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MICROBIOTA IN DEVELOPMENT AND STRESS MANAGEMENT

A thesis presented to the National University of Ireland for the degree of Doctor of Philosophy

By

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work (except as declared, hereafter), and has not been submitted for another degree, either at University College Cork or elsewhere.

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PUBLICATIONS

Review Articles

Kennedy, P.J., Murphy, A.B., Cryan, J.F., Ross, P.R., Dinan, T.G. and Stanton, C., 2016. Microbiome in brain function and mental health. *Trends in Food Science & Technology*, *57*, pp.289-301.

Scientific Articles

Scott, K.A., Ida, M., Peterson, V.L., Prenderville, J.A., Moloney, G.M., Izumo, T., Murphy, K., Murphy, A., Ross, R.P., Stanton, C. and Dinan, T.G., 2017. Revisiting Metchnikoff: Age-related alterations in microbiota-gut-brain axis in the mouse. *Brain, Behavior, and Immunity*.

Crowley, E., Long-Smith, C., Murphy, A., Patterson, E., Murphy, K., O'Gorman, D.,... & Nolan, Y. (2018). Dietary supplementation with a magnesium-rich marine mineral blend enhances the diversity of gastrointestinal microbiota. *Marine drugs*, *16* (6), 216.

Moloney, G., Long-Smith, C. M., Murphy, A., Dorland, D., Hojabri, S. F., Ramirez, L. O.,... & Fouhy, F. (2020). Improvements in Sleep Indices During exam stress due to Consumption of a Bifidobacterium Longum. *Brain, Behavior, & Immunity-Health*, 100174.

Bastiaanssen, T.F., Gururajan, A., van de Wouw, M., Moloney, G.M., Ritz, N.L., Long-Smith, C.M., Wiley, N.C., Murphy, A.B., Lyte, J.M., Fouhy, F. and Stanton, C., 2021. Volatility as a concept to understand the impact of stress on the microbiome. *Psychoneuroendocrinology*, *124*, p.105047.

Schellekens, Harriët, et al. "Bifidobacterium longum counters the effects of obesity: Partial successful translation from rodent to human." *EBioMedicine* 63 (2021): 103176.

Book Chapters

Chapter 5: Probiotics as curators of a healthy gut microbiota. Murphy, A.B., Dinan, T.G., Cryan, J.F., Stanton, C. and Ross, R.P in Hyland, N. and Stanton, C. eds., 2016. *The Gut-brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota*. Academic Press.

Conferences

Poster presentation at FEMS 2017, Valencia

"Examining the Influence Of Mode Of Delivery On The Gut Microbiota Composition In Adulthood"

THESIS ABSTRACT

The gut microbiota plays a significant role in health and development from birth and continues to affect several processes throughout life. The infant gut is unstable, and colonisation is influenced by a variety of factors. Many of these factors can contribute to an altered microbiota profile in infancy which can subsequently be associated with negative consequences later in life, due to interactions between the microbiota and the brain. The microbiota-gut-brain axis is a bi-directional communication network, which allows the microbiota to affect and influence a variety of psychological processes including mood and cognition. There is evidence to support the co-maturation of the infant brain with the early gut microbiota, and alterations in this parallel sequential maturation have been associated with long-term effects on brain signalling, immune, and metabolic function and mental health.

While many aspects of the microbiota-gut-brain axis are not covered in this thesis, an examination of participants from infancy to adulthood and a study of aged mice provides an insight into these populations. This thesis explores many of the early factors which contribute to gut microbiota disturbances and investigates the long-term consequences associated with these early microbiota maturation disruptions. Following on from this, a potential strategy to positively influence gut and brain health is considered. In Chapter 1, the use of probiotics and prebiotics as modulators of gut health are discussed, including methods of use, and previous investigations. Chapter 2 examines the microbiota-gut brain axis and details specific mechanisms involved in signalling pathways between the gut and the central nervous system (CNS).

Chapter 3 focuses on the gut microbiota composition of infants up to 24 weeks old who have been delivered by C-section and have received antibiotic treatment in the first four days of life. This observational study explores the immediate

consequences of these perinatal factors on the gut microbiota colonisation pattern, showing significantly different microbiota profiles between infants born vaginally, those born by C-section, and those who were also born by C-section and were treated with antibiotics. In Chapter 4, the long-term consequences of mode of delivery are examined. The gut microbiota composition of a cohort of 18-24-year-old males who were born by C-section is analysed and compared with those who were born vaginally. This investigation shows the significance of an altered microbiota during infancy on immune-brain signalling processes, as participants who were born by C-section exhibit increased vulnerability to psychological stress and anxiety.

Chapter 5 investigates the effects of a potential psychobiotic, *Bifidobacterium longum* 1714™, on stress, mood, and cognition in a healthy population. No differences are noted in gut microbiota profile, mood, or cognition in response to the intervention. However, a statistically significant difference is seen in sleep quality in participants consuming the strain, suggesting that this microbe may be beneficial to maintain sleep quality during periods of stress.

In Chapter 6, the caecal microbiota composition of aged (20-21 months old) and young mice (2-3 months old) was examined. The behavioural and psychological profiles of these mice were also assessed, as well as gut permeability and inflammation. It was found that older mice showed increased anxiety-like behaviours and reduced memory capabilities when compared to young mice. Additionally, the caecal microbiota profile differed between the two groups in terms of composition and gut permeability. This suggest that caecal microbiota changes seen in the aged mice are associated with aged-related behavioural and cognitive impairments.

Chapter 1

Probiotics as Potential Strategies to Restore a Healthy Gut Microbiota: Delivering the Solution

Amy Murphy is first author on this book chapter which is published in "The Gut-Brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota", Academic Press, 2016.

Abstract

In recent years, the influence of the gut microbiota on many aspects of human health has become clear. Accumulating evidence suggests that perturbations of the gut microbiota have consequences that extend beyond the gastrointestinal (GI) tract and have been associated with the development of several immunological, metabolic, and mental disorders. Consequently, the use of probiotics to modulate the gut microbiota has gained significant interest. In this review, we describe a typical healthy gut microbiota composition in both infants and adults, followed by the factors known to cause disturbances. Strategies to restore the gut microbiota using probiotics are discussed, with examples of food- and non-food based probiotic carriers, in addition to probiotic bacteria which affect brain function and mental health. The potential of probiotic therapy for a variety of conditions is evident however, further research is necessary to develop successful probiotic treatments focused on modulating the gut microbiota to maintain health or alleviate disease.

Introduction

The gut microbiota plays a vital role in human health and are essential for key processes such as nutrition, metabolism, and pathogen resistance (Lozupone et al., 2012). Significant advances in sequencing technologies have increased our understanding and appreciation of the complexity of the gut microbiota and the importance of a healthy gut ecosystem. Large-scale projects (Human Microbiome Project Consortium, 2012) have made significant progress in the characterisation of microbial communities in the gut that are crucial to human health. Defining the features of a healthy gut microbiota is essential to identify and examine the key alterations which can lead to disease.

The dominant influence of the gut microbiota in host physiology is becoming evident and thus, disruptions in the normal gut microbiota composition and function can have significant consequences for affected individuals. Microbiota disturbances are associated with an increased risk of disease development both in infants and adults (Lozupone et al., 2012). Moreover, a role for the gut microbiota in the regulation of several processes relating to brain function and mental health has emerged. Gut-brain interaction has emerged as an area of considerable investigation, with a multitude of preclinical studies suggesting that alterations in the gut-brain axis are involved in the development of several GI and psychiatric disorders such as irritable bowel syndrome (IBS), anxiety and depression (Mayer et al., 2015).

Given the prominent role of the gut microbiota in several functions that are essential to the maintenance of human health, modulation of the gut microbiota using functional food-based approaches is a rapidly growing area of research. Evidence from epidemiological and animal studies suggests a role for probiotics in the restoration of normal gut microbiota function (Derrien & van Hylckama Vlieg, 2015). Moreover,

probiotics have also been used as potential therapies for psychiatric disorders, with some promising initial results.

In this review, we describe the development of a healthy gut microbiota from infancy to adulthood, followed by the range of factors known to disrupt the composition in both infants and adults including mode of delivery, antibiotic treatment, diet, stress, and infection. We then examine the use of probiotics as a possible therapeutic strategy for the reversal of gut microbiota alterations and subsequently, the beneficial effects of probiotics on human health. Strategies for effective delivery of probiotics are discussed, with examples of food- and non-food based probiotic carriers. Finally, we describe preclinical and clinical studies where probiotics have been found to positively affect brain function and mental health.

Gut Microbiota Composition in Infants and Adults

The concept of a "placental microbiome" was previously proposed with studies reporting that the placenta contains non-pathogenic commensal bacteria with a similar profile to that of an oral microbiome (Aagaard et al., 2014). However, recent robust studies investigating this have shown that there is not enough evidence to support this (Kauperman et al., 2019; Gschwind et al., 2020; Olomu et al., 2020). At birth the infant gut is rapidly colonised by either maternal faecal and vaginal bacteria or microbes from the hospital environment depending on the mode of delivery (Ferretti et al., 2018). Initial colonisers of standard vaginally delivered (SVD) infants such as Enterobacteriaceae, *Staphylococcus* and *Streptococcus* consume oxygen in the gut which prepares the environment for the establishment of strict anaerobes such as *Bifidobacterium* and *Bacteroides* (Adlerberth & Wold, 2009; Penders et al., 2006). During the first months of life, the infant gut is unstable and can undergo many

compositional changes until approximately 2 years of age when the microbial community is similar to that of an adult (Sekirov et al., 2010).

Defining a healthy adult gut microbiota is vital to illustrate the compositional differences associated with disease (Lozupone et al., 2012). In recent years, due to substantial improvements in sequencing technologies, it has become clear that four main phyla (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria) dominate the healthy adult gut microbiota with members from the Firmicutes and Bacteroidetes phyla being most abundant (Eckburg et al., 2005; Qin et al., 2010; Turnbaugh et al., 2009). Although these four phyla make up a healthy phylogenetic core in adults, there is considerable inter-individual diversity at lower taxonomic levels with some studies suggesting that at species or strain level, the gut microbiota profile is highly unique and person-specific (Eckburg et al., 2005; Turnbaugh et al., 2010). It has been suggested that three enterotypes or core bacterial clusters exist based on the dominance of a particular genus - *Bacteroides*, *Prevotella* or *Ruminococcus* (Arumugam et al., 2011). However, this concept has been debated previously with suggestions that this approach may be an oversimplification of the gut microbiota composition (Jeffery et al., 2012). This is supported by a study in which Knights and colleagues reported that over the course of a year, a healthy individual's microbiota profile can fluctuate between clusters and is not restricted to a single enterotype (Knights et al., 2014). Alternatively, it has been proposed that rather than discrete groups or clusters, the gut microbiota composition is dynamic and should be viewed as a continuous gradient (Jeffery et al., 2012). It has been shown that some bacterial groups are bi-modally distributed; either being highly prevalent or nearly absent (Lahti et al., 2014). It was reported that these contrasting states are stable while intermediate abundance appears to be unstable. These stable states are associated with human health and can be

influenced by many host and environmental factors. Additionally, it has been stated that a combination of both diversity and functionality are necessary to maintain a stable microbiota.

Given that the microbiome (the gene pool of the microbiota) is highly conserved among individuals (Qin et al., 2010; Turnbaugh et al., 2009), the concept of functional redundancy has been suggested whereby essential processes may be carried out by several taxa (Jalanka-Tuovinen et al., 2011). Furthermore, it has been proposed that there are key species which may contribute considerably to the functional profile and play a vital role in maintaining gut microbiota stability (Flint et al., 2012).

Factors Disrupting the Gut Microbiota

It is well established that colonisation of the infant gut at birth influences normal development (Mueller et al., 2015). While caesarean-section (CS) delivery rates have increased rapidly over the last decade (Roberts et al., 2012), it has become apparent that CS delivery can greatly affect the infant gut colonisation pattern and can result in negative consequences for the infant. When compared with standard vaginally delivered (SVD) new-borns, infants born by CS are colonised with less beneficial microbes including lower numbers of *Bifidobacterium* and *Bacteroides* and higher numbers of potentially detrimental bacterial species such as *Clostridium difficile* (Biasucci et al., 2010; Penders et al., 2006). This disruption of maternal bacterial transmission in CS delivered infants was illustrated in a study which showed that in SVD infants, 72% of the initial bacterial species colonising the gut match species from the mother while only 41% of the maternal bacterial species are found in CS infants (Bäckhed et al., 2015). It has been suggested that these initial microbial alterations can persist for months (Bäckhed et al., 2015) or years after delivery (Jakobsson et al., 2014).

In addition, these colonisation disturbances at birth are associated with an increased risk of developing several disorders later in life such as inflammatory bowel disease, asthma, obesity, and type I diabetes (Hyde & Modi, 2012; Moore et al., 2019). Epidemiological studies have also suggested that that CS delivery may negatively affect normal CNS development (Juárez et al., 2008). It has been proposed that this influence is dependent on alterations in gut-brain signalling during critical neurodevelopmental time-windows (Borre et al., 2014). Given that microbiota disruption due to CS delivery can influence development and lead to an increased risk

of developing mental and immune related disorders, it is of relevance to investigate the use of early microbiota-targeted interventions to reduce later risk.

Antibiotic use is widespread with almost 50% of adults and 70% of children receiving antibiotics each year (Morgun et al., 2015). Antibiotic use in infancy can considerably disrupt the gut microbiota colonisation pattern and has been linked to childhood asthma and obesity (Murphy et al., 2014; Penders et al.,2011). Furthermore, short term treatment with antibiotics after birth has been shown to reduce bacterial diversity and has been associated with a significant reduction in *Bifidobacterium* spp. (Hussey et al., 2010; Savino et al., 2011) accompanied by increased levels of Proteobacteria (Fouhy et al., 2012). These initial disruptions can result in prolonged consequences and the altered microbiota composition can persist for at least 1 year after perinatal antibiotic exposure (Persaud et al., 2014). The adult gut microbiota is also susceptible to the effects of antibiotic use with some reports suggesting that the post-antibiotic compositional shift may take years to return to pre-treatment state, and in some cases, may never fully recover (Dethlefsen & Relman, 2011; Jakobsson et al., 2010; Jernberg et al., 2007). It has also been reported that intestinal disturbances due to antimicrobials can influence behaviour and brain chemistry (Bercik et al., 2011; Desbonnet et al., 2015). Indeed, antibiotics have been used positively to treat schizophrenia and depression. For example, Ghanizadeh and colleagues reported that using minocycline, which acts on both gram positive and negative bacteria, as an adjuvant treatment with risperidone yielded positive results when administered to schizophrenia patients, compared with placebo (Ghanizadeh et al., 2014).

Diet is a key factor affecting the colonisation and diversity of the human gut microbiota. From birth, the effects of diet can be seen between infants who are exclusively breastfed and infants who are formula fed (Timmerman et al., 2017).

Human breast milk contains a variety of lipids, proteins and an abundance of human milk oligosaccharides (HMOs) (Subramanian et al., 2015) which promote the growth of *Bifidobacterium* spp. and have been associated with many beneficial effects on health including enhanced intestinal barrier function (Weng et al., 2014) and improved response to vaccinations (Huda et al., 2014). Bacterial diversity and stability have been shown to differ between breastfed and formula fed infants, with breast fed infants having a lower overall bacterial diversity (Azad et al., 2013) but a more stable and uniform bacterial community (Bezirtzoglou et al., 2011). Formula feeding, on the other hand leads to increased levels of Verrucomicrobiaceae and *C. difficile* (Azad et al., 2013). It has been stated that once breastfeeding concludes and solid food is introduced, the microbial community dominated by *Lactobacillus, Bifidobacterium* and Enterobactericeae becomes colonised by *Clostridium* spp. and *Bacteroides* spp. (Bergström et al., 2014; Fallani et al., 2011). Several studies have shown strong links between adult diet and microbial diversity in the gut (Doré & Blottière, 2015). A diet rich in animal proteins and fat is associated with high levels of *Bacteroides* while individuals consuming a diet high in fibre, fruit and vegetables, and low in meat and dairy are dominated by *Prevotella* (Wu et al., 2011). Diet and gut microbiota composition have also been linked in older adults. Claesson and colleagues reported that elderly individuals in long-term residential care (who had a less diverse, high fat, low fibre diet) had higher levels of Bacteroidetes, while those individuals living in the community (low fat, high fibre diet) had higher proportions of Firmicutes (Claesson et al., 2012). Preclinical studies investigating a link between diet and depression-like symptoms in mice have shown that a diet high in saturated fat impacts on behaviour and immune function (Jørgensen et al., 2014). Furthermore, it has been shown that mice fed a high fat lard diet show impaired cognitive performance, increased brain

inflammation and decreases in cortical brain-derived neurotrophic factor (BDNF) (Pistell et al., 2010).

It is well known that geographical location can affect the gut microbiota composition in both infants and adults. De Filippo and colleagues reported that children consuming a high fibre diet in Burkina Faso have increased levels of *Prevotella* and *Xylanibacter* accompanied by a reduction in Firmicutes when compared with children from Italy (De Filippo et al., 2010). This finding is consistent with many of the other studies comparing the gut microbiota of westernised and non-westernised individuals. Consumption of a Western diet is characterised by the presence of *Faecalibacterium, Bacteroides, Ruminococcus* and *Bifidobacterium* while individuals consuming non-Western diets are dominated by *Prevotella* (Martínez et al., 2015). It has been argued that dietary habits are not entirely responsible for the differences found between the gut microbiota composition in Western societies and non-industrialised societies but that the variation is also influenced by modern lifestyle and cultural background (Martínez et al., 2015). However, the substantial influence of diet can be seen in a study comparing the gut microbiota of a hunter-gatherer community in northwest Tanzania to European individuals (Schnorr et al., 2014). The diet of the Hazda people is largely plant based which is evident in the composition of their gut microbiota; increased levels of *Prevotella* and *Treponema*. Furthermore, individuals in this region lack *Bifidobacterium* spp. in the gut bacterial community which is reflective of the absence of dairy and meat products in the diet (Schnorr et al., 2014). Geographical location appears to significantly affect the gut microbiota composition; however, the large proportion of this variation that can be attributed to the strong influence of dietary habits cannot be overlooked.

Stress can have a substantial effect on the gut microbiota composition (Cryan & Dinan, 2012). This is particularly evident in studies investigating the effects of prenatal stress and early life stress. Maternal stress during pregnancy has been shown to disrupt the gut microbiota in offspring and is also associated with a higher prevalence of neurodevelopmental disorders (Golubeva et al., 2015; Jašarević, Rodgers, & Bale, 2015). Early maternal separation can also result in a variety of behavioural and physiological alterations, accompanied by gut microbiota disturbances (O'Mahony et al., 2009). Additionally, chronic stress in adulthood has been associated with altered microbiota composition characterised by a decrease in *Bacteroides* spp. accompanied by an increase in *Clostridium* spp. (Cryan & Dinan, 2012).

Accumulating evidence has indicated that maternal infection during pregnancy is associated with brain and behavioural impairments in the offspring (Atladóttir et al., 2010; Brown & Derkits, 2010). Preclinical investigations have reported that offspring of mice infected with influenza show significant alterations in the expression of genes associated with neurodevelopmental disorders, accompanied by brain atrophy in several regions and altered levels of neurochemicals including serotonin and taurine (Fatemi et al., 2008). In addition, clinical studies have also reported that maternal hospitalisation with infection during pregnancy is associated with a higher risk of autism spectrum disorder (ASD) development in children (Lee et al., 2015). Some studies have shown that this risk is elevated if the infection occurs in the first and second trimesters (Atladóttir et al., 2010), with others reporting that timing did not influence the risk and that increased likelihood of ASD development in offspring is associated with infection at any time during gestation (Lee et al., 2015). Previously, it was shown that offspring of a maternal immune activation (MIA) mouse model show

GI abnormalities accompanied by alterations in gut microbiota composition (Hsiao et al., 2013) indicating that in addition to influencing behaviour, maternal infection can also impact the gut microbiota.

It is evident that a variety of factors can influence the gut microbiota of both the infant and adult and may contribute to alterations in brain function and behaviour. It is also of great importance to investigate new therapies such as those which may have the potential to reverse the gut microbiota disturbances, as well as prophylactic therapies which may help to prevent microbial alterations in the gut which can lead to disease.

Prebiotics and Probiotics

Potential Benefits of Prebiotics and Probiotics

It has become apparent that many neurological and GI conditions are linked with gut bacterial disruptions (Clemente et al.,, 2012). Dietary habits can have a large influence on the gut microbiota composition. Consequently, modulation of the gut microbiota through functional foods containing such ingredients as prebiotics and probiotics may be a promising intervention strategy to alleviate symptoms associated with GI and mental conditions.

The definition of prebiotics as determined by the International Scientific Association for Probiotics and Prebiotics is “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson et al., 2017). Prebiotics must be selectively utilised and have adequate evidence of health benefit. Prebiotics can act as substrates for probiotic bacteria in the gut such as *Lactobacillus* and *Bifidobacterium* (Gibson et al., 2017). For a specific food ingredient to be

classified as a prebiotic, there are certain criteria which need to be met. Firstly, it must be resistant to gastric acid secretion and to hydrolysis by mammalian digestive enzymes, it must be absorbed in the upper GI tract and fermented by the gut microbiota, and it must stimulate the growth or activity of beneficial gut bacteria (Gibson et al., 2017). The most well-known prebiotics include galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin (Al-Sheraji et al., 2013), and can be found naturally in a variety of foods including asparagus, garlic, tomato, and banana (Sangeetha et al., 2005). They can also be found in human breast milk as a substrate for *Bifidobacterium* (Morozov et al., 2018).

Prebiotics exert many positive effects on human health through the stimulation of beneficial microbes including improving host immunity, gut barrier function, and SCFA production while reducing potentially pathogenic bacteria (Slavin, 2013). Prebiotics have been shown to stimulate the bifidogenic effects of human breast milk and reduce the incidence of allergies and infection in infants for up to 2 years (Arslanoglu et al., 2008; Boehm & Moro, 2008). In addition, prebiotic treatment in mice has been shown to alter the caecal microbiota composition with differences seen in over 100 taxa when compared to controls (Everard et al., 2011). Prebiotics can be used in combination with probiotics (synbiotic) to enhance the effects of a probiotic. This has been shown to be more effective at altering the gut microbiota composition than using a probiotic alone (Saulnier et al.,, 2008).

Probiotics are live bacteria that can confer a beneficial effect on an individual when consumed in sufficient amounts (Butel, 2014). The most common probiotics are *Lactobacillus* spp. and *Bifidobacterium* spp. (Cammarota, Ianiro, Bibbò, & Gasbarrini, 2014). Probiotics exert a beneficial effect through several mechanisms including the inhibition of pathogenic species through the production of antimicrobial

compounds or direct competition for adhesive sites. Furthermore, they enhance epithelial barrier function and modulate immune responses (Power et al., 2014; El Aidy et al., 2015).

Probiotics have been reported to have a variety of beneficial effects on humans. For example, *Lactobacillus plantarum* 299 v and *Lactobacillus rhamnosus* GG increase the production of intestinal mucins which prevent adherence to epithelial cells by pathogenic microbes (Mack et al.,1999). *Lactobacillus* GG, *Bifidobacterium lactis* Bb-12 and *Saccharomyces boulardii* have also been reported to enhance IgA production and secretion (Rautava et al.,, 2006; Rodrigues et al., 2000). As IgA maintains homeostasis in the mucosal barrier, increased secretion can protect the epithelium and defend against pathogens (Mantis et al.,2011). Other studies have shown the protective effects of probiotics against pathogenic strains including the production of bacteriocins (Rea et al., 2011), and the ability to increase production of human beta-defensins (Kabeerdoss et al., 2011). Probiotics should have "Generally Recognised As Safe" (GRAS) status (Nagpal et al., 2012), be stable during processing and drying, and be capable of withstanding conditions encountered in the GI tract (Iannitti & Palmieri, 2010).

Delivery of Viable and Functional Probiotics

For a probiotic to exert beneficial effects, it must be remain viable and reach the GI tract in adequate numbers (at least 10^6-10^7 CFU/g) (Bosnea et al., 2009). Thus, there are many challenges in the successful delivery of probiotics, as the fate of ingested probiotics needs to be considered. Probiotic strains ingested orally pass through the GI tract where they are subject to low pH levels in the stomach and high enzyme levels in the duodenum. Following this, cells are exposed to bile, pancreatin

and lipase in the small intestine (Derrien & van Hylckama Vlieg, 2015). Survival in these harsh physiochemical conditions appears to be strain-specific with strains of *B. animalis, L. casei, L. rhamnosus* and *L. plantarum* proving to be most resilient (Derrien & van Hylckama Vlieg, 2015).

An attractive solution to increase viability of probiotic cells in the GI tract is encapsulation (Frakolaki et al., 2021). Microencapsulation must protect the probiotic cells from chemical degradation and must produce capsules large enough to allow for a sufficient bacterial load to be delivered (McClements et al., 2009). Moreover, the microencapsulation method needs to allow for controlled release of the probiotic into the GI tract (Cook et al., 2012). Microencapsulation of probiotics is performed using three main methods: extrusion, emulsion and spray drying (Kailasapathy, 2002). A range of materials are used for microencapsulation including alginate, gellan gum, chitosan, starch, and milk proteins (Burgain et al., 2011). The process involves the production of microcapsules through the formation of a water-in-oil emulsion, stabilised by surfactants. Microencapsulation using extrusion is a physical technique which projects a solution containing the probiotic cells through a nozzle at high pressure, while spray drying involves dissolving the probiotic and polymer matrix and subsequently atomising in heated air (de Vos et al., 2010). Spray freeze drying is also used in combination with cryoprotectants which produces microcapsules by drying a frozen sample under vacuum (Cook et al., 2012). Encapsulation is used to protect cells from heat and moisture which is frequent during processing of food products for probiotic delivery (D'Orazio et al., 2015).

The benefits of microencapsulation were highlighted in a study investigating the effects of this technique on the viability of several strains in conditions mimicking the gastrointestinal tract. Viability during storage and production was also examined

(D'Orazio et al., 2015). It was found that the non-encapsulated strains were destroyed on exposure to GI conditions as well as exposure to heat and osmotic stress. Chitosan-coated alginate microcapsules were found to significantly enhance protection and probiotic viability. The benefits of microbial encapsulation were demonstrated in a study which investigated the survival of commercial probiotics (Millette et al., 2013). Twenty-nine commercially available probiotics in various forms (fermented milk; powder; capsules, and yogurt) were assessed for bacterial viability in simulated gastric and intestinal fluids. It was found that probiotic capsules covered with an enteric coating had a higher rate of survival than uncoated forms. Only one fermented milk product and one probiotic powder showed high survival rates.

It is evident that further research is needed on viability of commercial probiotic products as the majority of those examined were unable to survive conditions consistent with the GI tract and thus, may not exert beneficial effects on the consumer. In addition to survival in the gut, probiotic strains must also reach the area in the GI tract where they can benefit the host. The addition of specific excipients can allow the probiotic to be released in a controlled manner. The use of chitosan has been shown to retain bacterial strains in vitro for over 2 hours in stimulated gastric and intestinal juices before release (Cook et al., 2011). However, given the timing of passage through the GI tract, these capsules would only allow for probiotic release into the small intestine, whereas the target area for various conditions is the large intestine (Cook et al., 2012). However, a study by Lin and colleagues described the use of alginate microcapsules coated with chitosan followed by a second coat of alginate and reported that these capsules were stable and remained intact in the GI tract of rats for 6 hours (Lin et al., 2008). It has been shown that microcapsules composed of alginate, xanthan

gum and carrageenan are very effective at enhancing probiotic survival (Ding & Shah, 2009).

Non-Food Based Probiotic Delivery

Probiotic products are currently available in a variety of non-food based forms including chewing gum, lozenges, sachets and capsules (Klayraung et al., 2009). Tablet based delivery systems have several benefits, including accurate dosage administration and targeted delivery to the site of action (e Silva et al., 2013). In addition, they are very stable during storage and easy to administer (Vorländer et al., 2020). A disadvantage of tablets, however, is the level of heat produced during compression, with temperatures reaching up to 60°C, which can be detrimental to the survival of many bacterial strains (Roueche et al.,, 2006). Several attempts have been made to prevent this damage using a variety of tablet excipients. Tablets have been shown to act as good carriers for probiotics (Mirzaeei et al., 2017). Several functional polymer compositions have been investigated for probiotic viability in simulated GI conditions. For example, a hydrophilic tablet was proposed containing carboxy-methyl high amylose starch (CM-HAS) and chitosan for the delivery of *L. rhamnosus* (Calinescu & Mateescu, 2008). It was found that alterations in the molecular weight and percentage of chitosan used affected the timing of bacterial release in simulated GI conditions and improved the percentage of bacteria delivered. This suggests that this formulation may have potential as a carrier for probiotics intended for colonic release. Klayraung and colleagues investigated the use of tablets as delivery systems for freeze-dried *L. fermentum* 2311 (Klayraung et al., 2009). It was found that tablets containing hydroxypropyl methylcellulose phthalate (HPMCP) were successful for enhancing survival of the probiotic bacterial strain (80% viability). Incorporation of sodium alginate further increased viability in simulated GI conditions (>90%) and the

tablets were found to have a slow disintegration time (approx. 5 hours). A novel tablet excipient was proposed by Poulin and colleagues (Poulin et al.,, 2011). These tablets containing *B. longum* HA-135 were assessed in simulated gastric conditions and it was found that increased compression during tablet formation decreased bacterial viability, while compression at 67 MPa resulted in tablets containing 10^9 viable cells. Viability decreased rapidly for non-compressed freeze-dried cells during gastric incubation. This was also true for tablets composed of β-lactoglobulin which dissolved after 30 minutes in gastric fluid. However, tablets were also prepared using succinylated β-lactoglobulin at 50% and 100%, resulting in survival of 10^7 CFU/tablet, two hours following exposure to gastric conditions. It was also noted that the tablets were stable for three months at 4°C. Therefore, manufacturing tablets at 67 MPa appears to be a stable pressure to allow for tablet formation but also to reduce probiotic viability loss.

Several other formulations have been examined as tablet based probiotic carriers (El Kadri et al., 2018) and have shown some promising results, including sodium alginate, hydroxypropylcellulose (Chan & Zhang, 2005), hydroxypropylmethylcellulose acetate succinate (Stadler & Viernstein, 2003), cellulose acetate phthalate in combination with sodium croscarmellose (e Silva et al., 2013), and a bi-layered mini-tablet-in-tablet system containing ovalbumin, lactose and eudragit S100 (Govender et al., 2015).

In addition to tablet carriers, there are a variety of well-known commercial probiotics available including Probio-Tec® probiotic strains available in a variety of dosage forms, BioGaia probiotic straws, chewable tablets and drops, Align (Proctor & Gamble); Florastor (Biocodex); Idoform/Bifiform (Ferrosan); Probiotica (McNeil Consumer Healthcare), and many others (Sreeja & Prajapati, 2013).

Food Based Probiotic Delivery

The demand for 'functional foods' which provide a health benefit to the consumer has gained popularity in previous years (Terpou et al., 2019)). In this respect, a variety of food products have been evaluated as delivery systems for probiotics (**see Table 1**). The dairy industry, in particular, has developed several probiotic carriers including fermented milks, yogurt, powdered infant milk, butter, cheese, mayonnaise and ice-cream (Cruz et al., 2009). Many factors need to be considered when developing functional food products harbouring viable probiotics, given that several aspects of food processing and storage can negatively affect the viability of probiotic strains. Factors including pH, the presence of salt, sugar and artificial flavourings, heat treatment and oxygen levels need to be taken into account (Tripathi & Giri, 2014). In addition to the stability of the probiotic strain, the sensory aspects of the food product also need to be examined. As such a high number of probiotic cells are necessary to exert a beneficial effect, this can result in some unpleasant flavours in the food product, such as the undesirable flavours associated with acetic or lactic acids produced by *Bifidobacterium*. This may require the addition of extra ingredients to mask undesirable flavours (Granato et al., 2010). Some common food products and their effectiveness as probiotic carriers are discussed below.

Yogurt

Fermented milks and yogurts have been used for the delivery of several probiotic strains (Terpou et al., 2019). As yogurts are natural carriers of bacteria and have a high consumer acceptance, they can be considered a good food based probiotic carrier (Sanders & Marco, 2010). Several studies have examined the sensory impact of the addition of probiotics to yogurt. Yogurt containing *L. rhamnosus* and *L. reuteri*

was found to have the same appearance, flavour, texture, and overall quality as non-probiotic yogurt (Hekmat & Reid, 2006). Similarly, probiotic Greek yogurt containing *L. paracasei* had a rich, smooth and traditional taste, and good acceptance among consumers (Maragkoudakis et al., 2006). However, it has been noted that there is a texture change in yogurt which has been modified with encapsulated probiotics (Fatima et al., 2020). Different types of yogurt have shown changes in probiotic viability levels; skimmed-set yogurt retains higher levels of viable probiotics than whole-set yogurt (Birollo et al., 2000). It has also been noted that plain yogurt sustains probiotic viability during storage better than fruit yogurts (Kailasapathy et al., 2008). This appears to be due to reductions in pH which occur after addition of fruit pulp (. Ranadheera et al., 2010). The addition of prebiotics such as inulin and fructooligosaccharides to yogurt products to create a synbiotic has also proven effective for probiotic viability enhancement (Capela et al., 2006). A study reported that probiotic yogurt containing *L. rhamnosus* GG, *B. lactis* Bb-12 and *L. acidophilus* La-5 was effective for the reduction of antibiotic-associated diarrhoea in children (Fox et al., 2015).

Although yogurt appears to be an effective probiotic carrier, there are many challenges during processing which need to be taken into consideration. Dairy products such as yogurt have a low pH and it has been suggested that previous strain exposure to lower pH values is useful to allow for acid tolerance during processing (Granato et al., 2010; Sanz, 2007). In addition, care should be taken to prevent the introduction of oxygen during processing as several anaerobic strains may lose viability upon exposure (Ahn et al., 2001). It is also necessary to consider the compatibility between starter cultures and probiotic strains (Vinderola et al., 2002).

Ice-cream and desserts

Ice-cream as a potential probiotic carrier has been demonstrated to provide greater protection than milk and yogurt (Ranadheera et al., 2012). It is also highly consumer friendly and is attractive to children and adults (Cruz et al., 2009). Ice-cream has several properties which makes it an effective probiotic carrier including the presence of milk proteins, fat, and lactose, in addition to a relatively high pH (5.5-6.5) (Cruz et al., 2009). Several studies have demonstrated the potential of ice-cream as a delivery vector for probiotics. Two different ice-cream formulations containing *L. acidophilus, L. agilis* and *L. rhamnosus* and either sucrose or aspartame were stable for 6 months stored at -20°C (Başyiğit et al., 2006). It was also found that the probiotic strains were resistant to a variety of conditions including bile salts and antibiotics. Ice-creams containing *L. johnsonii* La-1 with different fat and sugar amounts were found to retain high probiotic viability after 8 months of storage at -16°C and -28°C (Alamprese et al., 2005). *L. acidophilus* La-5 and *B. animalis* Bb-12 showed increased probiotic survival in ice-cream containing inulin and fructooligosaccharides (Akalın & Erişir, 2008). It was also noted that the prebiotic containing ice-cream was firmer and had less variation in melting properties compared with control product.

Several studies have examined the sensory properties of probiotic ice-cream and desserts. Ice-cream containing *L. acidophilus* and *B. lactis* was shown to have good overall sensory qualities and no "probiotic flavours" were detected (Akın et al., 2007). Strawberry ice-cream supplemented with *L. acidophilus* was found to be acceptable with the addition of fruit improving the taste, suggesting that acidic fruit may be a useful additive to ensure masking of any unpleasant "probiotic flavours" (Belgec Varder & Öksüz, 2007). A synbiotic chocolate mousse containing *L.*

paracasei and/or inulin was evaluated for sensory characteristics (Aragon-Alegro et al., 2007) and no significant differences were found between the probiotic, synbiotic and control mousse. However, upon sensory evaluation of each chocolate mousse, the probiotic mousse was preferred, followed by the synbiotic product. Sensory evaluation was performed on a probiotic coconut flan containing *B. lactis* and *L. paracasei* (Corrêa et al., 2008). While several sensory parameters, such as flavour and texture were found to be similar at 7, 14 and 21 days of storage, there was a tendency for preference of the probiotic flan over the control product.

