 3-indoxyl sulphate and hippurate, and therefore sequesters these molecules in the gut, preventing their absorption into the systemic circulation (see Figure 2). The results from an ongoing double-blinded placebo-controlled trial will reveal whether AB-2004 treatment proves to be effective for the manage-

ment of non-core behavioural symptoms and GI problems of ASD.

Other approaches have been employed to manipulate directly and profoundly the gut microbiota using faecal microbiota transplantation (FMT), as well as more subtle nutritional strategies (*e.g.*, psychobiotic diet, postbiotics) aimed at fortifying gut homeostasis and its microbial environment. Some preliminary FMT studies have shown positive preliminary results in ameliorating both autistic traits and GI symptoms (*Kang* et al., 2017; 2019; 2020). Successful examples of nutritional strategies have largely been limited to animal models of ASD. Current evidence supporting beneficial effects and long-term safety of microbiota-based interventions in infants at EL of ASD and young children with ASD are still limited.

THE MICROBIOTA-GUT-BRAIN AXIS, MATERNAL GUT MICROBIOTA AND NEURODEVELOPMENT

It has long been known that a complex bidirectional communication network exists between the gut and the CNS, known as the gut-brain axis, which includes the sympathetic and parasympathetic branches of the autonomic nervous system, the enteric nervous system and neuroendocrine and neuroimmune pathways. In recent decades, the gut microbiota has been identified as a crucial "third component" in the gut-brain crosstalk, thus leading to the current concept of the microbiota-gut brain axis. Communication along this axis involves multiple direct and indirect pathways, including neuronal circuits (*e.g.*, bidirectional vagus nerve-to-brain communication and the enteric nervous system), immune (*e.g.*, cytokines, monocyte trafficking), endocrine (*e.g.*, cortisol), and microbial-derived metabolites such as bacterial fermentation by-products such as short-chain fatty acids (SCFAs; propionate, butyrate, and acetate) (for a review, see: *Cryan* et al., 2019). However, the precise molecular mechanisms underlying the interactions between the gut microbiota and the developing brain remain to be elucidated.

It is now widely recognized that the maternal-foetal environment plays an important role in foetal brain and long-term neurodevelopmental trajectories, including susceptibility to neurodevelopmental and psychiatric disorders in childhood and adulthood (for a review, see: *Al-Haddad* et al., 2019). In recent years, the maternal gut microbiota has been associated not only with maternal health during pregnancy, but also with general foetal health and subsequent neurodevelopmental outcomes (for a review, see: *Bolte* et al., 2022). The developing foetus receives nutrients and other bioactive compounds from the maternal circulation, which could be profoundly influenced by the composition of the maternal gut microbes. Using GF mice, Braniste and colleagues investigated the impact of microbiota on the development of the BBB, which begins to develop early in foetal life and continues to mature during early postnatal life (*Braniste* et al., 2014). The authors found that GF mice displayed increased BBB permeability beginning in foetal life and continuing into adulthood, thus supporting a role of the maternal gut microbiota in BBB formation and maturation. The same study demonstrated that the SCFA butyrate could restore the integrity of the BBB in adult GF mice. Similarly, Erny and colleagues showed that a mix of SCFAs could also restore microglial malformation and immatu-

rity in adult GF mice (*Erny* et al., 2017). Recently, Kimura and colleagues demonstrated that SCFAs derived from the maternal gut microbiota could be translocated into the foetus and sensed through G protein-coupled receptors such as GPR41 in the developing sympathetic nervous system (*Kimura* et al., 2020). Crucially, they also showed that maternal-derived SCFAs are essential for the protection against high-fat-diet-induced metabolic syndrome and obesity in the offspring later in life (*Kimura* et al., 2020), thus highlighting the importance of the maternal gut microbiota in neurodevelopment. In another study, Vuong and colleagues recently showed that maternal microbial-derived metabolites shape foetal brain development and subsequent function (*Vuong* et al., 2020) and that this occurs in the absence of environmental challenges. These authors demonstrated that the maternal gut microbiota regulates metabolites (*e.g.*, trimethylamine-N-oxide, and hippurate), not only in the maternal serum, but also in the brains of the foetal offspring, promoting foetal thalamocortical axonogenesis (*Vuong* et al., 2020). These findings underscore the need to further characterize the influence that maternal microbial-derived metabolites or components have on the prenatal brain development of offspring and whether specific neural circuits in the developing brain are more sensitive to their modulation.

EMERGING ROLES OF THE BACTERIAL PEPTIDOGLYCAN SIGNALLING PATHWAY IN NEURODEVELOPMENT AND BEHAVIOUR

Traditionally, the translocation of bacterial cell wall components such as peptidoglycan (PGN, also called murein) motifs into the brain has mainly been considered in the context of a compromised BBB function (for example, bacterial or viral infections). Recent scientific discoveries revealing a previously unappreciated complexity of the human microbiota and its wide-ranging impact on health and disease have triggered a re-evaluation of our views about host-microbe interactions. The gut microbiota contains trillions of indigenous bacteria producing a diverse "peptidoglycome" that can disseminate systemically and reach peripheral organs (*Wheeler* et al., 2023). PGN is a unique and essential component of the bacterial cell wall that is absent in eukaryotes, which consists of glycan strands of two alternating β-1,4-linked sugars (N-acetylglucosamine and N-acetylmuramic acid) cross-linked by short peptides, containing two to five amino acids. It has highly dynamic structure that continuously undergoes remodelling, causing PGN motifs to be shed into the environment (*e.g.*, upon bacterial growth, replication, or death), a process termed PGN turnover. In mammals, PGN motifs are recognized by cytosolic NOD-like receptors (nucleotide-binding domain leucine-rich repeat containing receptors; Nod1 and Nod2) and PGN recognition proteins (PGRPs, Pglyrp1-4) (for a review see: *Mukherjee* et al., 2019; *Royet* et al., 2011). In a landmark study, Clarke and colleagues showed that the indigenous gut microbiota is a source of PGN which is translocated from the intestinal mucosa into circulation in the absence of pathogens (*Clarke* et al., 2010). Specifically, these authors showed that meso-diaminopimelic acid (meso-DAP)-type PGN, which is present in Gram-negative bacteria, could be translocated from the intestinal mucosa into neutrophils residing in bone marrow (*Clarke* et al., 2010).

Activation of the cytoplasmic pattern recognition receptors (PRR) Nod1 by meso-DAP-type PGN was sufficient to prime and restore neutrophil function in the bone marrow of mice with a manipulated microbiota (*i.e.*, GF or antibiotic-treated mice), revealing a previously undescribed role for PGN in priming systemic innate immunity in the absence of infection (*Clarke* et al., 2010).

In 2017, Arentsen and colleagues demonstrated that PGN fragments derived from the indigenous gut microbiota can be translocated into the developing brain and sensed by specific pattern recognition receptors (PRRs) of the innate immune system (*Arentsen* et al., 2017). Importantly, PGN levels in the brain increase in parallel with postnatal microbial colonization processes (*Arentsen* et al., 2017). Other groups have independently confirmed that PGN motifs from gut microbiota translocate across the intestinal barrier and can reach multiple peripheral organs, including the brain (*Wheeler* et al., 2023). Using expression-profiling techniques, Arentsen and colleagues also demonstrated that two families of PRRs that specifically detect PGN (*i.e.,* PGRPs and NOD-like receptors) and the PGN transporter PepT1 are highly expressed in the developing brain during specific windows of postnatal development in a brain-region and sex-dependent manner (*Arentsen* et al., 2017). Moreover, they showed that the absence of PGN-recognition protein 2 (Pglyrp2) leads to alterations in the expression of the autism risk genes c-Met and BDNF (brain-derived neurotrophic factor), which are implicated in the formation and modulation of brain circuits. Furthermore, juvenile Pglyrp2 knockout (KO) mice display immediate strong motivation to interact with same-sex novel social stimulus, without any alterations in anxiety-like behaviours or motor activity. Fascinatingly, this behavioural phenotype was more pronounced in male juvenile offspring. In another study, the same authors showed that the absence of Pglyrp2 leads to major sex-dependent alterations in motor and anxiety-like behaviour later in life (*Arentsen* et al., 2018). In this case, Pglyrp2 KO female mice, but not males, display increased levels of anxiety-like behaviour. However, they have better motor performance. These observations suggest that the modulatory effects of Pglyrp2 in the brain are highly dependent upon multiple host factors including age, sex, and domain-specific circuits in the brain. In an elegant study, Gabanyi and colleagues recently demonstrated that muramyl dipeptide (MDP)-type PGN (which is present in both Gram-positive and Gram-negative bacteria) regulates appetite and body temperature via activation of Nod2-expressing GABAergic neurons in the female hypothalamus (*Gabanyi* et al., 2022). Consistent with earlier studies by Arentsen and colleagues, these authors showed that Nod2 KO female mice, but not males, developed anxiety-like behaviour and metabolic dysregulation later in life (*Gabanyi* et al., 2022). Other studies have shown that selective deletion of Nod1 in intestinal epithelial cells increases the susceptibility of mice to stress-induced anxiety-like behaviour and cognitive impairment (*Pusceddu* et al., 2019). Taken together, these findings underscore the need to unravel the bacteria PGN pathways across different stages of brain development. In parallel, the potential influence of this pathway in typical and atypical human brain development could be explored by taking advantage of recent advances in brain organoid technologies.

CONCLUSIONS AND PERSPECTIVES

The gut microbiota is now recognized as a key regulator of brain development and behaviour, and a potential susceptibility factor in neurodevelopmental and psychiatric disorders such as ASD. Increasing evidence from both preclinical and clinical studies indicates that common perinatal risk factors (*e.g.*, stress, altered diet and antibiotic exposures) can perturb the maturation of the infant gut microbiota, with potential adverse consequences for early-life gut-brain interactions. However, it remains unclear whether the gut microbiota plays a role in the aetiology of neurodevelopmental disorders such as ASD. A recent preliminary study of infants at EL of ASD has provided strong evidence supporting the role of the gut microbiota on behavioural variability emerging around three years of age. There is an urgent need for multi-centre prospective longitudinal studies of infants at elevated risk for ASD from pregnancy until the first postnatal years of life, coupled with comprehensive metadata (*e.g.*, genetic information of infants and their families, dietary habits, perinatal risk factors, and relevant clinical outcomes) and multi-omics approaches.

The first years of postnatal life represent a period of rapid changes in brain structure and function, with the neonatal brain growing from about 36% to about 80-90% of its volume. During this period, the developing neural circuits in the brain undergo major changes, including massive outgrowth of dendrites and axons, rapid formation of new synapses (synaptogenesis), expansion of glial cells, and myelination. The striking increase in brain growth observed during this period, occurring in parallel with the maturation of the infant gut microbiota, indicates that this is not only a critical time-window of developmental vulnerability, but also a time of opportunity in which therapeutic interventions may have a maximal effect on neural circuits and subsequent functions. Consequently, there is a great need to unravel the precise molecular mechanisms underlying the communication between the gut microbiota and the developing brain to develop more targeted interventions for infants and young children at risk for neurodevelopmental and psychiatric disorders.

Recent studies have identified the bacterial PGN signalling pathway as a potential key regulator of gut microbiota–brain interactions in early life. From a clinical perspective, it would be important to explore potential associations between the PGN signalling pathway (from genetic variants in PGN-sensing molecules to dysregulation of the peptidoglycome) and neurodevelopmental disorders.

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LITERATURE

Adams, J.B., Johansen, L.J., Powell, L.D., Quig, D., and Rubin, R.A.: Gastrointestinal flora and gastrointestinal status in children with autism--comparisons to typical children and correlation with autism severity. BMC Gastroenterol. 11, 22 (2011).

Al-Haddad, B.J.S., Oler, E., Armistead, B., Elsayed, N.A., Weinberger, D.R., Bernier, R., Burd, I., Kapur, R., Jacobsson, B., Wang, C., Mysorekar, I., Rajagopal, L., and Adams Waldorf, K.M.: The fetal origins of mental illness. Am. J. Obstet. Gynecol. 221, 549-562 (2019).

Alamoudi, M.U., Hosie, S., Shindler, A.E., Wood, J.L., Franks, A.E., and Hill-Yardin, E.L.: Comparing the Gut Microbiome in Autism and Preclinical Models: A Systematic Review. Front. Cell. Infect. Microbiol. 12, 905841 (2022).

Andreo-Martinez, P., Rubio-Aparicio, M., Sanchez-Meca, J., Veas, A., and Martinez-Gonzalez, A.E.: A Meta-analysis of Gut Microbiota in Children with Autism. J. Autism. Dev. Disord. 52, 1374-1387 (2022).

Arentsen, T., Khalid, R., Qian, Y., and Diaz Heijtz, R.: Sex-dependent alterations in motor and anxiety-like behavior of aged bacterial peptidoglycan sensing molecule 2 knockout mice. Brain Behav. Immun. 67, 345-354 (2018).

Arentsen, T., Qian, Y., Gkotzis, S., Femenia, T., Wang, T., Udekwu, K., Forssberg, H., and Diaz Heijtz, R.: The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. Mol. Psychiatry 22, 257-266 (2017).

Bezawada, N., Phang, T.H., Hold, G.L., and Hansen, R.: Autism Spectrum Disorder and the Gut Microbiota in Children: A Systematic Review. Ann. Nutr. Metab. 76, 16-29 (2020).

Bolte, E.E., Moorshead, D., and Aagaard, K.M.: Maternal and early life exposures and their potential to influence development of the microbiome. Genome Med. 14, 4 (2022).

Borre, Y.E., O'Keeffe, G.W., Clarke, G., Stanton, C., Dinan, T.G., and Cryan, J.F.: Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol. Med. 20, 509-518 (2014).

Bourne, L., Mandy, W., and Bryant-Waugh, R.: Avoidant/restrictive food intake disorder and severe food selectivity in children and young people with autism: A scoping review. Dev. Med. Child Neurol. 64, 691-700 (2022).

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Toth, M., Korecka, A., Bakocevic, N., Ng, L.G., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B.T., Diamond, B., and Pettersson,S.: The gut microbiota influences blood-brain barrier permeability in mice. Sci. Transl. Med. 6, 263ra158 (2014).

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., and Cryan, J.F.: Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. USA 108, 16050-16055 (2011).

Campbell, A.S., Needham, B.D., Meyer, C.R., Tan, J., Conrad, M., Preston, G.M., Bolognani, F., Rao, S.G., Heussler, H., Griffith, R., Guastella, A.J., Janes, A.C., Frederick, B., Donabedian, D.H., and Mazmanian, S.K.: Safety and target engagement of an oral small-molecule sequestrant in adolescents with autism spectrum disorder: an open-label phase 1b/2a trial. Nat. Med. 28, 528-534 (2022).