Due to the high acceptance among testers and the probiotic viability noted during storage, ice-cream shows potential as a probiotic carrier. However, as with yogurt, many manufacturing and processing concerns need to be addressed. Most notable is the fact that oxygen incorporation is a key step in the manufacture of ice-cream and is vital for texture properties of the final product and thus, cannot be avoided (Sofjan & Hartel, 2004). Therefore, it may be necessary to select oxygen tolerant probiotic strains or use encapsulated probiotics during manufacture. It has been reported that microencapsulation of *L. casei* and *B. lactis* in ice-cream increased the probiotic survival by 30% (Homayouni et al., 2008). However, the effect of microencapsulation on the sensory and texture properties of the ice-cream should be considered.

Cheese

Several cheese varieties have been successfully used as probiotic delivery systems, including Cheddar cheese, goat cheese, Crescenza cheese, cottage cheese and fresh cheese (Ross et al., 2002). Cheese has a relatively high pH when compared to other food carriers and has a high fat content, which can help to protect the probiotics

during transit through the GI tract (Stanton et al., 1998). These properties provide a stable medium for the bacteria and may support long-term viability (Mushtaq et al., 2019). A variety of studies have examined the potential of cheese as probiotic carrier. For example, survival of *L. rhamnosus* and *L. acidophilus* was shown to be enhanced in simulated GI conditions (using GI tract and colon simulator) when present in Gouda cheese (Mäkeläinen et al., 2009). Furthermore, the cheese appeared to increase the levels of *Lactobacillus* in the simulation and increased the concentrations of fatty acids produced. Cheddar cheese containing *L. acidophilus, L. casei, L. paracasei* and *Bifidobacterium* spp. was produced, and strains showed high viability during manufacturing (Ong et al., 2006). This was also seen in a study containing the same probiotic species in Cheddar cheese (Ganesan et al., 2014). Two *L. salivarius* strains were shown to remain viable in fresh cheese for up to 21 days and their presence did not modify cheese texture (Cárdenas et al., 2014). Addition of *L. paracasei, B. bifidum* and *L. acidophilus* did not impact negatively on sensory properties after storage for 15 days at 5°C (Vinderola et al., 2009). This was noted in probiotic cheese supplemented with *L. fermentum* and *L. plantarum* with reports of similar flavour, texture, and appearance in both probiotic and control cheeses (Kılıç et al., 2009). Health benefits associated with the consumption of probiotic cheese include improvements in blood pressure, increased phagocytic activity and reduction of salivary yeast counts, based in clinical and animal studies (Lollo et al., 2015; Ouwehand et al., 2010).

Beverages and other probiotic foods

Many non-dairy and vegetarian foods have been used to deliver probiotics (Aspri et al., 2020; Min et al., 2019), including fruits (Lavermicocca et al., 2005), vegetables (Yoon et al., 2006), and cereal products (Setta et al., 2020; Helland et al.,

2004), as well as probiotic beverages, such as fruit and vegetable juices. It has been shown that several probiotic strains can tolerate the low pH environment of orange, pineapple and cranberry juices, surviving above 10^6 CFU/mL for at least 12 weeks (Sheehan et al., 2007). Carrot juice has been shown to promote the viability of *B. bifidum* and *B. lactis* for up to 24 hours (Kun et al., 2008). In addition, probiotics remain viable in tomato juice for up to 4 weeks at 4°C (Yoon et al., 2004). Although these juices may be potentially effective probiotic carriers, the sensory properties need to be examined, as strong or unpleasant flavours can result in a negative perception of probiotic juices (Granato et al., 2010). This was evident in a study by Luckow and Delahunty who reported that consumers were able to distinguish between orange juice containing probiotics and conventional orange juice and described the flavours as "medicinal", "dairy" and "dirty" (Luckow & Delahunty, 2004). Sensory properties of whey beverages were enhanced following incorporation of probiotic *B. longum* and *L. acidophilus*, (Zoellner et al., 2009), while fermented goat milk containing probiotic *L. acidophilus* and *B. bifidum* were also found to be highly acceptable (Martın-Diana et al., 2003; Vinderola et al., 2000).

Several other foods have been examined as probiotic carriers including soy cheese, soy milk and table olives (Peres et al., 2012; Min et al., 2019). The use of table olives is an interesting choice as a serving of 10-15 olives can carry 10^9-10^{10} *Lactobacillus* spp. which suggests that olives may be an effective probiotic delivery vector (Lavermicocca, 2006). The colonisation of the surface of olives was investigated using *L. paracasei* (De Bellis et al., 2010) and it was found to be successful. The addition of probiotics to vegetables provides an attractive option for consumers who are lactose intolerant or prefer non-dairy products. In addition, several

other fruits have been investigated as probiotic delivery vectors including strawberry and apple (Pérez-Cobas et al., 2013).

It is evident that both chemical composition and physical structure of the proposed food carrier are important parameters for probiotic viability. Moreover, slight changes in the fat and sugar composition can greatly impact bacterial survival (Ranadheera et al., 2010). In many of these food products, stability has been enhanced through the use of microencapsulation methods mentioned previously, with encapsulation proving to significantly enhance probiotic viability in comparison to free cells, during exposure to simulated gastric conditions and during storage (Capela et al., 2006; Dias et al., 2018; McMaster et al., 2005). However, further investigation is necessary to examine the sensory properties associated with encapsulated bacterial cells to enhance food products as optimal delivery systems for probiotic bacteria.

Table 1: Food products investigated as potential probiotic carriers

Carrier	Products	Probiotic	Reference
Dairy-based	Yogurt	*L. casei* *L. acidophilus, L. casei, B. bifidum*	(Aryana & McGrew, 2007) (Sendra et al., 2008)
	Ice-cream	*L. casei, B. lactis* *L. johnsonii*	(Homayouni et al., 2008) (Alamprese et al., 2002)
	Chocolate	*L. helveticus, L acidophilus*	(Possemiers et al., 2010)
	Whey protein drink	*B. breve, B. infantis, B. lactis, L. plantarum, L. casei, Streptococcus thermophilus*	(Dalev et al., 2006)
	Cheddar cheese	*B. longum, B. lactis, L. casei, L. acidophilus*	(Ong & Shah, 2009)
	Feta cheese	*L. acidophilus, B. lactis*	(Kailasapathy & Masondole, 2005)
Soy-based	Soymilk	*L. acidophilus, L. gasseri* *L. plantarum*	(Ewe et al., 2010) (Bao et al., 2012)
	Soy cream cheese	*L. acidophilus*	(Liong et al., 2009)
Fruit and vegetable based	Carrot juice	*B. lactis* Bb12, *B. bifidum* B7.1, B3.2	(Kun et al., 2008)
	Tomato, orange and grape juice	*L. plantarum, L. acidophilus* *L. casei* A4, *L. delbrueckii* D7	(Nagpal et al., 2012) (Yoon et al., 2004)
	Cabbage juice	*L. plantarum, L. acidophilus*	(Yoon et al., 2006)
	Banana puree	*L. acidophilus*	(Tsen et al., 2009)
	Blackcurrant juice	*L. plantarum* 299 v	(Luckow & Delahunty, 2004)
Oat based	Oat based drink	*L. plantarum* B28	(Angelov et al., 2006)
	Malt based drink	*L. reuteri*	(Kedia et al., 2007)
	Oat bran pudding	*Lactobacillus* and *Bifidobacteria*	(Blandino et al., 2003)

Gut Microbiota Modulation Using Probiotics and Prebiotics

Infant Microbiota Modulation

The use of probiotics during the perinatal period is an important area of investigation, due to the role of initial colonisation of the infant gut in health and disease prevention (Mueller et al., 2015). Disruption in the colonisation pattern of the infant microbiota during critical developmental windows can have long-lasting health consequences (Cox et al., 2014). As the gut microbiota in infancy is relatively simple and unstable, the use of probiotics could potentially have a large and long-lasting impact on the composition.

The administration of probiotics during pregnancy is still a somewhat new concept with relatively few clinical trials conducted to date. Many studies have focused on pregnancy outcomes and associated conditions including pre-term delivery, gestational diabetes, gestational weight gain and preeclampsia (Arango et al., 2015). Administration of *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* reduced the risk of atopic eczema in infants at 6 months (Bertelsen et al., 2014), while *L. rhamnosus* GG administration alone was also shown to reduce the risk of atopic eczema in children up to 7 years old (Kalliomäki et al., 2007). Beneficial effects of probiotics have also been noted for the treatment/prevention of allergies, diarrhoea, and necrotizing enterocolitis in pre-term infants (Di Gioia et al., 2014).

In a clinical trial, *Bifidobacterium breve* M-16 V and *Bifidobacterium longum* BB536 were given to pregnant mothers one month prior to delivery and to their infants for the first 6 months of life to assess the effects of a probiotic formulation on the prevention of allergic diseases (Enomoto et al., 2014). It was found that infants who showed symptoms of eczema/atopic dermatitis (AD) at 4 months had lower

proportions of Actinobacteria and higher proportions of Proteobacteria than those without symptoms. It was also noted that mothers given probiotics had a significantly lower abundance of Proteobacteria while infants in the probiotic group had higher levels of Bacteroidetes at 4 months of age. No differences in gut microbiota composition were seen between infants in the probiotic and placebo groups at 10 months of age. Interestingly, the occurrence of AD tended to be lower in the infants given probiotics at 4 months of age and was significantly reduced in this group at both 10 and 18 months. Several studies have indicated that children affected with allergic diseases have more *Clostridium* spp. accompanied by decreases in *Bifidobacterium* and Enterobacteriacae (Enomoto et al., 2014). Moreover, it is of interest to note that in this study, differences in microbial composition between AD symptomatic infants and healthy infants were apparent at 4 months but not at 10 months, suggesting that the early microbiota plays an important role in atopic dermatitis and related conditions. The gut microbiota modifications reported here suggest a role for *Bifidobacterium breve* M-16 V and *Bifidobacterium longum* BB536 during the perinatal period as a possible prophylactic treatment to reduce the risk of AD development. However, further analyses of the composition of the infant gut at lower taxonomic levels are necessary to examine the use of this probiotic formula as a possible intervention strategy.

Other studies have shown that the use of *L. rhamnosus* GG in both infants and pregnant mothers can result in higher levels of *Bifidobacterium*, specifically *B. longum* and *B. breve* (Gueimonde et al., 2006; Lahtinen et al., 2009). As breast milk is the gold standard for early infant nutrition (Hassiotou et al., 2013), due to the range of health benefits provided for the developing infant, interventions with strains targeted to the increase of beneficial microbes is a desirable outcome of probiotic treatment.

Moreover, it has been proposed that strains isolated from human breast milk for use in infant formula may be more beneficial than probiotics isolated from infant microbiota or fermented foods (Chassard et al., 2014; Korpela et al., 2018, Li et al., 2019). This may prove to be a promising avenue of investigation; however further clinical studies are necessary.

Adult Microbiota Modulation

Several clinical trials have examined the use of probiotics in adult cohorts to maintain health or alleviate disease (Lew et al., 2019; Bagga et al., 2018). A study by Zhang and colleagues investigated the use of the potential probiotic *Lactobacillus casei* Zhang which was isolated from fermented mare's milk (Zhang et al., 2014). The probiotic was administered to adults in the form of chewable tablets over a 28-day period. Significant differences in the gut microbiota composition were observed during and after treatment with the probiotic. It was found that consumption of *L. casei* Zhang was associated with increases in *Prevotella, Lactobacillus, Faecalibacterium, Propionibacterium* and *Bifidobacterium* while *Clostridium, Phascolarctobacterium, Serratia, Enterococcus, Shigella* and *Shewanella* either decreased in abundance or were eliminated during the treatment period. Similar clinical studies have also indicated that *Bifidobacterium lactis* and *L. casei* Shirota can result in increases of *Bifidobacterium* (Matsumoto et al., 2006). Administration of probiotic biscuits containing *L. helveticus* Barl3 and *B. longum* Bar33 to elderly subjects reversed age-related increases of opportunistic intestinal pathogens, such as *Clostridium* cluster XI, *Clostridium difficile, Clostridium perfringens, Enterococcus faecium* and *Campylobacter* (Rampelli et al., 2013). By reversing the increase of pathobionts in the gut associated with inflamm-ageing (Biagi et al., 2012), the probiotic may help to

maintain healthy gut function in elderly adults (Kim et al., 2021). The beneficial effect of probiotics on adults has been demonstrated in additional studies with reports concluding that four main probiotic treatments are effective for primary prevention of *Clostridium difficile* infection: *Saccharomyces boulardii, L. casei* DN1140011, a mixture of *L. acidophilus* and *B. bifidum*, and a mixture of *L. acidophilus*, *L. casei* and *L. rhamnosus* (McFarland, 2015). *L. rhamnosus* LC705, *L. rhamnosus* GG, *Propionibacterium freundenreichii* ssp. *shermanii* JS and *B.* animalis ssp. *lactis* Bb12 have also proven to be effective for IBS, with reductions in distension and abdominal pain reported, accompanied by stabilisation of the gut microbiota (Kajander et al., 2008).

It is important to note that while the clinical trials discussed here are examples of positive effects on the gut microbiota, there are a substantial number of studies where similar probiotic strains have had little or no effect (Cryan et al., 2019). There are many factors to consider when drawing conclusions about the effects of certain probiotics used in clinical studies. Considerable variability exists with respect to the potential probiotic used and the dosage. Similarly, the delivery of probiotics varies between studies with several examples of food based delivery and more conventional delivery such as tablets or capsules (Govender et al., 2014). The duration of administration also needs to be taken into consideration, in addition to the health status of subjects in the study. In healthy individuals, probiotic-induced gut microbiota modulation has been reported in some studies, while in others no effects were apparent. This has been noted in studies where the treatment conditions and strain did not differ between participants which suggests that in some cases, the effect of probiotics may depend on a person's own intestinal ecosystem before the intervention (Ferrario et al., 2014).

The Gut-Brain Axis and Probiotics

It is widely recognised that the bi-directional communication network between the gastrointestinal tract and the brain is vital for maintaining health (Cryan & O'Mahony, 2011). Disruption of the gut-brain axis can have important physiological and psychological consequences and has been implicated in a variety of disorders including IBS, ASD, Parkinson's disease, mood disorders and chronic pain (Mayer et al., 2015). Increasing evidence has accumulated supporting the view that the gut microbiota can substantially influence the brain and behaviour through the microbiota-gut-brain axis (Heijtz et al., 2011). Consequently, several studies have examined the use of probiotics for brain related disorders including anxiety, autism and depression (Borre et al., 2014). This has led to the emergence of a new field of probiotics, termed "psychobiotics" (Dinan et al., 2013). A psychobiotic is defined as "a live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness" (Dinan et al., 2013).

Preclinical studies

Given that certain bacteria can secrete neurochemicals such as gamma aminobutyric acid (GABA), serotonin, norepinephrine and dopamine which play an important role in brain function and behaviour, it has been proposed that these microbes may act as delivery vehicles for neuroactive compounds (Lyte, 2011). As dysfunction in neurotransmitter signalling has been associated with several neuropsychiatric conditions (Wall et al., 2014), the use of probiotic bacteria capable of producing these neuroactive metabolites is emerging as an attractive, novel treatment for a variety of disorders. GABA is the main inhibitory neurotransmitter in

the brain and disruptions in the GABAergic system have been linked to anxiety disorders and depression (Cryan & Kaupmann, 2005).

A pivotal study by Bravo and colleagues showed that long-term administration of the probiotic *L. rhamnosus* JB-1 to healthy mice resulted in alterations in GABA receptor expression (Bravo et al., 2011). Treatment with *L. rhamnosus* JB-1 decreased expression of $GABA_{B1b}$ subunit mRNA in the amygdala, hippocampus and locus coeruleus while increased expression was noted in the cortical areas. Furthermore, reductions in $GABA_{A\alpha2}$ expression were seen in the prefrontal cortex and amygdala accompanied by increases in the hippocampus. In addition to the physiological changes, the treated animals also showed behavioural changes with reductions in anxiety-like and depressive-like behaviours (Bravo et al., 2011). These GABA receptors have been implicated in stress and anxiety and decreases in $GABA_{B1b}$ are consistent with the $GABA_B$ receptor antagonists which have anti-depressant effects (Cryan & Slattery, 2010).

Serotonin is a metabolite of the essential amino acid tryptophan and plays a vital role in the regulation of several physiological functions (Kema et al., 2000). Low serotonin levels have been associated with depression and many currently available anti-depressant drugs result in increased concentrations in the brain (Wall et al., 2014). As serotonin synthesis is dependent on tryptophan availability, regulation of tryptophan has important implications. It has been shown that the probiotic *Bifidobacterium infantis* 35624 can increase plasma tryptophan levels in rats accompanied by elevated levels of kynurenic acid (Desbonnet et al., 2008). Interestingly, the kynureine pathway produces many neurotoxic metabolites including quinolinic acid (Orlando et al., 2001) but can also produce kynurenic acid which has been shown to have neuroprotective properties (Nemeth et al., 2005). In this study, the

authors proposed that the increased levels of plasma tryptophan accompanied by increases in kynurenic acid suggests that *B. infantis* 35624 can preferentially direct kynurenine to produce the neuroprotective kynurenic acid through an alternative pathway rather than neurotoxic metabolites (Desbonnet et al., 2008). As there is growing attention in the kynurenic pathway in relation to psychiatric conditions (O'Mahony et al., 2015), information on the potential anti-depressant effects of *B. infantis* 35624 is of great importance.

A subsequent study using this bacterial strain was carried out by Desbonnet and colleagues in a maternal separation (MS) model of depression (Desbonnet et al., 2010). Mice were maternally separated and treated with either *B. infantis* 35624 or a commonly prescribed anti-depressant, citalopram hydrobromide. Maternal separation was seen to induce behaviours indicative of a state of behavioural despair. In addition to these behavioural changes, biochemical changes were also apparent including decreases in noradrenaline (NA) in the brain, elevations in corticotrophin-releasing factor (CRF) expression in the amygdaloid cortex and enhanced IL-6 release following immune stimulation. MS mice treated with the probiotic showed restoration of NA concentrations in the brain but to a lesser extent than the group treated with the anti-depressant. This probiotic was also seen to reverse the behavioural abnormalities associated with MS and normalise the exaggerated IL-6 response. As these physiological changes seen in MS animals are consistent with stress and depression (Pace et al., 2006; Wong et al., 2000), further investigation into the use of *B. infantis* as a treatment option for mood disorders is necessary.

Savignac and colleagues investigated the potential of two *Bifidobacterium* strains, *B. longum* 1714 and *B. breve* 1205 on behaviour and physiology in BALB/c mice, an innately anxious strain (Savignac et al., 2014). Mice were given either *B.*

longum 1714, *B. breve* 1205, escitalopram or vehicle and subjected to a variety of tests relating to depression, anxiety and stress. *B. longum* 1714 was seen to reduce stress, anxiety, and depressive behaviours while *B. breve* 1205 reduced general anxiety behaviours and induced weight loss. It was also noted that both strains reduced anxiety-like behaviours to a greater extent than the anti-depressant, escitalopram. This finding is of great importance and may hold potential for patients who are resistant to currently available anti-depressant treatment (Savignac et al., 2014). Furthermore, it was also shown that this *B. longum* strain can also improve cognition in BALB/c mice (Savignac et al., 2015). This reinforces the view that the effects of commensal bacteria are highly strain-specific and thus the effects of a particular strain cannot be extrapolated from one probiotic species to another.

It is widely established that there is a high co-morbidity between psychiatric conditions such as anxiety and depression and gastrointestinal disorders such as irritable bowel syndrome and inflammatory bowel disorder (Kennedy et al., 2012; Walker et al., 2008). Co-occurring anxiety-like behaviour was investigated in a study by Bercik and colleagues (Bercik et al., 2011). Chronic colitis was induced in adult male mice using low dose dextran sodium sulphate (DSS) and the effects of a probiotics *B. longum* NCC3001 were examined. Colitis was shown to induce anxiety in the animals, as expected. The administration of the probiotic not only reversed the anxiety-like behaviour but also increased exploratory behaviour when compared with control mice. It was also demonstrated that the effect of *B. longum* is mediated by the vagus nerve. This is consistent with several other preclinical studies which have shown that vagal nerve integrity is important in mediating microbiota-brain interactions (de Lartigue et al., 2011; Goehler et al., 2008).

Several additional preclinical studies have demonstrated the positive effects of probiotics on mood and behaviour. For example, combination of *L. helveticus* R0052 and *B. longum* R0175 has been shown to positively affect post-mycocardial infarction (MI) behavioural symptoms in rats (symptoms which are consistent with depression in humans) and can restore intestinal barrier integrity in MI rats (Arseneault-Bréard et al., 2012). Positive effects on stress have also been noted in animal models after administration with *L. farciminis* (Ait-Belgnaoui et al., 2012), *L. helveticus* (Ohland et al., 2013) and a combination of *L. helveticus* and *B. longum* (Ait-Belgnaoui et al., 2014). Taken together, these studies provide compelling evidence for the possible use of psychobiotics for the treatment of brain related disorders. However, it is important to note that many underlying gut-to-brain signalling mechanisms remain unclear and caution is necessary when applying these findings to clinical populations.

Clinical studies

In contrast to the substantial literature investigating the use of psychobiotics in animal studies, clinical validation of the above findings is scarce. Examples of some clinical studies which have shown positive results are discussed. In a double-blind, placebo-controlled, randomised study by Diop and colleagues, the effects of a probiotic combination of *Lactobacillus helveticus* R0052 and *B. longum* R0175 on stress-induced gastrointestinal and psychological symptoms in healthy volunteers were investigated (Diop et al., 2008). Volunteers were eligible if they were affected by stress daily and had 2 or more stress-related symptoms. It was found that this probiotic combination helped to reduce stress-induced gastrointestinal symptoms such as abdominal pain, nausea and vomiting. However, the treatment did not have any

significant effects on other symptoms of stress including emotional, psychological, and mental stress, or sleeping patterns.

Following this, a double-blind placebo-controlled, randomised, parallel study was conducted using the same probiotic combination to investigate effects on distress, anxiety and depression in healthy men and women (Messaoudi et al., 2011). Participants were subject to a variety of tests including the Hopkins Symptom Checklist-90, Hospital Anxiety and Depression Scale, Perceived Stress Scale and Coping Checklist. Cortisol levels in urine were also measured. It was found that the probiotic formula helped to reduce anxiety and depression. Furthermore, cortisol levels in the treatment group decreased while levels in those consuming the placebo remained stable.

The effects of a probiotic drink containing *L. casei* Shirota were investigated in a double-blind placebo-controlled, randomised study containing 124 participants (Benton et al., 2007). The probiotic did not improve the overall mood in the participants, except for those individuals whose mood at baseline was initially low. Interestingly, participants in the test group reported an improvement in memory recall, however these effects were small. Although this probiotic did not improve mood among all the participants, patients suffering from depression are likely to have initially low baseline scores. This was examined in a subsequent study by Rao and colleagues who investigated the potential of *L. casei* Shirota to alleviate symptoms of depression and anxiety resultant from chronic fatigue syndrome (CFS) in a double-blind placebo-controlled, randomised pilot study (Rao et al., 2009). After an 8-week period, a significant improvement in anxiety was seen in the treatment group when compared to those consuming a placebo.

Consumption of a fermented milk drink containing a probiotic cocktail was shown to modulate brain activity in healthy women (Tillisch et al., 2013). The probiotic drink containing *Streptococcus thermophilus*, *Bifidobacterium lactis*, *Lactococcus lactis* and *Lactobacillus bulgaricus* was taken by participants twice daily. Functional magnetic resonance imaging (fMRI) was employed before and after the intervention period to examine brain responses. A standardised emotional faces attention task was used to examine changes in emotional regulation. Changes during the task were seen in the treatment group with activity reductions noted in several regions of the brain accompanied by conductivity changes.

A study investigated the use of a multispecies probiotic containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *L. casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* (W19 and W58) on cognitive reactivity to sad mood as well as anxiety and depressive symptoms (Steenbergen et al., 2015). The study was blind at three levels, placebo-controlled, randomised, and assessment was conducted pre- and post-intervention. Participants were assessed using a variety of questionnaires which investigated vulnerability to depression and the existence and severity of current depressive and anxiety symptoms. It was found that the treatment group showed reduced cognitive reactivity to sad mood. This probiotic therefore may help to reduce the negative or aggressive thoughts associated with depression.

Several additional clinical studies have been carried using probiotics and prebiotics to target a specific population and condition including aged individuals (Chung et al., 2014), IBS (Pinto-Sanchez et al., 2017), major depressive disorder (Akkasheh et al., 2016), and stress (Chong et al., 2019).

Although these studies present interesting evidence to support the use of probiotics as positive modifiers of brain function and mental health, much additional work is necessary to examine the underlying mechanisms of this modulation. Additionally, continued human intervention studies are necessary to examine the impact of probiotic strains on disorders associated brain-gut axis dysregulation with a view to developing potential disease specific bacterial treatments.

Conclusion

Consumer interest in probiotic containing functional foods and supplements has increased in recent years. This review examined the latest literature, with regard to what defines a healthy gut microbiota from infancy to adulthood, the factors which cause disruption and the effectiveness of probiotic interventions in prophylaxis, alongside strategies to improve probiotic delivery.

A stable microbiota appears to be the prerequisite for host health and while there is still much debate on what constitutes this stability in terms of bacterial composition. It is accepted that diversity and functionality are key elements to a healthy microbiota which has been suggested as being person-specific at species, or indeed, strain level. Disruptions in normal colonisation patterns increase the risk of disease development both in infancy and adulthood, and these effects are as far reaching as brain function. From womb to tomb, several factors can disrupt the microbiota, beginning at the very earliest stages of life from maternal infection during pregnancy to mode of delivery, antibiotic usage, diet, and stress.

Indeed, colonisation disturbances at birth or indeed as a result of antibiotic usage throughout life can persist for months or years and have been linked to disorders including IBD, asthma, obesity, diabetes, allergies and negatively affect CNS

development and function. Restoration of the gut microbiota using prebiotics and probiotics has emerged as an attractive intervention strategy. Indeed, in terms of probiotics, several clinical studies have yielded positive results ranging from reduction in diarrhoea, necrotizing enterocolitis and the development of allergies in pre-term infants and children in receipt of antibiotics, to an increase in beneficial gut microbiota profiles in adults with a concomitant reduction in intestinal pathogens and a proven ability to reduce the symptoms of IBS. However, not all studies have yielded such outcomes, with many failing to demonstrate the ability of probiotic interventions to positively impact human health. Several factors must be considered when selecting a potential probiotic which span the spectrum from strain selection to the individual's own ecosystem. For example, strains from human breast milk may be more beneficial for use in infant formula than strains isolated from the infant intestine (Chassard et al., 2014). The timing of delivery is also essential, with recent studies touting the benefits of probiotic administration during pregnancy.

Another major hurdle is the mode of probiotic delivery, since viable bacteria must reach the target site in sufficient numbers to exert an effect. Food based delivery systems are already in use and available on the market-place. Probiotic cheese and yogurts have the advantage of appealing to the "ready-to-go" consumer market. Interestingly, ice-cream has proven to be an effective matrix to protect bacterial viability. However, ice-cream itself is not considered a healthy option due to its high fat and sugar contents. In this respect, one must consider the conflicting message of such a product and ensure delivery of effective probiotic numbers in a healthy-size portion. In addition to dairy products, other food based options have emerged, including soy-based products, fruits and cereal, important alternatives for non-dairy consumers. Non-food based probiotic delivery systems have also gained acceptance

with a variety of tablets, powders, straws and chewing gums on the market. Such carriers have the advantage of eliminating many of the technological challenges associated with food vectors including interactions with starter cultures, detrimental pH levels and negative effects on sensory aspects. However, this method does not benefit from the advantages of a food based carrier which include growth promotion, increased probiotic viability and enhanced protection in the GI tract after consumption. In addition to carrier choice, methods for enhancing probiotic survival are necessary to ensure that sufficient numbers of bacterial cells are ingested and delivered to the target site, generally the large intestine. These include microencapsulation, protective agents, packaging materials which protect against oxygen and optimal storage conditions.

In conclusion, research continues to illustrate the benefits of a healthy gut microbiota on host health, linking gut microbiota perturbations to increased risk of disease. Probiotic intervention is a promising avenue to examine for restoring a stable microbiota and hence positively influencing health. However, the variability of effectiveness reported in clinical studies continues to be a cause for concern. Various factors can influence outcomes, ranging from the choice of probiotic strain to the processing procedures used for probiotic delivery. Carefully designed clinical trials are essential whereby selected probiotic strains, along with their carrier systems are tested in the appropriate population.

References

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors a unique microbiome. *Science Translational Medicine*, *6*(237), 237ra265-237ra265.

Adlerberth, I., & Wold, A. E. (2009). Establishment of the gut microbiota in Western infants. *Acta Paediatrica*, *98*(2), 229-238.

Ahn, J. B., Hwang, H.-J., & Park, J.-H. (2001). Physiological responses of oxygen-tolerant anaerobic Bifidobacterium longum under oxygen. *Journal of microbiology and biotechnology*, *11*(3), 443-451.

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L.,... Theodorou, V. (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology*, *37*(11), 1885-1895.

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C.,... Tompkins, T. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterology & Motility*, *26*(4), 510-520.

Akalın, A., & Erişir, D. (2008). Effects of Inulin and Oligofructose on the Rheological Characteristics and Probiotic Culture Survival in Low-Fat Probiotic Ice Cream. *Journal of Food Science*, *73*(4), M184-M188.

Akkasheh G, Kashani-Poor Z, Tajabadi-Ebrahimi M, Jafari P, Akbari H, Taghizadeh M, Memarzadeh MR, Asemi Z, Esmaillzadeh A. Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double-blind, placebo-controlled trial. *Nutrition* 32: 315–320, 2016.

Akın, M., Akın, M., & Kırmacı, Z. (2007). Effects of inulin and sugar levels on the viability of yogurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. *Food chemistry*, *104*(1), 93-99.

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., & Hassan, F. A. (2013). Prebiotics as functional foods: A review. *Journal of Functional Foods*, *5*(4), 1542-1553.

Alamprese, C., Foschino, R., Rossi, M., Pompei, C., & Corti, S. (2005). Effects of Lactobacillus rhamnosus GG addition in ice cream. *International journal of dairy technology*, *58*(4), 200-206.

Alamprese, C., Foschino, R., Rossi, M., Pompei, C., & Savani, L. (2002). Survival of Lactobacillus johnsonii La1 and influence of its addition in retail-manufactured ice cream produced with different sugar and fat concentrations. *International Dairy Journal*, *12*(2), 201-208.

Angelov, A., Gotcheva, V., Kuncheva, R., & Hristozova, T. (2006). Development of a new oat-based probiotic drink. *International journal of food microbiology, 112*(1), 75-80.

Aragon-Alegro, L. C., Alegro, J. H. A., Cardarelli, H. R., Chiu, M. C., & Saad, S. M. I. (2007). Potentially probiotic and synbiotic chocolate mousse. *LWT-Food Science and Technology, 40*(4), 669-675.

Arango, L. F. G., Barrett, H. L., Callaway, L. K., & Nitert, M. D. (2015). Probiotics and pregnancy. *Current diabetes reports, 15*(1), 1-9.

Arseneault-Bréard, J., Rondeau, I., Gilbert, K., Girard, S.-A., Tompkins, T. A., Godbout, R., & Rousseau, G. (2012). Combination of Lactobacillus helveticus R0052 and Bifidobacterium longum R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. *British Journal of Nutrition, 107*(12), 1793-1799.

Arslanoglu, S., Moro, G. E., Schmitt, J., Tandoi, L., Rizzardi, S., & Boehm, G. (2008). Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *The Journal of nutrition, 138*(6), 1091-1095.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R.,... Batto, J.-M. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174-180.

Aryana, K. J., & McGrew, P. (2007). Quality attributes of yogurt with Lactobacillus casei and various prebiotics. *LWT-Food Science and Technology, 40*(10), 1808-1814.

Aspri, M., Papademas, P., & Tsaltas, D. (2020). Review on non-dairy probiotics and their use in non-dairy based products. *Fermentation, 6*(1), 30.

Atladóttir, H. O., Thorsen, P., Østergaard, L., Schendel, D. E., Lemcke, S., Abdallah, M., & Parner, E. T. (2010). Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of autism and developmental disorders, 40*(12), 1423-1430.

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Chari, R. S.,... Kozyrskyj, A. L. (2013). Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *Canadian Medical Association Journal, 185*(5), 385-394.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P.,... Zhong, H. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell host & microbe, 17*(5), 690-703.

Bagga D, Reichert JL, Koschutnig K, Aigner CS, Holzer P, Koskinen K, Moissl-Eichinger C, Schöpf V. Probiotics drive gut microbiome triggering emotional brain signatures. *Gut Microbes* 9: 486–496, 2018

Bao, Y., Zhang, Y., Li, H., Liu, Y., Wang, S., Dong, X.,... Zhang, H. (2012). In vitro screen of Lactobacillus plantarum as probiotic bacteria and their fermented characteristics in soymilk. *Annals of microbiology, 62*(3), 1311-1320.

Başyiğit, G., Kuleaşan, H., & Karahan, A. G. (2006). Viability of human-derived probiotic lactobacilli in ice cream produced with sucrose and aspartame. *Journal of Industrial Microbiology and Biotechnology, 33*(9), 796-800.

Belgec Varder, N., & Öksüz, Ö. (2007). Artisan strawberry ice cream made with supplementation of Lactococci or Lactobacillus acidophilus. *Italian journal of food science, 19*(4), 403-412.

Benton, D., Williams, C., & Brown, A. (2007). Impact of consuming a milk drink containing a probiotic on mood and cognition. *European journal of clinical nutrition, 61*(3), 355-361.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J.,... McCoy, K. D. (2011). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology, 141*(2), 599-609. e593.

Bercik, P., Park, A., Sinclair, D., Khoshdel, A., Lu, J., Huang, X.,... Moine, D. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut–brain communication. *Neurogastroenterology & Motility, 23*(12), 1132-1139.

Bergström, A., Skov, T. H., Bahl, M. I., Roager, H. M., Christensen, L. B., Ejlerskov, K. T.,... Licht, T. R. (2014). Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. *Applied and environmental microbiology, 80*(9), 2889-2900.

Bertelsen, R. J., Brantsæter, A. L., Magnus, M. C., Haugen, M., Myhre, R., Jacobsson, B.,... London, S. J. (2014). Probiotic milk consumption in pregnancy and infancy and subsequent childhood allergic diseases. *Journal of Allergy and Clinical Immunology, 133*(1), 165-171. e168.

Bezirtzoglou, E., Tsiotsias, A., & Welling, G. W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe, 17*(6), 478-482.

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., & Brigidi, P. (2012). Ageing of the human metaorganism: the microbial counterpart. *Age, 34*(1), 247-267.

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., & Retetangos, C. (2010). Mode of delivery affects the bacterial community in the newborn gut. *Early human development, 86*(1), 13-15.

Birollo, G., Reinheimer, J., & Vinderola, C. (2000). Viability of lactic acid microflora in different types of yoghurt. *Food Research International, 33*(9), 799-805.

Blandino, A., Al-Aseeri, M., Pandiella, S., Cantero, D., & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International, 36*(6), 527-543.

Boehm, G., & Moro, G. (2008). Structural and functional aspects of prebiotics used in infant nutrition. *The Journal of nutrition, 138*(9), 1818S-1828S.

Borre, Y. E., Moloney, R. D., Clarke, G., Dinan, T. G., & Cryan, J. F. (2014). The impact of microbiota on brain and behavior: mechanisms & therapeutic potential. In *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease* (pp. 373-403): Springer.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in molecular medicine, 20*(9), 509-518.

Bosnea, L. A., Kourkoutas, Y., Albantaki, N., Tzia, C., Koutinas, A. A., & Kanellaki, M. (2009). Functionality of freeze-dried L. casei cells immobilized on wheat grains. *LWT-Food Science and Technology, 42*(10), 1696-1702.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G.,... Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences, 108*(38), 16050-16055.

Brown, A. S., & Derkits, E. J. (2010). Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. *The American journal of psychiatry*.

Burgain, J., Gaiani, C., Linder, M., & Scher, J. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering, 104*(4), 467-483.

Butel, M. J. (2014). Probiotics, gut microbiota and health. *Médecine et maladies infectieuses, 44*(1), 1-8.

Calinescu, C., & Mateescu, M. A. (2008). Carboxymethyl high amylose starch: Chitosan self-stabilized matrix for probiotic colon delivery. *European journal of pharmaceutics and biopharmaceutics, 70*(2), 582-589.

Cammarota, G., Ianiro, G., Bibbò, S., & Gasbarrini, A. (2014). Gut microbiota modulation: probiotics, antibiotics or fecal microbiota transplantation? *Internal and emergency medicine, 9*(4), 365-373.

Capela, P., Hay, T., & Shah, N. (2006). Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. *Food Research International, 39*(2), 203-211.

Cárdenas, N., Calzada, J., Peirotén, Á., Jiménez, E., Escudero, R., Rodríguez, J. M.,... Fernández, L. (2014). Development of a potential probiotic fresh cheese using two

Lactobacillus salivarius strains isolated from human milk. *BioMed research international, 2014*.

Chan, E. S., & Zhang, Z. (2005). Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. *Process Biochemistry, 40*(10), 3346-3351.

Chassard, C., de Wouters, T., & Lacroix, C. (2014). Probiotics tailored to the infant: a window of opportunity. *Current opinion in biotechnology, 26*, 141-147.

Chong HX, Yusoff NAA, Hor YY, Lew LC, Jaafar MH, Choi SB, Yusoff MSB, Wahid N, Abdullah MFIL, Zakaria N, Ong KL, Park YH, Liong MT. *Lactobacillus plantarum* DR7 alleviates stress and anxiety in adults: a randomised, double-blind, placebo controlled study. *Benef Microbes* 10: 355–373, 2019

Chung YC, Jin HM, Cui Y, Kim DS, Jung JM, Park JI, Jung ES, Choi EK, Chae SW. Fermented milk of *Lactobacillus helveticus* IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. *J Funct Foods* 10: 465–474, 2014.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S.,... O'Sullivan, O. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature, 488*(7410), 178-184.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270.

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*(7402), 207-214.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2011). Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. *Biomacromolecules, 12*(7), 2834-2840.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release, 162*(1), 56-67.

Corrêa, S., Castro, I. A., & Saad, S. M. (2008). Probiotic potential and sensory properties of coconut flan supplemented with Lactobacillus paracasei and Bifidobacterium lactis. *International journal of food science & technology, 43*(9), 1560-1568.

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I.,... Mahana, D. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell, 158*(4), 705-721.

Cruz, A. G., Antunes, A. E., Sousa, A. L. O., Faria, J. A., & Saad, S. M. (2009). Ice-cream as a probiotic food carrier. *Food Research International, 42*(9), 1233-1239.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience, 13*(10), 701-712.

Cryan, J. F., & Kaupmann, K. (2005). Don't worry 'B'happy!: a role for GABA B receptors in anxiety and depression. *Trends in pharmacological sciences, 26*(1), 36-43.

Cryan, J. F., & O'Mahony, S. (2011). The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterology & Motility, 23*(3), 187-192.

Cryan, J. F., & Slattery, D. A. (2010). GABA B receptors and depression: current status. *Advances in pharmacology, 58*, 427-451.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M., ... & Dinan, T. G. (2019). The microbiota-gut-brain axis. *Physiological reviews*.

D'Orazio, G., Di Gennaro, P., Boccarusso, M., Presti, I., Bizzaro, G., Giardina, S.,... La Ferla, B. (2015). Microencapsulation of new probiotic formulations for gastrointestinal delivery: in vitro study to assess viability and biological properties. *Applied microbiology and biotechnology*, 1-11.

Dalev, D., Bielecka, M., Troszynska, A., Ziajka, S., & Lamparski, G. (2006). Sensory quality of new probiotic beverages based on cheese whey and soy preparation. *Polish journal of food and nutrition sciences*(15/56).

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., & Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain Lactobacillus paracasei IMPC2. 1 in an industrial plant. *International journal of food microbiology, 140*(1), 6-13.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S.,... Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences, 107*(33), 14691-14696.

de Lartigue, G., de La Serre, C. B., & Raybould, H. E. (2011). Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. *Physiology & behavior, 105*(1), 100-105.

de Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal, 20*(4), 292-302.

Derrien, M., & van Hylckama Vlieg, J. E. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in microbiology, 23*(6), 354-366.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D.,... & Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: implications for brain and behaviour. *Brain, behavior, and immunity, 48*, 165-173.

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., & Dinan, T. G. (2008). The probiotic Bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. *Journal of psychiatric research*, *43*(2), 164-174.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J., & Dinan, T. (2010). Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience*, *170*(4), 1179-1188.

Dethlefsen, L., & Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4554-4561.

Di Gioia, D., Aloisio, I., Mazzola, G., & Biavati, B. (2014). Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Applied microbiology and biotechnology*, *98*(2), 563-577.

Dias, C. O., de Almeida, J. D. S. O., Pinto, S. S., de Oliveira Santana, F. C., Verruck, S., Müller, C. M. O., ... & Amboni, R. D. D. M. C. (2018). Development and physico-chemical characterization of microencapsulated bifidobacteria in passion fruit juice: A functional non-dairy product for probiotic delivery. *Food bioscience*, *24*, 26-36.

Dinan, T. G., Stanton, C., & Cryan, J. F. (2013). Psychobiotics: a novel class of psychotropic. *Biological psychiatry*, *74*(10), 720-726.

Ding, W., & Shah, N. P. (2009). Effect of various encapsulating materials on the stability of probiotic bacteria. *Journal of Food Science*, *74*(2), M100-M107.

Diop, L., Guillou, S., & Durand, H. (2008). Probiotic food supplement reduces stress-induced gastrointestinal symptoms in volunteers: a double-blind, placebo-controlled, randomized trial. *Nutrition Research*, *28*(1), 1-5.

Doré, J., & Blottière, H. (2015). The influence of diet on the gut microbiota and its consequences for health. *Current opinion in biotechnology*, *32*, 195-199.

e Silva, J. S., Sousa, S. C., Costa, P., Cerdeira, E., Amaral, M. H., Lobo, J. S.,... & Freitas, A. C. (2013). Development of probiotic tablets using microparticles: viability studies and stability studies. *Aaps Pharmscitech*, *14*(1), 121-127.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M.,... Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, *308*(5728), 1635-1638.

El Aidy, S., Dinan, T. G., & Cryan, J. F. (2015). Gut microbiota: the conductor in the orchestra of immune–neuroendocrine communication. *Clinical therapeutics*, *37*(5), 954-967.

El Kadri, H., Lalou, S., Mantzouridou, F., & Gkatzionis, K. (2018). Utilisation of water-in-oil-water (W1/O/W2) double emulsion in a set-type yogurt model for the delivery of probiotic Lactobacillus paracasei. *Food Research International*, *107*, 325-336.

Enomoto, T., Sowa, M., Nishimori, K., Shimazu, S., Yoshida, A., Yamada, K.,... Iwabuchi, N. (2014). Effects of bifidobacterial supplementation to pregnant women and infants in the prevention of allergy development in infants and on fecal microbiota. *Allergology International, 63*(4), 575-585.

Everard, A., Lazarevic, V., Derrien, M., Girard, M., Muccioli, G. G., Neyrinck, A. M.,... de Vos, W. M. (2011). Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. *Diabetes, 60*(11), 2775-2786.

Ewe, J.-A., Wan-Abdullah, W.-N., & Liong, M.-T. (2010). Viability and growth characteristics of Lactobacillus in soymilk supplemented with B-vitamins. *International journal of food sciences and nutrition, 61*(1), 87-107.

Fallani, M., Amarri, S., Uusijarvi, A., Adam, R., Khanna, S., Aguilera, M.,... Young, D. (2011). Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology, 157*(5), 1385-1392.

Fatemi, S. H., Reutiman, T. J., Folsom, T. D., Huang, H., Oishi, K., Mori, S.,... Sohr, R. (2008). Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders. *Schizophrenia research, 99*(1), 56-70.

Fatima, S. M., & Hekmat, S. (2020). Microbial and Sensory Analysis of Soy and Cow Milk-Based Yogurt as a Probiotic Matrix for Lactobacillus rhamnosus GR-1. *Fermentation*, *6*(3), 74.

Ferrario, C., Taverniti, V., Milani, C., Fiore, W., Laureati, M., De Noni, I.,... Guglielmetti, S. (2014). Modulation of fecal clostridiales bacteria and butyrate by probiotic intervention with Lactobacillus paracasei DG varies among healthy adults. *The Journal of nutrition, 144*(11), 1787-1796.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., ... & Segata, N. (2018). Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell host & microbe*, *24*(1), 133-145.Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology and Hepatology, 9*(10), 577-589.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M.,... Stanton, C. (2012). High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrobial agents and chemotherapy, 56*(11), 5811-5820.

Fox, M. J., Ahuja, K. D. K., Robertson, I. K., Ball, M. J., & Eri, R. D. (2015). Can probiotic yogurt prevent diarrhoea in children on antibiotics? A double-blind, randomised, placebo-controlled study. *BMJ open*, *5*(1).

Frakolaki, G., Giannou, V., Kekos, D., & Tzia, C. (2021). A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods. *Critical reviews in food science and nutrition*, *61*(9), 1515-1536.

Ganesan, B., Weimer, B., Pinzon, J., Dao Kong, N., Rompato, G., Brothersen, C., & McMahon, D. (2014). Probiotic bacteria survive in Cheddar cheese and modify populations of other lactic acid bacteria. *Journal of Applied Microbiology*, *116*(6), 1642-1656.

Ghanizadeh, A., Dehbozorgi, S., OmraniSigaroodi, M., & Rezaei, Z. (2014). Minocycline as add-on treatment decreases the negative symptoms of schizophrenia; a randomized placebo-controlled clinical trial. *Recent patents on inflammation & allergy drug discovery*, *8*(3), 211-215.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J.,... & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature reviews Gastroenterology & hepatology*, *14*(8), 491-502.

Goehler, L. E., Park, S. M., Opitz, N., Lyte, M., & Gaykema, R. P. (2008). Campylobacter jejuni infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain, behavior, and immunity*, *22*(3), 354-366.

Golubeva, A. V., Crampton, S., Desbonnet, L., Edge, D., O'Sullivan, O., Lomasney, K. W.,... Cryan, J. F. (2015). Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. *Psychoneuroendocrinology*, *60*, 58-74.

Govender, M., Choonara, Y. E., Kumar, P., du Toit, L. C., van Vuuren, S., & Pillay, V. (2014). A review of the advancements in probiotic delivery: Conventional vs. non-conventional formulations for intestinal flora supplementation. *Aaps PharmSciTech*, *15*(1), 29-43.

Govender, M., Choonara, Y. E., Vuuren, S., Kumar, P., Toit, L. C., & Pillay, V. (2015). A gastro-resistant ovalbumin bi-layered mini-tablet-in-tablet system for the delivery of Lactobacillus acidophilus probiotic to simulated human intestinal and colon conditions. *Journal of Pharmacy and Pharmacology*.

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. d. A. F., & Shah, N. P. (2010). Probiotic dairy products as functional foods. *Comprehensive Reviews in Food Science and Food Safety, 9*(5), 455-470.

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G., & Faria, J. A. (2010). Functional foods and nondairy probiotic food development: trends, concepts, and products. *Comprehensive Reviews in Food Science and Food Safety, 9*(3), 292-302.

Gschwind, R., Fournier, T., Kennedy, S., Tsatsaris, V., Cordier, A. G., Barbut, F., ... & Wydau-Dematteis, S. (2020). Evidence for contamination as the origin for bacteria found in human placenta rather than a microbiota. *Plos one, 15*(8), e0237232.

Gueimonde, M., Sakata, S., Kalliomäki, M., Isolauri, E., Benno, Y., & Salminen, S. (2006). Effect of maternal consumption of lactobacillus GG on transfer and establishment of fecal bifidobacterial microbiota in neonates. *Journal of Pediatric Gastroenterology and Nutrition, 42*(2), 166-170.

Hassiotou, F., Geddes, D. T., & Hartmann, P. E. (2013). Cells in human milk: state of the science. *Journal of Human Lactation, 29*(2), 171-182.

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A.,... Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences, 108*(7), 3047-3052.

Hekmat, S., & Reid, G. (2006). Sensory properties of probiotic yogurt is comparable to standard yogurt. *Nutrition Research, 26*(4), 163-166.

Helland, M. H., Wicklund, T., & Narvhus, J. A. (2004). Growth and metabolism of selected strains of probiotic bacteria in milk-and water-based cereal puddings. *International Dairy Journal, 14*(11), 957-965.

Homayouni, A., Azizi, A., Ehsani, M., Yarmand, M., & Razavi, S. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food chemistry, 111*(1), 50-55.

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T.,... Petrosino, J. F. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell, 155*(7), 1451-1463.

Huda, M. N., Lewis, Z., Kalanetra, K. M., Rashid, M., Ahmad, S. M., Raqib, R.,... Stephensen, C. B. (2014). Stool microbiota and vaccine responses of infants. *Pediatrics, 134*(2), e362-e372.

Hussey, S., Wall, R., Gruffman, E., O'Sullivan, L., Ryan, C. A., Murphy, B.,... Ross, R. P. (2010). Parenteral antibiotics reduce bifidobacteria colonization and diversity in neonates. *International journal of microbiology, 2011*.

Hyde, M. J., & Modi, N. (2012). The long-term effects of birth by caesarean section: the case for a randomised controlled trial. *Early human development, 88*(12), 943-949.

Iannitti, T., & Palmieri, B. (2010). Therapeutical use of probiotic formulations in clinical practice. *Clinical Nutrition, 29*(6), 701-725.

Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C.,... Andersson, A. F. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut, 63*(4), 559-566.

Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjölund-Karlsson, M., Jansson, J. K., & Engstrand, L. (2010). Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One, 5*(3), e9836.

Jalanka-Tuovinen, J., Salonen, A., Nikkilä, J., Immonen, O., Kekkonen, R., Lahti, L.,... de Vos, W. M. (2011). Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. *PloS one, 6*(7), e23035.

Jašarević, E., Rodgers, A. B., & Bale, T. L. (2015). A novel role for maternal stress and microbial transmission in early life programming and neurodevelopment. *Neurobiology of stress, 1*, 81-88.

Jeffery, I. B., Claesson, M. J., O'Toole, P. W., & Shanahan, F. (2012). Categorization of the gut microbiota: enterotypes or gradients? *Nature Reviews Microbiology, 10*(9), 591-592.

Jernberg, C., Löfmark, S., Edlund, C., & Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME journal, 1*(1), 56-66.

Jørgensen, B. P., Hansen, J. T., Krych, L., Larsen, C., Klein, A. B., Nielsen, D. S.,... & Sørensen, D. B. (2014). A possible link between food and mood: dietary impact on gut microbiota and behavior in BALB/c mice. *PloS one*, *9*(8), e103398.

Juárez, I., Gratton, A., & Flores, G. (2008). Ontogeny of altered dendritic morphology in the rat prefrontal cortex, hippocampus, and nucleus accumbens following Cesarean delivery and birth anoxia. *Journal of Comparative Neurology, 507*(5), 1734-1747.

Kabeerdoss, J., Devi, R. S., Mary, R. R., Prabhavathi, D., Vidya, R., Mechenro, J.,... & Ramakrishna, B. S. (2011). Effect of yoghurt containing Bifidobacterium lactis Bb12® on faecal excretion of secretory immunoglobulin A and human beta-defensin 2 in healthy adult volunteers. *Nutrition journal*, *10*(1), 1-4.

Kailasapathy, K. (2002). Microencapsulation of probiotic bacteria: technology and potential applications. *Current issues in intestinal microbiology, 3*(2), 39-48.

Kailasapathy, K., Harmstorf, I., & Phillips, M. (2008). Survival of Lactobacillus acidophilus and Bifidobacterium animalis ssp. lactis in stirred fruit yogurts. *LWT-Food Science and Technology, 41*(7), 1317-1322.

Kailasapathy, K., & Masondole, L. (2005). Survival of free and microencapsulated Lactobacillus acidophilus and Bifidobacterium lactis and their effect on texture of feta cheese. *Australian Journal of Dairy Technology, 60*(3), 252.

Kajander, K., Myllyluoma, E., RAJILIĆ-STOJANOVIĆ, M., Kyrönpalo, S., Rasmussen, M., Järvenpää, S.,... Korpela, R. (2008). Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. *Alimentary pharmacology & therapeutics, 27*(1), 48-57.

Kalliomäki, M., Salminen, S., Poussa, T., & Isolauri, E. (2007). Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. *Journal of Allergy and Clinical Immunology, 119*(4), 1019-1021.

Kedia, G., Wang, R., Patel, H., & Pandiella, S. S. (2007). Use of mixed cultures for the fermentation of cereal-based substrates with potential probiotic properties. *Process Biochemistry, 42*(1), 65-70.

Kema, I. P., de Vries, E. G., & Muskiet, F. A. (2000). Clinical chemistry of serotonin and metabolites. *Journal of Chromatography B: Biomedical Sciences and Applications, 747*(1), 33-48.

Kennedy, P. J., Clarke, G., Quigley, E. M., Groeger, J. A., Dinan, T. G., & Cryan, J. F. (2012). Gut memories: towards a cognitive neurobiology of irritable bowel syndrome. *Neuroscience & Biobehavioral Reviews, 36*(1), 310-340.

Kılıç, G. B., Kuleaşan, H., Eralp, İ., & Karahan, A. G. (2009). Manufacture of Turkish Beyaz cheese added with probiotic strains. *LWT-Food Science and Technology, 42*(5), 1003-1008.

Kim, C. S., Cha, L., Sim, M., Jung, S., Chun, W. Y., Baik, H. W., & Shin, D. M. (2021). Probiotic Supplementation Improves Cognitive Function and Mood with Changes in Gut Microbiota in Community-Dwelling Older Adults: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Trial. *The Journals of Gerontology: Series A, 76*(1), 32-40.

Klayraung, S., Viernstein, H., & Okonogi, S. (2009). Development of tablets containing probiotics: effects of formulation and processing parameters on bacterial viability. *International journal of pharmaceutics, 370*(1), 54-60.

Knights, D., Ward, T. L., McKinlay, C. E., Miller, H., Gonzalez, A., McDonald, D., & Knight, R. (2014). Rethinking "enterotypes". *Cell host & microbe, 16*(4), 433-437.

Korpela, K., Salonen, A., Vepsäläinen, O., Suomalainen, M., Kolmeder, C., Varjosalo, M., ... & de Vos, W. M. (2018). Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome, 6*(1), 1-11.

Kun, S., Rezessy-Szabó, J. M., Nguyen, Q. D., & Hoschke, Á. (2008). Changes of microbial population and some components in carrot juice during fermentation with selected Bifidobacterium strains. *Process Biochemistry, 43*(8), 816-821.

Kuperman, A. A., Zimmerman, A., Hamadia, S., Ziv, O., Gurevich, V., Fichtman, B., ... & Koren, O. (2020). Deep microbial analysis of multiple placentas shows no evidence for a placental microbiome. *BJOG: An International Journal of Obstetrics & Gynaecology, 127*(2), 159-169.

Lahti, L., Salojärvi, J., Salonen, A., Scheffer, M., & De Vos, W. M. (2014). Tipping elements in the human intestinal ecosystem. *Nature communications*, *5*(1), 1-10.

Lahtinen, S. J., Boyle, R. J., Kivivuori, S., Oppedisano, F., Smith, K. R., Robins-Browne, R.,... Tang, M. L. (2009). Prenatal probiotic administration can influence Bifidobacterium microbiota development in infants at high risk of allergy. *Journal of Allergy and Clinical Immunology, 123*(2), 499-501. e498.

Lavermicocca, P. (2006). Highlights on new food research. *Digestive and Liver Disease*, *38*, S295-S299.

Lavermicocca, P., Valerio, F., Lonigro, S. L., De Angelis, M., Morelli, L., Callegari, M. L.,... Visconti, A. (2005). Study of adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. *Applied and environmental microbiology, 71*(8), 4233-4240.

Lee, B. K., Magnusson, C., Gardner, R. M., Blomström, Å., Newschaffer, C. J., Burstyn, I.,... Dalman, C. (2015). Maternal hospitalization with infection during pregnancy and risk of autism spectrum disorders. *Brain, behavior, and immunity, 44*, 100-105.

Lew, L. C., Hor, Y. Y., Yusoff, N. A. A., Choi, S. B., Yusoff, M. S., Roslan, N. S., ... & Liong, M. T. (2019). Probiotic Lactobacillus plantarum P8 alleviated stress and anxiety while enhancing memory and cognition in stressed adults: a randomised, double-blind, placebo-controlled study. *Clinical Nutrition*, *38*(5), 2053-2064.

Li, L., Han, Z., Niu, X., Zhang, G., Jia, Y., Zhang, S., & He, C. (2019). Probiotic supplementation for prevention of atopic dermatitis in infants and children: a systematic review and meta-analysis. *American journal of clinical dermatology*, *20*(3), 367-377.

Lin, J., Yu, W., Liu, X., Xie, H., Wang, W., & Ma, X. (2008). In vitro and in vivo characterization of alginate-chitosan-alginate artificial microcapsules for therapeutic oral delivery of live bacterial cells. *Journal of bioscience and bioengineering, 105*(6), 660-665.

Liong, M. T., Easa, A. M., Lim, P. T., & Kang, J. Y. (2009). Survival, growth characteristics and bioactive potential of Lactobacillus acidophilus in a soy-based cream cheese. *Journal of the Science of Food and Agriculture, 89*(8), 1382-1391.

Lollo, P. C., Morato, P. N., Moura, C. S., Almada, C. N., Felicio, T. L., Esmerino, E. A.,... Raices, R. R. (2015). Hypertension parameters are attenuated by the continuous consumption of probiotic Minas cheese. *Food Research International, 76*, 611-617.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature, 489*(7415), 220-230.

Luckow, T., & Delahunty, C. (2004). Which juice is 'healthier'? A consumer study of probiotic non-dairy juice drinks. *Food Quality and Preference, 15*(7), 751-759.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays, 33*(8), 574-581.

Mack, D. R., Michail, S., Wei, S., McDougall, L., & Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic E. coliadherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 276*(4), G941-G950.

Mäkeläinen, H., Forssten, S., Olli, K., Granlund, L., Rautonen, N., & Ouwehand, A. (2009). Probiotic lactobacilli in a semi-soft cheese survive in the simulated human gastrointestinal tract. *International Dairy Journal, 19*(11), 675-683.

Mantis, N. J., Rol, N., & Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology, 4*(6), 603-611.

Maragkoudakis, P. A., Miaris, C., Rojez, P., Manalis, N., Magkanari, F., Kalantzopoulos, G., & Tsakalidou, E. (2006). Production of traditional Greek yoghurt using Lactobacillus strains with probiotic potential as starter adjuncts. *International Dairy Journal, 16*(1), 52-60.

Martın-Diana, A., Janer, C., Peláez, C., & Requena, T. (2003). Development of a fermented goat's milk containing probiotic bacteria. *International Dairy Journal, 13*(10), 827-833.

Martínez, I., Stegen, J. C., Maldonado-Gómez, M. X., Eren, A. M., Siba, P. M., Greenhill, A. R., & Walter, J. (2015). The gut microbiota of rural Papua New Guineans: composition, diversity patterns, and ecological processes. *Cell reports, 11*(4), 527-538.

Matsumoto, K., Takada, T., Shimizu, K., Kado, Y., Kawakami, K., Makino, I.,... Kajimoto, O. (2006). The effects of a probiotic milk product containing Lactobacillus casei strain Shirota on the defecation frequency and the intestinal microflora of sub-optimal health state volunteers: a randomized placebo-controlled cross-over study. *Bioscience and microflora, 25*(2), 39-48.

Mayer, E. A., Tillisch, K., & Gupta, A. (2015). Gut/brain axis and the microbiota. *The Journal of clinical investigation, 125*(3), 926-938.

McClements, D. J., Decker, E. A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition, 49*(6), 577-606.

McFarland, L. V. (2015). Probiotics for the Primary and Secondary Prevention of C. difficile Infections: A Meta-analysis and Systematic Review. *Antibiotics, 4*(2), 160-178.

McMaster, L., Kokott, S., Reid, S., & Abratt, V. (2005). Use of traditional African fermented beverages as delivery vehicles for Bifidobacterium lactis DSM 10140. *International journal of food microbiology, 102*(2), 231-237.

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A.,... Cazaubiel, M. (2011). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *British Journal of Nutrition, 105*(05), 755-764.

Millette, M., Nguyen, A., Amine, K. M., & Lacroix, M. (2013). Gastrointestinal survival of bacteria in commercial probiotic products. *International Journal of Probiotics & Prebiotics, 8*(4), 149.

Min, M., Bunt, C. R., Mason, S. L., & Hussain, M. A. (2019). Non-dairy probiotic food products: An emerging group of functional foods. *Critical reviews in food science and nutrition, 59*(16), 2626-2641.

Mirzaeei, S., & Tagheh, S. (2017). Formulation direct compression tablet of probiotic as vehicle for oral delivery. *Journal of Reports in Pharmaceutical Sciences*, *6*(1), 84.

Moore, R. E., & Townsend, S. D. (2019). Temporal development of the infant gut microbiome. *Open biology, 9*(9), 190128.

Morgun, A., Dzutsev, A., Dong, X., Greer, R. L., Sexton, D. J., Ravel, J.,... & Shulzhenko, N. (2015). Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks. *Gut*, *64*(11), 1732-1743.

Morozov, V., Hansman, G., Hanisch, F. G., Schroten, H., & Kunz, C. (2018). Human milk oligosaccharides as promising antivirals. *Molecular nutrition & food research, 62*(6), 1700679.

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The infant microbiome development: mom matters. *Trends in Molecular Medicine, 21*(2), 109-117.

Murphy, R., Stewart, A., Braithwaite, I., Beasley, R., Hancox, R., & Mitchell, E. (2014). Antibiotic treatment during infancy and increased body mass index in boys: an international cross-sectional study. *International journal of obesity, 38*(8), 1115-1119.

Mushtaq, M., Gani, A., & Masoodi, F. A. (2019). Himalayan cheese (Kalari/Kradi) fermented with different probiotic strains: In vitro investigation of nutraceutical properties. *LWT*, *104*, 53-60.

Nagpal, R., Kumar, A., & Kumar, M. (2012). Fortification and fermentation of fruit juices with probiotic lactobacilli. *Annals of microbiology*, *62*(4), 1573-1578.

Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., & Yadav, H. (2012). Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS microbiology letters*, *334*(1), 1-15.

Nemeth, H., Toldi, J., & Vécsei, L. (2005). Role of kynurenines in the central and peripherial nervous systems. *Current Neurovascular Research*, *2*(3), 249-260.

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A.-M., Quigley, E. M.,... Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological psychiatry*, *65*(3), 263-267.

O'Mahony, S., Clarke, G., Borre, Y., Dinan, T., & Cryan, J. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural brain research*, *277*, 32-48.

Ohland, C. L., Kish, L., Bell, H., Thiesen, A., Hotte, N., Pankiv, E., & Madsen, K. L. (2013). Effects of Lactobacillus helveticus on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. *Psychoneuroendocrinology*, *38*(9), 1738-1747.

Olomu, I. N., Pena-Cortes, L. C., Long, R. A., Vyas, A., Krichevskiy, O., Luellwitz, R., ... & Mulks, M. H. (2020). Elimination of "kitome" and "splashome" contamination results in lack of detection of a unique placental microbiome. *BMC microbiology*, *20*(1), 1-19.

Ong, L., Henriksson, A., & Shah, N. (2006). Development of probiotic Cheddar cheese containing Lactobacillus acidophilus, Lb. casei, Lb. paracasei and Bifidobacterium spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal*, *16*(5), 446-456.

Ong, L., & Shah, N. P. (2009). Probiotic Cheddar cheese: Influence of ripening temperatures on survival of probiotic microorganisms, cheese composition and organic acid profiles. *LWT-Food Science and Technology*, *42*(7), 1260-1268.

Orlando, L. R., Alsdorf, S. A., Penney, J. B., & Young, A. B. (2001). The role of group I and group II metabotropic glutamate receptors in modulation of striatal NMDA and quinolinic acid toxicity. *Experimental neurology*, *167*(1), 196-204.

Ouwehand, A. C., Ibrahim, F., & Forssten, S. D. (2010). Cheese as a carrier for probiotics: in vitro and human studies. *Australian Journal of Dairy Technology*, *65*(3), 165.

Pace, T. W., Mletzko, T. C., Alagbe, O., Musselman, D. L., Nemeroff, C. B., Miller, A. H., & Heim, C. M. (2006). Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. *American Journal of Psychiatry, 163*(9), 1630-1633.

Penders, J., Kummeling, I., & Thijs, C. (2011). Infant antibiotic use and wheeze and asthma risk: a systematic review and meta-analysis. *European Respiratory Journal, 38*(2), 295-302.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I.,... Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511-521.

Peres, C. M., Peres, C., Hernández-Mendoza, A., & Malcata, F. X. (2012). Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria–with an emphasis on table olives. *Trends in Food Science & Technology, 26*(1), 31-42.

Pérez-Cobas, A. E., Gosalbes, M. J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K.,... von Bergen, M. (2013). Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut, 62*(11), 1591-1601.

Persaud, R., Azad, M. B., Konya, T., Guttman, D. S., Chari, R. S., Sears, M. R.,... Investigators, C. S. (2014). Impact of perinatal antibiotic exposure on the infant gut microbiota at one year of age. *Allergy, Asthma & Clinical Immunology, 10*(Suppl 1), A31.

Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, Martin FP, Cominetti O, Welsh C, Rieder A, Traynor J, Gregory C, De Palma G, Pigrau M, Ford AC, Macri J, Berger B, Bergonzelli G, Surette MG, Collins SM, Moayyedi P, Bercik P. Probiotic *Bifidobacterium longum* NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome. *Gastroenterology* 153: 448–459.e8, 2017

Pistell, P. J., Morrison, C. D., Gupta, S., Knight, A. G., Keller, J. N., Ingram, D. K., & Bruce-Keller, A. J. (2010). Cognitive impairment following high fat diet consumption is associated with brain inflammation. *Journal of neuroimmunology, 219*(1), 25-32.

Possemiers, S., Marzorati, M., Verstraete, W., & Van de Wiele, T. (2010). Bacteria and chocolate: a successful combination for probiotic delivery. *International journal of food microbiology, 141*(1), 97-103.

Poulin, J.-F., Caillard, R., & Subirade, M. (2011). β-Lactoglobulin tablets as a suitable vehicle for protection and intestinal delivery of probiotic bacteria. *International journal of pharmaceutics, 405*(1), 47-54.

Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P., & Fitzgerald, G. F. (2014). Intestinal microbiota, diet and health. *British Journal of Nutrition*, *111*(3), 387-402.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C.,... Yamada, T. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, *464*(7285), 59-65.

Rampelli, S., Candela, M., Severgnini, M., Biagi, E., Turroni, S., Roselli, M.,... Brigidi, P. (2013). A probiotics-containing biscuit modulates the intestinal microbiota in the elderly. *The journal of nutrition, health & aging*, *17*(2), 166-172.

Ranadheera, C. S., Evans, C., Adams, M., & Baines, S. (2012). In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International*, *49*(2), 619-625.

Ranadheera, R., Baines, S., & Adams, M. (2010). Importance of food in probiotic efficacy. *Food Research International*, *43*(1), 1-7.

Rao, A. V., Bested, A. C., Beaulne, T. M., Katzman, M. A., Iorio, C., Berardi, J. M., & Logan, A. C. (2009). A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut Pathogens*, *1*(1), 1-6.

Rautava, S., Arvilommi, H., & Isolauri, E. (2006). Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. *Pediatric research*, *60*(2), 221-224.

Rea, M. C., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P. D.,... Ross, R. P. (2011). Effect of broad-and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4639-4644.

Roberts, C. L., Algert, C. S., Ford, J. B., Todd, A. L., & Morris, J. M. (2012). Pathways to a rising caesarean section rate: a population-based cohort study. *BMJ open*, *2*(5), e001725.

Rodrigues, A., Cara, D., Fretez, S., Cunha, F., Vieira, E., Nicoli, J., & Vieira, L. (2000). Saccharomyces boulardii stimulates sIgA production and the phagocytic system of gnotobiotic mice. *Journal of Applied Microbiology*, *89*(3), 404-414.

Ross, R., Fitzgerald, G., Collins, K., & Stanton, C. (2002). Cheese delivering biocultures--probiotic cheese. *Australian Journal of Dairy Technology*, *57*(2), 71.

Roueche, E., Serris, E., Thomas, G., & Périer-Camby, L. (2006). Influence of temperature on the compaction of an organic powder and the mechanical strength of tablets. *Powder technology*, *162*(2), 138-144.

Sanders, M. E., & Marco, M. L. (2010). Food formats for effective delivery of probiotics. *Annual Review of Food Science and Technology*, *1*, 65-85.

Sangeetha, P., Ramesh, M., & Prapulla, S. (2005). Recent trends in the microbial production, analysis and application of fructooligosaccharides. *Trends in Food Science & Technology, 16*(10), 442-457.

Sanz, Y. (2007). Ecological and functional implications of the acid-adaptation ability of Bifidobacterium: a way of selecting improved probiotic strains. *International Dairy Journal, 17*(11), 1284-1289.

Saulnier, D. M., Gibson, G. R., & Kolida, S. (2008). In vitro effects of selected synbiotics on the human faecal microbiota composition. *FEMS microbiology ecology, 66*(3), 516-527.

Savignac, H., Kiely, B., Dinan, T., & Cryan, J. (2014). Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterology & Motility, 26*(11), 1615-1627.

Savignac, H., Tramullas, M., Kiely, B., Dinan, T., & Cryan, J. (2015). Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behavioural brain research, 287*, 59-72.

Savino, F., Roana, J., Mandras, N., Tarasco, V., Locatelli, E., & Tullio, V. (2011). Faecal microbiota in breast-fed infants after antibiotic therapy. *Acta Paediatrica, 100*(1), 75-78.

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G.,... & Crittenden, A. N. (2014). Gut microbiome of the Hadza hunter-gatherers. *Nature communications, 5*(1), 1-12.

Sekirov, I., Russell, S. L., Antunes, L. C. M., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiological reviews, 90*(3), 859-904.

Sendra, E., Fayos, P., Lario, Y., Fernandez-Lopez, J., Sayas-Barbera, E., & Perez-Alvarez, J. A. (2008). Incorporation of citrus fibers in fermented milk containing probiotic bacteria. *Food microbiology, 25*(1), 13-21.

Setta, M. C., Matemu, A., & Mbega, E. R. (2020). Potential of probiotics from fermented cereal-based beverages in improving health of poor people in Africa. *Journal of Food Science and Technology*, 1-12.

Sheehan, V. M., Ross, P., & Fitzgerald, G. F. (2007). Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innovative Food Science & Emerging Technologies, 8*(2), 279-284.

Slavin, J. (2013). Fiber and prebiotics: mechanisms and health benefits. *Nutrients, 5*(4), 1417-1435.

Sofjan, R. P., & Hartel, R. W. (2004). Effects of overrun on structural and physical characteristics of ice cream. *International Dairy Journal, 14*(3), 255-262.

Sreeja, V., & Prajapati, J. B. (2013). Probiotic formulations: Application and status as pharmaceuticals—A review. *Probiotics and antimicrobial proteins, 5*(2), 81-91.

Stadler, M., & Viernstein, H. (2003). Optimization of a formulation containing viable lactic acid bacteria. *International journal of pharmaceutics, 256*(1), 117-122.

Stanton, C., Gardiner, G., Lynch, P., Collins, J., Fitzgerald, G., & Ross, R. (1998). Probiotic cheese. *International Dairy Journal, 8*(5), 491-496.