Chaidez, V., Hansen, R.L., and Hertz-Picciotto, I.: Gastrointestinal problems in children with autism, developmental delays or typical development. J. Autism Dev. Disord. 44, 1117-1127 (2014).

Chakraborty, P., Carpenter, K.L.H., Major, S., Deaver, M., Vermeer, S., Herold, B., Franz, L., Howard, J., and Dawson, G.: Gastrointestinal problems are associated with increased repetitive behaviors but not social communication difficulties in young children with autism spectrum disorders. Autism 25, 405-415 (2021).

Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y., and Weiser, J.N.: Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 16, 228-231 (2010).

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R.D., Shanahan, F., Dinan, T.G., and Cryan, J.F.: The microbiome-gut-brain axis during early life regulates the

hippocampal serotonergic system in a sex-dependent manner. Mol. Psychiatry 18, 666-673 (2013).

Cryan, J.F., O'Riordan, K.J., Cowan, C.S.M., Sandhu, K.V., Bastiaanssen, T.F.S., Boehme, M., Codagnone, M.G., Cussotto, S., Fulling, C., Golubeva, A.V., Guzzetta, K.E., Jaggar, M., Long-Smith, C.M., Lyte, J.M., Martin, J.A., Molinero-Perez, A., Moloney, G., Morelli, E., Morillas, E., O'Connor, R., Cruz-Pereira, J.S., Peterson, V.L., Rea, K., Ritz, N.L., Sherwin, E., Spichak, S., Teichman, E.M., van de Wouw, M., Ventura-Silva, A.P., Wallace-Fitzsimons, S.E., Hyland, N., Clarke, G., and Dinan, T.G.: The Microbiota-Gut-Brain Axis. Physiol. Rev. 99, 1877-2013 (2019).

Cryan, J.F., O'Riordan, K.J., Sandhu, K., Peterson, V., and Dinan, T.G.: The gut microbiome in neurological disorders. Lancet Neurol. 19, 179-194 (2020).

Davenport, E.R., Sanders, J.G., Song, S.J., Amato, K.R., Clark, A.G., and Knight, R.: The human microbiome in evolution. BMC Biol. 15, 127 (2017).

Diaz Heijtz, R.: Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. Semin. Fetal Neonatal Med. 21, 410-417 (2016).

Diaz Heijtz, R., Gressens, P., and Swann, J.R.: Targeting microbial metabolites to treat autism. Nat. Med. 28, 448-450 (2022).

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S.: Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. USA 108, 3047-3052 (2011).

Dominguez-Bello, M.G., Godoy-Vitorino, F., Knight, R., and Blaser, M.J.: Role of the microbiome in human development. Gut 68, 1108-1114 (2019).

Erny, D., Hrabe de Angelis, A.L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., Schwierzeck, V., Utermöhlen, O., Chun, E., Garrett, W.S., McCoy, K.D., Diefenbach, A., Staeheli, P., Stecher, B., Amit, I., and Prinz, M.: Host microbiota constantly control maturation and function of microglia in the CNS. Nat. Neurosci. 18, 965-977 (2015).

Erny, D., Hrabe de Angelis, A.L., and Prinz, M.: Communicating systems in the body: how microbiota and microglia cooperate. Immunology 150, 7-15 (2017).

Gabanyi, I., Lepousez, G., Wheeler, R., Vieites-Prado, A., Nissant, A., Chevalier, G., Wagner, S., Moigneu, C., Dulauroy, S., Hicham, S., Polomack, B., Verny, F., Rosenstiel, P., Renier, N., Gomperts Boneca, I., Eberl, G., and Lledo, P.-M.: Bacterial sensing via neuronal Nod2 regulates appetite and body temperature. Science 376, eabj3986 (2022).

Gamliel, I., Yirmiya, N., and Sigman, M.: The development of young siblings of children with autism from 4 to 54 months. J. Autism Dev. Disord. 37, 171-183 (2007).

Ganguly, K., Schinder, A.F., Wong, S.T., and Poo, M.: GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105, 521-532 (2001).

Henrick, B.M., Rodriguez, L., Lakshmikanth, T., Pou, C., Henckel, E., Arzoomand, A., Olin, A., Wang, J., Mikes, J., Tan, Z., Chen, Y., Ehrlich, A.M., Bernhardsson, A.K., Mugabo, C.H., Ambrosiani, Y., Gustafsson, A., Chew, S., Brown, H.K., Prambs, J., Bohlin, K., Mitchell, R.D., Underwood, M.A., Smilowitz, J.T., German, J.B., Frese, S.A., and Brodin, P.: Bifidobacteria-mediated immune system imprinting early in life. Cell 184, 3884-3898 (2021).

Hidalgo-Cantabrana, C., Delgado, S., Ruiz, L., Ruas-Madiedo, P., Sanchez, B., and Margolles, A.: Bifidobacteria and Their Health-Promoting Effects. Microbiol. Spectr. 5 (2017).

Hoban, A.E., Stilling, R.M., Ryan, F.J., Shanahan, F., Dinan, T.G., Claesson, M.J., Clarke, G., and Cryan, J.F.: Regulation of prefrontal cortex myelination by the microbiota. Transl. Psychiatry 6, e774 (2016).

Hooper, L.V., Littman, D.R., and Macpherson,

A.J.: Interactions between the microbiota and the immune system. Science 336, 1268-1273 (2012).

House, S.A., Goodman, D.C., Weinstein, S.J., Chang, C.H., Wasserman, J.R., and Morden, N.E.: Prescription Use among Children with Autism Spectrum Disorders in Northern New England: Intensity and Small Area Variation. J. Pediatr. 169, 277-283 (2016).

Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F., Patterson, P.H., and Mazmanian, S.K.: Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155, 1451-1463 (2013).

Hsiao, E.Y.: Gastrointestinal issues in autism spectrum disorder. Harv. Rev. Psychiatry 22, 104-111 (2014).

Iglesias-Vazquez, L., Van Ginkel Riba, G., Arija, V., and Canals, J.: Composition of Gut Microbiota in Children with Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. Nutrients 12, 792 (2020).

Jaggar, M., Rea, K., Spichak, S., Dinan, T.G., and Cryan, J.F.: You've got male: Sex and the microbiota-gut-brain axis across the lifespan. Front. Neuroendocrinol. 56, 100815 (2020).

Kang, D.W., Adams, J.B., Gregory, A.C., Borody, T., Chittick, L., Fasano, A., Khoruts, A., Geis, E., Maldonado, J., McDonough-Means, S., Pollard, E.L., Roux, S., Sadowsky, M.J., Schwarzberg Lipson, K., Sullivan, M.B., Caporaso, J.G., and Krajmalnik-Brown, R.: Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. Microbiome 5, 10 (2017).

Kang, D.W., Adams, J.B., Coleman, D.M., Pollard, E.L., Maldonado, J., McDonough-Means, S., Caporaso, J.G., and Krajmalnik-Brown, R.: Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. Sci. Rep. 9, 5821 (2019).

Kang, D.W., Adams, J.B., Vargason, T., Santiago, M., Hahn, J., and Krajmalnik-Brown, R.: Distinct Fecal and Plasma Metabolites in Children with Autism Spectrum Disorders and Their Modulation after Microbiota Transfer Therapy. mSphere 5, e00314-20 (2020).

Kimura, I., Miyamoto, J., Ohue-Kitano, R., Watanabe, K., Yamada, T., Onuki, M., Aoki, R., Isobe, Y., Kashihara, D., Inoue, D., Inaba, A., Takamura, Y., Taira, S., Kumaki, S., Watanabe, M., Ito, M., Nakagawa, F., Irie, J., Kakuta, H., Shinohara, M., Iwatsuki, K., Tsujimoto, G., Ohno, H., Arita, M., Itoh, H., and Hase, K.: Maternal gut microbiota in pregnancy influences offspring metabolic phenotype in mice. Science 367, eaaw8429 (2020).

Leader, G., Abberton, C., Cunningham, S., Gilmartin, K., Grudzien, M., Higgins, E., Joshi, L., Whelan, S., and Mannion, A.: Gastrointestinal Symptoms in Autism Spectrum Disorder: A Systematic Review. Nutrients 14, 1471 (2022).

Lees, E.A., Miyajima, F., Pirmohamed, M., and Carrol, E.D.: The role of Clostridium difficile in the paediatric and neonatal gut - a narrative review. Eur. J. Clin. Microbiol. Infect. Dis. 35, 1047-1057 (2016).

Lord, C., Elsabbagh, M., Baird, G., and Veenstra-Vanderweele, J.: Autism spectrum disorder. Lancet 392, 508-520 (2018).

Luck, B., Horvath, T.D., Engevik, K.A., Ruan, W., Haidacher, S.J., Hoch, K.M., Oezguen, N., Spinler, J.K., Haag, A.M., Versalovic, J., and Engevik, M.A.: Neurotransmitter Profiles Are Altered in the Gut and Brain of Mice Mono-Associated with Bifidobacterium dentium. Biomolecules 11, 1091 (2021).

Lum, G.R., Olson, C.A., and Hsiao, E.Y.: Emerging roles for the intestinal microbiome in epilepsy. Neurobiol. Dis. 135, 104576 (2020).

Madra, M., Ringel, R., and Margolis, K.G.: Gastrointestinal Issues and Autism Spectrum Disorder. Child Adolesc. Psychiatr. Clin. N. Am. 29, 501-513 (2020).

Marenco, S., Meyer, C., van der Veen, J.W., Zhang, Y., Kelly, R., Shen, J., Weinberger, D.R., Dickinson, D., and Berman, K.F.: Role

of gamma-amino-butyric acid in the dorsal anterior cingulate in age-associated changes in cognition. Neuropsychopharmacol. 43, 2285-2291 (2018).

Morton, J.T., Jin, D.M., Mills, R.H., Shao, Y., Rahman, G., McDonald, D., Zhu, Q., Balaban, M., Jiang, Y., Cantrell, K., Gonzalez, A., Carmel, J., Frankiensztajn, L.M., Martin-Brevet, S., Berding, K., Needham, B.D., Zurita, M.F., David, M., Averina, O.V., Kovtun, A.S., Noto, A., Mussap, M., Wang, M., Frank, D.N., Li, E., Zhou, W., Fanos, V., Danilenko, V.N., Wall, D.P., Cárdenas, P., Baldeón, M.E., Jacquemont, S., Koren, O., Elliott, E., Xavier, R.J., Mazmanian, S.K., Knight, R., Gilbert, J.A., Donovan, S.M., Lawley, T.D., Carpenter, B., Bonneau, R., and Taroncher-Oldenburg, G.: Multi-level analysis of the gut-brain axis shows autism spectrum disorder-associated molecular and microbial profiles. Nat. Neurosci. 26, 1208-1217 (2023).

Mukherjee, T., Hovingh, E.S., Foerster, E.G., Abdel-Nour, M., Philpott, D.J., and Girardin, S.E.: NOD1 and NOD2 in inflammation, immunity and disease. Arch. Biochem. Biophys. 670, 69-81 (2019).

Needham, B.D., Tang, W., and Wu, W.L.: Searching for the gut microbial contributing factors to social behavior in rodent models of autism spectrum disorder. Dev. Neurobiol. 78, 474-499 (2018).

Needham, B.D., Adame, M.D., Serena, G., Rose, D.R., Preston, G.M., Conrad, M.C., Campbell, A.S., Donabedian, D.H., Fasano, A., Ashwood, P., and Mazmanian, S.K.: Plasma and Fecal Metabolite Profiles in Autism Spectrum Disorder. Biol. Psychiatry 89, 451-462 (2021).

Needham, B.D., Funabashi, M., Adame, M.D., Wang, Z., Boktor, J.C., Haney, J., Wu, W.L., Rabut, C., Ladinsky, M.S., Hwang, S.J., Guo, Y., Zhu, Q., Griffiths, J.A., Knight, R., Bjorkman, P.J., Shapiro, M.G., Geschwind, D.H., Holschneider, D.P., Fischbach, M.A., and Mazmanian, S.K.: A gut-derived metabolite alters brain activity and anxiety behaviour in mice. Nature 602, 647-653 (2022).

Neufeld, K.M., Kang, N., Bienenstock, J., and Foster, J.A.: Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol. Motil. 23, 255-264 (2011).

Neuman, H., Forsythe, P., Uzan, A., Avni, O., and Koren, O.: Antibiotics in early life: dysbiosis and the damage done. FEMS Microbiol. Rev. 42, 489-499 (2018).

Niesler, B. and Rappold, G.A.: Emerging evidence for gene mutations driving both brain and gut dysfunction in autism spectrum disorder. Mol. Psychiatry 26, 1442-1444 (2021).

Otten, K., Keller, L., Puiu, A.A., Herpertz-Dahlmann, B., Seitz, J., Kohn, N., Edgar, J.C., Wagels, L., and Konrad, K.: Pre- and postnatal antibiotic exposure and risk of developing attention deficit hyperactivity disorder-A systematic review and meta-analysis combining evidence from human and animal studies. Neurosci. Biobehav. Rev. 140, 104776 (2022).

Ozonoff, S., Young, G.S., Belding, A., Hill, M., Hill, A., Hutman, T., Johnson, S., Miller, M., Rogers, S.J., Schwichtenberg, A.J., Steinfeld, M., and Losif, A.-M.: The broader autism phenotype in infancy: when does it emerge? J. Am. Acad. Child Adolesc. Psychiatry 53, 398-407 (2014).

Palmeira, O., Matos, L.R.B., Naslavsky, M.S., Bueno, H.M.S., Soler, J.P., Setubal, J.C., and Zatz, M.: Longitudinal 16S rRNA gut microbiota data of infant triplets show partial susceptibility to host genetics. iScience 25, 103861 (2022).

Pusceddu, M.M., Barboza, M., Keogh, C.E., Schneider, M., Stokes, P., Sladek, J.A., Kim, H.J.D., Torres-Fuentes, C., Goldfild, L.R., Gillis, S.E., Brust-Mascher, I., Rabasa, G., Wong, K.A., Lebrilla, C., Byndloss, M.X., Maisonneuve, C., Bäumler, A.J., Philpott, D.J., Ferrero, R.L., Barrett, K.E., Reardon, C., and Gareau, M.G.: Nod-like receptors are critical for gut-brain axis signalling in mice. J. Physiol. 597, 5777-5797 (2019).

Royet, J., Gupta, D., and Dziarski, R.: Peptidoglycan recognition proteins: modulators of the microbiome and inflammation.