Steenbergen, L., Sellaro, R., van Hemert, S., Bosch, J. A., & Colzato, L. S. (2015). A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, behavior, and immunity*, *48*, 258-264.

Subramanian, S., Blanton, L. V., Frese, S. A., Charbonneau, M., Mills, D. A., & Gordon, J. I. (2015). Cultivating Healthy Growth and Nutrition through the Gut Microbiota. *Cell, 161*(1), 36-48.

Terpou, A., Papadaki, A., Lappa, I. K., Kachrimanidou, V., Bosnea, L. A., & Kopsahelis, N. (2019). Probiotics in food systems: Significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients, 11*(7), 1591.

Tillisch, K., Labus, J., Kilpatrick, L., Jiang, Z., Stains, J., Ebrat, B.,... Naliboff, B. (2013). Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology, 144*(7), 1394-1401. e1394.

Timmerman, H. M., Rutten, N. B., Boekhorst, J., Saulnier, D. M., Kortman, G. A., Contractor, N., ... & Rijkers, G. T. (2017). Intestinal colonisation patterns in breastfed and formula-fed infants during the first 12 weeks of life reveal sequential microbiota signatures. *Scientific reports*, *7*(1), 1-10.

Tripathi, M., & Giri, S. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods, 9*, 225-241.

Tsen, J. H., Lin, Y. P., & King, V. (2009). Response surface methodology optimisation of immobilised Lactobacillus acidophilus banana puree fermentation. *International journal of food science & technology, 44*(1), 120-127.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E.,... Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature, 457*(7228), 480-484.

Turnbaugh, P. J., Quince, C., Faith, J. J., McHardy, A. C., Yatsunenko, T., Niazi, F.,... Knight, R. (2010). Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. *Proceedings of the National Academy of Sciences, 107*(16), 7503-7508.

Vinderola, C., Gueimonde, M., Delgado, T., Reinheimer, J., & De Los Reyes-Gavilan, C. (2000). Characteristics of carbonated fermented milk and survival of probiotic bacteria. *International Dairy Journal, 10*(3), 213-220.

Vinderola, C., Mocchiutti, P., & Reinheimer, J. (2002). Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. *Journal of Dairy Science, 85*(4), 721-729.

Vinderola, G., Prosello, W., Molinari, F., Ghiberto, D., & Reinheimer, J. (2009). Growth of Lactobacillus paracasei A13 in Argentinian probiotic cheese and its impact on the characteristics of the product. *International journal of food microbiology, 135*(2), 171-174.

Vorländer, K., Kampen, I., Finke, J. H., & Kwade, A. (2020). Along the Process Chain to Probiotic Tablets: Evaluation of Mechanical Impacts on Microbial Viability. *Pharmaceutics, 12*(1), 66.

Walker, J. R., Ediger, J. P., Graff, L. A., Greenfeld, J. M., Clara, I., Lix, L.,... McPhail, C. M. (2008). The Manitoba IBD cohort study: a population-based study of the prevalence of lifetime and 12-month anxiety and mood disorders. *The American journal of gastroenterology, 103*(8), 1989-1997.

Wall, R., Cryan, J. F., Ross, R. P., Fitzgerald, G. F., Dinan, T. G., & Stanton, C. (2014). Bacterial neuroactive compounds produced by psychobiotics. In *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease* (pp. 221-239): Springer.

Weng, M., Ganguli, K., Zhu, W., Shi, H. N., & Walker, W. A. (2014). Conditioned medium from Bifidobacteria infantis protects against Cronobacter sakazakii-induced intestinal inflammation in newborn mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 306*(9), G779-G787.

Wong, M.-L., Kling, M. A., Munson, P. J., Listwak, S., Licinio, J., Prolo, P.,... DeBellis, M. D. (2000). Pronounced and sustained central hypernoradrenergic function in major depression with melancholic features: relation to hypercortisolism and corticotropin-releasing hormone. *Proceedings of the National Academy of Sciences, 97*(1), 325-330.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A.,... Knight, R. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science, 334*(6052), 105-108.

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2004). Probiotication of tomato juice by lactic acid bacteria. *Journal of microbiology (Seoul, Korea), 42*(4), 315-318.

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. *Bioresource Technology, 97*(12), 1427-1430.

Zhang, J., Wang, L., Guo, Z., Sun, Z., Gesudu, Q., Kwok, L., & Zhang, H. (2014). 454 pyrosequencing reveals changes in the faecal microbiota of adults consuming Lactobacillus casei Zhang. *FEMS microbiology ecology, 88*(3), 612-622.

Zoellner, S., Cruz, A., Faria, J., Bolini, H., Moura, M., Carvalho, L., & Sant'ana, A. (2009). Whey beverage with acai pulp as a food carrier of probiotic bacteria. *Australian Journal of Dairy Technology*, *64*(2), 177.

Chapter 2

Microbiome in Brain Function and Mental Health

Amy Murphy is second author on this review which is published in Trends in Food Science and Technology, 2016

Abstract

Over recent years it has become evident that the physiological influence of the gut microbiota extends beyond the periphery to the central nervous system (CNS). Current data derived from preclinical studies indicate that the gut microbiota can influence CNS function. Despite limited attempts to translate these findings to clinical populations, emerging evidence suggests that alterations in the composition of the gut microbiota, across the lifespan, may have a fundamental role in the pathophysiology of a number of mental health disorders. Moreover, accumulating evidence demonstrates the central role of food consumed in programming gut microbiota composition, diversity and functionality throughout life.

In this review, we outline what is considered a healthy infant and adult gut microbiota composition followed by describing how the gut microbiota can influence the CNS via signalling pathways of the microbiota-gut-brain axis. Current findings from preclinical investigations, observation and intervention studies in humans, indicating the gut microbiota in brain function and mental health, are reviewed. Finally, we consider microbiota-targeted functional food interventions with potential application in promoting normal brain function.

Much work is yet to be carried out in determining the role of the gut microbiota in brain function and behaviour in human populations. Nevertheless, the potential for microbiota-targeted functional food interventions is evident. As new findings emerge in this rapidly developing field, it is envisaged that a greater understanding of microbe-brain interactions will herald a new era of psychotropic therapies to promote normal brain function and mental health.

Introduction

Although a key role for gut bacteria (the gut microbiota) in specific aspects of health and disease, such as regulating immune function and metabolic activity, has been recognised for some time (Sampson & Mazmanian, 2015). It has become apparent that the physiological influence of the microbiota extends beyond the periphery to the central nervous system (CNS). Clinically, the phenomenon of hepatic encephalopathy which, in the extreme, is characterised by dementia like symptoms that can be ameliorated by treatment with antibiotics (Strauss & da Costa, 1998) is often cited as a unique example of how perturbing the microbiota can influence brain function. It has also long been recognised that the acute phase reaction to microbial infection is accompanied by a number of neuro-immune mediated behavioural symptoms such as depressed mood and cognitive impairment (Dantzer, 2009). However, the realisation that not only pathogenic microorganisms, but the commensal/symbiotic gut bacteria can influence CNS activity has have led to a '*Paradigm Shift*' in brain and behavioural research (Mayer et al., 2014). Moreover, discoveries in microbe-brain interactions have revolutionised our approach to investigating psychopathology and spurred the development of microbiota-targeted interventions (psychobiotics) which hold great potential as a new therapeutic approach in mental health disorders (Dinan et al., 2013).

In this review we first provide an outline of what is, conceptually, considered a healthy infant and adult gut microbiota composition, followed by a description of the key pathways by which the gut microbiota signal to the brain to influence brain function and behaviour. Next, we review current evidence demonstrating a role for the gut microbiota in brain function and mental health, incorporating both preclinical and clinical studies. Finally, we discuss potential food based microbiota-targeted

approaches which have shown promise or have potential in the management of mental health.

Healthy Gut Microbiota Composition

Despite large variation in composition between healthy individuals due to a variety of environmental, physiological, genetic and psychological factors, a ‘core’ or ‘normal’ gut microbiota composition is emerging and despite large taxonomic diversity between individuals, colonisation trends can be observed in both infants and adults.

The infant gut is colonised during delivery, either from contact with the vaginal and faecal bacteria from the mother during standard vaginal delivery (SVD) or, in the case of caesarean-section (CS) delivery, from the hospital environment and maternal skin microbes (Borre et al., 2014). Culture-based studies have shown that at birth the healthy infant gut is initially colonised by facultative anaerobes such as Enterobacteriaceae, and once oxygen has been depleted and an anaerobic environment is present, strict anaerobes such as *Bifidobacterium* and *Bacteroides* appear (Adlerberth & Wold, 2009). Improvements in 16 S rRNA sequencing have led to a more accurate description of the gut microbiota composition inclusive of the substantial number of unculturable bacteria found in the gut. In healthy, vaginally delivered infants, the most prevalent initial bacterial groups include *Staphylococcus*, *Lactobacillus*, Enterobacteriaceae and *Bifidobacterium*, followed by later increases in the abundance of *Veillonella* and *Lachnospiraceae* and a decline in *Staphylococcus* (Palmer et al., 2007; Ferretti et al., 2018). During infancy, the composition of the gut microbial community is unstable and dynamic and undergoes a variety of changes

before resembling an adult gut microbiota at approximately 2 years of age, after the introduction of solid foods (Borre et al., 2014).

What constitutes a 'healthy microbiota' in adults is not entirely clear and indeed may be person-specific. Nevertheless, many advances have been made in defining a healthy phylogenetic core, which have led to a consensus that the healthy adult gut is dominated by Firmicutes, Bacteroidetes, Actinobacteria, and Verrucomicrobia (Human Microbiome Project Consortium, 2012). Within these phyla there is still large inter-individual diversity, with each person harbouring a unique microbiota profile (Moore et al., 2019).

It has been proposed that the gut microbiota can be categorised into three core clusters or enterotypes (Arumugam et al., 2011). These are broad clusters which are defined by the presence of a particular bacterial genus - *Bacteroides*, *Prevotella* or *Ruminococcus.* However, this approach to defining the gut microbiota composition is the subject of ongoing debate and is questionable when considering that over the course of year a healthy individual's microbiota can vary between clusters and is not strictly defined within one enterotype (Knights et al., 2014). Alternatively, rather than a taxonomic core, the composition may be viewed as a core set of functional profiles, in which some key bacterial species may contribute significantly to the functional profile and play an important role in health and disease (Flint et al., 2012).

A range of factors are known to disrupt the infant and adult gut microbiota composition which have been extensively reviewed elsewhere (Rodriguez, 2015), and include mode of delivery at birth, antibiotic treatment, diet, stress, infection and host genetics. The extent to which each of these factors influences brain function and mental health via disrupting the gut microbiota composition is not entirely clear and is currently an area of intensive investigation. Nevertheless, the following sections

integrate current findings in which these factors are implicated in brain function and behaviour via interactions with the gut microbiota.

The Microbiota-Gut-Brain Axis

The microbiota-gut-brain axis is a bi-directional communication network encompassing the central nervous system (CNS), sympathetic and parasympathetic branches of the autonomic nervous system (ANS), the enteric nervous system (ENS), neuroendocrine and neuroimmune pathways, and the gut microbiota (Cryan & Dinan, 2012). A complex reflexive network of efferent fibers projecting to the gastrointestinal (GI) tract and afferent fibers that project to a number of interconnected regions of the CNS facilitate communication within the axis (Dinan et al., 2015). Bi-directional communication along neural, hormonal and immune pathways thus enables the brain to influence secretory, sensory, and motor functions of the GI tract, and conversely, signals arising from the viscera to influence CNS activity (Aziz & Thompson, 1998).

Although interactions between the brain and enteric nervous system have been the focus of intense study over the past two decades, particularly in the context of functional GI disorders such as irritable bowel syndrome, over recent years there has been growing recognition that the gut microbiota has a dominant influence on signalling along this axis (Figure 1) (Cryan & Dinan, 2012; Mayer et al., 2014; Sampson & Mazmanian, 2015).

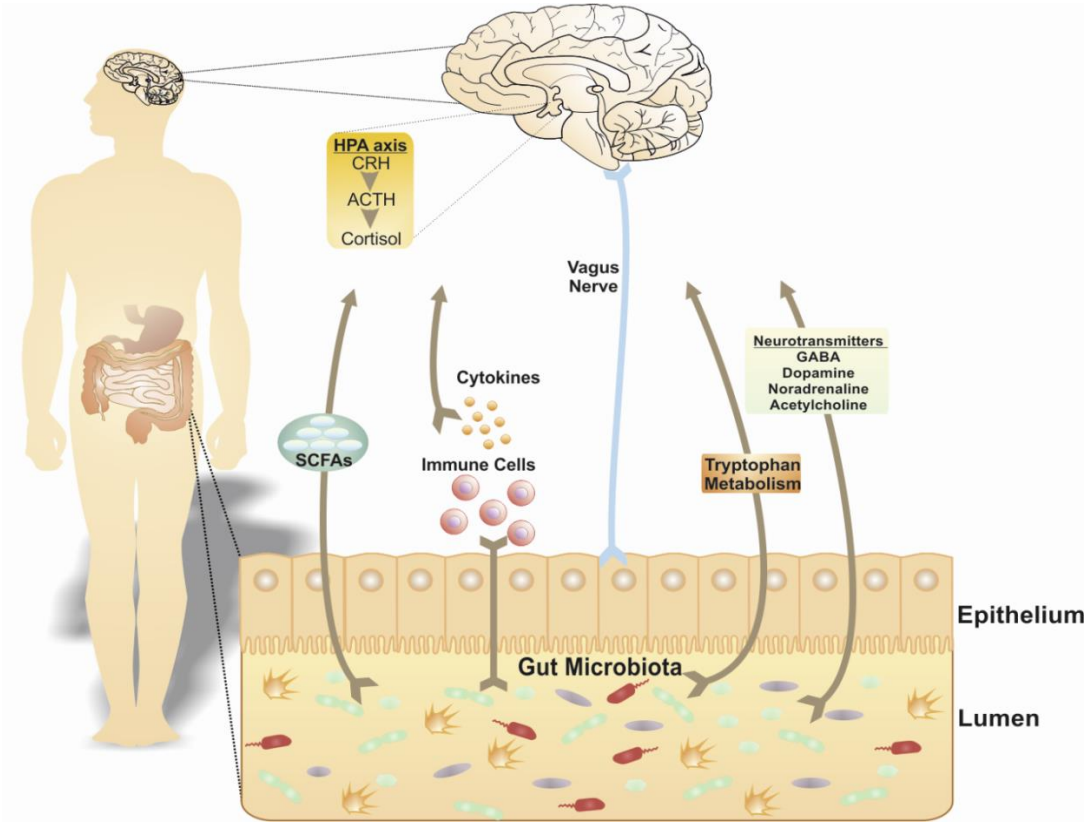


Figure 1. The Microbiota-Gut-Brain Axis. There are a number of pathways through which the gut microbiota can signal to the brain. These include, regulating immune activity and thus the production of inflammatory cytokines which can impact directly on the brain or can, stimulate the HPA axis to produce CRH, ACTH and cortisol; by mediating tryptophan metabolism which may alter the downstream production of serotonin quinolinic and kynurenic acid; through the production of SCFAs such as butyric, acetic and propionic acid; and by the production of neurotransmitters. Afferent signaling pathways of the vagus nerve are crucial in mediating the effects of the gut microbiota on brain function and behavior. **Abbreviations:** ACTH, adrenocorticotropin hormone; CRH, corticotropin-releasing hormone; GABA, gamma-aminobutyric acid; HPA, hypothalamic-pituitary-adrenal; SFCAs, short-chain fatty acids.

Microbe to Brain Signalling Pathways

The Vagus Nerve

The vagus is the major nerve mediating parasympathetic activity of the autonomic nervous system. Vagal afferent sensory neurons relay information from the GI tract to the nucleus of the solitary tract, which projects to the thalamus, hypothalamus, locus coeruleus, amygdala and periaqueductal grey (Wang et al., 2007). A number of preclinical studies have demonstrated afferent pathways of the vagus nerve are fundamental in mediating the effects of the gut microbiota on brain function and behaviour. For example, in a landmark preclinical study with conventional mice, treatment with the probiotic *Lactobacillus rhamnosus* (JB-1) reduced anxiety and depressive-like behaviour and stress-induced corticosterone levels, however, these behavioural effects were not evident in vagotimized mice (Bravo et al., 2011). Despite the importance of vagal pathways, it must be noted that vagotomy does not mediate all effects of the microbiota on brain function and behaviour (Bercik et al., 2011), and the mechanisms underlying vagal mediated microbiota-brain interactions have not yet been determined (Cryan & Dinan, 2012; Cryan et al., 2019).

Microbial Regulation of Neuro-immune Signaling

Bacterial colonisation of the gut during early life influences normal development and maturation of the immune system, and across the lifespan, the gut microbiota regulate innate and adaptive immune responses (Shanahan & Quigley, 2014). A previous ground-breaking report has elegantly demonstrated that maturation and function of microglia- the tissue macrophages of the CNS- are under control of

the gut microbiota (Erny et al., 2015). Of note, this effect was demonstrated in both germ-free (GF) and conventional mice following ablation of the gut microbiota with antibiotics. Microglia have been implicated in the pathophysiology of a host of neurodegenerative and psychiatric disorders and as such, this study has major implications for our understanding of how the microbiota interact with CNS immune activity to affect brain function and mental health (Schafer et al., 2015). Finally, pro-inflammatory cytokines released from macrophages following immune activation can act on receptors of afferent pathways of the vagus nerve to signal to the CNS, or if released systemically following a GI immune response, they may influence the CNS via other mechanisms including, direct stimulation of the hypothalamic-pituitary-adrenal (HPA) axis at the anterior pituitary or hypothalamus, active transport via saturable transport molecules, passage through leaky portions of the BBB or by activation of endothelial cells (Dantzer, 2009).

Microbiota-Mediated Tryptophan Metabolism

Tryptophan is an essential amino acid which is the precursor molecule to serotonin (5-HT), kynurenine, and metabolites of the kynurenine pathway (Clarke et al., 2012). Despite a focus on the 5-HT system in brain-gut axis and psychiatric disorders, only around 5% of systemic tryptophan is metabolised into 5-HT, while around 90% is metabolised along the kynurenine pathway (O'Mahony et al., 2015). The rate of tryptophan metabolism along the kynurenine pathway is dependent on expression of indoleamine-2,3-dioxygenase (IDO), found in all tissues, and tryptophan-2,3-dioxygenase (TDO) which is localised to the liver (Clarke et al., 2012). IDO expression can be induced by the action of inflammatory cytokines (Interferon-γ in particular), and TDO expression by glucocorticoids (O'Mahony et al., 2015). As

noted previously, the microbiota regulates host immune function, and as will be discussed below, can modulate HPA axis activity and the production of glucocorticoids (Cryan & Dinan, 2012). Thus, the gut microbiota can affect the rate of tryptophan metabolism along the kynurenine pathway. Indeed, an informative study conducted in GF animals indicates that peripheral tryptophan levels are under microbial control (Clarke et al., 2012; Patel et al., 2011). Moreover, the probiotic *Bifidobacterium infantis* was found to increase plasma tryptophan and kynurenine levels in a preclinical study in rats (Desbonnet et al., 2008). This has important implications for brain function and behaviour; downstream metabolites of the kynurenine pathway- kynurenic acid and quinolinic acid- have been shown to be neuroactive and can act on *N*-methyl-D-asparate receptors and nicotinic receptors in the CNS, and as such, there is increased interest in the role of the kynurenine pathway in psychiatric disorders and neurodegenerative disease (O'Mahony et al., 2015).

The mechanisms by which the gut microbiota regulate tryptophan metabolism have not been fully elucidated. However, delineating this process may be pivotal in understanding microbiota-brain interactions in brain function and mental health.

Microbial Control of Neuroendocrine Function

A seminal report by Sudo and colleagues was the first to demonstrate that the HPA axis, the core neuroendocrine system in humans and rodents, is under microbial control (Sudo et al., 2004). GF mice exhibited an exaggerated corticosterone and ACTH response to mild-restraint stress in comparison to specific pathogen free controls (Sudo et al., 2004). Importantly, the exaggerated HPA axis response in GF mice could be partly normalised by reconstitution with faecal matter from specific pathogen free mice, and fully normalised, in a time-dependent manner, by

monoassociation with *Bifidobacterium infantis* (Sudo et al., 2004). In addition to elegantly demonstrating the intricate interplay between microbes and neuroendocrine function, this study also highlights two critical points; that a microbiota during development is essential for normal HPA axis function in later life; and that there are critical neurodevelopmental time-windows during which microbes must colonise the gut for development of a normal stress response (Dinan et al., 2015; Cryan et al., 2019). This finding has subsequently been replicated by independent investigators (Clarke et al., 2013).

These findings are important when considering that HPA axis dysfunction is a key factor in the pathophysiology of psychiatric disorders such as depression (O'Brien et al., 2004) and functional GI disorders with marked psychiatric co-morbidity, such as irritable bowel syndrome (IBS) (Kennedy et al., 2014). However, to date, it is unknown if microbiota-targeted interventions have positive effects on HPA axis function in psychiatric populations. Nevertheless, promising findings from preliminary studies with healthy control participants indicate that pre and probiotic interventions can modulate HPA axis functioning (Messaoudi et al., 2011; Schmidt et al., 2015).

Microbial Neuro-metabolite Production

Gut bacteria can produce a number of neuroactive compounds which have potential to influence brain function and mental health (Dinan et al., 2013). For example, *in vitro* studies have demonstrated that *Candida, Streptococcus, Escherichia* and *Enterococcus* species produce 5-HT; *Bacillus* and *Serratia* species produce dopamine; *Escherichia, Bacillus, Saccharomyces* species produce noradrenaline; *Lactobacillus* species produce Acetylcholine (Lyte, 2011), and *Lactobacillus* and

Bifidobacterium secrete GABA (Barrett et al., 2012). Given the key role of these neurotransmitters in all aspects of brain function and behaviour, that gut bacteria secrete these is a striking finding. However, replicating this *in-vivo* still poses a significant challenge and the mechanism(s) by which neurotransmitters secreted by gut bacteria can influence the CNS is unclear at present as some neurotransmitters such as GABA do not cross the blood brain barrier (Hyland & Cryan, 2010). Nevertheless, utilising neuroactive metabolite secreting bacteria to promote normal brain function and mental health is a promising avenue of investigation.

Microbial Short-Chain Fatty Acid Production and Brain Function

Short chain fatty acids (SCFAs), such as butyric, acetic and propionic acid are produced by microbial fermentation of complex carbohydrates in the GI tract (Macfarlane & Macfarlane, 2003; Gill et al., 2018). SCFAs can cross the BBB and have been shown to possess some neuroactive properties (Sampson & Mazmanian, 2015). For instance, a high dose of propionic acid administered direct to the CNS (MacFabe et al., 2011) or peripherally (Foley et al., 2014), in rats, induces a range of behavioural alterations relevant to neurodevelopmental disorders. Butyrate, on the other hand, has potential pro-cognitive and beneficial behavioural properties via epigenetic mechanisms, due to potent inhibition of histone deacetylase (Stilling et al., 2014). SCFAs also regulate immune activity locally in the GI tract which may have downstream effects on CNS function (Dantzer, 2009). Moreover, the study by Enry and colleagues which reported that maturation of microglia within the CNS of GF animals was impaired, also demonstrated that treatment with a mix of SCFAs could rescue microglial function in the same manner as re-colonisation of a complex microbiota (Erny et al., 2015).

Microbes, Neurotransmitters & Neurophysiology

Clear mechanistic links between microbiota-mediated levels of peripheral biochemical parameters and central neurobiological changes are difficult to establish. Nevertheless, it is clear that the microbiota, and specific bacterial species, can regulate central neurotransmitter levels and receptor expression. For example, GF mice have decreased 5-HT levels and 5-HT_{1A} receptor expression in the amygdala and hippocampus (Bercik et al., 2011; Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011), increased 5-HT, noradrenaline and dopamine turnover in the striatum (Heijtz et al., 2011), and increased activity-related transcriptional pathways in the amygdala (Stilling et al., 2015). In conventional mice, treatment with the probiotic *L. rhamnosus* (JB-1) led to a number of brain region specific changes in GABA receptor expression; $GABA_{B1b}$ mRNA was increased in cortical regions, but reduced in the hippocampus, amygdala, and locus coeruleus; $GABA_{A\alpha2}$ mRNA expression was reduced in the prefrontal cortex and amygdala, but increased in the hippocampus (Bravo et al., 2011). Many of these neurochemical changes likely regulate numerous neurophysiological process which are thought to underlie key aspects of brain function and behaviour, such as hippocampal neurogenesis (Ogbonnaya et al., 2015).

In summary, there is clear evidence that the gut microbiota can signal to the CNS and also regulate many neurobiological processes that underlie the full spectrum of behavioural, emotional and cognitive functions. Much of this evidence is, by necessity, gleaned from preclinical studies in rodents and the extent to which the mechanisms described above, through which the gut microbiota influence brain function and behaviour translates to human physiology is not yet fully understood.

Nevertheless, the following sections outline what is currently understood as regards microbiota-brain interactions in healthy individuals and clinical populations.

The Microbiota in Brain-Gut Axis and Psychiatric Disorders

Irritable bowel syndrome (IBS): The prototypical brain-gut axis disorder

IBS is a stress-related, brain-gut axis disorder in which GI symptoms are accompanied by functional and structural brain abnormalities, HPA axis dysfunction, subtle cognitive impairment and significant psychiatric co-morbidity (Kennedy et al., 2014). The role of the microbiota in the pathogenesis of GI and extra-intestinal symptoms in IBS has received significant attention over recent years with a growing body of evidence that the diversity, stability and metabolic activity of the gut microbiome is altered in IBS when compared to healthy individuals (Jeffery et al., 2012; Enck et al., 2018). At the phylum level, a relatively consistent finding across studies determining the gut microbiota composition in IBS is decreased abundance of Bacteroidetes and increased Firmicutes (Jeffery et al., 2012). Although the functional relevance of alterations in the composition of specific microbial species in IBS is not clear, correlational studies have documented a relationship between *Ruminococcus-torques* related bacterial phylotype and *Firmicutes, Gammaproteobacteria and Verrucomicrobia* phylum and symptom scores (Kennedy et al., 2014). In addition, some probiotic strains have shown efficacy in ameliorating symptoms in patients with IBS (Clarke et al., 2012). Visceral pain hypersensitivity has long been considered a key feature of symptomatology in IBS and may be underpinned by abnormal CNS processing of signals arising from the GI tract (Tillisch & Labus, 2011). It is noteworthy then, that in rodents, perturbing the gut microbiota with antibiotic

treatment in early life can induce visceral hypersensitivity (O'Mahony et al., 2014), while probiotic strains such as *B. infantis* 35624 can alleviate hypersensitivity (McKernan et al., 2010).

Although a clear link between alterations in the gut microbiota and other centrally mediated features of IBS, including psychiatric co-morbidity, HPA axis dysfunction and cognitive impairment is yet to be established, it is conceivable that an altered gut microbiota composition may underlie the manifestation of extra-intestinal CNS symptoms in the disorder (Kennedy et al., 2014).

Microbes, Mood & Anxiety

Major depression is a highly prevalent, debilitating, mental health condition, affecting up to 1 in 5 adults and is associated with marked symptom severity, chronicity, role impairment and substantial socioeconomic cost (Kessler et al., 2003). Major depression is rarely a principal diagnosis and up to 60% of patients have a co-morbid anxiety disorder (Kessler et al., 2003). As such, the role of the gut microbiota in depression and anxiety is considered together.

Stress is a key factor in the pathophysiology of depression and patients exhibit HPA axis dysfunction as evidenced by elevated cortisol levels, increased cerebrospinal fluid levels of corticotrophin-releasing factor (CRF) and impaired negative feedback in response to the dexamethasone challenge test (O'Brien et al., 2004). In addition, elevated plasma pro-inflammatory cytokines levels have been documented by a number of studies in individuals with depression (O'Brien et al., 2004). Given that the gut microbiota can regulate HPA axis and immune activity (Shanahan & Quigley, 2014; Sudo et al., 2004), in addition to other physiological processes involved in depression and anxiety, such as tryptophan metabolism

(Dantzer, 2009), a prominent role for the microbiota in depressive and anxiety disorders is likely (Dinan & Cryan, 2013; Lew et al., 2019).

Using a number of approaches, it has been shown in preclinical studies, with some consistency, that the gut microbiota leverages a significant influence on depressive and anxiety-like behaviour. For example, GF mice exhibit both decreased (Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011) and increased (Bercik et al., 2011) anxiety-like behaviour, which can be normalised by bacterial colonisation prior to critical neurodevelopmental time-windows (Clarke et al., 2013). Treatment with the probiotic *B. breve* 1205 (Savignac et al., 2014), or ablation of the gut microbiota through administration of an antibiotic cocktail (Desbonnet et al., 2015 reduces anxiety-like behaviour in conventional mice. Moreover, it has been shown that an anxiety-like behavioural phenotype can be transferred from one mouse strain to another via faecal microbiota transplantation (Bercik et al., 2011). Finally, studies examining the role of the gut microbiota in depressive-like behaviour in rodents indicate that specific probiotic strains such as *B.infantis* (Desbonnet et al., 2008), *L. rhamnosus* (Bravo et al., 2011) and a cocktail of *L. helveticus* and *B. longum* (Messaoudi et al., 2011) possess potential anti-depressant qualities.

Investigations with healthy human volunteers lend some support to this preclinical data indicating a microbial influence on mood and anxiety. For example, probiotic treatment has been shown to reduce self-reported depression (Messaoudi et al., 2011), increase self-reported happiness in participants with low baseline mood (D. Benton et al., 2007) and decrease ruminative thinking (Steenbergen et al., 2015). Moreover, intervention with a probiotic combination of *L. helveticus* and *B. longum* reduced 24-hour urinary cortisol output (Messaoudi et al., 2011) and 4-week treatment with a prebiotic reduced the cortisol awakening response (Schmidt et al., 2015) in

healthy volunteers. Finally, at the cognitive level, a prebiotic intervention increased response time to positive versus negative stimuli in an emotional attention task (Schmidt et al., 2015), while a brain imaging study showed that 4-week intake of a fermented milk product containing probiotic modulates functional brain activity during a similar task (Tillisch et al., 2013).

In a Norwegian sample of 37 patients with a diagnosis of major depression and 18 healthy control participants, no significant group difference in overall gut microbial diversity or richness was found, but an underrepresentation of *Bacteroidales* was associated with depression (Naseribafrouei et al., 2014). In contrast, an independent report documented increased bacterial α-diversity in patients with major depression who were 'actively' (Hamilton Rating Scale For Depression (HAMDS) score ≥20) depressed, in comparison to patients who had responded to treatment (HAMDS score ≥20 at baseline with a 50% reduction after 4 weeks) and healthy controls (Jiang et al., 2015). At the phylum level, reduced levels of Firmicutes and increased Bacteroidetes, Proteobacteria, and Actinobacteria were found in patients with major depression, regardless of treatment status. Furthermore, differences in specific genera were documented, with increased Enterobacteriaceae and *Alistipes*, but decreased abundance of *Faecalibacterium* which negatively correlated with HAMDS scores (Jiang et al., 2015).

With limited data available in clinical populations, it is not possible to draw meaningful conclusions as to the nature of microbiota alterations in patients with depressive and anxiety disorders. Moreover, conflicting findings between studies is perhaps not surprising as considering major depression or anxiety disorders as singular clinical entities does not capture the breadth and inter-individual variability of symptoms patients may exhibit. Future studies with well phenotyped patient cohorts

are needed to fully determine if mood and anxiety disorders are characterised by an altered gut microbiota composition in general, and if specific microbial compositions are associated with specific symptomatology.

Microbiota, Social Behaviour and Autism

Autism spectrum disorders (ASD) are neurodevelopmental disorders typified by marked deficits in reciprocal social interaction, communication and language development, and restricted, repetitive patterns of activities and behaviours (Dinan et al., 2015; Masi et al., 2017). A role for the gut microbiota in neurodevelopmental disorders such as ASD has become a topic of significant interest over recent years (Borre et al., 2014). Clinically, this interest has been spurred by the recognition that a subset of individuals with ASD suffer problematic GI symptoms, an altered composition of the gut microbiota and increased intestinal permeability (Julio-Pieper et al., 2014; Kang et al., 2018).

In parallel, preclinical studies with GF animals have demonstrated that in the complete absence of a microbiota, rodents exhibit impairments in social behaviours relevant to ASD (Desbonnet et al., 2014). Of note, bacterial colonisation of the gut in post-weaning GF animals can normalise impaired social novelty preference, with no effect on other aspects of social-cognitive function (Desbonnet et al., 2014). As has been indicated for other types of behaviour such as anxiety and stress responsiveness, studies with GF animals suggest that the presence or absence of the gut microbiota during critical neurodevelopmental windows can modulate the expression of normal or abnormal social behaviours in later life.

Maternal infection during pregnancy is a known risk factor for ASD (Atladóttir et al., 2010), and the maternal immune activation (MIA) mouse model produces adult

offspring exhibiting both ASD like behavioural deficits and GI disturbances, including increased gut permeability and an altered gut microbiota (Malkova et al., 2012). Interestingly, in this model treatment, *Bacteroides fragilis* can repair intestinal barrier function and normalise deficits in communicative, stereotypic and anxiety-like behaviours (Hsiao et al., 2013).

Previous epidemiological studies suggest that mode of delivery at birth (C-section *vs.* vaginal) may also influence normal central nervous system (CNS) development leading to cognitive and motor problems during infancy (Al Khalaf et al., 2015). Furthermore, CS delivery has been associated with an increased risk of developing ASD (Curran et al., 2015). However, future studies are needed to determine if such associations may be better accounted for by genetic or environmental factors (Curran et al., 2015).

Taken together, the preclinical and clinical evidence suggests the gut microbiota significantly influence neurodevelopment and may have a role in the pathophysiology of ASD. However, as with other mental health disorders, clinical studies to determine the efficacy of microbiota-targeted therapies in ASD are lacking. Over a decade ago, it was shown in a small sample of children with regressive-onset autism, that treatment with oral vancomycin could improve symptoms (Sandler et al., 2000). The beneficial effect of this antibiotic was not apparent following the cessation of treatment at follow-up, and of course, long-term antibiotic treatment is not a viable therapeutic strategy in ASD. Nevertheless, when taken with the preclinical work which has been conducted to date, developing safe approaches to targeting the gut microbiota through food based approaches, to ameliorate symptoms in ASD has potential, and presents an exciting and hopeful prospect in new treatment approaches for these individuals.

The Gut Microbiota and Age-related Cognitive Decline

As the world's ageing population is rapidly expanding, it has become a major societal goal to promote health and wellbeing in later life, and a number of innovative strategies have been implemented over recent years (Rechel et al., 2013). A prominent consequence of ageing is a steady decline in cognitive functions including the ability to encode new episodic memories, working memory/executive functions and processing speed (Deary et al., 2009). Ageing is accompanied by a plethora of age-related neurobiological changes, including altered HPA axis function, decreased neurotransmitter/neurotrophin concentration and receptor expression, and increased oxidative stress (Prenderville et al., 2015). Moreover, the concept of inflamm-ageing, indicating a general increase of inflammatory tone during ageing, has been championed as a key process mediating many of the age-related changes occurring in the CNS and periphery (Franceschi et al., 2000; Kim et al., 2021). In a seminal study, the ELDERMET consortium identified that age-related change in the composition of the gut microbiota in elderly individuals was associated with number of health parameters including inflammation and cognitive function (Claesson et al., 2012). Based on these findings, it is tempting to speculate that the gut microbiota plays a significant role in age-related cognitive decline (Mahmoudian Dehkordi et al., 2019). Further lines of evidence lend weight to this speculation. For instance, heightened immune activity and HPA axis dysfunction are considered to be key in pathological cognitive decline during ageing (Prenderville et al., 2015), and as outlined above, both systems are influenced by the gut microbiota. A number of studies utilising various approaches have demonstrated that the microbiota can regulate some aspects of cognitive function (Dinan et al., 2015) and a double-blind randomised controlled trial

in a small sample of healthy elderly adults revealed that treatment with a *Lactobacillus helveticus* probiotic strain had some positive effects on attentional performance (Chung et al., 2014).