Nat. Rev. Immunol. 11, 837-851 (2011).
Sandler, R.H., Finegold, S.M., Bolte, E.R., Buchanan, C.P., Maxwell, A.P., Vaisanen, M.L., Nelson, M.N., and Wexler, H.M.: Short-term benefit from oral vancomycin treatment of regressive-onset autism. J. Child. Neurol. 15, 429-435 (2000).
Sgritta, M., Dooling, S.W., Buffington, S.A., Momin, E.N., Francis, M.B., Britton, R.A., and Costa-Mattioli, M.: Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. Neuron 101, 246-259 (2019).
Sharon, G., Cruz, N.J., Kang, D.W., Gandal, M.J., Wang, B., Kim, Y.M., Zink, E.M., Casey, C.P., Taylor, B.C., Lane, C.J., Bramer, L.M., Isern, N.G., Hoyt, D.W., Noecker, C., Sweredoski, M.J., Moradian, A., Borenstein, E., Jansson, J.K., Knight, R., Metz, T.O., Lois, C., Geschwind, D.H., Krajmalnik-Brown, R., and Mazmanian, S.K.: Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. Cell 177, 1600-1618 (2019).
Swann, J.R., Spitzer, S.O., and Diaz Heijtz, R.: Developmental Signatures of Microbiota-Derived Metabolites in the Mouse Brain. Metabolites 10, 172 (2020).
Tabouy, L., Getselter, D., Ziv, O., Karpuj, M., Tabouy, T., Lukic, I., Maayouf, R., Werbner, N., Ben-Amram, H., Nuriel-Ohayon, M., Koren, O., and Elliot, E.: Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. Brain Behav. Immun. 73, 310-319 (2018).
Thion, M.S., Low, D., Silvin, A., Chen, J., Grisel, P., Schulte-Schrepping, J., Blecher, R., Ulas, T., Squarzoni, P., Hoeffel, G., Coulpier, F., Siopi, E., David, F.S., Scholz, C., Shihui, F., Lum, J., Amoyo, A.A., Larbi, A., Poidinger, M., Buttgereit, A., Lledo, P.-M., Greter, M., Chan, J.K.Y., Amit, I., Beyer, M., Schultze, J.L., Schlitzer, A., Pettersson, S., Ginhoux, F., and Garel, S.: Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner. Cell 172, 500-516 (2018).
Vuong, H.E., Pronovost, G.N., Williams, D.W., Coley, E.J.L., Siegler, E.L., Qiu, A., Kazantsev, M., Wilson, C.J., Rendon, T., and Hsiao, E.Y.: The maternal microbiome modulates fetal neurodevelopment in mice. Nature 586, 281-286 (2020).
Wang, L.W., Tancredi, D.J., and Thomas, D.W.: The prevalence of gastrointestinal problems in children across the United States with autism spectrum disorders from families with multiple affected members. J. Dev. Behav. Pediatr. 32, 351-360 (2011).
Wheeler, R., Bastos, P.A.D., Disson, O., Rifflet, A., Gabanyi, I., Spielbauer, J., Berard, M., Lecuit, M., and Boneca, I.G.: Microbiota-induced active translocation of peptidoglycan across the intestinal barrier dictates its within-host dissemination. Proc. Natl. Acad. Sci. USA 120, e2209936120 (2023).
Xu, M., Xu, X., Li, J., and Li, F.: Association Between Gut Microbiota and Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. Front. Psychiatry 10, 473 (2019).
Yap, C.X., Henders, A.K., Alvares, G.A., Wood, D.L.A., Krause, L., Tyson, G.W., Restuadi, R., Wallace, L., McLaren, T., Hansell, N.K., Cleary, D., Grove, R., Hafekost, C., Harun, A., Holdsworth, H., Jellett, R., Khan, F., Lawson, L.P., Leslie, J., Frenk, M.L., Masi, A., Mathew, N.E., Muniandy, M., Nothard, M., Miller, J.L., Nunn, L., Holtmann, G., Strike, L.T., de Zubicaray, G.I., Thompson, P.M., McMahon, K.L., Wright, M.J., Visscher, P.M., Dawson, P.A., Dissanayake, C., Eapen, V., Heussler, H.S., McRae, A.F., Whitehouse, A.J.O., Wray, N.R., and Gratten, J.: Autism-related dietary preferences mediate autism-gut microbiome associations. Cell 184, 5916-5931 (2021).
Zeidan, J., Fombonne, E., Scorah, J., Ibrahim, A., Durkin, M.S., Saxena, S., Yusuf, A., Shih, A., and Elsabbagh, M.: Global prevalence of autism: A systematic review update. Autism Res. 15, 778-790 (2022).
Zhao, H., Mao, X., Zhu, C., Zou, X., Peng, F., Yang, W., Li, B., Li, G., Ge, T., and Cui, R.:

GABAergic System Dysfunction in Autism Spectrum Disorders. Front. Cell Dev. Biol. 9, 781327 (2021).

Zheng, Y., Bek, M.K., Prince, N.Z., Peralta Marzal, L.N., Garssen, J., Perez Pardo, P., and Kraneveld, A.D.: The Role of Bacterial-Derived Aromatic Amino Acids Metabolites Relevant in Autism Spectrum Disorders: A Comprehensive Review. Front. Neurosci. 15, 738220 (2021).

Zuffa, S., Schimmel, P., Gonzalez-Santana, A., Belzer, C., Knol, J., Bolte, S., Falck-Ytter, T., Forssberg, H., Swann, J., and Diaz Heijtz, R.: Early-life differences in the gut microbiota composition and functionality of infants at elevated likelihood of developing autism spectrum disorder. Transl. Psychiatry 13, 257 (2023).

GUT MICROBIOME: THE MISSING INGREDIENT IN CELIAC DISEASE?

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SUMMARY

Immune mediated diseases, including celiac disease, are increasingly prevalent affecting 1% of most populations worldwide. Celiac disease is characterized by a loss of oral tolerance to the prolamin family of proteins in wheat, rye, and barley, collectively called "gluten" in genetically susceptible individuals. The only available treatment is a strict gluten-free diet for life, which is difficult to adhere to and not always effective. Genes and gluten are necessary but insufficient to develop celiac disease, as a minority of those individuals genetically at risk will develop it. Microbes, both in the form of infections or alterations in the intestinal microbiome, are considered key additional environmental factors, but the mechanisms remain unclear. Gluten proteins are resistant to proteolysis by host enzymes. Thus, in this review we emphasize the metabolic capacity of the small intestinal microbiota and discuss how it can be altered to facilitate gluten degradation in patients with celiac disease. We also discuss the potential role of other metabolic alterations that can contribute to a proinflammatory environment, such as altered tryptophan metabolism and defective activation of the Aryl hydrocarbon receptor (AhR) pathway. Understanding precise microbiota-driven mechanisms in celiac disease will help develop adjuvant therapies to the gluten-free diet based on optimal microbial modulation of gluten protein metabolism or AhR activation.

A MODEL DISEASE TO UNDERSTAND HOW ENVIRONMENTAL FACTORS MODULATE AUTOIMMUNITY

Celiac disease is an autoimmune condition, triggered by the ingestion of gluten, the collective name given to storage proteins in wheat, rye, and barley. The disease is strongly associated with HLA-DQ, and almost all celiac patients carry the human leukocyte antigen HLA-DQ2.5, HLA-DQ2.2 or HLA-DQ8 (*Iversen* and *Sollid*, 2023). Celiac disease onset can occur at any age, and its presentation may relate to the genetic load, with DQ2.5 homozygosis associated with greater risk for childhood onset, and additional environmental triggers such as infections, antibiotics, especially if concomitant with high gluten exposure (*Verdu* et al., 2015). Once developed, autoimmune features include production of specific autoantibodies called anti-tissue transglutaminase (anti-tTG IgA), and immune-mediated killing of enterocytes. Pathologically, the hallmark lesion is the blunting of small intestinal villi and crypt hyperplasia (atrophy). Clinically, symptomatic celiac disease associates with nutritional deficiencies, and gastrointestinal symptoms, including changes in bowel habits and abdominal pain. However, patients, especially in adulthood, can present with extra-intestinal, and systemic manifestations such as

psychiatric and neurological symptoms, anaemia, infertility, osteoporosis, among others (*Catassi* et al., 2022). Potential celiac disease is characterized by activation of the adaptive immune response with persistently positive anti-tTG IgA antibodies and normal-appearing duodenal mucosa. Unfortunately, asymptomatic cases, despite the presence of small intestinal atrophy are common, and these patients are difficult to diagnose until complications arise related to micronutrient deficiencies (*Theethira* et al., 2014), bone fractures and even the higher incidence of certain cancers (*Catassi* et al., 2022). There is currently no pharmacological treatment for celiac disease, which is managed by a strict gluten-free diet (GFD) for life.

Compared with other autoimmune diseases, such as type 1 diabetes (T1D) or multiple sclerosis, celiac disease is the only one where the main necessary environmental driver, gluten, has been identified, and in which the autoimmune features such as gluten-specific $CD4^+$T cells, anti-tTG antibodies and immune mediated epithelial cell killing, can be turned "on" or "off" by its ingestion or the GFD, respectively. Gluten is a mixture of proteins, that in wheat include gliadins and glutenins, secalins in rye, and hordeins in barley. All gluten proteins are resistant to complete degradation by mammalian enzymes, which results in the production of large peptides with immunogenic sequences, such as the 33-mer peptide, that trigger inflammation in celiac patients (*Shan* et al., 2022). Partially digested gluten peptides translocate the mucosal barrier and are deamidated by tTG (*Dieterich* et al., 1997). Gluten peptide deamidation increases their affinity to HLA-DQ2 or DQ8 in antigen-presenting cells, initiating the T-cell-mediated inflammation. HLA-DQ2 or DQ8 is therefore the second, necessary factor to develop celiac disease. About 30-40% of the worldwide population carry one, or a combination of these genes, and therefore are "at risk" to develop it when consuming a gluten. However only a minority of those at risk (3-4%), and consuming gluten, will develop celiac disease. This, together with the fact celiac disease prevalence has quadrupled in the past 40 years, in parallel with the increase in prevalence with other autoimmune conditions, such as T1D with which it associates (*Verdu* and *Danska*, 2018), suggests other environmental factors are at play.

THE EMERGING ROLE OF THE MICROBIOME IN CELIAC DISEASE PATHOGENESIS

Both viral and bacterial infections have been associated with higher celiac disease risk, and this phenomenon has been reviewed extensively elsewhere (*Caminero* et al., 2019). In the past decade, microbiome alterations have been described in celiac patients (*Verdu* and *Schuppan*, 2021), but the mechanisms through which changes in human-associated microbial communities influence disease pathogenesis have been elusive.

ROLE OF MICROBIAL METABOLISM IN CELIAC DISEASE

We have shown that duodenal bacteria participate in gluten metabolism, and that depending on the type of bacteria present, microbial enzymes may yield peptides with enhanced or reduced immunogenicity. We found that elastase

from the opportunistic pathogen *Pseudomonas aeruginosa* degrades gluten, producing immunogenic peptides that better translocate the epithelial barrier, and stimulate gluten-specific T cells from patients with celiac disease. On the other hand, *Lactobacillus* species, isolated from a healthy control, further detoxify the *P. aeruginosa* generated immunogenic peptides (*Caminero* et al., 2016). These findings are supported by our follow-up study, where we showed that although duodenal microbiota composition in active CeD patients differs from that in non-celiac controls, the changes are specific to localized regions of the small proximal intestine, namely first, second and third portion of the duodenum. Moreover, duodenal microbiota from celiac patients also had an altered predicted gluten proteolytic profile, which was location specific. The altered proteolytic profile translated to functional differences *in vivo*, as mice colonized with duodenal microbiota from active celiac patients had impaired capacity to degrade gluten, while mice colonized with duodenal microbiota from control subjects, including those with genetic predisposition for celiac disease, effectively degraded gluten (*Constante* et al., 2022). Thus, taken together, these results indicate that microbial gluten metabolism is a) location specific and, b) associated with the condition of active celiac disease. It is possible altered microbial "glutenasic" function is both driver and consequence of inflammation in the small intestine. This insight has clear preventive and therapeutic implications. This mechanism could be targeted for preventive or therapeutic purposes, based on increasing the effectiveness of gluten detoxification by microbes adapted to survive in the duodenum.

In addition to microbial gluten metabolism, other pathways of altered microbial metabolism could be at play in celiac disease. Tryptophan is an essential amino acid provided by foods such as poultry and cruciferous vegetables. After digestion, tryptophan becomes available for further metabolism by the host through the kynurenine or serotonin pathway, or by certain gut microbes, such as lactobacilli. Microbial metabolism of tryptophan results in the production of indole and its derivatives (indole-3-aldehyde (IAld), indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-acetaldehyde (IAAld), indole-3-lactic acid (ILA) and indole-acrylic acid), as well as tryptamine, many of which are ligands for the aryl hydrocarbon receptor (AhR) (*Agus* et al., 2018). AhR signalling contributes to maintaining intestinal homeostasis through its role in epithelial renewal and maintaining barrier integrity. Thus, the indole pathway, mainly dictated by microbial metabolism of tryptophan, is beneficial for the host. Because host and microbial tryptophan metabolic pathways are interconnected and in equilibrium, a reduction in the microbial metabolic pathway of tryptophan could provide more substrate to the remaining two metabolic pathways. Indeed, this has been shown in several chronic inflammatory diseases, where an increase in the kynurenine pathway, has been demonstrated and proposed to be proinflammatory (*Tennyson* et al., 2016; *Natividad* et al., 2018). However, a recent paper has suggested some kynurenine metabolites could play an anti-inflammatory role in IBD (*Michaudel* et al., 2023). Thus, interventions that target tryptophan metabolic pathways need to consider the complexity of its metabolism, the location of disease and thus dietary supplementation may be an attractive approach, especially for small intestinal conditions, as most microbe-dietary interactions (except for fermentable fibres) will occur in the upper gastrointestinal tract.

We demonstrated that patients with active celiac disease have decreased AhR expression in the duodenum (*Natividad* et al., 2018). We also showed that the microbiota of active CeD have impaired capacity to metabolize tryptophan to produce AhR ligands, resulting in reduced capacity of the microbiota to activate the AhR pathway, and reduced expression of host genes related to AhR activation. Lower abundance of lactobacilli, known AhR ligand producers, has been described in the small intestine and faeces of patients with active celiac disease. AhR ligand production, AhR activity, and expression of AhR pathway genes were only partially restored 2 years after a GFD. We also demonstrated that in mice expressing the celiac disease risk gene, DQ8, a diet supplemented with 1% tryptophan increased lactobacilli abundance and indole production while ameliorating gluten immunopathology. Finally, in a mouse model, tryptophan supplementation increased endogenous lactobacilli, increased AhR ligands (indoles) and increased AhR activation in the small intestine (*Natividad* et al., 2018). Moreover, tryptophan supplementation improved gluten immunopathology in mice expressing celiac disease risk genes. A clinical trial is underway (https://clinicaltrials.gov/study/NCT03566238) to study the efficacy of tryptophan supplementation in celiac patients that are non-responsive to a gluten-free diet for more than 1 year. We hypothesize that tryptophan supplementation will lead to resolution of persistent symptoms in celiac disease, while increasing taxa that metabolize tryptophan and restore AhR signalling.