Further clinical interventions studies are needed to determine the potential pro-cognitive effects of microbiota-targeted interventions in elderly individuals, either in healthy or clinical populations (Kim et al., 2021). Nevertheless, as preclinical data indicate that the gut microbiota can mediate various aspects of cognitive performance, there is clear potential for microbial targeted prophylactic interventions to tackle age-related cognitive decline and promote normal cognitive function in later life.

Microbiota-Targeted Functional Foods for Brain Health

Prebiotics and Probiotics

As the studies reviewed above indicate, modulation of the gut microbiota using dietary intervention, in particular with prebiotics and probiotics (Korpela et al., 2018), is a promising intervention strategy in promoting normal brain function and mental health.

However, to progress the field, it is of great importance to understand the mechanisms by which prebiotics and probiotic species exert their effect on brain function and behaviour. Prebiotics are may exert a beneficial brain effect through improving host immunity, enhancing SCFA production, reducing potentially pathogenic microbes and improving gut barrier function (Slavin, 2013). *Lactobacillus* spp. and *Bifidobacterium* spp. are the most commonly used probiotics and may act via a number of mechanisms to alter the gut microbiota of the host in order to improve brain health, including production of antimicrobial compounds, reduction of the luminal pH through the production of SCFA, competitive exclusion (which involves

preventing other microbes from adhering to epithelial cells), production of growth substrates such as vitamins and exopolysaccharides, enhanced barrier function, and modulation of immune responses (Power et al., 2014, Wang et al., 2019). Future studies to fully understand the mechanism of prebiotic and probiotic interventions for brain function and behaviour are needed to determine those species which may have potential application in mental health disorders.

Table 2.1: Human trials investigating the effects of probiotic and prebiotic interventions on mood and cognition

Probiotic/prebiotic	Objectives	Study design	Sample size	Participant age (years)	Probiotic delivery	Findings	Reference
Bifidobacterium bifidum W23 *Bifidobacterium lactis* W52 *Lactobacillus acidophilus* W37 *Lactobacillus brevis* W63 *Lactobacillus casei* W56 *Lactobacillus salivarius* W24 and (W19 and W58)	Investigate whether a multispecies probiotic may reduce cognitive reactivity in non-depressed individuals	-Triple-blind, placebo-controlled, randomised, pre- and post-intervention assessment -4 week intervention	40	20.2 ± 2.4	-2 g/day freeze-dried powder -2.5 x 10^9 CFU/g	-Participants who received the multispecies probiotic showed reduced overall cognitive reactivity to sad mood -Participants showed reduced aggressive and ruminative thoughts in response to sad mood	(Steenbergen et al., 2015)
Lactobacillus helveticus R0052 *Bifidobacterium longum* R0175	Effects of the probiotic formulation on anxiety, stress, depression and coping strategies in healthy individuals	-Double-blind, placebo-controlled, randomised, parallel -30 day intervention	55	30 - 60	-1.5 g/day probiotic stick -3 x 10^9 CFU/stick	-Probiotic treatment showed a beneficial effect on general signs of anxiety and depression	(Messaoudi et al., 2011)
Bifidobacterium lactis, *Streptococcus thermophilus*, *Lactococcus lactis* *Lactobacillus bulgaricus*	Investigate whether consumption of a fermented milk product with probiotic alters brain intrinsic connectivity or responses to emotional attention tasks in healthy women	-Single centre, randomised, controlled, parallel-arm design -4 week intervention	36	18 - 55	-Fermented milk -125 g pot consumed twice daily -1.25 x 10^{10} CFU/cup *B. lactis* -1.2 x 10^9 CFU/cup *S. thermophilus and L. bulgaricus*	-Intake of fermented milk product with probiotic affected brain activity in regions controlling central processing of emotion and sensation	(Tillisch et al., 2013)
Lactobacillus casei Shirota	Effect of consumption of a probiotic containing milk drink on mood and memory	-Double-blind, placebo-controlled, randomised -3 week intervention	124	48 - 79	-Probiotic milk drink -65 ml pots 10^8 CFU/ml *L. casei* Shirota	-Probiotic consumption improved mood in participants whose mood was initially poor	(Benton et al., 2007)
Lactobacillus casei Shirota	Effect of probiotic intervention on symptoms of depression and anxiety in adults with chronic fatigue syndrome	-Double-blind, placebo-controlled, randomised -8 week intervention	35	18 - 65	-Sachet containing 8 x 10^9 CFU *L. casei* Shirota -Participants consumed three sachets per day (24 x 10^9 CFU/day)	- Increase in *Lactobacillus* and *Bifidobacterium* in participants taking the probiotic -Decrease in anxiety symptoms in comparison to the placebo	(Rao et al., 2009)
Oligofrucrose-encriched inulin	Examine the acute effects of oligofructose-enriched inulin on mood and cognitive performance	-Double-blind, placebo-controlled -4 hour period in the laboratory (test sessions before and after intervention)	47	19 - 30	-Sachet containing 5 mg of inulin in de-caffeinated tea or coffee at 9am	-Participants who consumed inulin felt happier, had less indigestion and were less hungry. They also showed improvements in episodic memory (recall and recognition)	(Smith et al., 2015)

Polyphenols

Polyphenols are a large group of compounds naturally occurring in plants and a variety of foods, including citrus fruits, cocoa, red wine, tea and coffee (Gomez-Pinilla & Tyagi, 2013). Based on their structure, the main classes of polyphenols are phenolic acids, flavonoids, lignans, and stilbenes (Valdes et al., 2015). Large-scale epidemiological investigations suggest that a diet rich in polyphenols may help maintain normal brain function and mental health (Letenneur et al., 2007). Interventional studies in humans provide some supportive evidence for this epidemiological data (see Table 2.2). Although a number of mechanisms, including anti-inflammatory, anti-oxidant and modulation of enzyme activity have been proposed to account for the positive CNS effects of polyphenols (Letenneur et al., 2007), these actions are presumed to be indirect as bio-availability of native polyphenols is low (Crozier et al., 2009). Approximately 90-95% of total dietary polyphenols accumulate in the large intestine where they are broken down into less complex metabolites by the gut microbiota (Selma et al., 2009). Conversely, polyphenol metabolites modulate gut microbiota composition. For example, black and green tea (epigallocatechin, epicatechin, catechin) have been shown to affect the growth of *Helicobacter pylori, Staphylococcus aureus, Salmonella typhimurium, Listeria monocytogenes,* while other polyphenols have been shown to promote the growth of beneficial bacteria, such as *Bifidobacterium* spp, in animal models and in human studies (Duda-Chodak et al., 2015).

It is likely that the beneficial CNS effects of polyphenol compounds are mediated, at least in part, by interactions with the gut microbiota (Schaffer & Halliwell, 2012). As such, one key consideration when developing polyphenol interventions to promote normal brain function and mental health, is the large inter-

individual variation in gut microbiota composition which may significantly affect polyphenol bioefficacy (Selma et al., 2009). Furthermore, it is not entirely clear which specific constituents of polyphenol rich food exert beneficial effects on brain function. Nevertheless, future studies to determine the positive effects of polyphenol-microbe interactions on brain function and mental health may prove to be a fruitful therapeutic approach.

Table 2.2: Human studies investigating the effects of polyphenol based interventions on cognitive performance

Source of polyphenol	Major polyphenols	Study aims	Study design	Sample size	Mean Age (years)	Findings	Reference
Concord grape juice	Proanthocyanins Anthocyanins	Effect of Concord grape juice on memory performance in older adults with age-related memory decline	-Double-blind, randomised, placebo-controlled -7 control subjects and 5 test subjects given grape juice -Duration : 12 weeks	12	78.2±5	Significant improvement in verbal learning with trends toward improved spatial memory	(Krikorian et al., 2010)
Blueberry juice	Hydroxycinnamic acid ester Chlorogenic acid Cyanidin 3-glucoside	Effect of blueberry juice on memory performance in older adults with age-related memory decline	-Single-blind, placebo-controlled -7 control subjects and 9 test subjects -Duration : 12 weeks	16	80.2 ± 6.3	Paired associate learning and word list recall were significantly improved. Trends toward reduced depressive symptoms were seen	(Krikorian et al., 2010)
Dark chocolate drink	Cocoa polyphenols - Epicatechin, Catechin	Effects of cocoa polyphenols on cognition and mood in healthy middle-aged adults	-Double-blind, randomised, placebo-controlled -Participants given either drinks with a high dose of cocoa polyphenols (500 mg), a low dose (250 mg) or placebo (0 mg) -Duration : 30 days	72	40–65	Significant increases in calmness and contentedness seen for the group given the high dose of cocoa polyphenols but not for the low group or the placebo No improvement in cognition seen for any group	(Pase et al., 2013)
Dark chocolate	Cocoa flavanols (CF)	Investigated whether visual and cognitive function is influenced by an acute dose of CF in young adults	-Single-blind, randomised, counterbalanced, cross-over -Consumed 35 g of dark chocolate (720 mg CF) and were subjected to cognitive/visual tests 2 hours later -One week later consumed 35 g of white chocolate and subjected to same cognitive/visual tests	30	18-25	Improvements in visual function, spatial memory and performance in individuals after CF consumption	(Field et al., 2011)
Cocoa drink	Cocoa flavanols (CF)	Impact of cocoa flavanol (CF) consumption on cognitive function in elderly individuals with mild cognitive impairment	-Double-blind, randomised, parallel -Participants were given a dairy-based cocoa drink once daily containing either a high dose of CF (990 mg/serving), intermediate (520 mg/serving), or low (45 mg/serving) -Duration : 8 weeks	90	65-82	Improvements in cognitive performance were seen in individuals give either high or intermediate doses of CF but not in the group given the low dose	(Desideri et al., 2012)
Tablet form	Isoflavones	Effects of isoflavones on mood and cognitive function in postmenopausal women	-Double-blind, randomised, cross-over, placebo-controlled -Participants given either phytoestrogens tablets (600-mg tablets containing 60 mg of isoflavones) or placebo tablets -1 tablet/day -Duration: 6 months on phytoestrogens/placebo and then those given a placebo were given phytoestrogens and those given phytoestrogens were given a placebo for another 6 months	78	49-50	Better cognitive performance compared to placebo Individuals reported feeling less depressed when taking the phytoestrogens 49 individuals preferred the phytoestrogens over placebo	(Casini et al., 2006)
Cocoa drink	Cocoa flavanols (CF)	Investigate the relationship between cerebral blood flow and a single acute dose of flavanol-rich cocoa in young adults Investigate the behavioural and fMRI response to a cognitive task	- Double-blind, randomised, placebo-controlled -Participants received a high flavanol cocoa drink (172 mg flavanols per drink) for 5 days before one fMRI session and a low flavanol cocoa drink (13 mg flavanols per drink) before the second fMRI -Duration : One drink/day for 5 days before each scan with the final drink 1.5 hours before the scan	16	18-30	High flavanol comsumption was seen to increase the cerebralblood flow to grey matter No effects on behavioural responses or heart rate were seen	(Francis et al., 2006)
Tablet form	Restaverol and piperine	Investigate whether piperine is capable of enhancing the bioefficacy of resveratrol Subsequent effect on cerebral blood flow and cognitive performance in healthy adults	-Double-blind, randomised, cross-over, placebo-controlled -Three study visits -The participants received three single-dose treatments -The three treatments comprised two capsules, with each combination delivering a placebo, 250 mg of trans-resveratrol or 250 mg of trans-resveratrol plus 20 mg of piperine	23	19-34	Participants given 250 mg trans-resveratrol showed no changes in cerebral blood flow during cognitive tests Co-administration of the same dose of resveratrol with 20 mg piperine resulted in significantly increased cerebral blood flow. There were no significant differences in any cognitive or mood measures	(Wightman et al., 2014)

Omega-3 Polyunsaturated Fatty Acids

A major characteristic of the modern Western diet is an increased consumption of pro-inflammatory omega-6 polyunsaturated fatty acids (PUFA) relative to anti-inflammatory omega-3 PUFA (Simopoulos, 2002). A such, a diet consisting of a greater intake of omega-6 PUFA than omega-3 PUFA has been associated with a number of inflammatory related diseases (Simopoulos, 2002). Dietary intakes of omega-3 PUFA, in particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been studied in the context of almost every aspect of brain function and mental health, from neurodevelopment to age-related cognitive decline, and the manifestation and treatment of all the major psychiatric disorders (Freeman et al., 2006; Baxter et al., 2019).

Epidemiological data suggest that dietary intake of omega-3 is protective against unipolar and bipolar depression (Freeman et al., 2006), and dementia (Huang et al., 2005). Although concern has been raised over the methodological quality of clinical trials conducted in this field (Bloch & Hannestad, 2012), EPA and DHA have purportedly shown some efficacy when used an as adjunct therapy in patients with major depression and schizophrenia (Freeman et al., 2006). In addition, a previous meta-analysis indicates that omega-3 intervention is beneficial in preventing age-related cognitive decline (Zhang et al., 2015).

Preclincial studies have outlined many potential mechanisms by which omega-3 PUFAs may act in the CNS to promote normal brain function and mental health. These include regulation of monaminergic neurotransmission and BDNF levels, modulating HPA axis activity and reducing peripheral and central inflammatory

activity (Pusceddu et al., 2015). Many of these central effects may be mediated by the gut microbiota. For example, *in-vitro* and animal studies suggest that PUFA can promote the growth of *Lactobacillus* strains and conversely *Lactobacillus* can modulate PUFA absorption (Laparra & Sanz, 2010). Interestingly, it has been shown that *in-vivo* treatment of conventional mice with *B. breve* NCIMB 702258 increases brain levels of DHA (Wall et al., 2012). In support of this preclinical data, supplementing infant formula with *Bifidobacterium* Bb-12 increased plasma alpha-linolenic acid in a small sample of infants with atopic eczema (Kankaanpää et al., 2002).

In summary, the evidence indicates that PUFA may have a number of physiological effects which are beneficial to brain function and mental health which may be mediated via interactions with the gut microbiota (Table 2.3).

Omega 3 PUFA	Objectives	Study design	Sample size	Participant age (years)	Findings	Reference
Fish oil capsules	Effect of omega-3 supplementation on cognition and physiology in healthy participants	- Double-blind, placebo-controlled, randomised -35 day intervention -Treatment group consumed 4 g of fish oil per day containing 2.8 g omega-3 PUFA (1.6 g EPA and 0.8 g DHA and 0.4 g other types of omega-3 PUFA: alpha-linolenic, stearidonic, eicosatetraeonic and docosapentaenoic acid) -Participants were tested on day 1 and day 35 of the study	33	22 - 51	-Omega 3 supplementation associated with improvements in reactivity, attention and cognitive performances -Improvements in mood state were also seen in the treatment group	(Fontani et al., 2005)
EPA-enriched oil capsules	Investigate the efficacy of EPA as a treatment for schizophrenia	-Double-blind, placebo-controlled, randomised -Treatment group given 2 g/day EPA -Patients were still given conventional anti-psychotic medication if this was necessary	30	34.4± 8.5 – treatment 36.7 ± 8.1 – placebo	-In the placebo group, all patients required conventional anti-psychotic medication at the end of the study while 6 patients in the treatment group were not taking medication -Treatment group also had significantly lower scores on the Positive and Negative Syndrome Scale (PANSS)	(Peet et al., 2001)
Fish oil capsules	Assess the effects of omega-3 long chain PUFA supplementation during pregnancy on infants	-Double-blind, placebo-controlled, randomised -Pregnant mothers given supplementation from 20 weeks gestation until delivery -Treatment group received 1.1 g/day EPA and 2.2 g/day DHA	72 infants 83 mothers	Infants : 2.5 Mothers : 30.9 ± 3.7 – treatment 32.6 ± 3.6 – placebo group	-Significant positive correlation between the eye and hand coordination score at 34 months and omega-3 PUFA composition of cord blood erythrocytes -Omega-6 levels negatively correlated with eye and hand coordination	(Dunstan et al., 2008)
Fish oil capsules	Investigate the effects of omega-3 supplementation on depression-relevant cognitive functioning in healthy individuals	-Double-blind, placebo-controlled, randomised -4 week intervention -Participants given 3 g fish oil/day in 3 soft capsules -Each test participant consumed 2.3 g omega-3 PUFA:1.74 g EPA, 0.25 g DHA	54	22.2 ± 3.6 – treatment group 22.6 ± 4.1 – placebo group	-Treatment group made fewer risk-averse decisions compared with the placebo -Participants in treatment group also showed improved scores on a control/perfectionism scale of cognitive reactivity measure	(Antypa et al., 2009)

Fish oil capsules	Determine whether long chain omega-3 PUFA can reduce the rate of progression to first-episode psychotic disorder in young adults at ultra-high risk of psychosis	-Double-blind, placebo-controlled, randomised -12 week intervention period followed by 40 week monitoring period -Treatment group received 700 mg of EPA/day and 480 mg of DHA/day -Coconut oil was used as placebo	76	13 - 25	-Treatment group showed reduction in rate of transition to psychosis accompanied by symptomatic and functional improvements during the 40-week monitoring period -By the end of the monitoring period, 2 of 41 participants in the treatment group transitioned to psychosis compared with 11 of 40 in the placebo group	(Amminger et al., 2010)
Fish oil supplements containing a 7:1 ratio of EPA to DHA	Examine whether omega-3 supplementation can attenuate loneliness-related episodic memory problems	-Placebo-controlled, randomised -Participants were given either the placebo, 1.25 g/day of omega-3, or 2.50 g/day	138	40 - 85	-Lonelier participants consuming the higher dose supplement had better verbal episodic memory compared with lonelier participants consuming the placebo -Improvements in plasma omega-6:omega-3 ratio were related to better immediate and long-delay free recall	(Jaremka et al., 2014)

Table 2.3: Human trials investigating the effects of omega-3 PUFA interventions on brain health and cognition

Other Dietary Factors That May Impact Brain and Behaviour by Modulating the Gut Microbiota

Previous evidence has indicated that food additives such as emulsifiers and food colorants may alter the gut microbiota composition and impact negatively on host health (He et al., 2013). For example, administration of the food emulsifiers, carboxymethylcellulose (CMC) and polysorbate-80 (P80) to normal mice induced low-grade inflammation, metabolic syndrome and induced colitis when administered to a genetically predisposed mouse strain (*Il10−/−* and *Tlr5−*; (Chassaing et al., 2015)). Moreover, administration of CMC and P80 altered the gut microbial composition in normal mice, and FMT from normal mice induced metabolic syndrome and low-grade inflammation in GF animals (Chassaing et al., 2015), thus demonstrating that changes in the gut microbiota were necessary for the metabolic changes to occur following CMC and P80 treatment. It is yet to be determined if this startling finding translates to humans. Nevertheless, it will be important to understand if these emulsifiers in food, such as CMC and P80, impact not only on metabolic function, but brain function and behaviour. Although the impact of other food additives such as colorants on the gut microbiota, have not been extensively studied, there is evidence that some may alter the microbial composition (Pan et al., 2012). It is of note then, that symptoms of the neurodevelopmental disorder attention deficit hyperactivity disorder (ADHD) show a modest response to dietary exclusion of food colorants (Sonuga-Barke et al., 2013). This raises the intriguing possibility that food colorants may induce symptoms in ADHD via modulation of pathways of the microbiota-gut-brain axis. However, this speculation clearly needs much further investigation.

Emerging evidence that food additives can alter the composition of the gut microbiota must be considered in the context of how these microbial changes might impact on brain function and behaviour. Exclusion of such additives from an individual's diet should be considered in complement to additions of functional foods which are beneficial for microbial composition and thus brain function and behaviour.

Future Trends & Conclusions

As the growing body of evidence outlined herein indicates, the gut microbiota can influence mammalian brain development and function, ultimately affecting numerous psychological processes such as mood, emotion, social interaction and cognitive function. The preclinical data are strong in this regard, and studies in rodents will continue to be fundamental in providing a mechanistic understanding of microbiota-brain interactions. Despite limited attempts to translate these findings to humans, emerging data suggest that the microbiota can regulate certain aspects of emotional and neuropsychological functioning.

Pre and probiotic interventions in healthy human adults have demonstrated that some bacterial species exert positive effects on emotion, cognition and HPA axis function. While these data are encouraging, such studies have been limited in their scope of psychological assessment (e.g., mood, emotion, cognition) are confounded by lack of specific controls (e.g., diet) and without a comprehensive analysis of a range of biological parameters (e.g., faecal microbiota, metabolomic analysis, inflammatory markers) to provide a mechanistic account of the physiological processes mediating a gut microbiota influence on brain function. Future intervention studies in healthy participants, which are adequately powered must rigorously employ a range of

biological and psychological measures to fully determine the effects of pre or probiotic species, on specific aspects of brain function and behaviour.

In clinical populations, current observational data indicate that the gut microbiota composition is altered in individuals with depression, ASD and IBS. Future studies are needed to clearly define the nature of gut microbiota changes in these disorders, and to understand if these disorders are characterised by a specific microbial signature. Such studies will help to narrow the search for bacterial species that can be targeted for therapeutic benefit. Determining cause and effect will be problematic in defining a microbial signature in any mental health disorder, i.e., is the altered microbiota composition a cause or a consequence of mental health problems? As such, randomised controlled trials with well phenotyped psychiatric populations are clearly warranted.

Developing our understanding of how the gut microbiota influence brain function and behaviour at the extremes of life is key priority of ongoing and future research. Preclinical studies with GF rodents have demonstrated that the composition of the gut microbiota in early life can significantly influence neurodevelopment and subsequent cognitive and behavioural function. In parallel, there is supporting evidence from epidemiological studies that environmental factors which disrupt the gut microbiota in early life, such as birth by C-section, are associated with altered neurodevelopmental functioning. Although there has been relatively less focus on old age, preliminary evidence that age-related cognitive decline is influenced by the gut microbiota composition indicates that microbiota-targeted therapies may prove to be of great importance in promoting healthy brain ageing.

Finally, the extent to which functional foods (prebiotics, probiotics, omega-3 PUFAs and polyphenols) can be employed as stand-alone nutritional solutions to

promote normal brain function and mental health or will be effective as adjunct to current pharmacological therapies, will be important to determine in future studies with clinical populations. As will identifying the potential negative impact of certain food additives such as emulsifiers and colorants.

As a rapidly developing field, new findings are continually emerging which bolster our knowledge of how the gut microbiota influence brain function and behaviour. Despite significant gains over the past decade in understanding the brain mechanisms underlying the development and manifestation of most major psychiatric disorders, few advances have been made in the discovery of novel CNS acting agents. Future use of paraprobiotics (Enck et al., 2020) or psychobiotics, which target pathways of microbiota-gut-brain axis, represent a new era in psychotropic therapies and hold great promise in promoting normal brain function and mental health across the lifespan.

References

Adlerberth, I., & Wold, A. E. (2009). Establishment of the gut microbiota in Western infants. *Acta Paediatrica, 98*(2), 229-238.

Al Khalaf, S. Y., O'Neill, S. M., O'Keeffe, L. M., Henriksen, T. B., Kenny, L. C., Cryan, J. F., & Khashan, A. S. (2015). The impact of obstetric mode of delivery on childhood behavior. *Social psychiatry and psychiatric epidemiology, 50*(10), 1557–1567.

Amminger, G., Schäfer, M. R., Papageorgiou, K., & et al. (2010). Long-chain ω-3 fatty acids for indicated prevention of psychotic disorders: A randomized, placebo-controlled trial. *Archives of General Psychiatry, 67*(2), 146-154.

Antypa, N., Van der Does, A., Smelt, A., & Rogers, R. (2009). Omega-3 fatty acids (fish-oil) and depression-related cognition in healthy volunteers. *Journal of Psychopharmacology, 23*(7), 831-840.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R.,... Batto, J.-M. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174-180.

Atladóttir, H. O., Thorsen, P., Østergaard, L., Schendel, D. E., Lemcke, S., Abdallah, M., & Parner, E. T. (2010). Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of autism and developmental disorders, 40*(12), 1423-1430.

Aziz, Q., & Thompson, D. G. (1998). Brain-gut axis in health and disease. *Gastroenterology, 114*(3), 559-578.

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., & Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology, 113*(2), 411-417.

Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C., & Schmidt, T. M. (2019). Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. *MBio, 10*(1), e02566-18.

Benton, D., Williams, C., & Brown, A. (2007). Impact of consuming a milk drink containing a probiotic on mood and cognition. *European journal of clinical nutrition, 61*(3), 355–361.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J.,... McCoy, K. D. (2011). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology, 141*(2), 599-609. e593.

Bloch, M. H., & Hannestad, J. (2012). Omega-3 fatty acids for the treatment of depression: systematic review and meta-analysis. *Molecular psychiatry*, *17*(12), 1272–1282.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in molecular medicine, 20*(9), 509-518.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G.,... Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences, 108*(38), 16050-16055.

Casini, M. L., Marelli, G., Papaleo, E., Ferrari, A., D'Ambrosio, F., & Unfer, V. (2006). Psychological assessment of the effects of treatment with phytoestrogens on postmenopausal women: a randomized, double-blind, crossover, placebo-controlled study. *Fertility and sterility, 85*(4), 972-978.

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature, 519*(7541), 92-96.

Chung, Y.-C., Jin, H.-M., Cui, Y., Kim, D. S., Jung, J. M., Park, J.-I.,... Chae, S.-W. (2014). Fermented milk of Lactobacillus helveticus IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. *Journal of Functional Foods, 10*(0), 465-474.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S.,... O'Toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature, 488*(7410), 178-184.

Clarke, G., Cryan, J. F., Dinan, T. G., & Quigley, E. M. (2012). Review article: probiotics for the treatment of irritable bowel syndrome--focus on lactic acid bacteria. *Alimentary pharmacology & therapeutics*, *35*(4), 403–413.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., Dinan, T. G., & Cryan, J. F. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular psychiatry*, *18*(6), 666–673.

Clarke, G., McKernan, D. P., Gaszner, G., Quigley, E. M., Cryan, J. F., & Dinan, T. G. (2012). A Distinct Profile of Tryptophan Metabolism along the Kynurenine Pathway Downstream of Toll-Like Receptor Activation in Irritable Bowel Syndrome. *Frontiers in pharmacology*, *3*, 90.

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*(7402), 207-214.

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural product reports, 26*(8), 1001-1043.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience, 13*(10), 701-712.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M., ... & Dinan, T. G. (2019). The microbiota-gut-brain axis. *Physiological reviews*.

Curran, E. A., Dalman, C., Kearney, P. M., & et al. (2015). Association between obstetric mode of delivery and autism spectrum disorder: A population-based sibling design study. *JAMA Psychiatry*.

Curran, E. A., O'Neill, S. M., Cryan, J. F., Kenny, L. C., Dinan, T. G., Khashan, A. S., & Kearney, P. M. (2015). Research Review: Birth by caesarean section and development of autism spectrum disorder and attention-deficit/hyperactivity disorder: a systematic review and meta-analysis. *Journal of Child Psychology and Psychiatry, 56*(5), 500-508.

Dantzer R. (2009). Cytokine, sickness behavior, and depression. *Immunology and allergy clinics of North America*, *29*(2), 247–264.

Deary, I. J., Corley, J., Gow, A. J., Harris, S. E., Houlihan, L. M., Marioni, R. E., Penke, L., Rafnsson, S. B., & Starr, J. M. (2009). Age-associated cognitive decline. *British medical bulletin*, *92*, 135–152.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., & Cryan, J. F. (2014). Microbiota is essential for social development in the mouse. *Molecular psychiatry*, *19*(2), 146–148.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D., Cotter, P. D., Dinan, T. G., & Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity*, *48*, 165–173.

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., & Dinan, T. G. (2008). The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. *Journal of psychiatric research*, *43*(2), 164–174.

Desideri, G., Kwik-Uribe, C., Grassi, D., Necozione, S., Ghiadoni, L., Mastroiacovo, D.,... Lechiara, M. C. (2012). Benefits in Cognitive Function, Blood Pressure, and Insulin Resistance Through Cocoa Flavanol Consumption in Elderly Subjects With Mild

Cognitive Impairment The Cocoa, Cognition, and Aging (CoCoA) Study. *Hypertension, 60*(3), 794-801.

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 3047–3052.

Dinan, T. G., & Cryan, J. F. (2012). Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. *Psychoneuroendocrinology, 37*(9), 1369-1378.

Dinan, T. G., & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression?. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*, *25*(9), 713–719.

Dinan, T. G., Stanton, C., & Cryan, J. F. (2013). Psychobiotics: a novel class of psychotropic. *Biological psychiatry*, *74*(10), 720–726.

Dinan, T. G., Stilling, R. M., Stanton, C., & Cryan, J. F. (2015). Collective unconscious: How gut microbes shape human behavior. *Journal of Psychiatric Research, 63*, 1-9.

Duda-Chodak, A., Tarko, T., Satora, P., & Sroka, P. (2015). Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. *European journal of nutrition, 54*(3), 325-341.

Dunstan, J. A., Simmer, K., Dixon, G., & Prescott, S. L. (2008). Cognitive assessment of children at age 2½ years after maternal fish oil supplementation in pregnancy: a randomised controlled trial. *Archives of Disease in Childhood - Fetal and Neonatal Edition, 93*(1), F45-F50.

Enck, P., & Mazurak, N. (2018). Dysbiosis in functional bowel disorders. *Annals of Nutrition and Metabolism*, *72*(4), 296-306.

Enck, P., Schemann, M., & Frieling, T. (2020). Paraprobiotics for irritable bowel syndrome: all that glitters is not gold. *The Lancet Gastroenterology & Hepatology*, *5*(9), 797.

Erny, D., Hrabě 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., & Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nature neuroscience*, *18*(7), 965–977.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., ... & Segata, N. (2018). Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell host & microbe, 24*(1), 133-145.

Field, D. T., Williams, C. M., & Butler, L. T. (2011). Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions. *Physiology & behavior, 103*(3), 255-260.

Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology and Hepatology, 9*(10), 577-589.

Foley, K. A., Ossenkopp, K.-P., Kavaliers, M., & MacFabe, D. F. (2014). Pre- and Neonatal Exposure to Lipopolysaccharide or the Enteric Metabolite, Propionic Acid, Alters Development and Behavior in Adolescent Rats in a Sexually Dimorphic Manner. *PLoS ONE, 9*(1), e87072.

Fontani, G., Corradeschi, F., Felici, A., Alfatti, F., Migliorini, S., & Lodi, L. (2005). Cognitive and physiological effects of Omega-3 polyunsaturated fatty acid supplementation in healthy subjects. *European journal of clinical investigation, 35*(11), 691-699.

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., & De Benedictis, G. (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences, 908*, 244–254.

Francis, S., Head, K., Morris, P., & Macdonald, I. (2006). The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. *Journal of cardiovascular pharmacology, 47*, S215-S220.

Freeman, M. P., Hibbeln, J. R., Wisner, K. L., Davis, J. M., Mischoulon, D., Peet, M., Keck, P. E., Jr, Marangell, L. B., Richardson, A. J., Lake, J., & Stoll, A. L. (2006). Omega-3 fatty acids: evidence basis for treatment and future research in psychiatry. *The Journal of clinical psychiatry, 67*(12), 1954–1967.

Gill, P. A., Van Zelm, M. C., Muir, J. G., & Gibson, P. R. (2018). Short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Alimentary pharmacology & therapeutics, 48*(1), 15-34.

Gomez-Pinilla, F., & Tyagi, E. (2013). Diet and cognition: interplay between cell metabolism and neuronal plasticity. *Current opinion in clinical nutrition and metabolic care, 16*(6), 726-733.

He, X., Marco, M. L., & Slupsky, C. M. (2013). Emerging aspects of food and nutrition on gut microbiota. *Journal of agricultural and food chemistry*, *61*(40), 9559-9574.

Hsiao, Elaine Y., McBride, Sara W., Hsien, S., Sharon, G., Hyde, Embriette R., McCue, T.,... Mazmanian, Sarkis K. (2013). Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders. *Cell*, *155*(7), 1451-1463.

Huang, T., Zandi, P., Tucker, K., Fitzpatrick, A., Kuller, L., Fried, L.,... Carlson, M. (2005). Benefits of fatty fish on dementia risk are stronger for those without APOE ε4. *Neurology*, *65*(9), 1409-1414.

Hyland, N. P., & Cryan, J. F. (2010). A Gut Feeling about GABA: Focus on GABA(B) Receptors. *Frontiers in pharmacology*, *1*, 124.

Jaremka, L. M., Derry, H. M., Bornstein, R., Prakash, R. S., Peng, J., Belury, M. A.,... Kiecolt-Glaser, J. K. (2014). Omega-3 supplementation and loneliness-related memory problems: secondary analyses of a randomized controlled trial. *Psychosomatic medicine*, *76*(8), 650-658.

Jeffery, I. B., O'Toole, P. W., Ohman, L., Claesson, M. J., Deane, J., Quigley, E. M., & Simren, M. (2012). An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut*, *61*(7), 997-1006.

Jeffery, I. B., Quigley, E. M., Öhman, L., Simrén, M., & O'Toole, P. W. (2012). The microbiota link to irritable bowel syndrome: an emerging story. *Gut Microbes*, *3*(6), 572-576.

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., Wang, W., Tang, W., Tan, Z., Shi, J., Li, L., & Ruan, B. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain, behavior, and immunity*, *48*, 186–194.

Julio-Pieper, M., Bravo, J. A., Aliaga, E., & Gotteland, M. (2014). Review article: intestinal barrier dysfunction and central nervous system disorders--a controversial association. *Alimentary pharmacology & therapeutics*, *40*(10), 1187–1201.

Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M, Lozupone CA, Hahn J, Adams JB, Krajmalnik-Brown R. Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. *Anaerobe* 49: 121–131, 2018.

Kankaanpää, P. E., Yang, B., Kallio, H. P., Isolauri, E., & Salminen, S. J. (2002). Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants. *The Journal of Nutritional Biochemistry*, *13*(6), 364-369.

Kennedy, P. J., Cryan, J. F., Dinan, T. G., & Clarke, G. (2014). Irritable bowel syndrome: A microbiome-gut-brain axis disorder? *World journal of gastroenterology: WJG, 20*(39), 14105.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K. R., Rush, A. J., Walters, E. E., Wang, P. S., & National Comorbidity Survey Replication (2003). The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA, 289*(23), 3095–3105.

Kim, C. S., Cha, L., Sim, M., Jung, S., Chun, W. Y., Baik, H. W., & Shin, D. M. (2021). Probiotic Supplementation Improves Cognitive Function and Mood with Changes in Gut Microbiota in Community-Dwelling Older Adults: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Trial. *The Journals of Gerontology: Series A, 76*(1), 32-40.

Knights, D., Ward, T. L., McKinlay, C. E., Miller, H., Gonzalez, A., McDonald, D., & Knight, R. (2014). Rethinking "enterotypes". *Cell host & microbe, 16*(4), 433-437.

Korpela, K., Salonen, A., Vepsäläinen, O., Suomalainen, M., Kolmeder, C., Varjosalo, M., ... & de Vos, W. M. (2018). Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome, 6*(1), 1-11.

Krikorian, R., Nash, T. A., Shidler, M. D., Shukitt-Hale, B., & Joseph, J. A. (2010). Concord grape juice supplementation improves memory function in older adults with mild cognitive impairment. *British Journal of Nutrition, 103*(05), 730-734.

Krikorian, R., Shidler, M. D., Nash, T. A., Kalt, W., Vinqvist-Tymchuk, M. R., Shukitt-Hale, B., & Joseph, J. A. (2010). Blueberry Supplementation Improves Memory in Older Adults†. *Journal of agricultural and food chemistry, 58*(7), 3996-4000.

Laparra, J. M., & Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research, 61*(3), 219-225.

Letenneur, L., Proust-Lima, C., Le Gouge, A., Dartigues, J. F., & Barberger-Gateau, P. (2007). Flavonoid intake and cognitive decline over a 10-year period. *American journal of epidemiology, 165*(12), 1364–1371.

Lew, L. C., Hor, Y. Y., Yusoff, N. A. A., Choi, S. B., Yusoff, M. S., Roslan, N. S., ... & Liong, M. T. (2019). Probiotic Lactobacillus plantarum P8 alleviated stress and anxiety while enhancing memory and cognition in stressed adults: a randomised, double-blind, placebo-controlled study. *Clinical Nutrition, 38*(5), 2053-2064.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays, 33*(8), 574-581.

MacFabe, D. F., Cain, N. E., Boon, F., Ossenkopp, K. P., & Cain, D. P. (2011). Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. *Behavioural brain research, 217*(1), 47–54.

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society, 62*(01), 67-72.

Mahmoudian Dehkordi, S., Arnold, M., Nho, K., Ahmad, S., Jia, W., Xie, G., ... & Alzheimer Disease Metabolomics Consortium. (2019). Altered bile acid profile associates with cognitive impairment in Alzheimer's disease—an emerging role for gut microbiome. *Alzheimer's & Dementia, 15*(1), 76-92.

Malkova, N. V., Yu, C. Z., Hsiao, E. Y., Moore, M. J., & Patterson, P. H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, behavior, and immunity, 26*(4), 607–616.

Masi A, DeMayo MM, Glozier N, Guastella AJ. An Overview of Autism Spectrum Disorder, Heterogeneity and Treatment Options. *Neurosci Bull* 33: 183–193, 2017.

Mayer, E., Knight, R., Mazmanian, S. K., Cryan, J. F., & Tillisch, K. (2014). Gut microbes and the brain: paradigm shift in neuroscience. *The Journal of Neuroscience, 34*(46), 15490-15496.

McKernan, D., Fitzgerald, P., Dinan, T., & Cryan, J. (2010). The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. *Neurogastroenterology & Motility, 22*(9), 1029-e1268.

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J. F., Rougeot, C., Pichelin, M., Cazaubiel, M., & Cazaubiel, J. M. (2011). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *The British journal of nutrition, 105*(5), 755–764.

Moore, R. E., & Townsend, S. D. (2019). Temporal development of the infant gut microbiome. *Open biology, 9*(9), 190128.

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linløkken, A., Wilson, R., & Rudi, K. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterology & Motility, 26*(8), 1155-1162.

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society, 23*(3), 255–e119.

O'Brien, S. M., Scott, L. V., & Dinan, T. G. (2004). Cytokines: abnormalities in major depression and implications for pharmacological treatment. *Human Psychopharmacology: clinical and experimental, 19*(6), 397-403.

O'Mahony, S. M., Felice, V. D., Nally, K., Savignac, H. M., Claesson, M. J., Scully, P.,... Cryan, J. F. (2014). Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience, 277*, 885-901.

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., & Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural Brain Research, 277*(0), 32-48.

Ogbonnaya, E. S., Clarke, G., Shanahan, F., Dinan, T. G., Cryan, J. F., & O'Leary, O. F. (2015). Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biological psychiatry, 78*(4), e7–e9.

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., & Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol, 5*(7), e177.

Pan, H., Feng, J., He, G. X., Cerniglia, C. E., & Chen, H. (2012). Evaluation of impact of exposure of Sudan azo dyes and their metabolites on human intestinal bacteria. *Anaerobe, 18*(4), 445-453.

Pase, M. P., Scholey, A. B., Pipingas, A., Kras, M., Nolidin, K., Gibbs, A.,... Stough, C. (2013). Cocoa polyphenols enhance positive mood states but not cognitive performance: a randomized, placebo-controlled trial. *Journal of Psychopharmacology, 27*(5), 451-458.

Patel, B. A. (2011). Electroanalytical approaches to study signaling mechanisms in the gastrointestinal tract. *Neurogastroenterology & Motility, 23*(7), 595-605.

Peet, M., Brind, J., Ramchand, C., Shah, S., & Vankar, G. (2001). Two double-blind placebo-controlled pilot studies of eicosapentaenoic acid in the treatment of schizophrenia. *Schizophrenia research, 49*(3), 243-251.

Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P., & Fitzgerald, G. F. (2014). Intestinal microbiota, diet and health. *The British journal of nutrition, 111*(3), 387–402.

Prenderville, J. A., Kennedy, P. J., Dinan, T. G., & Cryan, J. F. (2015). Adding fuel to the fire: the impact of stress on the ageing brain. *Trends in neurosciences*, *38*(1), 13–25.

Pusceddu, M. M., Kelly, P., Ariffin, N., Cryan, J. F., Clarke, G., & Dinan, T. G. (2015). n-3 PUFAs have beneficial effects on anxiety and cognition in female rats: Effects of early life stress. *Psychoneuroendocrinology, 58,* 79-90.

Rao, A. V., Bested, A. C., Beaulne, T. M., Katzman, M. A., Iorio, C., Berardi, J. M., & Logan, A. C. (2009). A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut Pathogens, 1*(1), 1-6.

Rechel, B., Grundy, E., Robine, J. M., Cylus, J., Mackenbach, J. P., Knai, C., & McKee, M. (2013). Ageing in the European Union. *Lancet, 381*(9874), 1312-1322.

Sampson, Timothy R., & Mazmanian, Sarkis K. (2015). Control of Brain Development, Function, and Behavior by the Microbiome. *Cell Host & Microbe, 17*(5), 565-576.

Sandler, R. H., Finegold, S. M., Bolte, E. R., Buchanan, C. P., Maxwell, A. P., Väisänen, M. L., Nelson, M. N., & Wexler, H. M. (2000). Short-term benefit from oral vancomycin treatment of regressive-onset autism. *Journal of child neurology*, *15*(7), 429–435.

Savignac, H. M., Kiely, B., Dinan, T. G., & Cryan, J. F. (2014). Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*, *26*(11), 1615–1627.

Schaffer, S., & Halliwell, B. (2012). Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations. *Genes & nutrition*, *7*(2), 99–109.

Schafer, D. P., & Stevens, B. (2015). Microglia function in central nervous system development and plasticity. *Cold Spring Harbor perspectives in biology*, *7*(10), a020545.

Schmidt, K., Cowen, P. J., Harmer, C. J., Tzortzis, G., Errington, S., & Burnet, P. W. (2015). Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. *Psychopharmacology*, *232*(10), 1793–1801.

Selma, M. V., Espin, J. C., & Tomas-Barberan, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *Journal of agricultural and food chemistry*, *57*(15), 6485-6501.

Shanahan, F., & Quigley, E. M. (2014). Manipulation of the microbiota for treatment of IBS and IBD-challenges and controversies. *Gastroenterology*, *146*(6), 1554–1563.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & pharmacotherapy, 56*(8), 365-379.

Slavin, J. (2013). Fiber and prebiotics: mechanisms and health benefits. *Nutrients, 5*(4), 1417-1435.

Smith, A. P., Sutherland, D., & Hewlett, P. (2015). An Investigation of the Acute Effects of Oligofructose-Enriched Inulin on Subjective Wellbeing, Mood and Cognitive Performance. *Nutrients, 7*(11), 8887-8896.

Sonuga-Barke, E. J., Brandeis, D., Cortese, S., Daley, D., Ferrin, M., Holtmann, M., Stevenson, J., Danckaerts, M., van der Oord, S., Döpfner, M., Dittmann, R. W., Simonoff, E., Zuddas, A., Banaschewski, T., Buitelaar, J., Coghill, D., Hollis, C., Konofal, E., Lecendreux, M., Wong, I. C., … European ADHD Guidelines Group (2013). Nonpharmacological interventions for ADHD: systematic review and meta-analyses of randomized controlled trials of dietary and psychological treatments. *The American journal of psychiatry*, *170*(3), 275–289.

Steenbergen, L., Sellaro, R., van Hemert, S., Bosch, J. A., & Colzato, L. S. (2015). A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, behavior, and immunity*, *48*, 258–264.

Stilling, R. M., Dinan, T. G., & Cryan, J. F. (2014). Microbial genes, brain & behaviour - epigenetic regulation of the gut-brain axis. *Genes, brain, and behavior*, *13*(1), 69–86.

Stilling, R. M., Ryan, F. J., Hoban, A. E., Shanahan, F., Clarke, G., Claesson, M. J., Dinan, T. G., & Cryan, J. F. (2015). Microbes & neurodevelopment--Absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. *Brain, behavior, and immunity*, *50*, 209–220.

Strauss, E., & da Costa, M. F. (1998). The importance of bacterial infections as precipating factors of chronic hepatic encephalopathy in cirrhosis. *Hepatogastroenterology, 45*(21), 900-904.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., Kubo, C., & Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *The Journal of physiology*, *558*(Pt 1), 263–275.

Tillisch, K., Labus, J., Kilpatrick, L., Jiang, Z., Stains, J., Ebrat, B.,... Mayer, E. A. (2013). Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology, 144*(7), 1394-1401, 1401 e1391-1394.

Tillisch, K., & Labus, J. S. (2011). Advances in imaging the brain-gut axis: functional gastrointestinal disorders. *Gastroenterology, 140*(2), 407-411 e401.

Valdés, L., Cuervo, A., Salazar, N., Ruas-Madiedo, P., Gueimonde, M., & González, S. (2015). The relationship between phenolic compounds from diet and microbiota: impact on human health. *Food & function, 6*(8), 2424–2439.

Wall, R., Marques, T. M., O'Sullivan, O., Ross, R. P., Shanahan, F., Quigley, E. M., Dinan, T. G., Kiely, B., Fitzgerald, G. F., Cotter, P. D., Fouhy, F., & Stanton, C. (2012). Contrasting effects of Bifidobacterium breve NCIMB 702258 and Bifidobacterium breve DPC 6330 on the composition of murine brain fatty acids and gut microbiota. *The American journal of clinical nutrition*, *95*(5), 1278–1287.

Wang, F. B., & Powley, T. L. (2007). Vagal innervation of intestines: afferent pathways mapped with new en bloc horseradish peroxidase adaptation. *Cell and tissue research*, *329*(2), 221-230.

Wang, H., Braun, C., Murphy, E. F., & Enck, P. (2019). Bifidobacterium longum 1714™ strain modulates brain activity of healthy volunteers during social stress. *The American journal of gastroenterology*, *114*(7), 1152.

Wightman, E. L., Reay, J. L., Haskell, C. F., Williamson, G., Dew, T. P., & Kennedy, D. O. (2014). Effects of resveratrol alone or in combination with piperine on cerebral blood flow parameters and cognitive performance in human subjects: a randomised, double-blind, placebo-controlled, cross-over investigation. *British Journal of Nutrition, 112*(02), 203-213.

Zhang, X. W., Hou, W. S., Li, M., & Tang, Z. Y. (2016). Omega-3 fatty acids and risk of cognitive decline in the elderly: a meta-analysis of randomized controlled trials. *Aging clinical and experimental research*, *28*(1), 165–166.

CHAPTER 3

INFLUENCE OF C-SECTION BIRTH MODE AND ANTIBIOTIC EXPOSURE ON THE INFANT GUT

CHAPTER CONTRIBUTIONS:

Amy Murphy

- planned sample processing methods and specific sample use within study design,
- collected stool samples,
- carried out DNA extraction,
- planned and prepared 16 S compositional sequencing library,
- planned and carried out culture-based work and isolated specific microbes,
- planned and carried out anaerobic culture isolation,
- planned and carried out statistical analyses,
- significantly contributed to manuscript preparation.

Bioinformatic analysis was completed by Dr. Fiona Fouhy

Abstract

Several factors can disrupt the normal colonisation of the infant gut including mode of delivery and antibiotic treatment in early life. Caesarean-section (CS) birth can significantly alter the infant gut microbiota composition compared with vaginal delivery in the early weeks of life, due to the lack of contact during birth between the new-born and maternal vaginal and intestinal microbes. This disruption is associated with lower numbers of beneficial bacteria such as *Bifidobacterium* and *Bacteroides* when compared with standard vaginally delivered infants and has been associated with an increased risk of developing several disorders later in life such as inflammatory bowel disease, asthma and type I diabetes. The use of antibiotics in early life can also have a considerable negative impact on the infant gut microbiota with compositional changes persisting for at least 1 year after treatment. Evidence suggests that these initial perturbations of the infant gut microbiota can have long-lasting effects on health such as increased rates of obesity, metabolic and neurodevelopmental disorders.

This study aimed to examine the influence of such perinatal factors on the development of the infant microbiota composition, particularly, the impact of CS birth mode coupled with antibiotic exposure following birth on the developing gut microbiota of infants over 24 weeks was assessed. After examining the gut microbiota composition of three groups of infants (infants born vaginally, infants born by CS who had received antibiotics and infants born by CS who had not received antibiotic treatment), it was found that antibiotic exposure and CS delivery had a significant impact on the infant gut, with differences noted in the relative abundances of many taxa along with changes in alpha diversity and beta diversity when compared to infants born vaginally who were not exposed to antibiotics

Introduction

The infant gut microbiota is dynamic and can be influenced by a variety of different factors (Marques et al., 2010). As previously mentioned, the infant gut is highly susceptible to compositional changes as a consequence of events which can occur before birth and in early life (Mueller et al., 2015). These include birth mode, antibiotic treatment, maternal stress or infection, gestational age and diet (Milani et al., 2017). It is of great importance to understand the initial gut microbiota colonisation pattern of healthy infants and the composition alterations which can occur due to these perinatal factors.

It is recognised that infant gut microbiota development coincides with neurodevelopmental windows (Borre et al., 2014). Development during these periods is critical as dysregulation can result in long-lasting consequences. It has also been suggested that initial early life microbiota colonisation of the infant gut can have profound influences on stress and psychological development later in life (Heijtz et al., 2011). Development of the brain in utero and into infancy is a methodical and highly regulated process involving growth of axons and dendrites, formation and remodelling of synapses and myelination. While the brain is undergoing these rapid changes in infancy, similarly the infant gut microbiota is maturing and being colonised by various microbes in a regulated sequence. As these two developmental periods are highly dynamic and fluid, there is a window where disruption can occur before the brain and gut reach a more stable, fixed state (O'Mahony et al., 2017).

It has been established that birth by CS can significantly alter the infant gut microbiota and result in long-term health consequences. Vaginally delivered infants acquire maternal vaginal microbes as they pass through the birth canal, including *Lactobacillus* and *Prevotella*. C-section delivered infants, however, do not come in

contact with the mother's vaginal microbiome, instead they are colonised by maternal skin microbes or bacteria present in the hospital environment (Penders et al., 2006). Infants delivered by CS are frequently given antibiotics prophylactically, as mothers who deliver via CS are at a higher risk for infection (Opøien et al., 2007).

Early antibiotic treatment can have a profound and long-lasting effect on the infant gut and subsequently, the development of the immune and metabolic systems (Ajslev et al., 2011). Many studies have reported that the infant gut microbiota does not recover and return to a pre-antibiotic state for months after the antibiotic treatment has concluded (Fouhy et al., 2012). Antibiotic treatment in infancy has been associated with the development of several conditions later in childhood, including asthma, allergic disease and obesity (Mueller et al., 2015).

Early microbiota perturbations due to CS delivery and antibiotic treatment interrupt the co-maturation and crosstalk between the brain and gut, which can lead to significant long-term psychological consequences (Yang et al., 2016). While it has been shown that CS delivery and repeated early antibiotic exposure result in significant infant gut microbiota perturbations, with such substantial gut microbiota alterations, it is often difficult to isolate the effects of CS and antibiotics alone. To address this, samples collected from infants delivered by CS who received antibiotic treatment (CS+ABX) were compared with infants born by CS who had not received antibiotic treatment (CS), as well as infants born vaginally (SVD). This allowed for examination of the effects of CS alone when compared with infants delivered by CS and treated with antibiotics. It was found that both of these factors had a significant effect on infant gut microbiota maturation, when compared with vaginally delivered infants It was also noted that several specific differences occurred between the

antibiotic-treated and non-treated CS groups, which suggests that many of these changes were driven by antibiotic exposure.

Materials and Methods

Participants and sample collection

Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Infants born by C-section and treated with antibiotics for the first four days of life were recruited between January 2015 and January 2018 from Cork University Maternity Hospital (CUMH). Additional information was collected including feeding type, antibiotic treatment, weight, head circumference and incidence of illness. Faecal samples were collected from infants at 1, 4, 8 and 24 weeks of life. Fresh faecal samples were collected and stored at 4°C and transported in temperature-controlled collection bags to the lab for processing.

The gut microbiota of these infants was compared with a subset of infants who were born vaginally, and a subset of infants born by CS who had not received antibiotic treatment in the first four days of life from the INFANTMET study (Hill et al., 2017). DNA samples collected from these infants at Week 1, 4, 8 and 24 were prepared for 16 S MiSeq sequencing with the CS+ABX cohort. The faecal samples from the CS and SVD cohort have previously been sequenced, however, as this analysis was performed in a different laboratory and using different library preparation steps and primers, these infant samples were re-sequenced with the CS+ABX cohort to maintain consistency. Culture-based analysis was also carried out following the protocols used in the previous cohort to allow for comparison.

Culture-Dependent Analysis

Fresh faecal samples were weighed and serially diluted in maximum recovery diluent (Fluka, Sigma Aldrich, Ireland) from 10^{-1} to 10^{-8}. Bifidobacteria were enumerated by spread-plating serial dilutions onto de Man, Rogosa, Sharpe (MRS) agar (Difco, Becton-Dickenson Ltd., Ireland), which had been modified by adding 0.05% L-cysteine hydrochloride (Sigma Aldrich, Ireland), 100 ug/ml mupirocin (Sigma Aldrich, Ireland) and 50 units of nystatin (Sigma Aldrich, Ireland). Agar plates were incubated anaerobically for three days at 37°C. *Lactobacillus* selective (LBS) agar (Difco, Becton-Dickenson Ltd., Ireland), supplemented with 50 units of nystatin was used to enumerate lactobacilli. Agar plates were incubated anaerobically for five days at 37°C. Total anaerobic bacteria were enumerated by spread-plating onto Wilkins Chalgren agar (WCA) (Sigma Aldrich, Ireland) supplemented with 50 units of nystatin and 7% defibrinated horse blood (Cruinn Diagnostics Ltd., Ireland). Agar plates were then incubated anaerobically for five days at 37°C. Brain Heart Infusion (BHI) agar supplemented with 50 units of nystatin was used to enumerate total aerobic bacteria. These were also incubated anaerobically for five days at 37°C.

DNA Extraction

DNA was extracted from adult faecal samples using the RBB method (Yu & Morrison, 2004). A 0.2 g faecal sample was weighed and added to 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm diameter sterile zirconia beads plus a single 2.5 mm diameter bead (BioSpec Products, Bartlesville, USA). To this, 1 ml of lysis buffer was added (500 mM NaCl, 50 mM tris-HCL, pH 8.0, 50 mM EDTA and 4% sodium dodecyl sulphate (SDS)). Each sample was then homogenised using a Mini-Beadbeater™, BioSpec Products, Bartlesville, OK, USA) at maximum speed for 3 min and incubated at 70° C for 15

mins to lyse the cells. Samples were then centrifuged for 5 min at 16,000 x g and the supernatant was transferred to a fresh Eppendorf tube. The bead beating, heating and centrifugation steps were repeated using 300 µl of lysis buffer and the supernatant was pooled. Following this, 260 µl of 7.5 M ammonium acetate was added and the samples were vortexed and incubated on ice for 5 min. Isopropanol was added to precipitate the DNA and samples were centrifuged to pellet the nucleic acid. The pellets were then washed with 70% ethanol and allowed to dry before being dissolved in 100 µl TE buffer. The DNA was treated with RNAse and Proteinase K and washed with Qiagen buffers AW1 and AW2 using columns provided in the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK).

The DNA was then eluted in 200 µl Buffer ATE. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland).

16S Compositional Sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

The protocol involved two PCR reactions on the extracted DNA. The DNA was first amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

Reverse primer

5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT CTAATCC).

Each reaction contained 2.5 µl genomic DNA, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following programme: 95°C x 3 min, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, 72°C x 5 min and held at 4°C. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland). Following this, a second PCR reaction was performed on the purified DNA using two indexing primers per sample (Illumina Nextera XT indexing primers, Illumina, Netherlands). Each reaction contained 5 µl purified DNA, 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix and 10µl PCR grade water. PCR amplification was completed using the previous programme but with only 8 amplification cycles instead of 25. PCR products were visualised and purified as described above. Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Teagasc using standard Illumina sequencing protocols.

Microbiome Statistical Analysis

All data were analysed and graphed using either R-3.3.1, Microsoft Excel or GraphPad Prism (Version 5). Data are expressed as means ± standard error of the mean

(SEM). Statistical analysis was carried out using the Kruskal-Wallis test or the Wilcoxon signed-rank test for paired data to identify significant differences between the groups. Differences were considered significant at $p < 0.05$. Correction of *p*-values was performed using the Benjamini-Hochberg method (FDR <0.05). The vegan package was used for Bray-Curtis based MDS analysis, and the Adonis function in vegan was used for PERMANOVA in beta diversity.

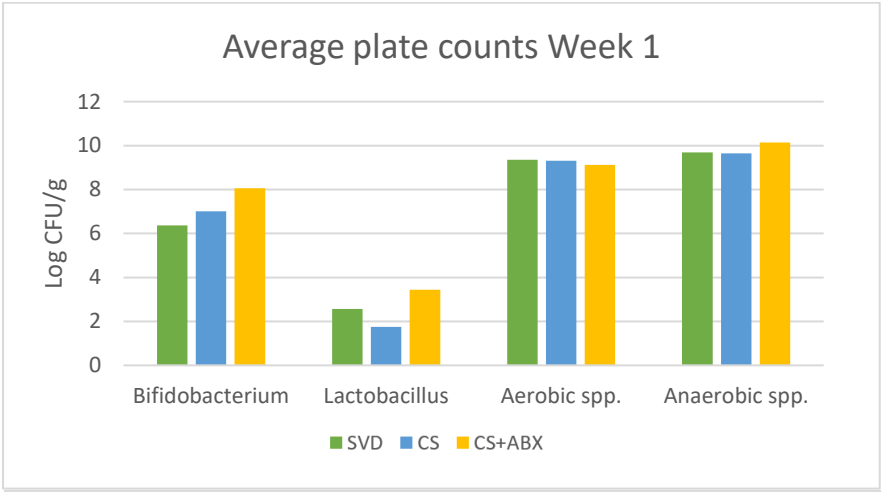
Bioinformatic Analysis

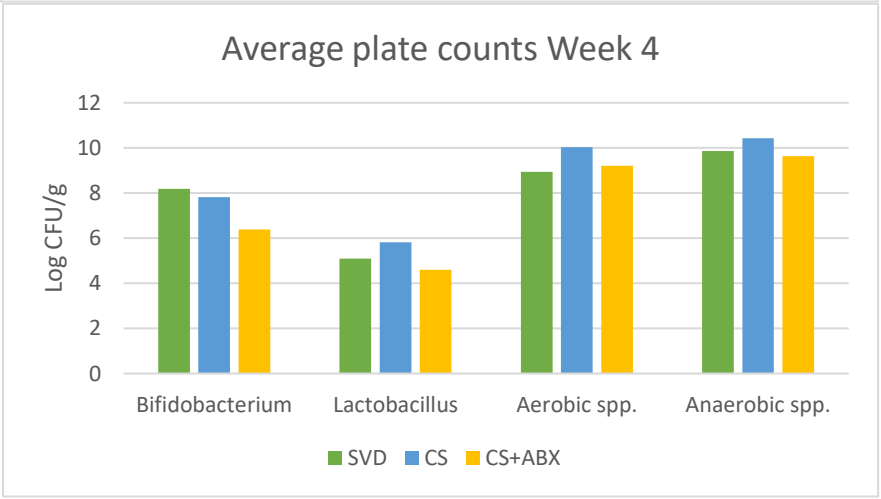
In brief, the 16S rRNA gene amplicon sequences were processed through a bioinformatics pipeline. Resulting 300 bp paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies; (Magoč & Salzberg, 2011)). Further sequence read processing was performed using QIIME (Version 1.8.0.) including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds (QIIME allows analysis of high-throughput community sequencing data). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH (Version 7, 64-bit, search and clustering orders of magnitude faster than BLAST; (Edgar, 2010). OTU sequences were aligned using PyNAST (PyNAST: python nearest alignment space termination; (Caporaso et al., 2009), a flexible tool for aligning sequences to a template alignment) and taxonomy was determined using the SILVA SSURef database release 111 (Quast et al., 2012), at 97% similarity. Alpha diversity estimates were calculated using QIIME. Beta diversity was calculated using Bray-Curtis based multidimensional scaling (MDS) analysis of faecal microbiota.

Results

Culture Dependent Analysis

Stool samples from infants at each time-point were diluted in MRD and plated onto four different types of media. Following incubation, plates were removed, and colonies were counted. Statistical analyses were not performed on these samples as the number of infants in each group was too small.





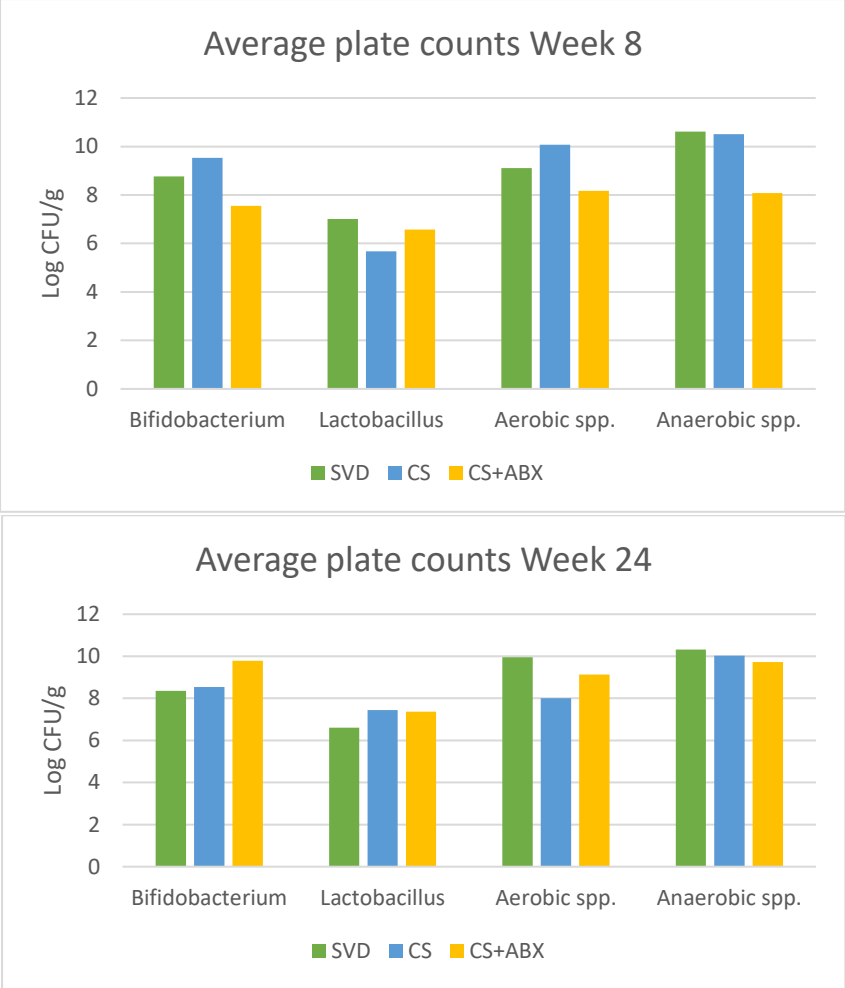


Figure 3.1

Enumeration of Bifidobacterium, Lactobacillus, total aerobes and total anaerobes at Week 1, 4, 8 and 24. Results are presented as Log CFU/g.

16S Compositional Sequencing

Following 16 S sequencing, there were differences seen between the groups at each taxonomic level, throughout the time-points (see Table 3.1 for overview of significant differences). It was seen when comparing the gut microbiota of the three groups (SVD: Standard Vaginally Delivered, CS: C-section and CS+ABX: C-section plus antibiotic treatment) during Week 1 at the phylum level, Bacteroidetes differed significantly between the infants delivered by CS and the infants who were born via

SVD (p=0.005), and, to a lesser extent, between the infants delivered by CS and those also delivered by CS but who received antibiotics in the first four days of life (p=0.02). Relative abundance of Bacteroidetes was seen to be 11% in SVD infants, but less than 1% in both CS groups (Figure 3.2 A). Additionally, at Week 1, there was a higher relative abundance of Verrucomicrobia present in the CS+ABX group when compared to the CS group (p=0.04) (Figure 3.2 B). By Week 4, these differences were no longer apparent. However, there was a difference in relative abundance of Firmicutes at Week 4 between the SVD group and both CS groups (Figure 3.2 C). At Week 8, there were no differences at phylum level. One significantly different phylum was noted at Week 24 between the SVD infants and the CS+ABX with higher levels of Actinobacteria in the CS+ABX infants (p=0.009) (Figure 3.2 D).

At family level, during Week 1, significant differences in Corynebacteriales were seen between SVD infants and CS+ABX (Figure 3.3 A). This difference was also noted at Week 4, with an additional significant difference in relative abundance between SVD and CS infants (Figure 3.4 A). Bacteroidales levels differed at Week 1 between SVD infants and both CS groups (Figure 3.3 B). This difference was seen again at Week 4, however, only between the SVD and CS+ABX group (Figure 3.4 B). These differences in Corynebacteriales and Bacteroidales did not occur beyond the Week 4 time-point. Flavobacteriales differed significantly between CS and CS+ABX at Week 1 and persisted to the Week 24 time-point (Figures 3.3 C, 3.4 C, 3.5 B and 3.6 B). Additional differences were noted at Week 1 for Bacillales, Erysipelotrichales, Selenomonadales, Rhizobiales and Verrucomicrobiales, all of which were resolved by Week 4 (Figure 3.3 D, E, F, H and K). Caulobacterales differed at Week 1 and Week 4 between SVD and CS+ABX (Figures 3.3 G and 3.4 D). This difference was absent at Week 8 but interestingly had returned by Week 24 (Figure 3.6 D). Significant

differences were noted for Burkholderiales and Pseudomonadales at Week 1 between SVD and both CS groups (Figure 3.3 I and J). Pseudomonadales was no longer significant after Week 8, however the differences in the relative abundance of Burkholderiales was still seen at Week 24. Other family level differences included Propionibacteriales which only differed at Week 4 between CS and CS+ABX. Additional family differences emerged later including Lactobacillales which began to differ at Week 8 and continued to Week 24. Bifidobacteriales and Pasteurellales only showed significant differences at the Week 24 time-point (Figure 3.6 A and J).

At the genus level, many differences in genera were seen between groups throughout the first 24 weeks of life. At Week 1, differences were noted which did not persist to Week 4 in the following genera: *Leucobacter, Staphyloccus, Enterococcus, Eubacterium, Dorea, Lachnospira, Eubacterium.coprostanoligenes.group, Proteus, Pseudomonas* and *Akkermansia* (Figure 3.7). There were changes in relative abundance of additional genera between the groups which were also seen to continue to the Week 4 time-point, including *Rhodococcus, Flavobacterium, Dolosigranulum, Pseudomonas* and a genus of the Phyllobacteriaceae family (Figure 3.8). Only three genera were significantly different at three of the four time-points sampled: *Bacteroides, Burkholderia.Paraburkholderia* and *Veillonella* with just *Chryseobacterium* noted to be consistently significantly higher in the CS+ABX group at all four time-points.

When examining the overall average phylum level relative abundance between the groups from Week 1 to Week 24 (Figure 3.11), three main phyla dominate throughout the time-points (Proteobacteria, Firmicutes and Actinobacteria). Bacteroides is present at between 10-20% relative abundance in the SVD infants, increasing in prevalence with infant age. Both CS groups do not show a relative

abundance of Bacteroides higher than 7% at any time-point. At family level (Figure 3.12), the dominant taxa are Bifidobacteriales, Lactobacillales, Clostridiales, Enterobacteriales, and to a lesser extent Bacteroidales, Bacillales, Erysipelotrichales, and Selenomonadales. The differences in abundances of many of these taxa are resolved by Week 24 where the two CS groups resemble that of the infants born vaginally. This is also seen at genus level. At Week 1, 19 taxa are significantly different between the groups, while at Week 24, many of these initial differences have resolved, however changes do persist 9 genera (Figure 3.13).

Alpha diversity, including species richness, evenness, diversity and observed species, was examined between groups at each time-point (Figure 3.14). At Week 1, it was noted that there was a difference in the Chao 1 index between infants born by C-section (CS) and those born by C-section and treated with antibiotics (CS+ABX) ($p=0.01$). This difference in Chao 1 was also statistically significant at Week 1 between the CS group and the SVD group ($p=0.01$). Similarly, changes between these groups were also seen with respect to observed species at Week 1, with CS infants showing lower observed species than both the CS+ABX and SVD groups. A significant difference in phylogenetic diversity between the infants delivered by CS and those delivered by SVD was also seen at Week 1, again with the CS infants showing reduced diversity. This reduction in phylogenetic diversity also continued to Week 4 between the CS and SVD groups. No other statistically significant differences were noted between the groups at Week 1, 4, 8 or 24 for any additional alpha diversity measures.

When examining beta diversity, which was calculated using Bray-Curtis based multidimensional scaling (MDS), it was noted that at each time-point, the samples clustered based on mode of delivery (Figure 3.15).

Table 3.1: Significantly different taxa at each time-point between CS, CS+ABX and SVD infants

Phylum	Week 1	Week 4	Week 8	Week 24
Bacteroidetes	✓			
Verrucomicrobia	✓			
Firmicutes		✓		
Actinobacteria				✓

Family	Week 1	Week 4	Week 8	Week 24
Corynebacteriales	✓	✓		
Bacteroidales	✓	✓		
Flavobacteriales	✓	✓	✓	✓
Bacillales	✓			
Erysipelotrichales	✓			
Selenomonadales	✓			
Caulobacterales	✓	✓		✓
Rhizobiales	✓			
Burkholderiales	✓	✓	✓	✓
Pseudomonadales	✓	✓	✓	
Verrucomicrobiales	✓			
Desulfovibrionales		✓		
Propionibacteriales			✓	
Lactobacillales			✓	✓
Bifidobacteriales				✓
Pasteurellales				✓

Genus	Week 1	Week 4	Week 8	Week 24
Rhodococcus	✓	✓		
Leucobacter	✓			
Bacteroides	✓	✓		✓
Chryseobacterium	✓	✓	✓	✓
Flavobacterium	✓	✓		
Staphylococcus	✓			
Dolosigranulum	✓	✓		
Enterococcus	✓			

Eubacterium	✓			
Dorea	✓			
Lachnospira	✓			
Eubacterium.coprostanoligenes group	✓			
Veillonella	✓		✓	✓
Phyllobacteriaceae.Other	✓	✓		
Burkholderia.Paraburkholderia	✓	✓		✓
Proteus	✓			
Acinetobacter	✓			
Pseudomonas	✓	✓		
Akkermansia	✓			
Lactobacillus		✓		
Hungatella		✓		
Butyricicoccus		✓		
Ruminiclostridium.5		✓		
Ruminococcaceae uncultured		✓		✓
Caulobacter		✓		✓
Gemella		✓		
Corynebacterium.1		✓		
Desulfovibrionaceae.Other		✓		
Enterobacteriaceae.Other		✓	✓	
Roseburia			✓	
Escherichia.Shigella			✓	
Klebsiella			✓	
Porphyromonas			✓	
Acinetobacter			✓	
Bifidobacterium				✓
Bacteroides				✓
Alistipes				✓
Dialister				✓
Haemophilus				✓

Table 3.1

Significantly different taxa noted at each time-point at phylum, family and genus levels. Green ticks signify a statistically significant difference in taxa between the groups.