LIMITATIONS OF THE GFD AND NEW THERAPIES IN CED

The only treatment for celiac disease is a strict gluten-free diet (GFD) and dietary compliance is essential, not only for intestinal mucosal recovery and alleviation of symptoms, but also for the prevention of complications such as anaemia, osteoporosis, and small bowel lymphoma (*Michaudel* et al., 2023). However, a GFD is an imperfect treatment, as it is difficult to follow and expensive, resulting in high non-adherence rates. Accidental contamination is common and small amounts of gluten (~50 mg) cause inflammation (*Lindfors* et al, 2019; *Lamas* et al., 2020). Mucosal recovery after starting a GFD is slow, and more than 60% of patients have persistent mucosal inflammation even after 5 years of a GFD (*Silvester* et al., 2020). This is clinically important because long-term, low-grade mucosal injury increases bone fracture risk and nutritional deficiencies. In addition, a large proportion of celiac disease patients are non-responders to a GFD or become symptomatic after initial response (*Catassi* et al., 2007; *Rubio-Tapia* et al., 2010; *Oza* et al., 2016; *Stasi* et al., 2016). Annual health care costs are higher in celiac disease than in non-celiac patients, mainly related to poorly controlled disease. Celiac disease therefore has a significant health, social, and economic burden, highlighting the need for novel or adjuvant therapeutics in addition to GFD. Importantly, celiac disease patients have a very high perceived burden of treatment and desire an adjuvant treatment to the GFD (*Leffler* et al., 2007; *See* et al., 20156; *Pinto-Sanchez* et al., 2015). This reality presents a clearly unmet need for patients with celiac disease which is also evidenced by the intense research and investment into the development of adjuvant therapies to the GFD.

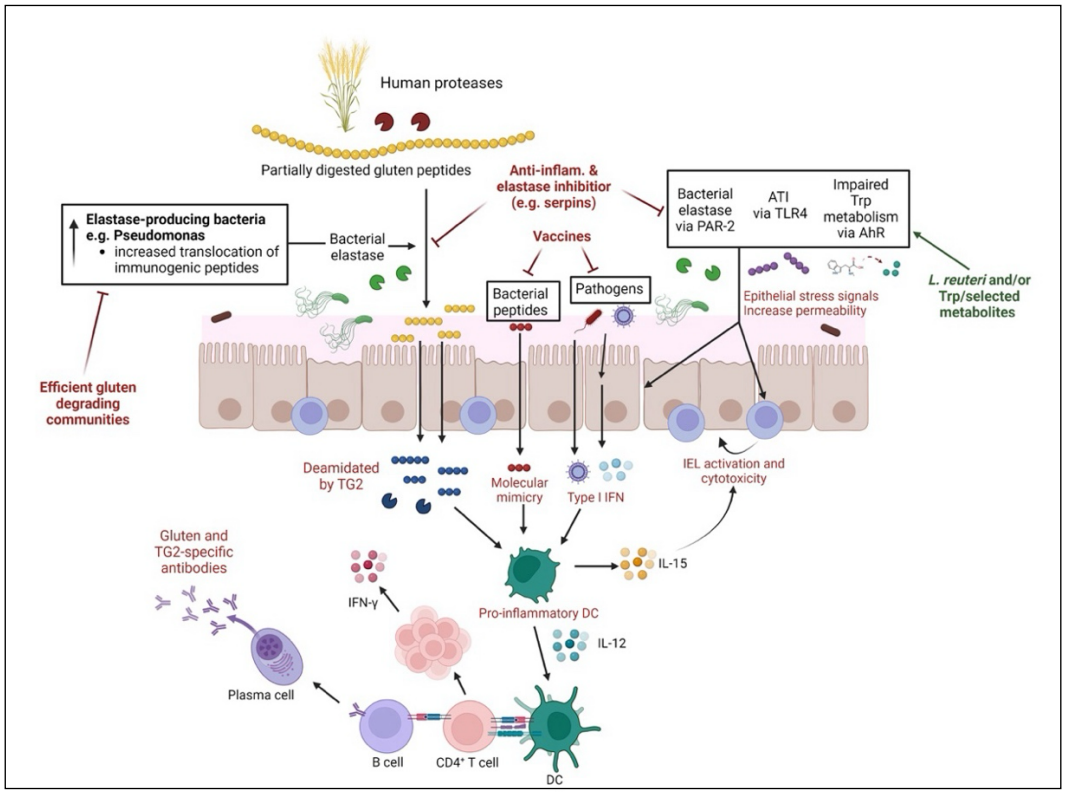

Figure 1: Potential Microbial Therapeutic targets for celiac disease. Owing to the detailed knowledge of celiac immunobiology and microbe-gluten interactions, several druggable targets have emerged. These include, development of gluten degrading communities that effectively detoxify gluten immunogenic sequences, inhibitors of bacterial elastase (e.g. serpins), pre- or probiotic combinations of tryptophan metabolite producers and vaccines aiming at preventing viral infections that could act as additional triggers of autoimmunity. (Adapted from: *Verdu* and *Schuppan*, Nat. Struct. Mol. Biol. 27, 5-7, 2020).

Major pharmaceutical companies have launched pipelines to develop adjunct therapies to the GFD (*Guandalini* et al., 2016) that currently include tTG inhibitors (*Pinto-Sanchez* et al, 2021), TAK-062 a computationally designed enzyme that targets 2 gluten proteins (*McCarville* et al, 2015), TAK-101 a gluten protein shielded within a polymer-based nanoparticle that aims to restore tolerance to gluten (*Schuppan* et al., 2021), and anti-IL-15 antibodies to limit intestinal epithelial cell destruction and for the treatment of refractory celiac disease (*Pultz* et al., 2021) in clinical trials. The use of probiotics for the treatment of celiac disease have previously been proposed (*Cellier* et al., 2019; *Murray* et al., 2023) and shown to be safe if the probiotic is certified gluten free, but this application has not been based on deep knowledge of mechanisms of action, and currently no recommendations of specific probiotics can be made (*De Angelis* et al., 2006). Based on our basic and translational results, we propose to target dysregulated microbial metabolism by developing and characterizing duodenal communities that efficiently degrade gluten or enhance AhR activation, through the supplementation of tryptophan or specific indole metabolites (Figure 1).

MAIN CONCLUSIONS

The small intestinal microbiome has emerged in the last decade as a recognized cofactor in celiac disease pathogenesis. Celiac disease is a unique autoimmune disorder, with a *known* environmental trigger (gluten) that is responsible for the generation of $CD4^+$ T cell mediated inflammation and development of autoantibodies in genetically at-risk individuals. Immune mediated killing of intestinal epithelial cells contributes to intestinal atrophy, and cofactors that are independent of gluten may modulate risk. Thus, celiac disease is an ideal model disease to advance microbiomics and therapeutic microbiology towards clinical applications by targeting precise microbial mechanisms that impact key steps in its pathogenesis.

LITERATURE

Agus, A., Planchais, J., and Sokol, H.: Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe 23, 716-724 (2018).

Caminero, A., Galipeau, H.J., McCarville, J.L., Johnston, C.W., Bernier, S.P., Russell, A.K., Jury, J., Herran, A.R., Casqueiro, J., Tye-Din, J.A., Surette, M.G., Magarvey, N.A., Schuppan, D., and Verdu, E.F.: Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. Gastroenterology 151, 670-683 (2016).

Caminero, A., Meisel, M., Jabri, B., and Verdu, E.F.: Mechanisms by which gut microorganisms influence food sensitivities. Nat. Rev. Gastroenterol. Hepatol. 16, 7-18 (2019).

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., and Fasano, A.: A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. Am. J. Clin. Nutr. 85, 160-166 (2007).

Catassi, C., Verdu, E.F., Bai, J.C., and Lionetti, E.: Coeliac disease. Lancet 399(10344), 2413-2426 (2022).

Cellier, C., Bouma, G., van Gils, T., Khater, S., Malamut, G., Crespo, L., Collin, P., Green, P.H.R., Crowe, S.E., Tsuji, W., Butz, E., Cerf-Bensussan, N., Macintyre, E., Parnes, J.R., Leon, F., Hermine, O., Mulder, C.J.; RCD-II Study Group Investigators. Safety and efficacy of AMG 714 in patients with type 2 refractory coeliac disease: a phase 2a, randomised, double-blind, placebo-controlled, parallel-group study. Lancet Gastroenterol. Hepatol. 4, 960-970 (2019).

Constante, M., Libertucci, J., Galipeau, H.J., Szamosi, J.C., Rueda, G., Miranda, P.M., Pinto-Sanchez, M.I., Southward, C.M., Rossi, L., Fontes, M.E., Chirdo, F.G., Surette, M.G., Bercik, P., Caminero, A., and Verdu, E.F.: Biogeographic Variation and Functional Pathways of the Gut Microbiota in Celiac Disease. Gastroenterology 163, 1351-1363 (2022).

De Angelis, M., Rizzello, C.G., Fasano, A., Clemente, M.G., De Simone, C., Silano, M., De Vincenzi, M., Losito, I., and Gobbetti, M.: VSL#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue. Biochim. Biophys. Acta 1762, 80-93 (2006).

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., and Schuppan, D.: Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat. Med. 3, 797-801 (1997).

Guandalini, S., Tundia, N., Thakkar, R., Macaulay, D., Essenmacher, .K, and Fuldeore, M.: Direct Costs in Patients with Celiac Disease in the USA: A Retrospective

Claims Analysis. Dig. Dis. Sci. 61, 2823-2830 (2016).

Iversen, R. and Sollid, L.M.: The Immunobiology and Pathogenesis of Celiac Disease. Annu. Rev. Pathol. 18, 47-70 (2023).

Lamas, B., Hernandez-Galan, L., Galipeau, H.J., Constante, M., Clarizio, A., Jury, J., Breyner, N.M., Caminero, A., Rueda, G., Hayes, C.L., McCarville, J.L., Bermudez Brito, M., Planchais, J., Rolhion, N., Murray, J.A., Langella, P., Loonen, L.M.P., Wells, J.M., Bercik, P., Sokol, H., and Verdu, E.F.: Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation. Sci. Transl. Med. 12 (566), eaba0624 (2020).

Leffler, D.A., Dennis, M., Hyett, B., Kelly, E., Schuppan, D., and Kelly, C.P.: Etiologies and predictors of diagnosis in nonresponsive celiac disease. Clin. Gastroenterol. Hepatol. 5, 445-450 (2007).

Lindfors, K., Ciacci, C., Kurppa, K., Lundin, K.E.A., Makharia, G.K., Mearin, M.L., Murray, J.A., Verdu, E.F., and Kaukinen, K.: Coeliac disease. Nat. Rev. Dis. Primers 5, 3 (2019).

McCarville, J.L., Caminero, A., and Verdu, E.F.: Pharmacological approaches in celiac disease. Curr. Opin. Pharmacol. 25, 7-12 (2015).

Michaudel, C., Danne, C., Agus, A., Magniez, A., Aucouturier, A., Spatz, M., Lefevre, A., Kirchgesner, J., Rolhion, N., Wang, Y., Lavelle, A., Galbert, C., Da Costa, G., Poirier, M., Lapière, A., Planchais, J., Nádvorník, P., Illes, P., Oeuvray, C., Creusot, L., Michel, M.L., Benech, N., Bourrier, A., Nion-Larmurier, I., Landman, C., Richard, M.L., Emond, P., Seksik, P., Beaugerie, L., Arguello, R.R., Moulin, D., Mani, S., Dvorák, Z., Bermúdez-Humarán, L.G., Langella, P., and Sokol, H.: Rewiring the altered tryptophan metabolism as a novel therapeutic strategy in inflammatory bowel diseases. Gut 72, 1296-1307 (2023).

Murray, J.A., Wassaf, D., Dunn, K., Arora, S., Winkle, P., Stacey, H., Cooper, S., Goldstein, K.E., Manchanda, R., Kontos, S., and Grebe, K.M.: Safety and tolerability of KAN-101, a liver-targeted immune tolerance therapy, in patients with coeliac disease (ACeD): a phase 1 trial. Lancet Gastroenterol. Hepatol. S2468-1253(23)00107-3 (2023).

Natividad, J.M., Agus, A., Planchais, J., Lamas, B., Jarry, A.C., Martin, R., Michel, M.L., Chong-Nguyen, C., Roussel, R., Straube, M., Jegou, S., McQuitty, C., Le Gall, M., da Costa, G., Lecornet, E., Michaudel, C., Modoux, M., Glodt, J., Bridonneau, C., Sovran, B., Dupraz, L., Bado, A., Richard, M.L., Langella, P., Hansel, B., Launay, J.M., Xavier, R.J., Duboc, H., and Sokol, H.: Impaired Aryl Hydrocarbon Receptor Ligand Production by the Gut Microbiota Is a Key Factor in Metabolic Syndrome. Cell Metab. 28, 737-749 (2018).

Oza, S.S., Akbari, M., Kelly, C.P., Hansen, J., Theethira, T., Tariq, S., Dennis, M., and Leffler, D.A.: Socioeconomic Risk Factors for Celiac Disease Burden and Symptoms. J. Clin. Gastroenterol. 50, 307-312 (2016).

Pinto-Sanchez, M.I., Verdu, E.F., Gordillo, M.C., Bai, J.C., Birch, S., Moayyedi, P., and Bercik, P.: Tax-deductible provisions for gluten-free diet in Canada compared with systems for gluten-free diet coverage available in various countries. Can. J. Gastroenterol. Hepatol. 29, 104-110 (2015).

Pinto-Sanchez, M.I., Silvester, J.A., Lebwohl, B., Leffler, D.A., Anderson, R.P., Therrien, A., Kelly, C.P., and Verdu, E.F.: Society for the Study of Celiac Disease position statement on gaps and opportunities in coeliac disease. Nat. Rev. Gastroenterol. Hepatol. 18, 875-884 (2021).

Pultz, I.S., Hill, M., Vitanza, J.M., Wolf, C., Saaby, L., Liu, T., Winkle, P., and Leffler, D.A.: Gluten Degradation, Pharmacokinetics, Safety, and Tolerability of TAK-062, an Engineered Enzyme to Treat Celiac Disease. Gastroenterology 161, 81-93 (2021).

Rubio-Tapia, A., Rahim, M.W., See, J.A., Lahr, B.D., Wu, T.T., and Murray, J.A.: Mucosal recovery and mortality in adults with celiac disease after treatment with a gluten-free

diet. Am. J. Gastroenterol. 105, 1412-1420 (2010).

Schuppan, D., Mäki, M., Lundin, K.E.A., Isola, J., Friesing-Sosnik, T., Taavela, J., Popp, A., Koskenpato, J., Langhorst, J., Hovde, Ø., Lähdeaho, M.L., Fusco, S., Schumann, M., Török, H.P., Kupcinskas, J., Zopf, Y., Lohse, A.W., Scheinin, M., Kull, K., Biedermann, L., Byrnes, V., Stallmach, A., Jahnsen, J., Zeitz, J., Mohrbacher, R., Greinwald, R.; CEC.-3 Trial Group: A Randomized Trial of a Transglutaminase 2 Inhibitor for Celiac Disease. N. Engl. J. Med. 385, 35-45 (2021).

See, J.A., Kaukinen, K., Makharia, G.K., Gibson, P.R., and Murray, J.A.: Practical insights into gluten-free diets. Nat. Rev. Gastroenterol. Hepatol. 12, 580-591 (2015).

Seiler, C.L., Kiflen, M., Stefanolo, J.P., Bai, J.C., Bercik, P., Kelly, C.P., Verdu, E.F., Moayyedi, P. and Pinto-Sanchez, M.I.: Probiotics for Celiac Disease: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Am. J. Gastroenterol. 115, 1584-1595 (2020).

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., and Khosla, C.: Structural basis for gluten intolerance in celiac sprue. Science 97, 2275-2279 (2022).

Silvester, J.A., Comino, I., Kelly, C.P., Sousa, C., Duerksen, D.R.; DOGGIE BAG Study Group.: Most Patients With Celiac Disease on Gluten-Free Diets Consume Measurable Amounts of Gluten. Gastroenterology 158, 1497-1499 (2020).

Smecuol, E., Constante, M., Temprano, M.P., Costa, A.F., Moreno, M.L., Pinto-Sanchez, M.I., Vázquez, H., Stefanolo, J.P., Gonzalez, A.F., D'Adamo, C.R., Niveloni, S.I., Mauriño, E., Verdu, E.F., and Bai, J.C.: Effect of Bifidobacterium infantis NLS super strain in symptomatic coeliac disease patients on long-term gluten-free diet - an exploratory study. Benef. Microbes 11, 527-534 (2020).

Stasi, E., Marafini, I., Caruso, R., Soderino, F., Angelucci, E., Del Vecchio Blanco, G., Paoluzi, O.A., Calabrese, E., Sedda, S., Zorzi, F., Pallone, F., and Monteleone, G.: Frequency and Cause of Persistent Symptoms in Celiac Disease Patients on a Long-term Gluten-free Diet. J. Clin. Gastroenterol. 50, 239-243 (2016).

Tennyson, C.A., Simpson, S., Lebwohl, B., Lewis, S., and Green, P.H.: Interest in medical therapy for celiac disease. Therap. Adv. Gastroenterol. 6, 358-364 (2013).

Theethira, T.G., Dennis, M., and Leffler, D.A.: Nutritional consequences of celiac disease and the gluten-free diet. Expert Rev. Gastroenterol. Hepatol. 8, 123-129 (2014).

Verdu, E.F., Galipeau, H.J., and Jabri, B.: Novel players in coeliac disease pathogenesis: role of the gut microbiota. Nat. Rev. Gastroenterol. Hepatol. 12, 497-506 (2015).

Verdu, E.F. and Danska, J.S.: Common ground: shared risk factors for type 1 diabetes and celiac disease. Nat Immunol. 19, 685-695 (2018).

Verdu, E.F. and Schuppan, D.: Co-factors, Microbes, and Immunogenetics in Celiac Disease to Guide Novel Approaches for Diagnosis and Treatment. Gastroenterology 161, 1395-1411 (2021).

NEUROIMMUNE MECHANISMS IN THE MICROBIOTA-GUT-BRAIN AXIS

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INTRODUCTION

Gut microbiota has been increasingly recognized as a key factor in human health and disease, shaping and educating our immune system, participating in nutrient digestion and absorption, affecting host metabolism, playing key roles in gastrointestinal tract function, and even affecting distant organs, such as the brain (*Collins* et al., 2012; *Macpherson* et al., 2023). There are many pathways through which the gut microbiome can communicate with the brain, including the immune system, neuroendocrine system as well as using direct communication with the neural system (*Collins* et al., 2012).

GUT-BRAIN COMMUNICATION

Bacteria can signal through immune cells, as neurons express receptors for many cytokines and chemokines, which help to shape the function and development of the neural system (*Deverman* and *Patterson*, 2009). Multiple studies demonstrated that bacteria influence the activation of peripheral immune cells, thus regulating responses in neuroinflammation, brain injury, autoimmunity and neurogenesis (*Fung* et al., 2017).

Serotonin is one of the most abundant neurotransmitters in the gut and the brain, with effects on mood, cognition, and memory. Most of the human serotonin is being produced within the gut by the enteroendocrine cells (*O'Mahony* et al., 2015), and the intestinal microbiome can affect this production. It has been shown that indigenous spore-forming bacteria promote serotonin biosynthesis from colonic enterochromaffin cells, which supply 5-HT to the mucosa, lumen and circulating platelets, significantly impacting host physiology (*Yano* et al., 2015).

Gut bacteria produce many bioactive molecules that can affect the neural system as byproducts of bacterial fermentation, including short chain fatty acids (SCFAs), but they can also produce neurotransmitters found in mammals, including acetylcholine and GABA (*Lyte*, 2014). *Yunes* and colleagues showed that among 135 strains of human-derived Lactobacilli and Bifidobacteria strains, 43% had ability to produce GABA from its precursor monosodium glutamate (*Yunes* et al., 2016). Furthermore, the genes underlying GABA production, gadB and gadC, were also identified in the phyla Bacteroidetes, Proteobacterium and Firmicutes, indicating that the ability to produce GABA are widely distributed among many gut-derived bacterial species.

Gut bacteria can signal directly to the brain through neural pathways, such as the vagus nerve, which likely serves as an early danger signal during infection (*Goehler* et al., 2005), but can also mediate beneficial effects of probiotics

on anxiety-like behaviour (*Bercik* et al., 2011a; *Bravo* et al., 2011). The communication between the gut and the brain is bi-directional, as neural mediators, as well as stress can affect the composition and function of the gut microbiome. For example, adherence of *Escherichia coli* O157:H7 to murine caecal mucosa can be modulated by norepinephrine and dopamine, suggesting that stress exposure may influence host susceptibility to enteric infections (*Chen* et al., 2003). Furthermore, chronic social stress alters the composition of the gut microbiome and exacerbates *Citrobacter rodentium*-induced inflammation (*Galley* et al., 2017), which can be improved by treatment with probiotics.

The gut microbiome affects the function as well as the structure of the brain. Compared to conventionally raised mice, germ-free mice display more exploratory (anxiolytic) and less depressive-like behaviour associated with altered expression of multiple genes regulating neurotransmitter and neurotrophin production (*Bercik* et al., 2011b; *Diaz Heijtz* et al., 2011). Germ-free mice also have altered blood-brain barriers, changes in morphology of the amygdala and hippocampus, altered myelination profiles and plasticity, and global defects in microglia (*Braniste* et al., 2014; *Hoban* et al., 2016; *Luczynski* et al., 2016; *Erny* et al., 2017). Interestingly, most of these abnormalities, including behaviour, normalize after bacterial colonization. However, it is unknown what specific neuroimmune mechanisms mediate and initiate these changes.

THE GUT MICROBIOME AND BEHAVIOUR

We studied mouse behaviour, in germ-free conditions and at several time points after colonization with either complex or simple microbiota. We confirmed that germ-free mice display more exploratory and less depressive-like behaviour, as assessed by the light preference and the tail suspension tests, respectively, compared to mice born and raised conventionally (in the presence of microbiota). Colonization of adult germ-free mice with either complex (Specific Pathogen Free) or simple (Altered Schaedler Flora) microbiota normalized their behaviour and altered expression of brain neurotrophins; these changes were observed already at 2 weeks post-colonization. Interestingly, monocolonization of mice with a laboratory strain of *E. coli* was sufficient to normalize their behaviour and brain chemistry.

We found that this behavioural normalization was dependent on the activation of the innate, but not the adaptive, arm of the immune system through TLR and NOD signalling, and that intestinal dendritic cells but not macrophages played a key role in this process. Finally, we discovered that the normalization of behaviour was associated with many changes in the neuroimmune system, both in the gut and the brain, which included altered expression of multiple brain proteins involved in neural plasticity (*Philip* and *Kraimi*, unpublished data).

Although these results were obtained in mice, the observed impact of the bacterial colonization on the neuroimmune system can be likely extrapolated to humans, bearing on psychiatric conditions, in which altered innate immune signalling has been implicated (*Ratajczak* et al., 2018). Indeed, our results raise the possibility that perturbation of initial (postnatal) microbiota-host cross-talk could affect normal brain development and function. In that respect, a large population-based study

found that bacterial infections and use of antibiotics at very early age increase risk of developing psychiatric diseases later in life (*Köhler-Forsberg* et al., 2019).

THE GUT MICROBIOME AND PAIN

Apart from mood and behaviour, the gut microbiome has been shown to affect perception of somatic and visceral pain (*Verdú* et al., 2006; *Amaral* et al., 2008; *Yang* and *Chiu*, 2017), although the mechanisms are not fully understood. We have recently shown in patients with irritable bowel syndrome (IBS), a disorder where microbiome plays a key pathophysiological role, that altering intake of poorly digestible dietary fibre improves gut symptoms, mainly abdominal pain (*McIntosh* et al., 2017). This was associated with decrease in urinary histamine, which correlated with severity and frequency of pain (*Keshteliu* et al., 2019), as well as changes in the gut microbiota profiles (*McIntosh* et al., 2017). Histamine is a neuroimmune mediator, produced by mast cells and basophils, known to be involved in the control of intestinal permeability and visceral sensitivity (*Boeckxstaens* and *Wouters*, 2017). However, histamine can be produced by gut bacteria as many microbes possess the histidine decarboxylase (*hdc*) gene encoding the enzyme capable of converting histidine into histamine (*Takahashi* et al., 2003; *Fiorani* et al., 2023).

To investigate the putative role of gut microbiome in the abdominal pain in IBS patients we employed our previously developed microbiota-humanized mouse model, that mimics many features of IBS, including altered gastrointestinal transit, low grade inflammation, increased intestinal permeability and anxiety-like behaviour (*De Palma* et al., 2017). We chose faecal microbiota samples from IBS patients with either high or low urinary histamine, and used samples from healthy volunteers as control. Mice were then placed on human-like diet with high content fermentable fibre, and their visceral sensitivity was assessed three weeks later.

Although all mice colonized with IBS microbiota displayed greater bowel distension compared to mice with healthy control microbiota, only mice colonized with microbiota from patients with high urinary histamine displayed higher pain responses to colorectal distension (*De Palma* et al., 2022). When incubating caecal contents of these mice with excess histidine, we found 40 times higher production of histamine, compared to caecal contents of mice colonized with microbiota from healthy volunteer or patients with low urinary histamine. We identified a specific bacterium, *Klebsiella aerogenes* MQ, which generated up to 100 times more histamine than any other strain investigated.

When examining host responses to bacterial histamine, we found a greater density of colonic mast cells, which were often co-localized with neural cells. Interestingly, mast cell hyperplasia and co-localization with neural fibres were previously shown in colonic biopsies from patients with IBS, and this finding correlated with severity of abdominal pain (*Barbara* et al., 2004). Using immunohistochemistry, we found that the H4 receptor (H4R), but not H1, H2 or H3 receptor, expression was upregulated in mice with high bacterial histamine and was present in multiple cell types, including goblet cells, mast cells, lymphocytes, macrophages and enteroendocrine cells. Treatment with a

H4R antagonist normalized both pain responses and mast cell levels in mice colonized with histamine producing microbiota.

Placing mice with histamine producing microbiota on a low fermentable diet decreased pain signalling and mast cell levels, which was associated with decrease in colonic lactic acid producing bacteria and lactic acid, a main determinant of colonic pH. We found that *in vitro* histamine production by *K. aerogenes MQ* was pH dependent, in agreement with previous reports (*Landete* and *De las Rivas*, 2008), with optimal pH of 6.5 We thus incubated *K. aerogenes MQ* with lactobacilli and colonized germ-free mice with mixture of lactobacilli and *K. aerogenes MQ*. In both instances, histamine production dramatically decreased suggesting that fermentable fibre creates an ideal environment for histamine production, thus explaining results from our clinical trial (*McIntosh* et al., 2017).

Altogether, our data suggest that bacterial histamine can trigger chronic abdominal pain, at least in a subset of patients with IBS. Based on our own data and a publicly available dataset (*Mars* et al., 2020), we estimate that between 15 and 25% of IBS patients have high histamine producing microbiota. Identifying this subgroup will be crucial as these patients may truly benefit from a low fermentable diet, and presence of *K. aerogenes* or bacteria with similar *hdc* activity in the gut could guide dietary recommendations, microbiota-directed therapies, or possible use of H4 receptor antagonists.

CONCLUSIONS

In summary, accumulating evidence suggest that gut bacteria can interact with both the peripheral and the central neural system, either directly or through immune or endocrine systems. Better understanding of these mechanisms and their implications are likely to help in management of patients with chronic gastrointestinal diseases, as well as neuropsychiatric disorders, in which microbiota has an important pathophysiological role.

LITERATURE

Amaral, F.A., Sachs, D., Costa, V.V., Fagundes, C.T., Cisalpino, D., Cunha, T.M., Ferreira, S.H., Cunha, F.Q., Silva, T.A., Nicoli, J.R., Vieira, L.Q., Souza, D.G., and Teixeira, M.M.: Commensal microbiota is fundamental for the development of inflammatory pain. Proc. Natl. Acad. Sci. USA 105, 2193-2197 (2008).

Barbara, G., Stanghellini, V., De Giorgio, R., Cremon, C., Cottrell, G.S., Santini, D., Pasquinelli, G., Morselli-Labate, A.M., Grady, E.F., Bunnett, N.W., Collins, S.M., and Corinaldesi, R.: Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 126, 693-702 (2004).