Statistically significant taxa across taxonomic levels and timepoints

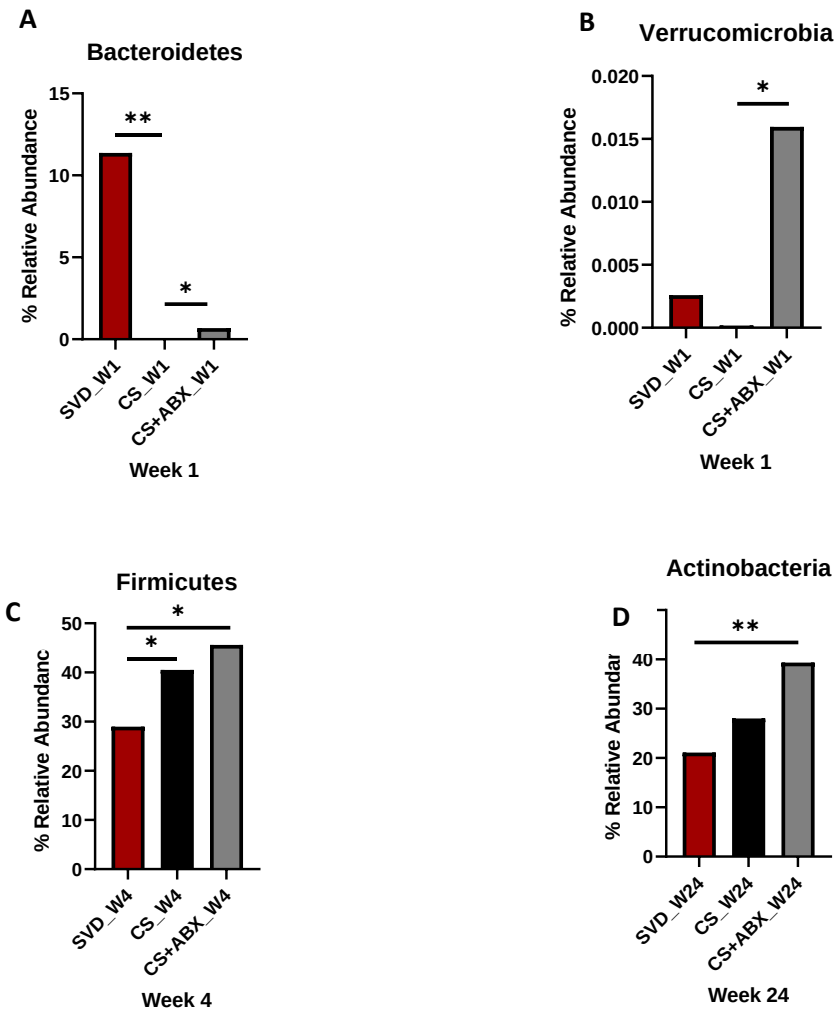


Figure 3.2

Significantly different phyla at Week 1 (A and B), Week 4 (C) and Week 24 (D). No differences were noted between phyla present at Week 8.

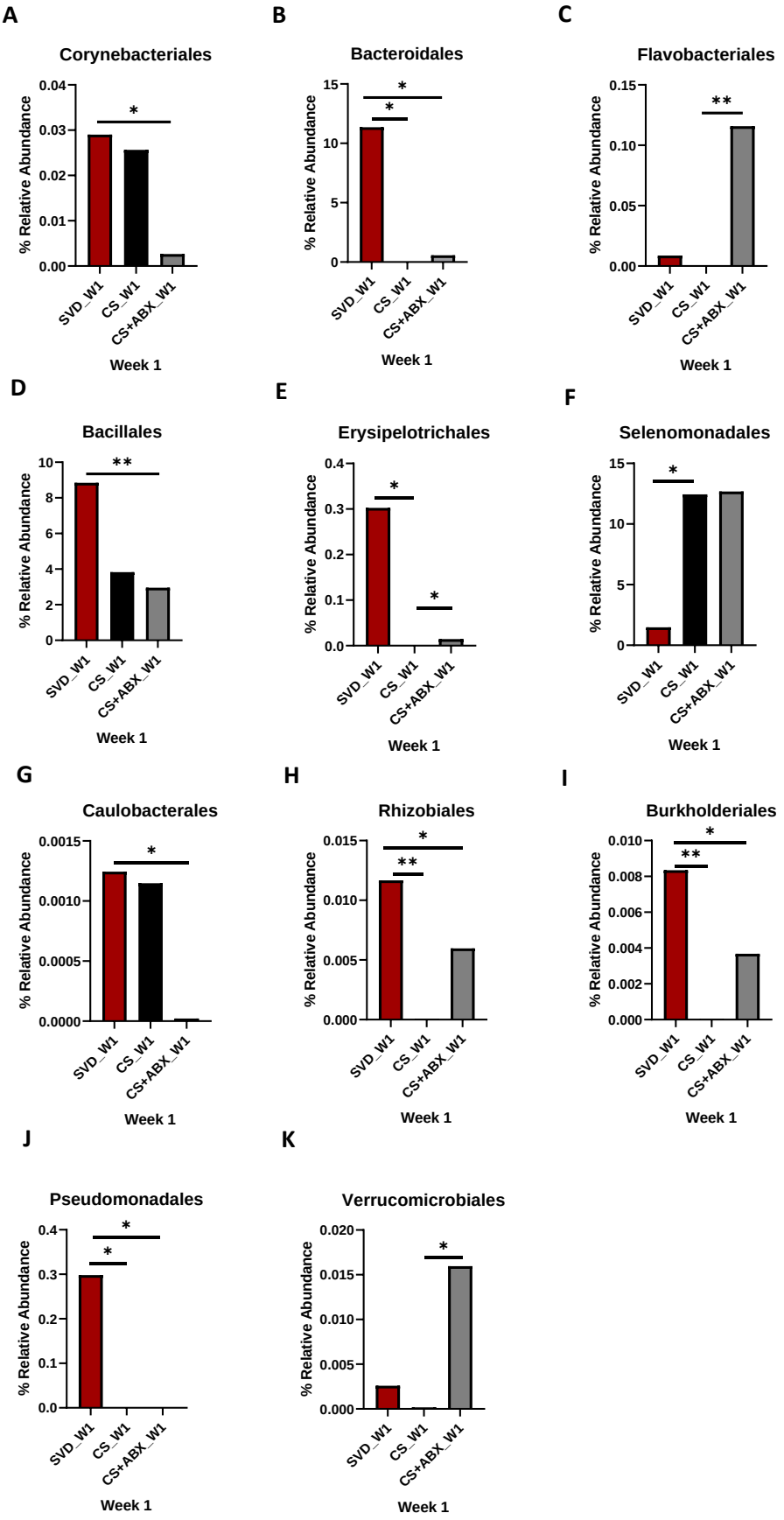


Figure 3.3 Significantly different taxa at family level at Week 1

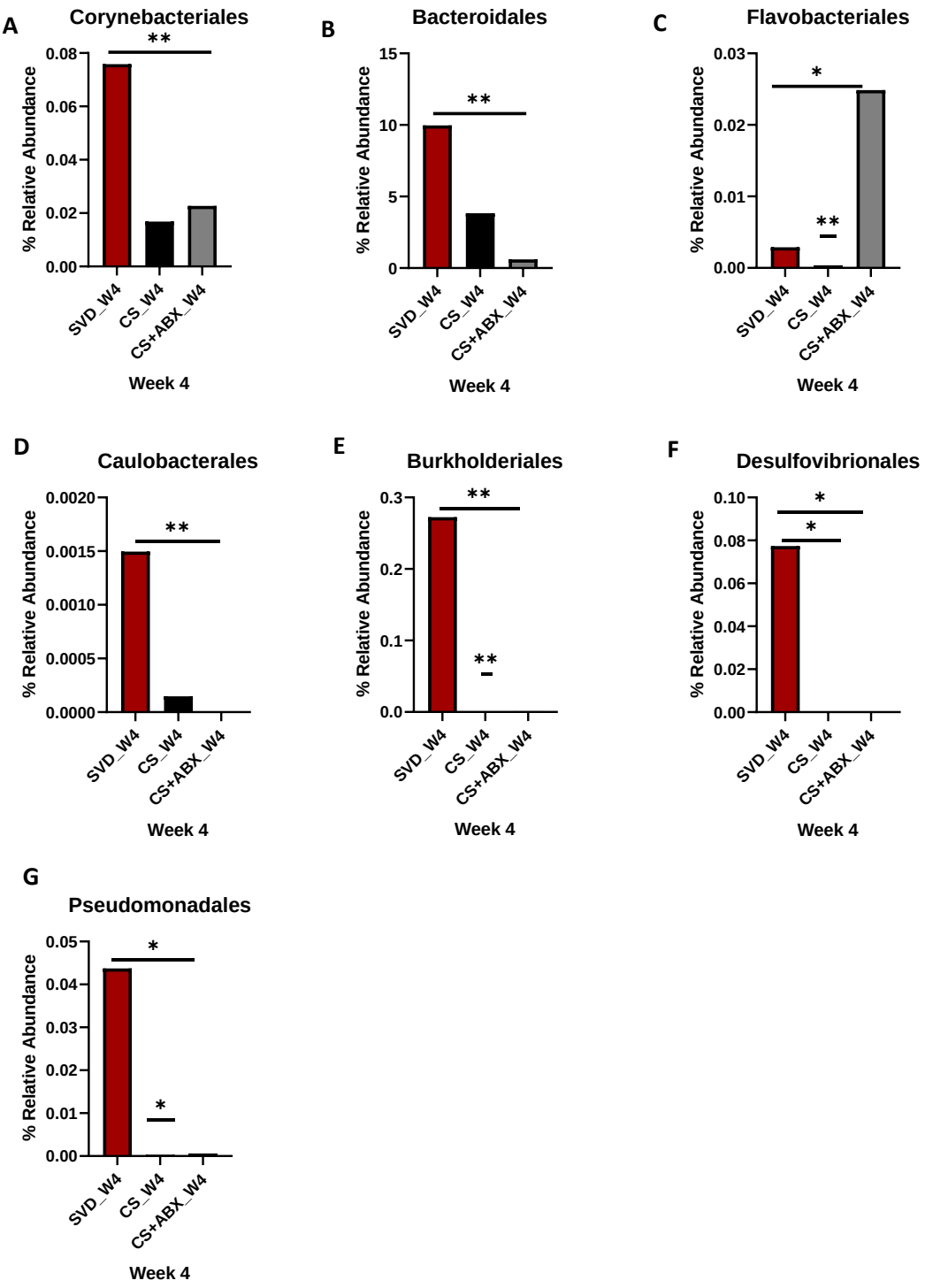


Figure 3.4. Significantly different taxa at family level at Week 4

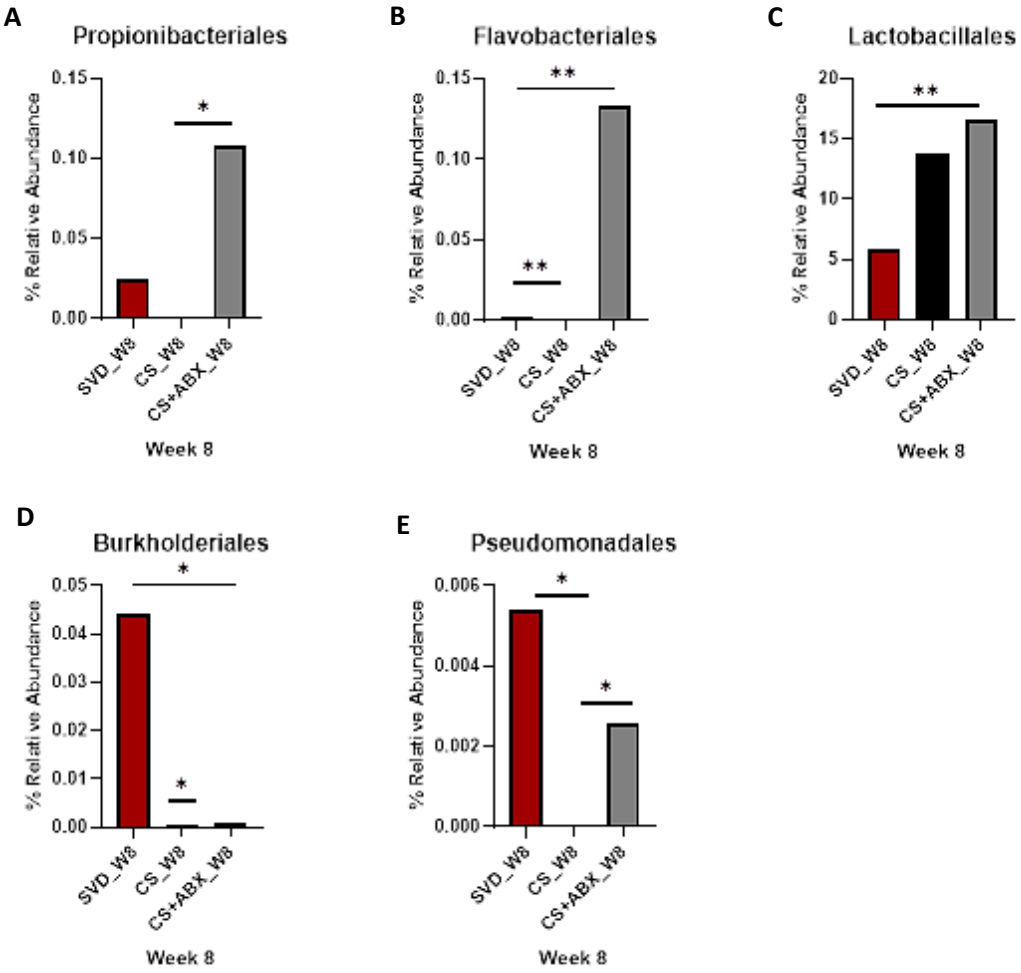


Figure 3.5. Significantly different taxa at family level at Week 8

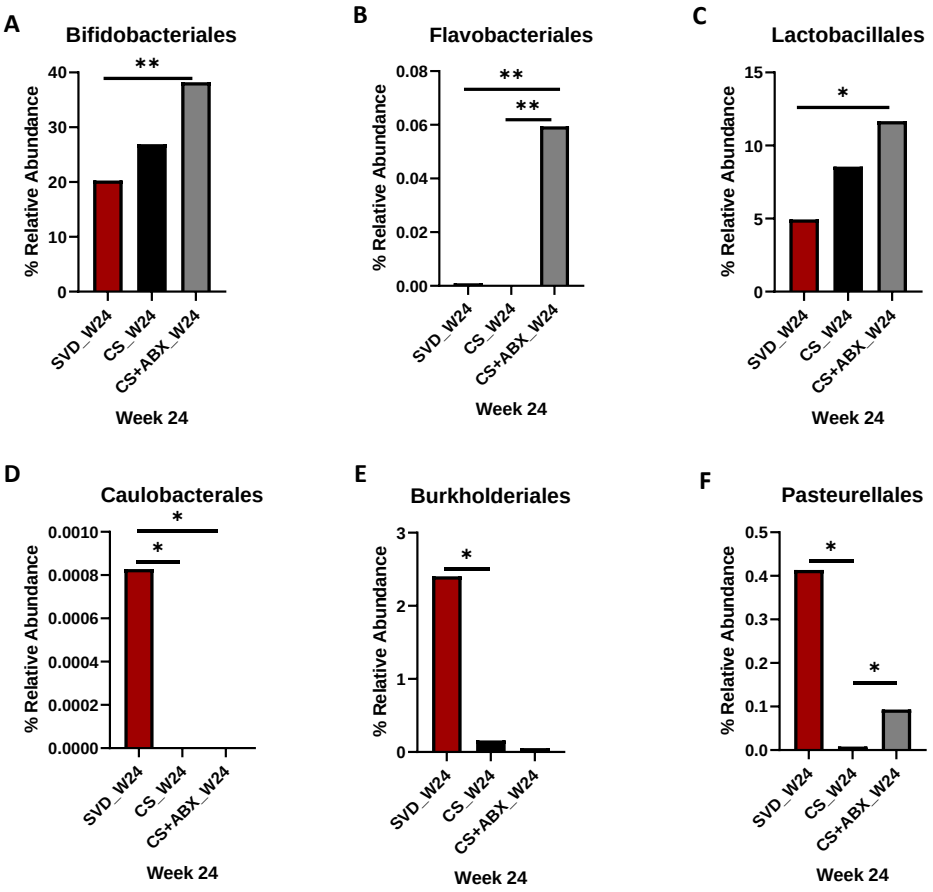
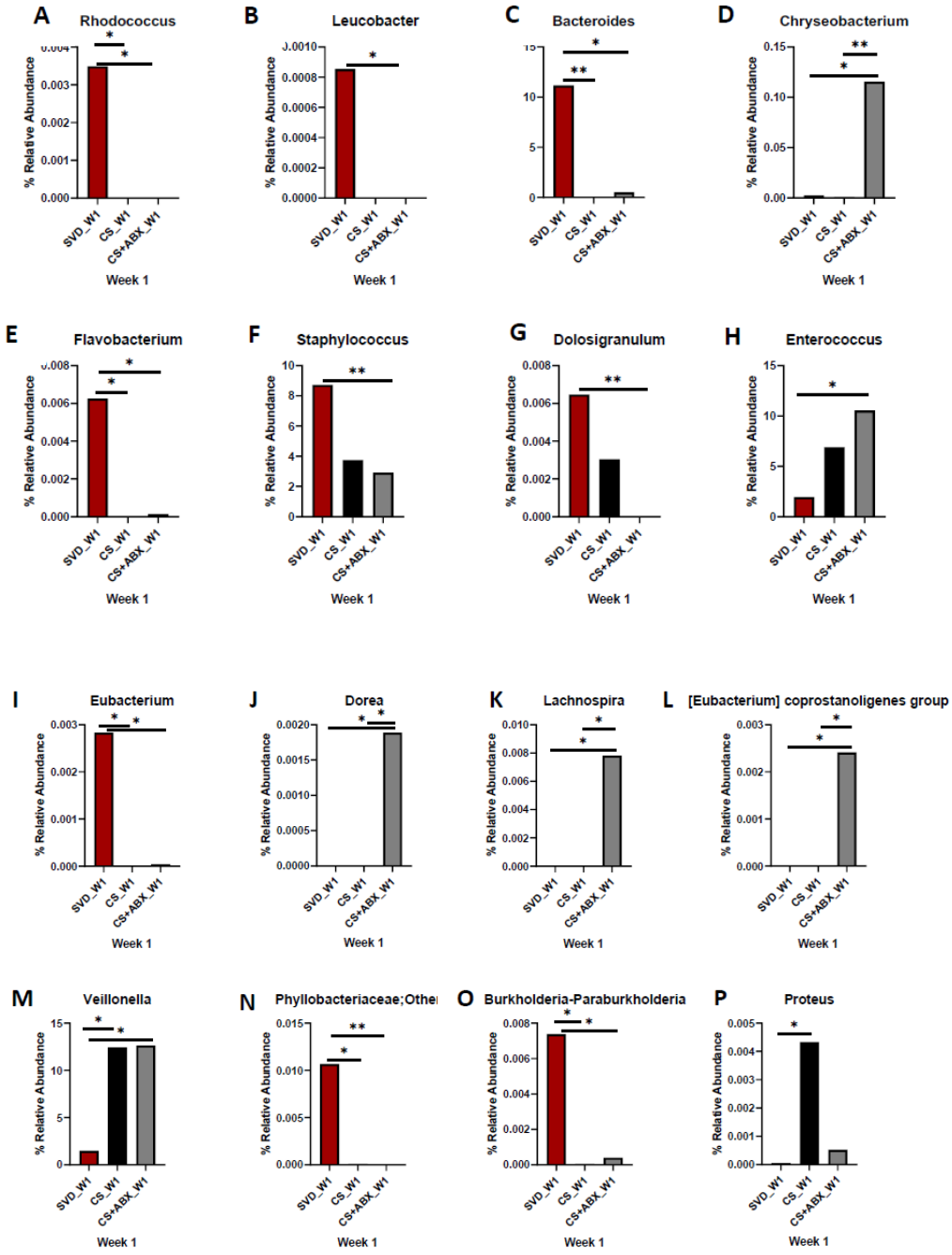


Figure 3.6. Significantly different taxa at family level at Week 24


A
Rhodococcus
B
Leucobacter
C
Bacteroides
D
Chryseobacterium
E
Flavobacterium
F
Staphylococcus
G
Dolosigranulum
H
Enterococcus
I
Eubacterium
J
Dorea
K
Lachnospira
L
[Eubacterium] coprostanoligenes group
M
Veillonella
N
Phyllobacteriaceae;Othe
O
Burkholderia-Paraburkholderia
P
Proteus
% Relative Abundance
SVD_W1
CS_W1
CS+ABX_W1
Week 1