Bercik, P., Park, A.J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, Y., Blennerhassett, P.A., Fahnestock, M., Moine, D., Berger, B., Huizinga, J.D., Kunze, W., McLean, P.G., Bergonzelli, G.E., Collins, S.M., and Verdu, E.F.: The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol. Motil. 23, 1132-1139 (2011a).

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., Macri, J., McCoy, K.D., Verdu, E.F., and Collins, S.M.: The intestinal microbiota affect central levels of brain-derived

neurotropic factor and behavior in mice. Gastroenterology 141, 599-609 (2011b).

Boeckxstaens, G.E. and Wouters, M.M.: Neuroimmune factors in functional gastrointestinal disorders: A focus on irritable bowel syndrome. Neurogastroenterol. Motil. 29 (2017).

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., Korecka, A., Bakocevic, N., Ng, L.G., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B.T., Diamond, B., and Pettersson, S.: The gut microbiota influences blood-brain barrier permeability in mice. Sci. Transl. Med. 6, 263ra158 (2014).

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., and Cryan, J.F.: Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. USA 108, 16050-16055 (2011).

Chen, C., Brown, D.R., Xie, Y., Green, B.T., and Lyte, M.: Catecholamines modulate *Escherichia coli* O157:H7 adherence to murine cecal mucosa. Shock 20, 183-188 (2003).

Collins, S.M., Surette, M., and Bercik, P.: The interplay between the intestinal microbiota and the brain. Nat. Rev. Microbiol. 10, 735-742 (2012).

De Palma, G., Lynch, M.D., Lu, J., Dang, V.T., Deng, Y., Jury, J., Umeh, G., Miranda, P.M., Pigrau Pastor, M., Sidani, S., Pinto-Sanchez, M.I., Philip, V., McLean, P.G., Hagelsieb, M.G., Surette, M.G., Bergonzelli, G.E., Verdu, E.F., Britz-McKibbin, P., Neufeld, J.D., Collins, S.M., and Bercik, P.: Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. Sci. Transl. Med. 9, eaaf6397 (2017).

De Palma, G., Shimbori, C., Reed, D.E., Yu, Y., Rabbia, V., Lu, J., Jimenez-Vargas, N., Sessenwein, J., Lopez-Lopez, C., Pigrau, M., Jaramillo-Polanco, J., Zhang, Y., Baerg, L., Manzar, A., Pujo, J., Bai, X., Pinto-Sanchez, M.I., Caminero, A., Madsen, K., Surette, M.G., Beyak, M., Lomax, A.E., Verdu, E.F., Collins, S.M., Vanner, S.J., and Bercik, P.: Histamine production by the gut microbiota induces visceral hyperalgesia through histamine 4 receptor signaling in mice. Sci. Transl. Med. 14, eabj1895 (2022).

Deverman, B.E. and Patterson, P.H.: Cytokines and CNS development. Neuron 64, 61-78 (2009).

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S.: Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. USA 108, 3047-3052 (2011).

Erny, D., Hrabě de Angelis, A.L., and Prinz, M.: Communicating systems in the body: how microbiota and microglia cooperate. Immunology 150, 7-15 (2017).

Fiorani, M., Del Vecchio, L.E., Dargenio, P., Kaitsas, F., Rozera, T., Porcari, S., Gasbarrini, A., Cammarota, G., and Ianiro, G.: Histamine-producing bacteria and their role in gastrointestinal disorders. Expert. Rev. Gastroenterol. Hepatol. 17, 709-718 (2023).

Fung, T.C., Olson, CA, Hsiao, E.Y.: Interactions between the microbiota, immune and nervous systems in health and disease. Nat. Neurosci. 20, 145-155 (2017).

Galley, J.D., Mackos, A.R., Varaljay, V.A., Bailey, M.T.: Stressor exposure has prolonged effects on colonic microbial community structure in Citrobacter rodentium-challenged mice. Sci. Rep. 7, 45012 (2017).

Goehler, L.E., Gaykema, R.P., Opitz, N., Reddaway, R., Badr, N., and Lyte, M.: Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with *Campylobacter jejuni*. Brain Behav. Immun. 19, 334-344 (2005).

Hoban, A.E., Stilling, R.M., Ryan, F.J., Shanahan, F., Dinan, T.G., Claesson, M.J., Clarke, G., and Cryan, J.F.: Regulation of prefrontal cortex myelination by the microbiota. Transl. Psychiatry 6, e774 (2016).

Keshteli, A.H., Madsen, K.L., Mandal, R., Boeckxstaens, G.E., Bercik, P., De Palma, G., Reed, D.E., Wishart, D., Vanner, S., and

Dieleman, L.A.: Comparison of the metabolomic profiles of irritable bowel syndrome patients with ulcerative colitis patients and healthy controls: new insights into pathophysiology and potential biomarkers. Aliment. Pharmacol. Ther. 49, 723-732 (2019).

Köhler-Forsberg, O., Petersen, L., Gasse, C., Mortensen, P.B., Dalsgaard, S., Yolken, R.H., Mors, O., and Benros, M.E.: A Nationwide Study in Denmark of the Association Between Treated Infections and the Subsequent Risk of Treated Mental Disorders in Children and Adolescents. JAMA Psychiatry 76, 271-279 (2019).

Landete, J.M., De las Rivas, B., Marcobal, A., and Munoz, R.: Updated molecular knowledge about histamine biosynthesis by bacteria. Crit. Rev. Food Sci. Nutr. 48, 697-714 (2008).

Luczynski, P., Whelan, S.O., O'Sullivan, C., Clarke, G., Shanahan, F., Dinan, T.G., and Cryan, J.F.: Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus. Eur. J. Neurosci. 44, 2654-2666 (2016).

Lyte, M.: Microbial endocrinology and the microbiota-gut-brain axis. Adv. Exp. Med. Biol. 817, 3-24 (2014).

Macpherson, A.J., Pachnis, V., and Prinz, M.: Boundaries and integration between microbiota, the nervous system, and immunity. Immunity 56, 1712-1726 (2023).

Mars, R.A.T., Yang, Y., Ward, T., Houtti, M., Priya, S., Lekatz, H.R., Tang, X., Sun, Z., Kalari, K.R., Korem, T., Bhattarai, Y., Zheng, T., Bar, N., Frost, G., Johnson, A.J., van Treuren, W., Han, S., Ordog, T., Grover, M., Sonnenburg, J., D'Amato, M., Camilleri, M., Elinav, E., Segal, E., Blekhman, R., Farrugia, G., Swann, J.R., Knights, D., and Kashyap, P.C.: Longitudinal Multi-omics Reveals Subset-Specific Mechanisms Underlying Irritable Bowel Syndrome. Cell 182, 1460-1473 (2020).

McIntosh, K., Reed, D.E., Schneider, T., Dang, F., Keshteli, A.H., De Palma, G., Madsen, K., Bercik, P., and Vanner, S.: FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial. Gut 66, 1241-1251 (2017).

O'Mahony, S.M., Clarke, G., Borre, Y.E., Dinan, T.G., and Cryan, J.F.: Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav. Brain Res. 277, 32-48 (2015).

Ratajczak, M.Z., Pedziwiatr, D., Cymer, M., Kucia, M., Kucharska-Mazur, J., and Samochowiec, J.: Sterile Inflammation of Brain, due to Activation of Innate Immunity, as a Culprit in Psychiatric Disorders. Front. Psychiatry 9, 60 (2018).

Takahashi, H., Kimura, B., Yoshikawa, M., and Fujii, T.: Cloning and sequencing of the histidine decarboxylase genes of gram-negative, histamine-producing bacteria and their application in detection and identification of these organisms in fish. Appl. Environ. Microbiol. 69, 2568-2579 (2003).

Verdú, E.F., Bercik, P., Verma-Gandhu, M., Huang, X.X., Blennerhassett, P., Jackson, W., Mao, Y., Wang, L., Rochat, F., and Collins, S.M.: Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55, 182-190 (2006).

Yang, N.J. and Chiu, I.M.: Bacterial Signaling to the Nervous System through Toxins and Metabolites. J. Mol. Biol. 429, 587-605 (2017).

Yano, J.M., Yu K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L., Nagler, C.R., Ismagilov, R.F., Mazmanian, S.K., and Hsiao, E.Y.: Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 161, 264-276 (2015).

Yunes, R.A., Poluektova, E.U., Dyachkova, M.S., Klimina, K.M., Kovtun, A.S., Averina, O.V., Orlova, V.S., and Danilenko, V.N.: GABA production and structure of gadB/gadC genes in Lactobacillus and Bifidobacterium strains from human microbiota. Anaerobe 42, 197-204 (2016).

IN THE SHADOW OF JOHN BIENENSTOCK: TARGETING THE ENTERIC NERVOUS SYSTEM TO TREAT NEURODEGENERATIVE DISEASES

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INTRODUCTION

John Bienenstock has been colleague of mine for over a decade and with whom I have shared many inspiring discussions across the broad range of basic science and medicine. My contribution to the 2023 Old Herborn University Seminar focuses on our mutual research interests that have led to several successful clinical trials.

I first met John at a 2010 meeting of the Broad Research Foundation in Los Angeles at which recipients of grants awarded in the area of inflammatory bowel disease presented their work. At that meeting he presented data demonstrating that probiotic bacteria influenced behaviour/mood by stimulating signals within the enteric nervous system(ENS) that communicated to brain centres via the vagal nerves. The studies were subsequently published in 2011 (*Bravo* et al., 2011).

Ingestion of a *Lactobacillus* strain regulates emotional behaviour and central GABA receptor expression in a mouse via the vagus nerve). The basic theme was that microbes positioned within certain portions of the GI tract of the mouse could have a profound impact on central neurological symptoms. Most surprisingly, the effect of the bacteria appeared to depend on electrical signals transmitted from the intestine to the brain via the vagal nerves. These studies profoundly altered my understanding of the "Gut-Brain Axis." Previously I had assumed that the gut and brain communicated principally through circulating chemicals such as hormones or bioactive compounds. John's studies now extended the communication circuitry to electrical impulses between the ENS and the central nervous system (CNS).

PARKINSON'S DISEASE

Sometime later a family member of mine was diagnosed with Parkinson's disease (PD). This event led me to review the current understanding of the natural history and pathophysiology of this condition. In medical school, I was taught that PD first appeared clinically when the dopamine-rich neurons within the substantia nigra died off. Characteristic intra-neuronal inclusions called Lewy bodies were pathognomonic findings. I knew that dopamine replacement, achieved through the oral administration of L-DOPA, a dopamine prodrug, was a standard therapy. I had not realized that about 25 years ago a protein, alpha-synuclein (aS), had been linked to PD by genetic analysis of several rare families. Furthermore, aS was identified within the Lewy body and present in elevated amounts in both the peripheral nervous system and CNS of advanced cases of PD. Most curiously, I learned of the work of Braak, who had demonstrated through histology of the neural tissues of individuals

with PD ranging from the earliest symptoms of the disease to the most advanced, that aS inclusions appeared in the ENS many years before severe movement deficits were clinically evident. As the disease progressed aS accumulation appeared to progressively move, via the vagus, into the dorsal motor nucleus, and cranially, eventually reaching the substantia nigra. Braak hypothesized that PD actually begins in the ENS of the proximal GI tract through the accumulation of aS, which then is slowly transported cranially via the vagal nerves (*Braak* et al., 2003).

Braak's observation and hypothesis were supported by careful re-evaluation of the natural history of PD. Surprisingly, the vast majority of patients with PD experienced severe constipation many years before the onset of the classic signs and symptoms of PD. Indeed, several non-motor symptoms were identified that strongly predicted the eventual onset of PD, most robustly, REM behaviour sleep disorder (*Postuma* et al., 2012). Indeed, in the absence of other confounding medical conditions, isolated REM behaviour disorder is associated with a greater than 95% risk of developing PD (*Mahowald* and *Schenck*, 2013). In fact, upon questioning my family member I learned that he had suffered from both bowel issues and REM behaviour disorder for many years.

SQUALAMINE

As I read more deeply about aS, I learned that this 145-residue protein was unstructured when in aqueous solution and described as "intrinsically disordered." Due to the presence of several runs of lysine residues within the N-terminal portion of the protein, aS bound avidly to membranes that displayed negatively charged phospholipid headgroups (*Perni* et al., 2017). Upon binding the amino-terminal portion, aS adopted an alpha-helical configuration, while the carboxy-terminal portion remained free in solution. As the surface density of the monomeric aS molecules increased, closely positioned neighbours could aggregate forming membrane-active oligomers, which in turn could damage the physical integrity of the membrane (*Perni* et al., 2017). As it turned out, I had discovered a class of cationic sterols in the liver of the dogfish shark in the late 1990s and had been studying their biophysical properties as well as their pharmacology ever since (*Moore* et al., 1993; *Rao* et al., 2000). Indeed, one of these compounds, squalamine had entered several clinical trials for cancer and diabetic retinopathy some years earlier. Squalamine is a C27 bile salt in which the C24 hydroxyl is esterified with a sulphate, the C7 position contains an alpha-OH group, the C5 an alpha hydrogen, and the C3 position an alpha spermidine. The compound binds avidly to membrane surfaces composed of precisely the same phospholipids as does aS, and upon binding had been shown to displace positively charged peptides and proteins without disturbing the physical integrity of the membrane.

With colleagues at the NIH and Cambridge we showed that squalamine could displace aS from neuronal membranes, prevent aggregation, protect neuronal cells from lethal cytolysis caused by neurotoxic aggregates of aS (*Perni* et al., 2017). Exposure of a PD model of *C. elegans*, expressing a mutant aggregating form of aS, prevented both the formation of aS aggregates and the onset of paralysis in a dose-dependent fashion.

As these studies were progressing, I formed Enterin along with Dr. Denise

Barbut. Together we drafted the roadmap to the clinic with the intent of evaluating squalamine as a potential treatment for PD. Since we knew that most patients with PD suffered from constipation, and that aetiology of their dysmotility was likely due to accumulation of aS within the ENS, causing neuronal dysfunction, oral administration of squalamine might correct their constipation.

Most exciting, however, was the possibility, inspired by our many discussions with John and his colleague Wolf Kunze, that by restoring the electrical activity of the ENS we could restore communication between the ENS and the CNS. If, in fact, the aS-compromised ENS of the PD patient no longer communicated with brain, centres in the brain that normally received neuronal signals from the GI tract might atrophy, as occurs usually when an end organ is de-afferented. If so, as we corrected the constipation in PD patients, we might see improvements in other neurological symptoms.

PRECLINICAL STUDIES

With John and Wolf, along with Wolf's prior mentor, John Furness, we orally administered squalamine to mice engineered to express an aggregating form of human aS (*West* et al., 2020). Squalamine administration stimulated intestinal motility, as we had expected. Electrophysiological studies of the ENS, specifically the electrical activity of the intrinsic primary afferent neuron (IPAN), demonstrated that oral administration had restored their previously depressed excitability to normal, thus providing a mechanistic explanation for the improvement in intestinal motility.

In the next series of studies, we examined the impact of oral administration of squalamine in the PD mouse on the electrical signals directed to the brain via the vagal nerves (*West* et al., 2019). In these experiments single fibres were teased out of a branch of the mesenteric branch of the vagus, a parasympathetic stimulus was introduced onto the ENS, and the resulting electrical activity monitored with respect to amplitude, frequency and duration of excitation. We demonstrated that exposure of the ENS to squalamine dramatically stimulated the intensity and duration of signals directed from the ENS via the vagus to the brain.

From the time that John had shown that certain strains of *Lactobacillus* could induce a vagally transmitted signal that calmed mice he and Wolf believed that the electrical signals carried information. These signals were not "noise", but rather "music," characterized by specific patterns of frequency and amplitudes. John and Wolf described these patterns as the "SSRI" code, since their ongoing studies had demonstrated similarities between the signal patterns stimulated by the application of either the calming *Lactobacillus* strain or several selective serotonin reuptake inhibitors. The paper that describes John's thoughts was published in a series of experiments that included squalamine which, like the probiotic and an SSRI therapeutic, stimulated an SSRI-like vagal signal (*West* et al., 2021). Whole brain cFos imaging of mice following oral administration squalamine confirmed that extensive areas of the brain were either inhibited or activated by the compound. Since squalamine is not absorbed from the GI tract after oral administration, the effects observed result exclusively from communication between gut and brain.

CLINICAL TRIALS

In parallel with these studies, Enterin conducted two clinical trials, evaluating ENT-01 for the daily oral treatment of Parkinson's disease associated constipation over a 28-day period. (*Hauser* et, al., 2019). ENT-01 is a chemical stable crystalline phosphate salt of squalamine (which is why it was the preferred salt) that dissolves into the active squalamine zwitterion in the stomach. The first clinical trial, RASMET (https://clinicaltrials.gov/study/NCT03047629, was an open label study that evaluated the safety, tolerability, pharmacokinetics of ENT-01. In addition, we monitored effects on constipation as well as hallucinations and dementia in the subset of patients that had these symptoms. In addition, we monitored circadian rhythm and sleep parameters. As we reported, the RASMET trial demonstrated that ENT-01 corrected the constipation in a dose-dependent manner, very much reproducing the observations made with John and Wolf in the preclinical study. We also observed that in patients who had suffered from hallucinations, ENT-01 treatment had a dramatic effect, in some cases completely eliminating them for many weeks following cessation of treatment. Similarly in those patients suffering from dementia, significant improvement was observed during the 28-day treatment period. As in the case of hallucinations, cognitive functions continued to improve for several weeks after treatment had stopped. We monitored circadian rhythm through the measurement of skin temperature at the wrist using a wireless continuous recording device. Circadian rhythm is maintained through a complex circuit governed by the master clock within the hypothalamus, the suprachiasmatic nucleus. By monitoring circadian rhythm we can assess the "health" of the "beating" hypothalamus. Remarkably, the disturbed circadian rhythms of the untreated patients were corrected in a dose dependent fashion by oral administration of ENT-01. When we thought about it, after seeing the clinical data the effects on circadian rhythm made sense. The primary function of the circadian rhythm is to synchronize physical activity and hunger with the particular light/dark cycle appropriate for a species. Perhaps properly synchronized signals from the GI tract induced by a meal might be required to sustain a normal circadian rhythm. If so, by clearing the aS from the ENS, ENT-01 might be restoring necessary input to central circadian circuitry.

The RASMET clinical trial was followed by double blind randomized placebo-controlled Phase 2a study, KARMET (https://clinicaltrials.gov/study/NCT03781791. The study enrolled about 150 patients, each administered either placebo or ENT-01 for a period of 28 days. It successfully replicated the results of the RASMET trial. Constipation was corrected ($p<0.0001$), symptoms of both dementia and hallucinations were improved, and all effects of ENT-01 persisted for weeks after treatment ended. The study was published in the Annals of Internal Medicine (*Camilleri* et al., 2022). ENT-01 is now positioned to enter Phase 3 for PD associated constipation, Phase 2 for PD dementia, and Phase 2 for PD psychosis.

APPRECIATION

Both Denise and I have had the great pleasure and honour having worked with John and Wolf on the scientific journey I have described. John's

enthusiasm, the breadth of his knowledge of medicine and physiology made our collaboration intellectually exhilarating. If he disagreed with us, he told us directly, but was also ready to be corrected. This ability to converse freely in science, so smoothly and effortlessly is a rare characteristic and it made our friendship with John so very special. We miss him.

LITERATURE

Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., and Braak, E.: Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197-211 (2003).

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., Cryan, J.F.: Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. USA 108, 16050-16055 (2011).

Camilleri, M., Subramanian, T., Pagan, F., Isaacson, S., Gil, R., Hauser, R.A., Feldman, M., Goldstein, M., Kumar, R., Truong, D., Chhabria, N., Walter, B.L., Eskenazi, J., Riesenberg, R., Burdick, D., Tse, W., Molho, E., Robottom, B., Bhatia, P., Kadimi, S., Klos, K., Shprecher, D., Marquez-Mendoza, O., Hidalgo, G., Grill, S., Li, G., Mandell, H., Hughes, M., Stephenson, S., Vandersluis, J., Pfeffer, M., Duker, A., Shivkumar, V., Kinney, W., MacDougall, J., Zasloff, M., and Barbut, D.: Oral ENT-01 Targets Enteric Neurons to Treat Constipation in Parkinson Disease : A Randomized Controlled Trial. Ann. Intern. Med. 175, 1666-1674 (2022).

Hauser, R.A., Sutherland, D., Madrid, J.A., Rol, M.A., Frucht, S., Isaacson, S., Pagan, F., Maddux, B.N., Li, G., Tse, W., Walter, B.L., Kumar, R., Kremens, D., Lew, M.F., Ellenbogen, A., Oguh, O., Vasquez, A., Kinney, W., Lowery, M., Resnick, M., Huff, N., Posner, J., Ballman, K.V., Harvey, B.E., Camilleri, M., Zasloff, M., and Barbut, D.: Targeting neurons in the gastrointestinal tract to treat Parkinson's disease. Clin. Park. Relat. Disord. 1, 2-7 (2019).

Mahowald, M.W. and Schenck, C.H.: REM sleep behaviour disorder: a marker of synucleinopathy. Lancet Neurol. 12, 417-419 (2013).

Moore, K.S., Wehrli, S., Roder, H., Rogers, M., Forrest Jr., J.N., McCrimmon, D., and Zasloff, M.: Squalamine: an aminosterol antibiotic from the shark. Proc. Natl. Acad. Sci. USA 90, 1354-1358 (1993).

Perni, M., Galvagnion, C., Maltsev, A., Meisl, G., Müller, M.B.D., Challa, P.K., Kirkegaard, J.B., Flagmeier, P., Cohen, S.I.A., Cascella, R., Chen, S.W., Limbocker, R., Sormanni, P., Heller, G.T., Aprile, F.A., Cremades, N., Cecchi, C., Chiti, F., Nollen, E.A.A., Knowles, T.P.J., Vendruscolo, M., Bax, A., Zasloff, M., and Dobson, C.M.: A natural product inhibits the initiation of α-synuclein aggregation and suppresses its toxicity. Proc. Natl. Acad Sci. USA 114, E1009-E1017 (2017).

Postuma, R.B., Gagnon, J.F., and Montplaisir, J.Y.: REM sleep behavior disorder: from dreams to neurodegeneration. Neurobiol. Dis. 46, 553-558 (2012).

Rao, M.N., Shinnar, A.E., Noecker, L.A., Chao, T.L., Feibush, B., Snyder, B., Sharkansky, I., Sarkahian, A., Zhang, X., Jones, S.R., Kinney, W.A., and Zasloff, M.: Aminosterols from the dogfish shark Squalus acanthias. J. Nat. Prod. 63, 631-635 (2000).

West, C.L., Amin, J.Y., Farhin, S., Stanisz, A.M., Mao, Y.-K., and Kunze, W.A.: Colonic Motility and Jejunal Vagal Afferent Firing Rates Are Decreased in Aged Adult Male Mice and Can Be Restored by an Aminosterol. Front. Neurosci. 13, 955 (2019).

West, C.L., Mao, Y.-K., Delungahawatta, T., Amin, J.Y., Farhin, S., McQuade, R.M., Diwakarla, S., Pustovit, R., Stanisz, A.M., Bienenstock, J., Barbut, D., Zasloff, M., Furness,J.B., and Kunze, W.A.: Squalamine

Restores the Function of the Enteric Nervous System in Mouse Models of Parkinson's Disease. J. Parkinsons Dis. 10, 1477-1491 (2020).

West, C.L., McVey Neufeld, K.-A., Mao, Y.-K., Stanisz, A.M., Forsythe, P., Bienenstock, J., Barbut, D., Zasloff, M., and Kunze, W.A.: Identification of SSRI-evoked antidepressant sensory signals by decoding vagus nerve activity. Sci. Rep. 11, 21139 (20

MICROBES AND MAMMALIAN BIOLOGY – A SUMMARY OF OLD HERBORN UNIVERSITY SEMINAR 35

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INTRODUCTION – MAMMAL AS HOST AND BENEFICIARY

Our annual tradition of the Old Herborn University Seminar (OHUS) series highlighted our microbial world yet again in 2023. In this latest seminar, our emphasis was on the mammalian host, in contrast to plants as hosts in OHUS 33. In OHUS 35, our aim was to explore the bidirectional interplay of mammals and microbes, while considering mammalian species as both hosts for diverse microbes and as beneficiaries of microbes in their roles as messengers and catalysts. In the broader context of mammalian physiology and systems biology, it is striking how profoundly mammal-associated microbes impact early life development, local and systemic immunity, and the communication stream between the gut and the central nervous system.

Evidence supports exposure to microbes and/or microbial metabolites during foetal development and immediately after birth. During the neonatal period, infancy and early life, the microbial ecosystem fluctuates and expands gradually in terms of microbial composition and function. The intestinal microbiome fluctuates dramatically during infancy and the first 3 years of human life, and it continues to evolve and change during childhood. We know that the human microbiome reaches a relatively steady-state or equilibrium by early adulthood, but these microbial communities continue to change or shift during adulthood depending on the influences of diet, medications, hygiene and a diverse array of xenobiotic compounds in the environment (built or natural). Microbes are intimately associated with different body sites throughout the mammalian lifespan, and ongoing shifts in microbial composition and function may contribute to the nature of the aging process.

In addition to changes over time during an animal's lifespan, we must consider fluctuations in microbial composition and function at different body sites (in space) and whether microbes send signals extending beyond local targets to remote locations. Shifts in microbial ecosystems at specific body sites such as the oral cavity may impact the placenta via the bloodstream. Another example prominently displayed at OHUS 35 was the nature of communication between the intestinal microbiome and the brain via the peripheral / central nervous system. This microbiome-gut-brain axis highlights the importance of microbe-generated signals affecting remote targets in mammalian systems via bidirectional communication.

GROWTH, DEVELOPMENT AND INFECTIONS

Kjersti Aagaard and colleagues described the importance of maternal-foetal communication during early mammalian development. The "Developmental Origins of Health and Disease" hypothesis (*Barker*, 1986) links maternal exposures during pregnancy to adverse outcomes later in life for offspring. The mammalian microbiome

serves as a mediator and messenger conveying signals from maternal exposures such as diet with lasting effects on foetal and early postnatal life development. In nonhuman primates, a high fat maternal diet during pregnancy has lasting effects on microbial composition and function after birth (*Ma* et al., 2014; *Bolte* et al., 2022). The effects of the high fat diet on the gut microbiome seem to be dependent on the diet quality, and not the number of calories. Another example in humans of the potentially profound impact of dietary compounds is the example of the sugar alcohol, xylitol, and its application to prevention of preterm birth in humans (*Aagaard* et al., 2022). Xylitol in chewing gum may be used preferentially as a carbohydrate source by pathogens in the oral microbiome, thereby suppressing proliferation of pathogenic streptococci and oral inflammation (e.g., periodontitis). Suppression of oral inflammation may reduce the production of inflammatory cytokines and lower the risk of preterm birth perhaps by suppression of systemic inflammation. Martin Schwarzer cited studies by Gehrig and colleagues (*Gehrig* et al., 2019) showing that changes in therapeutic diets could result in increased abundances of growth-promoting bacterial taxa in juvenile mice, piglets and children. These findings highlight the potential importance of diet as a strategy to shift the biology of the microbiome in a more favourable direction for the mammalian host.

Microbial communities at different body sites may have cumulative effects on biological processes and different remote effects dependent on resident microbes and microbial mediators. Shifts in the oral microbiome may yield remote effects on the placenta and the foetus. We've come to appreciate the presence of low biomass microbial communities in body sites such as the placenta and the vagina, and in different body fluids such as amniotic fluid during pregnancy. We know that pathogenic microbes within the oral microbiome can spread via the bloodstream to infect remote sites such the placenta or the heart, so we cannot discount the possibility of bacterial colonization in one body site affecting another. Within amniotic fluid, the combination of human milk oligosaccharides and bacterial composition might affect the likelihood of preterm birth or timing of labour and delivery. Priming of the immune system and foetal immune development may be impacted by the various signals emanating from the microbiome. Martin Schwarzer and colleagues identified a single beneficial microbe, a *Lactiplantibacillus plantarum* strain (LpWJL), that can single-handedly rescue a growth-deficient phenotype in mice on a low protein diet. The problem gets flipped. Instead of studying how diet modifies the composition and function of the microbiome, (*Schwarzer* et al., 2016) studied how supplementation with a single gut microbe (LpWJL) rescues the stunted phenotype in mice as a result of chronic undernutrition. The punchline is that a cell wall component of this bacterial strain stimulates the pattern recognition receptor (NOD2) in the intestinal epithelium and promotes growth hormone (GH) signalling via insulin-like growth factor 1 (IGF-1) in the liver. Changes in microbial composition in the intestine result in modulation of signalling pathways in liver and muscle (remote effects) resulting in improved growth in early life.

Luigina Romani and colleagues elaborated on microbe:microbe interactions across bacterial and fungal kingdom boundaries. In addition to considerations of commensal bacteria and bacterial pathogens at different body sites, bacterial and fungal species (emphasis on yeast) interact with each other. *Candida albicans* can be considered a commensal of the gut microbiome, and *C. albicans* can modulate signalling pathways in dendritic cells and promote immune tolerance (*Romani* et al., 2002; *Romani*, 2011). *C. albicans* can also modulate bacterial composition in the gut microbiome, so we are left to conclude that bacteria and fungi are dynamically fluctuating

over time in terms of relative abundances. One example of a signal is the compound p-cresol that is produced by *C. difficile* and suppresses hyphal growth by *C. albicans* (*van Leeuwen* et al., 2016), demonstrating cross-kingdom signalling within the intestinal microbiome. Tripartite interactions between bacteria, fungi and mammals provide fascinating examples of cross-communication and influences on development of the immune system. The bacterial commensal organism, *L. reuteri*, can utilize the amino acid tryptophan (Trp) in the intestine and produces indole-3-aldehyde (*Morgan* et al., 2023). Indole-3-aldehyde is a ligand for the aryl hydrocarbon receptor (AhR) which is a key receptor of microbial signals within the intestinal epithelium. AhR signaling results in release of IL-22 by innate lymphoid cells, resulting in suppression of *C. albicans* proliferation via release of antimicrobial peptides. The key message is that bacterial metabolism of dietary components (amino acids) can promote immune function and resistance to fungal pathogens.

MICROBIOME-GUT-BRAIN AXIS

Microbes can have a profound impact on remote body sites in mammalian systems. A prime example is the microbiome – gut – brain axis and emerging insights with respect to communication between intestinal microbes and the mammalian central nervous system. Rochellys Diaz Heijtz described contributions of gut microbes and intestinal microbiology to neurodevelopment and autism spectrum disorder of childhood. As already mentioned in this summary, gut microbes may contribute to fundamental processes in prenatal and postnatal mammalian development. Postnatal microbial colonization promotes establishment of gut homeostasis, angiogenesis and immune system development (*Hooper* et al., 2012). The gut microbiota influences a wide array of neurodevelopmental processes such as the maturation of microglia and synaptogenesis (*Diaz Heijtz* et al., 2011). Interestingly, the metabolic capacity of gut microbes to produce metabolites such as vitamins B9 (folate) and B12 in childhood may support their contributions to neurodevelopment (*Hollister* et al., 2015). Similar to the work presented by Martin Schwarzer whereby cell wall components from *L. plantarum* bind to pattern recognition receptors such as NOD2, Rochellys Diaz Heijtz presented signalling pathways that included bacterial peptidoglycan (PGN) fragments interacting with NOD-like receptors and PGN recognition proteins. PGN can prime systemic innate immunity by interacting with Nod1 (*Clarke* et al., 2010). In a fascinating twist to the story, PGN recognition proteins were expressed in the developing mammalian brain. PGN recognition protein-2 (Pglyrp2) appears to modulate expression of autism risk genes in mouse models and may affect formation of brain circuits. Both Pglyrp2 and NOD2 appear to modulate mammalian brain function and behaviour, and such findings add evidence to the importance of microbial components in neurodevelopment and biology of the mammalian brain.

Intestinal microbes may produce neurotransmitters such as y-aminobutyric acid (GABA) and histamine, and gut microbes may stimulate mammalian enteroendocrine cells to produce serotonin. Rochellys Diaz Heijtz discussed the potential importance of microbial GABA in neurodevelopment with low levels of faecal GABA in young children associated with elevated risk for developing autism spectrum disorder. Gut microbes such as *Bifidobacterium* spp. may produce GABA and other gut microbes such as Clostridia may consume GABA so the relative balance of GABA producers and consumers may affect the relative risk of autism spectrum disorder. In addition to

GABA, histamine may be produced by intestinal microbes and contribute to abdominal (visceral) pain in irritable bowel syndrome (IBS) due to signalling via the histamine 4 receptor (H4R). Premysl Bercik described intriguing studies whereby mice colonized with IBS microbiota displayed greater visceral pain responses due to colonic distension (*De Palma* et al., 2022). One microbial strain, *Klebsiella aerogenes* MQ, was identified as an avid producer of microbial histamine in the gut. Clearly, production and release of neurotransmitters and amino acid metabolites such as GABA and histamine could have important consequences for neurodevelopment and pain signalling.

Autism spectrum disorder and Parkinson's disease represent well-known medical disorders with prominent neurologic/psychiatric and gastrointestinal features. Michael Zasloff described fundamental studies documenting the importance of gastrointestinal symptoms such as constipation and intestinal alpha-synuclein (aS) inclusions in Parkinson's disease. The gut-brain connection was emphasized by the Braak hypothesis of aS accumulation in the enteric nervous system as a possible initiator of Parkinson's disease pathogenesis (*Braak* et al., 2003). The cationic sterol, squalamine, was identified as a compound that could displace aS from neuronal membranes and serve as a drug candidate to ameliorate constipation and improve neurologic symptoms. In collaboration with John Bienenstock's team, Michael Zasloff and his team showed that squalamine could enhance intestinal motility and stimulate signalling from the enteric nervous system (ENS) to the brain in mammalian (mouse) models (*West* et al., 2019). These applications have been extended to human clinical trials. An important concept that was highlighted as part of our tribute to John Bienenstock was the notion of the "SSRI"code whereby distinct stimuli such as squalamine, probiotic lactobacilli, or a SSRI therapeutic all transmitted SSRI-like signals via the vagus nerve (*West* et al., 2021).

CELIAC DISEASE : DIET, MICROBIOME AND IMMUNITY

Elena Verdu shared insights regarding diet-microbiome-immune system interactions in the context of celiac disease. Celiac disease is instructive as a human disease model whereby specific dietary components contribute directly to human gastrointestinal pathology. Specific proteins from cereal grains known as gluten trigger intestinal inflammation in susceptible individuals (*Shan* et al., 2022). Deamidation of gluten peptides increases their affinity for HLA surface molecules – HLA-DQ2 and HLA-DQ8 – and highlights the importance of autoimmune responses associated with specific MHC class II antigens (specifically, HLA-DQ). The microbiome participates in gluten metabolism, and duodenal microbes are known to generate gluten peptides with enhanced or reduced immunogenicity. Elena Verdu raised examples of microbes such as *Pseudomonas aeruginosa* that produce proteases cleaving gluten into peptides with greater immunogenicity. Countering this phenomenon, other microbes such as *Lactobacillus rhamnosus* or *Lactobacillus fermentum* from healthy controls digested gluten to peptides with reduced immunogenicity. These findings highlight the importance of a relative balance in microbial composition as a predisposing or protective factor for autoimmunity. Gut microbes can profoundly affect digestion of dietary components such as plant proteins, thereby influencing protein metabolism and local immunity.

Beyond luminal proteins and peptides, amino acids such as tryptophan may be metabolized to specific signalling com-

pounds including indole derivatives. Luigina Romani highlighted indole derivatives from tryptophan metabolism as key elements of antifungal defence systems in mammals, while Elena Verdu mentioned indoles as key signals maintaining intestinal homeostasis and suppressing inflammation (*Verdu* et al., 2015). Indoles augment antifungal defence and modulate inflammation by signalling through a common pathway via the AhR. Tryptophan metabolism yields distinct compounds such as serotonin, kynurenine and indole derivatives, and the gut microbiome plays a pivotal role in these bioconversions from a single amino acid. Clearly dietary protein may yield peptides that stimulate the immune system, possibly resulting in immunopathology, or amino acids that can be converted to a variety of bioactive microbial metabolites. These experimental insights provide opportunities for new therapeutic strategies such as engineered bacterial enzymes, new probiotics that may suppress specific bacterial proteases, indole-generating microbes or host enzyme inhibitors.

CONCLUSIONS

OHUS 35 highlighted the importance of the mammalian microbiome in mammalian systems biology. The findings and insights shared at **OHUS 35** strongly support the holobiont concept that includes both microbial and mammalian cells as cellular components of one integrated mammalian system. Microbes and their corresponding enzymes and metabolites clearly contribute to early life development (both prenatal and postnatal) and proper growth and maturation. The microbiome-gut-brain axis has become a central theme in neurobiology and gastroenterology, while emphasizing the ways in which gut microbes can affect neurodevelopment and brain function remotely. Microbes may produce neurotransmitters or stimulate mammalian cells to produce neurotransmitters, and bidirectional communication via nerves such as the vagus highlight how the microbiome interacts directly with the central nervous system and vice-versa. The coordination of signalling via the microbiome as a bridge between diet and host is evident when considering the impact of diet during pregnancy or during pathologic states such as celiac disease. Microbes may modulate immunity by altering metabolism of dietary components, and microbes may also regulate visceral pain signalling by converting amino acids to signalling metabolites. Specific host receptors such as H4R and AhR may provide mechanistic pathways to explain microbial communication with mammalian cells. In short, we have witnessed many examples showing how the mammalian microbiome can profoundly impact mammalian biology. Our long-time colleague in Herborn and pioneering scientist, John Bienenstock (1936-2022), https://www.old-herborn-university.de/in-memoriam-john-bienenstock-1936-2022/, would not be surprised today to see so many insights extending from his seminal work in mucosal immunology and the gut-brain axis. As we close 2023 and OHUS 35, we say good-bye in appreciation once again to Professor John Bienenstock and to Old Herborn University Seminar 35.

LITERATURE

Aagaard, K.M, Valentine, G.C, Antony, K.M, Sangi-Haghpeykar, H., Chirwa, R., Dumba, M., Petro, S., Nanthuru, D., Shope, C., Mlotha-Namarika, J., Wilkinson, J.P., Aagaard, J., Aagaard, E.J., Seferovic, M.D., and Levison, J.: PPaX: Cluster randomized

trial of xylitol chewing gum on prevention of preterm birth in Malawi. Am. J. Obstet. Gynecol. 226, Suppl. 1, S777 (2022).

Barker, D.: Infant mortality, childhood nutrition, and ischaemic heart disease in england and wales. Lancet 327, 1077-1081 (1986).

Bolte, E. E., Moorshead, D., and Aagaard, K.M.: Maternal and early life exposures and their potential to influence development of the microbiome. Genome Med. 14, 4 (2022)

Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., and Braak, E.: Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197-211 (2003).

Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y., and Weiser, J.N.: Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 16, 228-231 (2010).

De Palma, G., Shimbori, C., Reed, D.E., Yu, Y., Rabbia, V., Lu, J., Jimenez-Vargas, N., Sessenwein, J., Lopez-Lopez, C., Pigrau, M., Jaramillo-Polanco, J., Zhang, Y., Baerg, L., Manzar, A., Pujo, J., Bai, X., Pinto-Sanchez, M.I., Caminero, A., Madsen, K., Surette, M.G., Beyak, M., Lomax, A.E., Verdu, E.F., Collins, S.M., Vanner, S.J., and Bercik, P.: Histamine production by the gut microbiota induces visceral hyperalgesia through histamine 4 receptor signaling in mice. Sci. Transl. Med. 14, eabj1895 (2022).

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S.: Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. USA 108, 3047-3052 (2011).

Gehrig, J.L., Venkatesh, S., Chang, H.W., Hibberd, M.C., Kung, V.L., Cheng, J., Chen, R.Y., Subramanian, S., Cowardin, C.A., Meier, M.F., O'Donnell, D., Talcott, M., Spears, L.D., Semenkovich, C.F., Henrissat, B., Giannone, R.J., Hettich, R.L., Ilkayeva, O., Muehlbauer, M., Newgard, C.B., Sawyer, C., Head, R.D., Rodionov, D.A., Arzamasov, A.A., Leyn, S.A., Osterman, A.L., Hossain, M.I., Islam, M., Choudhury, N., Sarker, S.A., Huq, S., Mahmud, I., Mostafa, I., Mahfuz, M., Barratt, M.J., Ahmed, T., and Gordon, J.I.: Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. Science, 365 (6449) (2019).

Hollister, B., Reihle, K., Luna, R.A., Weidler, E.M., Rubio-Gonzalez, M., Mistretta, T.A., Raza, S., Doddapaneni, H.V., Metcalf, G.A., Muzny, D.M., Gibbs, R.A., Petrosino, J.F., Shulman, R.J., and Versalovic, J.: Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome 3, 36 (2015).

Hooper, L.V., Littman, D.R., and Macpherson, A.J.: Interactions between the microbiota and the immune system. Science 336, 1268-1273 (2012)

Ma, J., Prince, A.L., Bader, D., Hu, M., Ganu, R., Baquero, K., Blundell, P., Harris, R.A., Frias, A.E., Grove, K.L. and Aagaard, K.M.: High-fat maternal diet during pregnancy persistently alters the offspring microbiome in a primate model. Nat. Commun. 5, 3889 (2014).

Morgan, E.W., Dong, F., Annalora, A.J., Murray, I.A., Wolfe, T., Erickson, R., Gowda, K., Amin, S.G., Petersen, K.S., Kris-Etherton, P.M., Marcus, C.B., Walk, S.T., Patterson, A.D., and Perdew, G.H.: Contribution of Circulating Host and Microbial Tryptophan Metabolites Toward Ah Receptor Activation. Int. J. Tryptophan Res. 16, 11786469231182510 (2023).

Romani, L., Bistoni, F., and Puccetti, P.: Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol. 10, 508-514 (2002).

Romani, L.: Immunity to fungal infections. Nat. Rev. Immunol. 11, 275-288 (2011).

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., and Khosla, C.: Structural basis for gluten intolerance in celiac sprue. Science 97, 2275-2279 (2022).

Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova, P., Martino, M.E., Balmand, S., Hudcovic, T., Heddi, A., Rieusset, J., Kozakova, H., Vidal, H., and Leulier, F.:

Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. Science, 351, 854-857 (2016)
van Leeuwen, P.T., van der Peet, J.M., Bikker, F.J., Hoogenkamp, M.A., Oliveira Paiva, A.M., Kostidis, S., Mayboroda, O.A., Smits, W.K., and Krom, B.P.: Interspecies Interactions between Clostridium difficile and Candida albicans. mSphere 1, e00187-16 (2016).
Verdu, E.F., Galipeau, H.J., and Jabri, B.: Novel players in coeliac disease pathogenesis: role of the gut microbiota. Nat. Rev. Gastroenterol. Hepatol. 12, 497-506 (2015).
West, C.L., Amin, J.Y., Farhin, S., Stanisz, A.M., Mao, Y.-K., and Kunze, W.A.: Colonic Motility and Jejunal Vagal Afferent Firing Rates Are Decreased in Aged Adult Male Mice and Can Be Restored by an Aminosterol. Front. Neurosci. 13, 955 (2019).
West, C.L., McVey Neufeld, K.-A., Mao, Y.-K., Stanisz, A.M., Forsythe, P., Bienenstock, J., Barbut, D., Zasloff, M., and Kunze, W.A.: Identification of SSRI-evoked antidepressant sensory signals by decoding vagus nerve activity. Sci. Rep. 11, 21139 (2021).