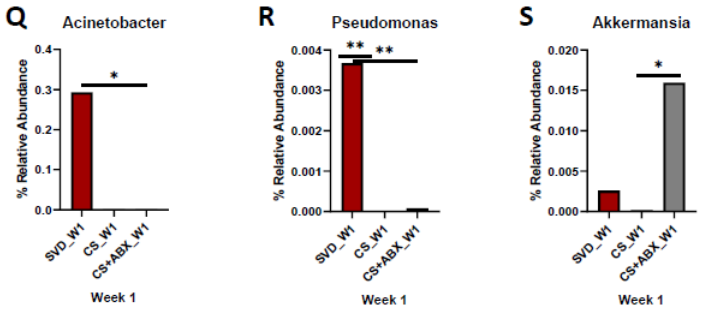


Figure 3.7 Significantly different taxa at genus level at Week 1

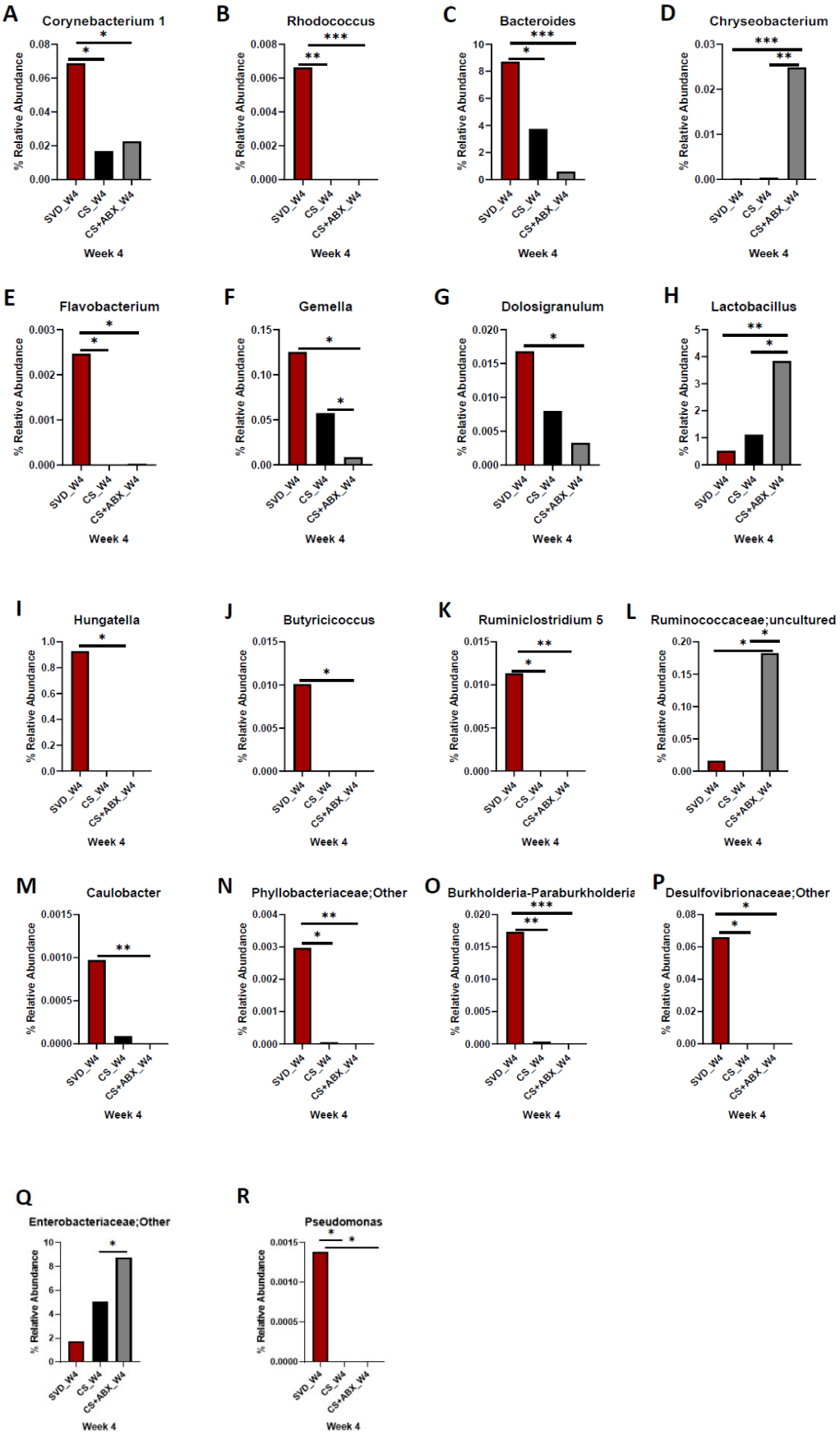


Figure 3.8. Significantly different taxa at genus level at Week 4

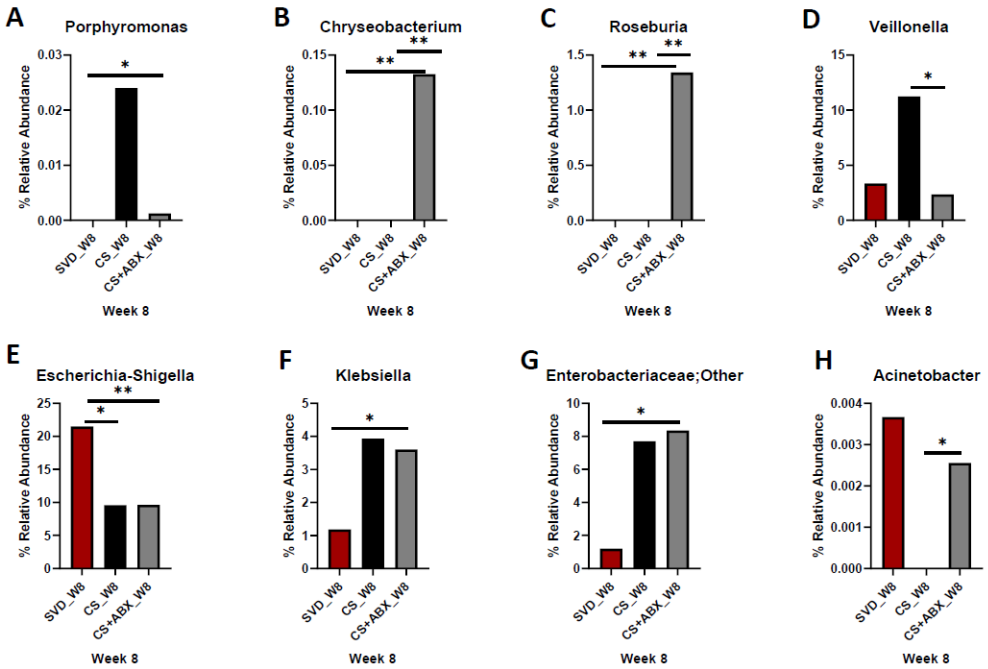


Figure 3.9. Significantly different taxa at genus level at Week 8

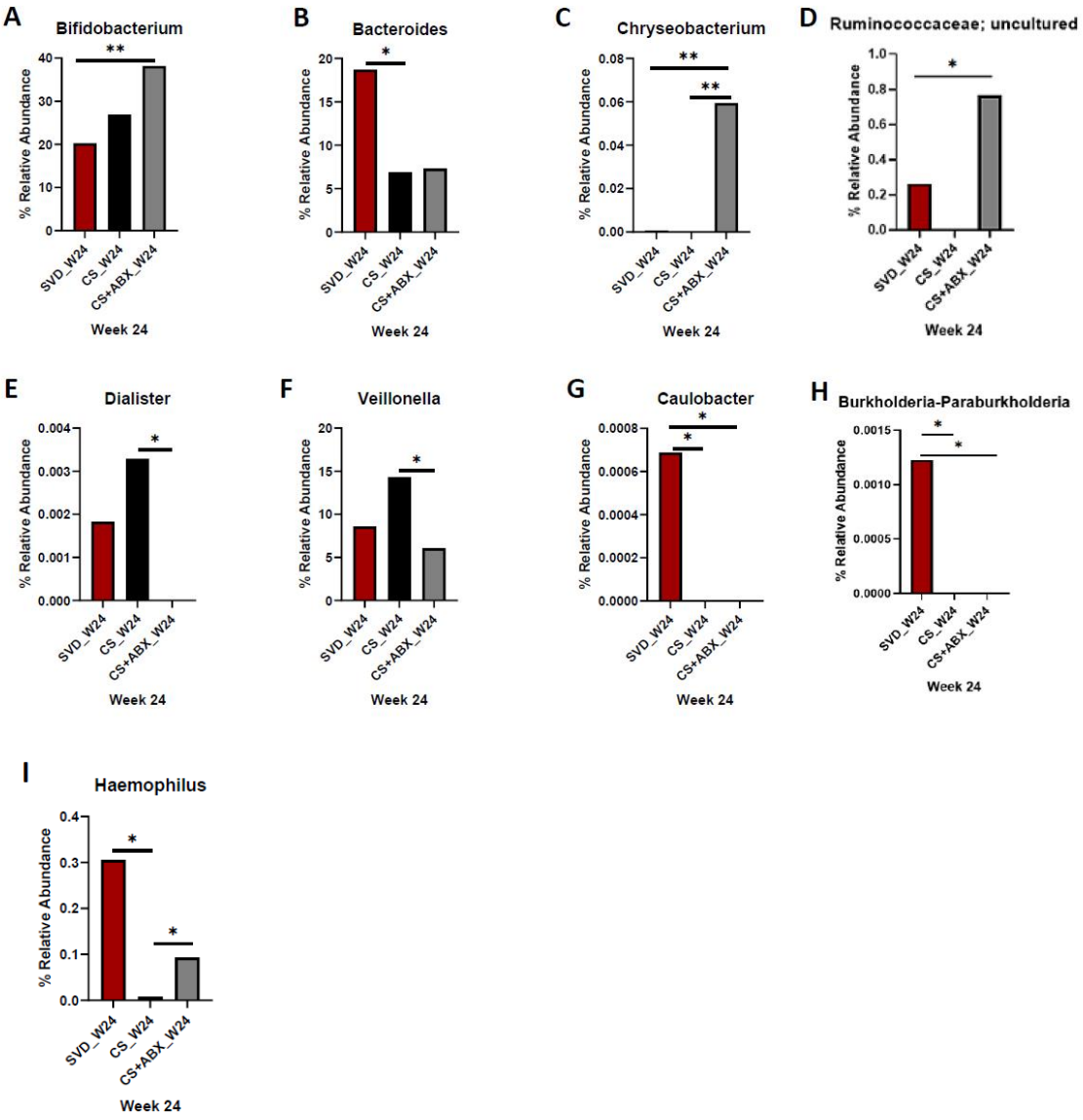


Figure 3.10. Significantly different taxa at genus level at Week 24

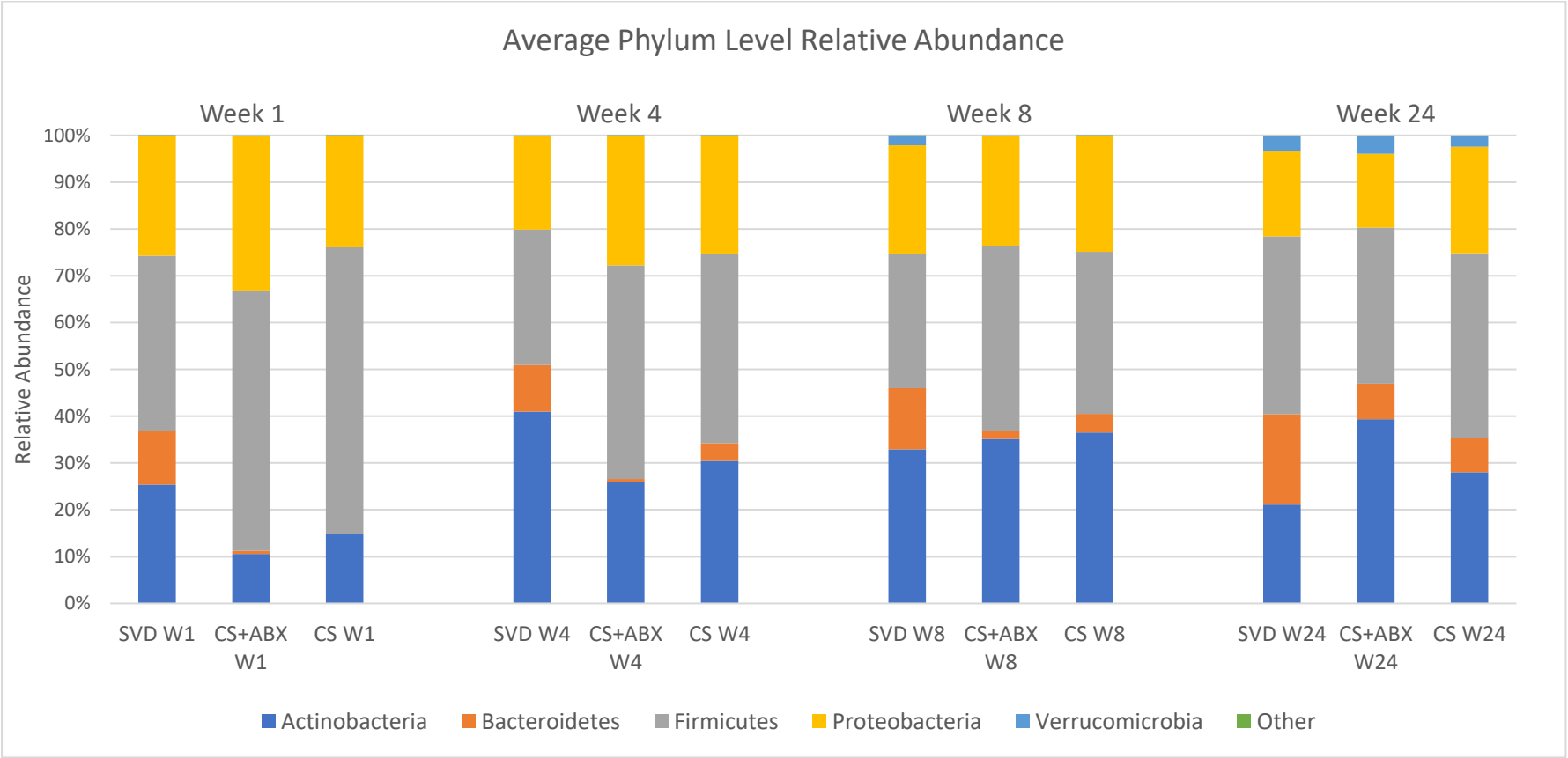


Figure 3.11. Average relative abundance at phylum level

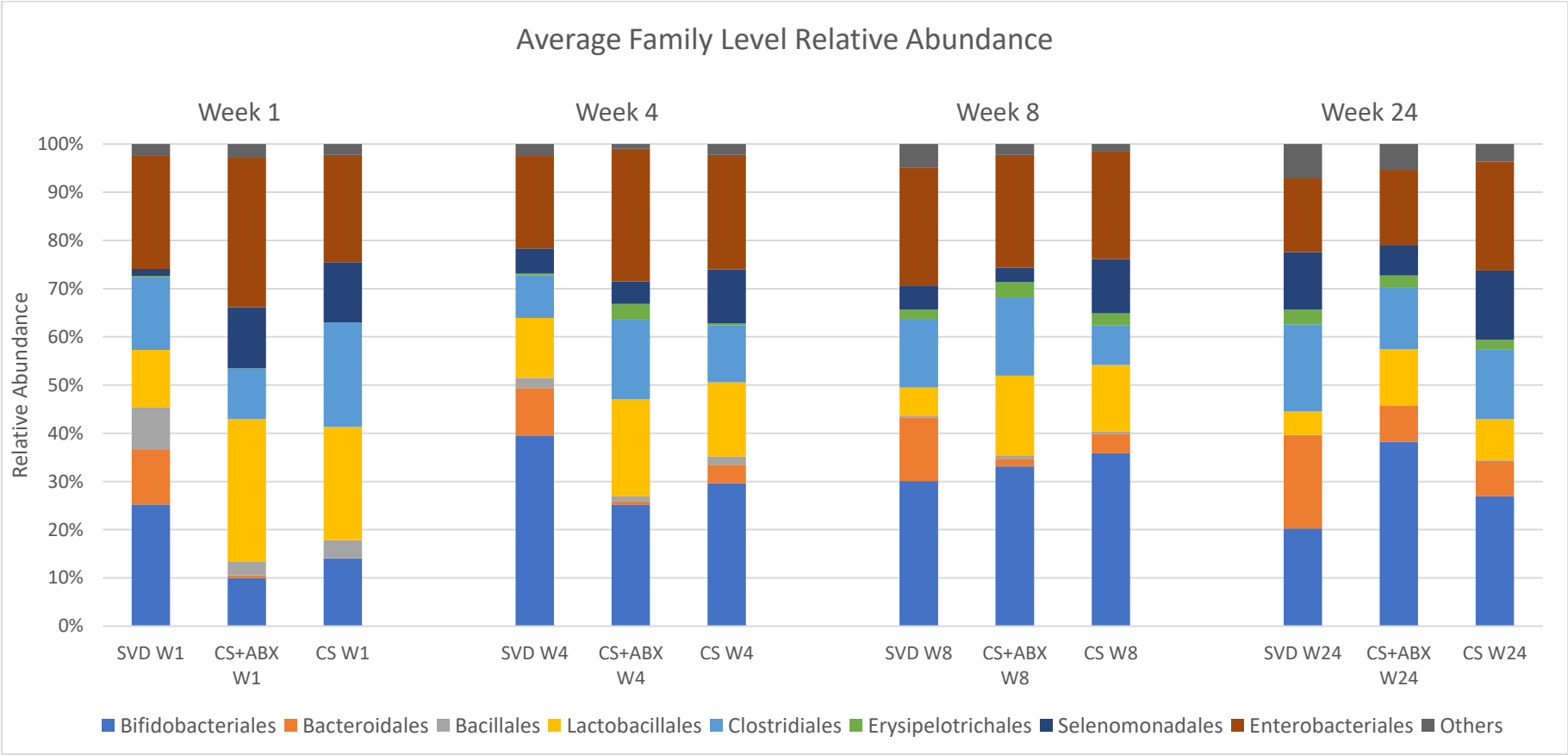


Figure 3.12 Average relative abundance at family level

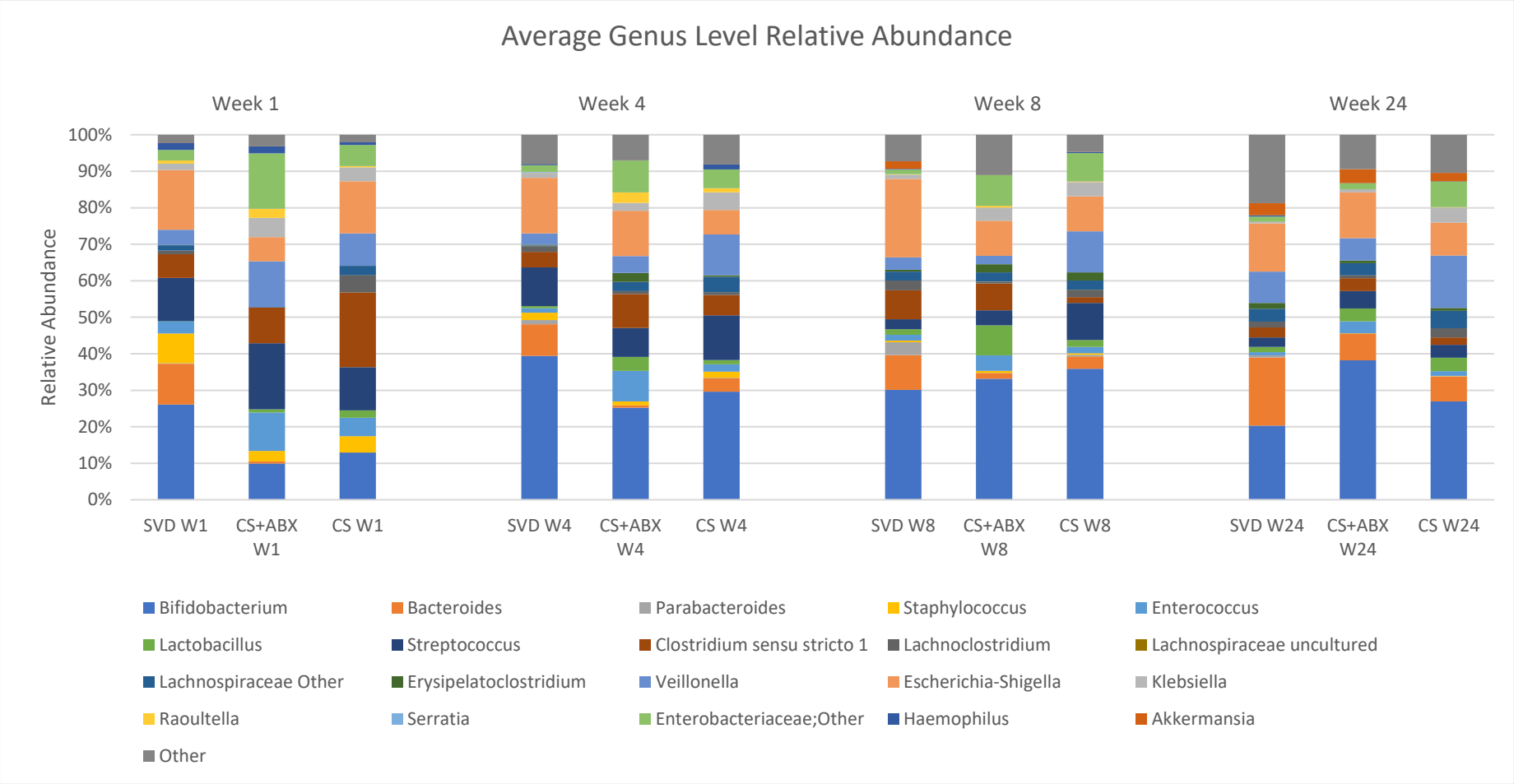


Figure 3.13 Average relative abundance at genus level

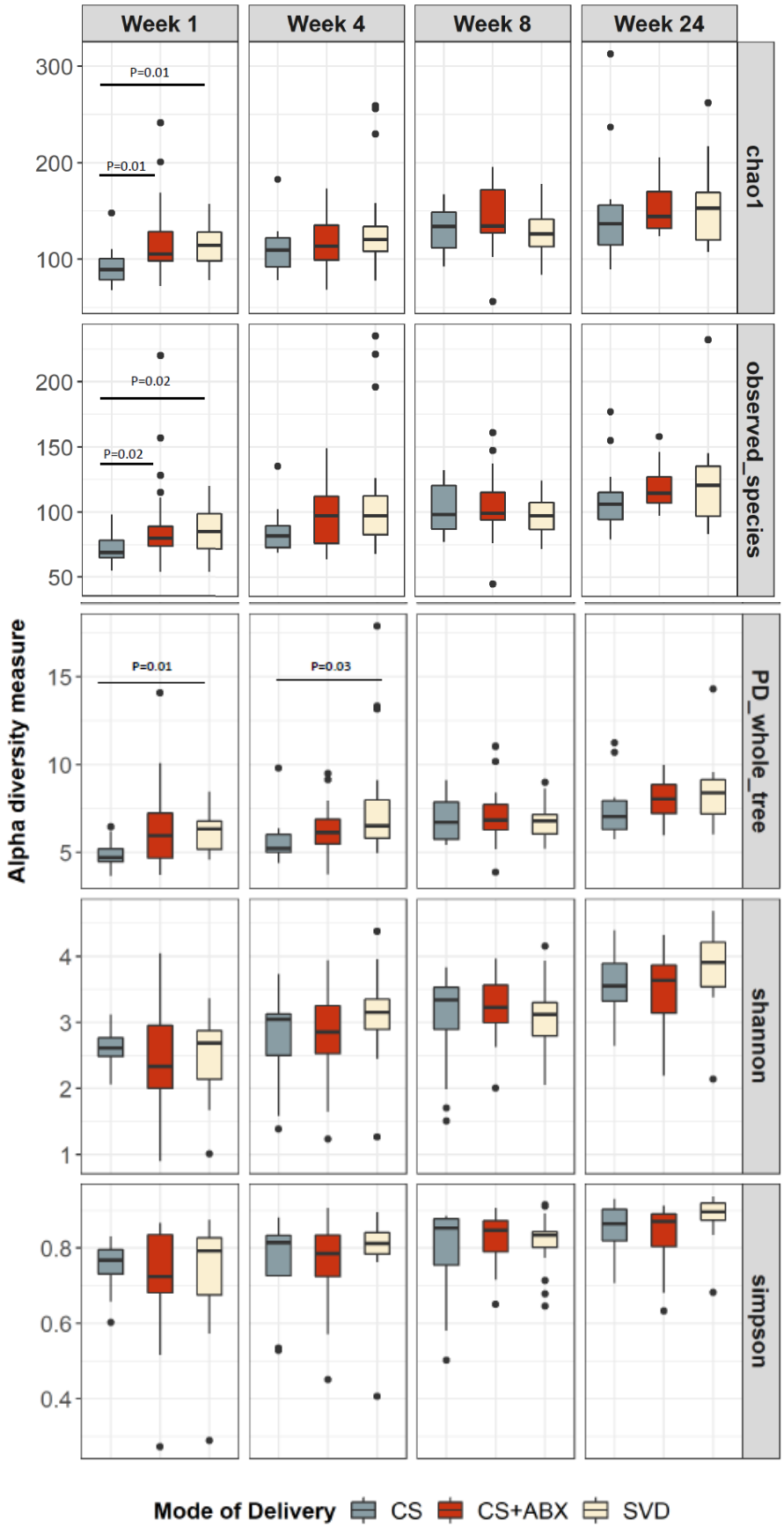


Figure 3.14 Alpha diversity between the groups throughout the time-points

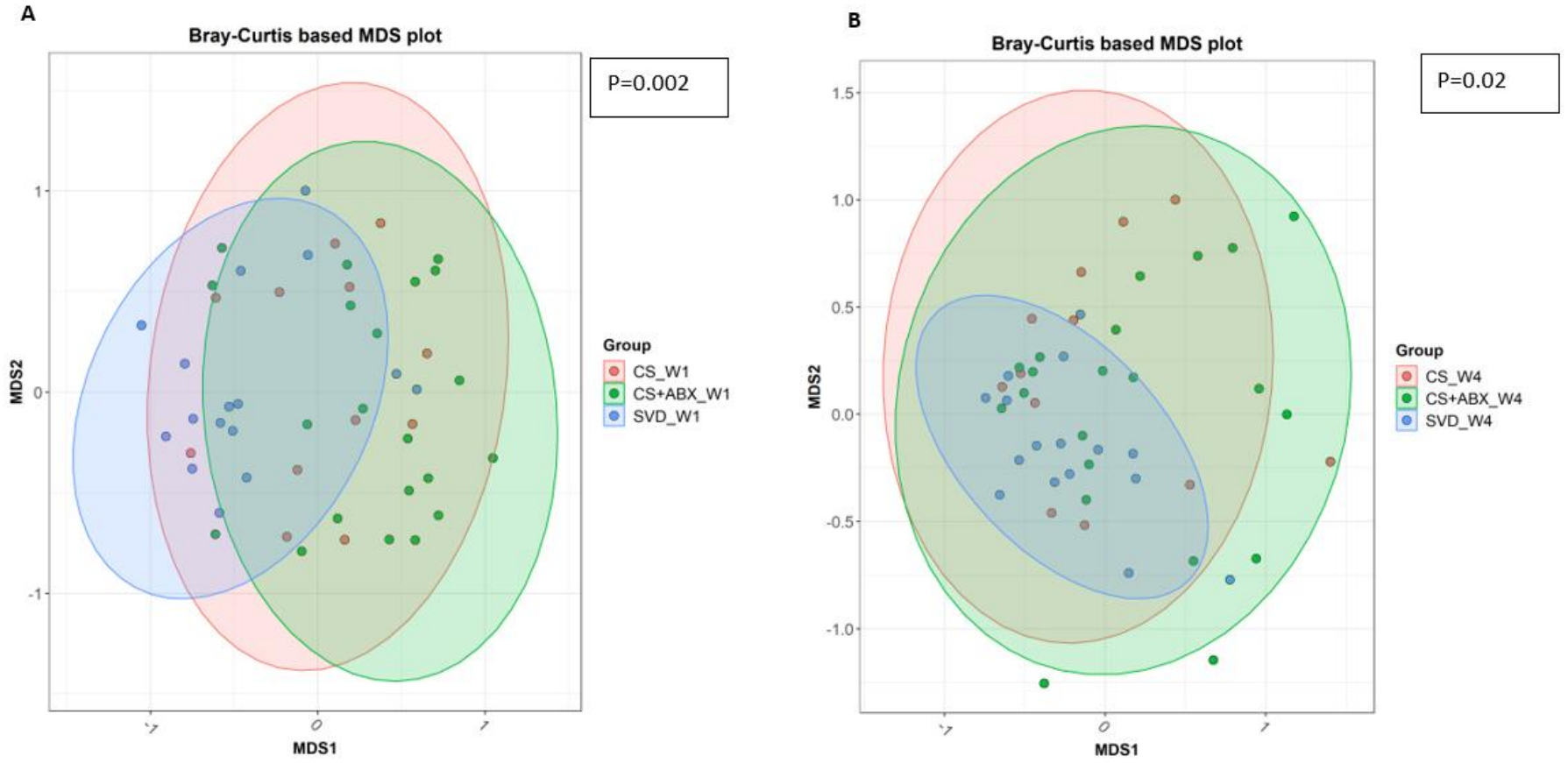


Figure 3.15 A and B: Beta diversity between the groups at Week 1 and Week 4

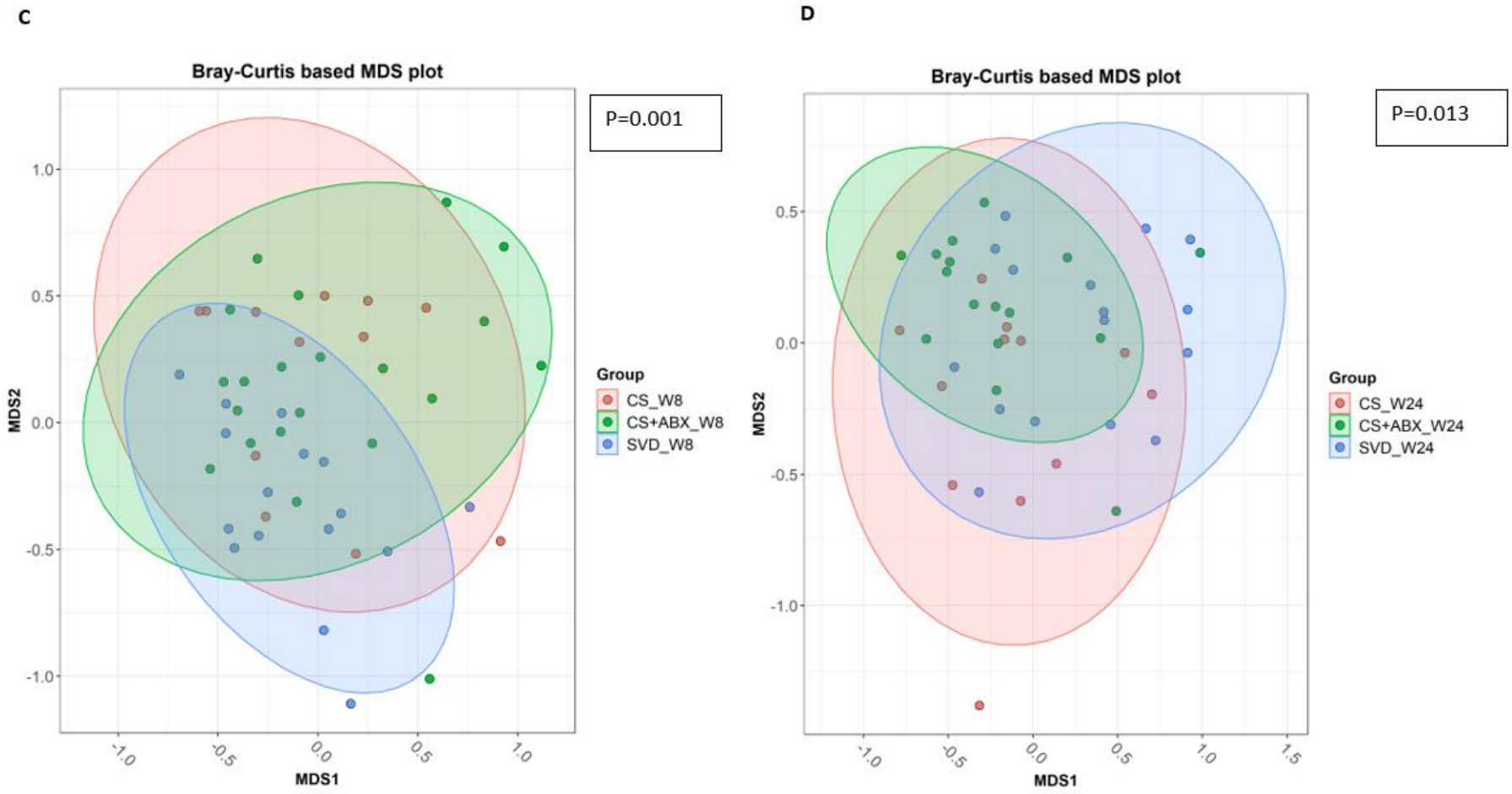


Figure 3.15 C and D: Beta diversity between the groups at Week 8 and Week 24

Discussion

It has been established that perinatal factors can significantly affect the gut microbiota progression in infancy (Penders et al., 2006). C-section delivery has previously been shown to result in significant disruption in the typical infant gut early colonisation pattern. Infants delivered by C-section are initially colonised by microbes that are commonly found on skin or in the hospital environment such as *Staphylococcus* and *Corynebacterium* (Ximenez & Torres, 2017). This bypassing of contact with the maternal birth canal results in a significant lack of colonisation by maternal vaginal microbes (Wang et al., 2019). Antibiotic exposure during infancy has previously been associated with the reduction of beneficial microbes (Hussey et al., 2011) and the development of allergic diseases and asthma in childhood (Risnes et al., 2010).

In this study, we investigated the impact of CS birth mode coupled with antibiotic exposure following birth, on the developing gut microbiota of infants over 24 weeks. As both CS birth mode and antibiotic exposure following birth have previously been shown to contribute to a significantly altered microbiota profile in infancy, it is not surprising that the CS+ABX infants have a distinctly altered microbiota composition at birth. Additionally, it is noted that many of these disturbances persist until at least the first 24 weeks of life.

Following compositional sequencing at weeks 1, 4, 8 and 24 of life, when comparing the gut microbiota of the three groups (SVD: Standard Vaginally Delivered, CS: C-section and CS+ABX: C-section plus antibiotic treatment), it was found that the most extensive changes were seen at Week 1 with a statistically significant decrease in Bacteroidetes in the CS and CS+ABX groups when compared

to the SVD group (Figure 3.2 A). Bacteroidetes was reduced from 11.3% in the SVD infants to lower than 1% in both other groups. This reduction in Bacteroidetes has previously been noted in infants born by C-section, with delayed colonisation found to occur in the first two years of life (Jakobsson et al., 2014). This was reflected at family level with a reduction in the relative abundance of Bacteroidales in both the CS and CS+ABX groups at weeks 1 and 4 (Figure 3.3 B and 3.4 B), and again for the genus Bacteroides at weeks 1 and 4 (Figure 3.7 C and 3.8 C). As Bacteroides has been reported to be transmitted from mother to infant during vaginally delivery, it is not surprising that this genus is reduced in both CS groups. Bacteroides has also been noted to play a role in the early immune system (Gregory et al., 2015). Therefore, this delayed colonisation may potentially be associated with the development of conditions in childhood that have an immune system component. Additionally, while there is a reduction in the relative abundance of Bacteroides in the CS group compared to the SVD group throughout the taxonomic levels, at Week 4 the CS group appears to be recovering slightly and colonisation increases (Figure 3.8 C). This is not seen in the CS+ABX group, as the relative abundance remains under 1%. This may be explained by the additional antibiotic usage in this group, with effects persisting and continuing to delay Bacteroides colonisation.

At phylum level, during Week 1, there was an increase in the relative abundance of Verrucomicrobia (Figure 3.2 B). This phylum makes up a small percentage of the overall relative abundance of the infant gut. However, increases in Verrucomicrobia have been seen previously following the administration of broad-spectrum antibiotics (Dubourg et al., 2013). This increase was also shown at family

level during Week 1 for Verrucomicrobiales between the CS and CS+ABX groups (Figure 3.3 K).

Reflecting the differences in the Bacteroidetes phylum during Week 1, there was an increase in the family Flavobacteriales between the groups (Figure 3.3 C). This increase continued to remain significant between the CS+ABX group and both other groups until Week 24. This family has previously been seen to be increased in infants treated with antibiotics (Zou et al., 2018) and given the significance at all time-points in just the CS+ABX group, it is not unreasonable to suggest that the higher relative abundance of Flavobacteriales may be associated with antibiotic exposure at birth. This also appears to be the case for the colonisation of Chryseobacterium (Figures 3.7 D, 3.8 D, 3.9 B and 3.10 C).

Many of the significant differences seen in this study reinforce previous work that investigated the gut microbiota composition of infants delivered by C-section. C-section has previously been associated with a lower relative abundance of Bacteroidetes and a higher relative abundance of Firmicutes (Rutayisire et al., 2016). This is similar to what we see in this study, as Bacteroidetes is significantly reduced in both CS groups at Week 1, while Firmicutes is increased in both CS groups when compared with SVD infants at Week 4. This was also true for the increase in Veillonella seen at Week 24 (Figure 3.10 F) and Klebsiella at Week 8 (Figure 3.9 F). Similar increases have been noted previously (Dogra et al., 2015; Hesla et al., 2014). Enterococcus has also previously been shown to be significantly increased in infants delivered by C-section (Jakobsson et al., 2014). This is also seen in this study, however, it must be noted that the addition of the CS+ABX group showed an

additional substantial colonisation increase in this genus, which was significantly different than the infants delivered vaginally at Week 1 (Figure 3.7 H).

Given that this study compares infants delivered by C-section with infants who also undergo this mode of delivery but are treated with antibiotics, it allows examination of those taxa that are significantly different in the CS+ABX group, but not the CS group, when compared with SVD infants. This can be seen in the relative abundance of Chryseobacterium at weeks 1, 4, 8 and 24. The lack of colonisation by this microbe in the SVD and CS infants may suggest that the presence of this genus is driven by antibiotic exposure. This can also be seen in the relative abundance of Dorea, Lachnosapira and [Eubacterium] coprostanoligenes group (Figure 3.7 J, K and L). Additionally, we can see from the data that some genera are present in SVD and CS groups but specifically reduced in the CS+ABX group, suggesting that CS alone does not result in a loss of abundance. This can be seen in the abundance of Dolosigranulum at Week 1 (Figure 3.7 G) and Gemella at Week 4 (Figure 3.8 F).

When examining the alpha diversity (richness and evenness among individual samples) between infants delivered by SVD, CS and CS+ABX at Week 1, a difference was seen between the SVD group and the CS+ABX group and also between the CS and CS+ABX group for the chao1 diversity measure, with the CS+ABX significantly reduced compared to the other two groups (Figure 3.14). Similarly, at Week 1 for the number of observed species, there was also a significant reduction in the CS+ABX group when compared to the two others. The CS+ABX group was also shown to be reduced for the phylogenetic diversity (PD whole tree) at Week 1 when compared with the SVD infants. No other differences were noted at Week 1. The differences seen at Week 1 were resolved by Week 4, apart from phylogenetic diversity. No differences

were noted between the groups for the Shannon or Simpson diversity measures at any time-point.

Overall, there was a reduction in alpha diversity between the infants delivered vaginally and the two other CS groups, however, this was mostly resolved by week four of life. Low alpha diversity has previously been reported as a hallmark of infants born by C-section (Wang et al., 2020). In this study, we see a further reduction of alpha diversity in the infants who were treated with antibiotics. This has been widely reported for both infants and adults and can occur regardless of the specific antibiotic type (Di Gioia et al., 2014; Dubourg et al., 2013; Fouhy et al., 2012). However, due to the unstable nature of the infant gut microbiome, antibiotic-associated changes can take considerably longer to resolve (Yassour et al., 2016).

In this study, the specific effect of antibiotic treatment on the early gut microbiota composition was apparent, with delays in colonisation and increases in microbial groups that are not present in the gut of vaginally delivered infants. Interestingly, while the significant differences in alpha diversity appear to resolve after the first four weeks of life, we can see that longer lasting effects can occur for specific microbes. The influence of antibiotics on certain groups of microbes can have significant consequences, for example, a reduction in short chain fatty acid producing-microbes can potentially affect the maturation of the infant immune system (Bokulich et al., 2016).

When examining beta diversity, at each time-point, the groups cluster based on their relatedness to each other. The samples cluster or separate based on their delivery mode and previously assigned groups (SVD, CS, CS+ABX) at each time-point (Figure 3.15). At Week 1, the groups cluster tightly with similar samples,

particularly the SVD group (Figure 3.15 A). At Week 4, the two CS groups overlap but are still separating from each other, while the SVD group remains significantly separated from the other groups (Figure 3.15 B). During weeks 8 and 24, the groups are still clustering based on mode of delivery. However, at Week 24, while they are still diverging from each other, there is some increasing overlap when the groups begin to show more similarity to the infants born by vaginally delivered. The PCA plots show that while the alpha diversity of the samples may not be significant at Week 24, and many of the taxa may not show significantly different levels of relative abundance, the groups are still more similar to each other based on their mode of delivery than the infants in the other groups until at least Week 24.

Following the analysis of the three groups of infants, when examining the impact of mode of delivery and antibiotic exposure, it is evident that these factors contribute significantly to disturbances in the infant gut microbiota profile. Throughout the compositional data, the significant impact of both C-section delivery and antibiotic treatment was apparent, whether it results in a complete lack of colonisation by certain microbes that are present in infants delivered vaginally, a reduction based on antibiotics specifically, or an increase in microbes that are not typically present in the infant gut after vaginal delivery. Additionally, changes are seen in both alpha and beta diversity which can persist until at least the first 24 weeks of life. These initial gut microbiota disturbances can significantly alter the colonisation pattern of the infant gut and have been associated with several negative consequences during childhood. Long-lasting C-section- and antibiotic- driven gut microbiota disturbances have been shown to increase the risk of developing allergies and asthma (Bager et al., 2008; Thavagnanam et al., 2008), obesity and type-1 diabetes (Iizumi et

al., 2017) in childhood, potentially due to the lack of protective effect that early colonising microbes can provide.

Here we have detailed some of the specific microbes which may contribute to these long-lasting detrimental effects. Many of the altered taxa reported here due to mode of delivery or antibiotic exposure are consistent with previous findings, however, we have examined those microbes that are colonising the gut at very low relative abundances and potentially are driven by early antibiotic exposure. These low relative abundance colonisers should be examined further, to examine the effect these may have on the overall microbial profile and whether they are exclusively driven by antibiotic exposure in infants.

Further analysis is warranted in this area to disentangle the specific effects of each insult on the developing infant gut. Additional data should be taken into consideration including antibiotic type and treatment duration, elective versus emergency C-section, inflammatory profile, psychological and cognitive assessments and feeding regime. In addition, comprehensive data should be collected on maternal health and any other potential factors that may contribute to the immediate disturbances of the early infant gut microbiota and the associated negative consequences in childhood.

References

Ajslev, T. A., Andersen, C. S., Gamborg, M., Sørensen, T. I. A., & Jess, T. (2011). Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *International journal of obesity, 35*(4), 522-529.

Bager, P., Wohlfahrt, J., & Westergaard, T. (2008). Caesarean delivery and risk of atopy and allergic disesase: meta-analyses. *Clinical & Experimental Allergy, 38*(4), 634-642.

Bokulich, N. A., Chung, J., Battaglia, T., Henderson, N., Jay, M., Li, H.,... Blaser, M. J. (2016). Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Science Translational Medicine, 8*(343), 343ra82.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in Molecular Medicine, 20*(9), 509-518.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2009). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics, 26*(2), 266-267.

Di Gioia, D., Aloisio, I., Mazzola, G., & Biavati, B. (2014). Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Applied microbiology and biotechnology, 98*(2), 563-577.

Dogra, S., Sakwinska, O., Soh, S. E., Ngom-Bru, C., Brück, W. M., Berger, B., Brüssow, H., Lee, Y. S., Yap, F., Chong, Y. S., Godfrey, K. M., Holbrook, J. D., & GUSTO Study Group (2015). Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. *mBio, 6*(1), e02419-14.

Dubourg, G., Lagier, J.-C., Armougom, F., Robert, C., Audoly, G., Papazian, L., & Raoult, D. (2013). High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment. *International Journal of Antimicrobial Agents, 41*(2), 149-155.

Edgar R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England), 26*(19), 2460–2461.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M.,... Stanton, C. (2012). High-throughput sequencing reveals the incomplete, short-term recovery of

infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrobial agents and chemotherapy, 56*(11), 5811-5820.

Gregory, K. E., LaPlante, R. D., Shan, G., Kumar, D. V., & Gregas, M. (2015). Mode of Birth Influences Preterm Infant Intestinal Colonization With Bacteroides Over the Early Neonatal Period. *Advances in neonatal care : official journal of the National Association of Neonatal Nurses, 15*(6), 386-393.

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A.,... Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences, 108*(7), 3047-3052.

Hesla, H. M., Stenius, F., Jäderlund, L., Nelson, R., Engstrand, L., Alm, J., & Dicksved, J. (2014). Impact of lifestyle on the gut microbiota of healthy infants and their mothers–the ALADDIN birth cohort. *FEMS microbiology ecology, 90*(3), 791-801.

Hussey, S., Wall, R., Gruffman, E., O'Sullivan, L., Ryan, C. A., Murphy, B.,... Ross, R. P. (2011). Parenteral Antibiotics Reduce Bifidobacteria Colonization and Diversity in Neonates. *International journal of microbiology, 2011*, 130574.

Iizumi, T., Battaglia, T., Ruiz, V., & Perez Perez, G. I. (2017). Gut Microbiome and Antibiotics. *Archives of Medical Research, 48*(8), 727-734.

Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C.,... Andersson, A. F. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. *Gut, 63*(4), 559.

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A., & Stanton, C. (2010). Programming infant gut microbiota: influence of dietary and environmental factors. *Current opinion in biotechnology, 21*(2), 149-156.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiology and molecular biology reviews : MMBR, 81*(4), e00036-17.

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The infant microbiome development: mom matters. *Trends in Molecular Medicine, 21*(2), 109-117.

O'Mahony, S. M., Clarke, G., Dinan, T., & Cryan, J. (2017). Early-life adversity and brain development: Is the microbiome a missing piece of the puzzle? *Neuroscience, 342*, 37-54.

Opøien, H. K., Valbø, A., Grinde-Andersen, A., & Walberg, M. (2007). Post-cesarean surgical site infections according to CDC standards: rates and risk factors. A prospective cohort study. *Acta obstetricia et gynecologica Scandinavica*, *86*(9), 1097–1102.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I.,... Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511-521.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(Database issue), D590–D596.

Risnes, K. R., Belanger, K., Murk, W., & Bracken, M. B. (2010). Antibiotic Exposure by 6 Months and Asthma and Allergy at 6 Years: Findings in a Cohort of 1,401 US Children. *American Journal of Epidemiology, 173*(3), 310-318.

Rutayisire, E., Huang, K., Liu, Y., & Tao, F. (2016). The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. *BMC Gastroenterology, 16*(1), 86.

Thavagnanam, S., Fleming, J., Bromley, A., Shields, M. D., & Cardwell, C. (2008). A meta-analysis of the association between Caesarean section and childhood asthma. *Clinical & Experimental Allergy, 38*(4), 629-633.

Wang, S., Ryan, C. A., Boyaval, P., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). Maternal Vertical Transmission Affecting Early-life Microbiota Development. *Trends in microbiology, 28*(1), 28-45.

Ximenez, C., & Torres, J. (2017). Development of Microbiota in Infants and its Role in Maturation of Gut Mucosa and Immune System. *Archives of Medical Research, 48*(8), 666-680.

Yang, I., Corwin, E. J., Brennan, P. A., Jordan, S., Murphy, J. R., & Dunlop, A. (2016). The Infant Microbiome: Implications for Infant Health and Neurocognitive Development. *Nursing research, 65*(1), 76-88.

Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A.-M., Härkönen, T., Ryhänen, S. J.,... Gevers, D. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Science Translational Medicine, 8*(343), 343ra81.

Zou, Z.-H., Liu, D., Li, H.-D., Zhu, D.-P., He, Y., Hou, T., & Yu, J.-L. (2018). Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. *Annals of Clinical Microbiology and Antimicrobials, 17*(1), 9.

CHAPTER 4

BIRTH BY CAESAREAN-SECTION HAS LONG-TERM PSYCHOLOGICAL CONSEQUENCES

CHAPTER CONTRIBUTIONS:

Amy Murphy

- planned sample quantity, timing, and processing methods,
- collected all stool samples,
- carried out DNA extraction,
- planned and prepared 16 S compositional sequencing library,
- prepared faecal water,
- planned and carried out culture-based work and isolated specific microbes,
- planned and carried out statistical analyses,
- significantly contributed to manuscript preparation.

Bioinformatic analysis was completed by Dr. Kiera Healy

Dr. Paul Kennedy and Dr. Caitriona Long-Smith conducted psychological analyses and contributed to manuscript preparation

Abstract

Colonisation of the infant gut early in life has an important role in directing development of the immune system, nervous system and brain. It is well established that birth by Caesarean (C)-section disrupts the normal colonisation of the infant gut due to the lack of contact between the new-born and maternal vaginal and intestinal microbes. Indeed, there is accumulating evidence that C-section delivery bypasses stress and immune priming and can have many long-lasting health effects.

The long-term effects of C-section birth were investigated by examining the gut microbiota of a cohort of matched healthy young male university students aged between 18 and 24, either born vaginally (N=39) or by C-section (N=37) during a Non-Stress period and during university exam stress. The Trier Social Stress Test (TSST) was also used to investigate the hypothalamic, pituitary, adrenal (HPA) axis, inflammatory and psychological responses to acute psychosocial stress. Participants completed a battery of cognitive tests and self-reported measures assessing mood, anxiety and perceived stress. Saliva, blood and stool samples were collected for analysis of cortisol, peripheral immune profile and microbiota analyses.

The gut microbiota of C-section and vaginally-born subjects showed similar pair-wise relative abundances at phylum, family and genus levels. Additionally, diversity levels were not influenced by mode of delivery. However, participants born by C-section showed increased psychological vulnerability to acute psychosocial stress and exam stress. In conclusion, this study reveals that differences in gut microbial profiles that are found between infants born by C-section versus those vaginally delivered do not persist into young adulthood. However, the long-term impact of birth by C-section on psychological measures in young adulthood has

significant implications for determining vulnerability to mental health-related disorders.

Introduction

Birth by Caesarean-section (C-section) disrupts the normal microbial colonisation of the gut, in addition to preventing early stress- and immune-priming, increasing the risk of metabolic and immune disorders. The microbiota-brain-gut axis plays an important role in psychological processes, but the impact of birth by C-section on psychological processes is largely unknown (Dominguez-Bello et al., 2010).

The timing of developmental processes in the brain is highly precise and occurs in distinct phases (Ben-Ari, 2013). Early colonisation and maturation of the gut microbiome occurs in a similarly defined manner. Alterations during these key developmental periods of the microbiota-gut-brain axis have been associated with long-term consequences in brain signalling and can influence mental health later in life (Borre et al., 2014). As the natural progression and maturation of the gut microbiota occurs in tandem with immune and brain development, the absence of key microbes during this developmental period could potentially have long-term effects.

C-section delivery can affect the normal colonisation pattern due to lack of vaginal microbiota transfer between the mother and the infant. However, these microbial alterations appear to be largely resolved by early childhood, with the gut microbiota of children who were born by C-section resembling that of children who were born vaginally (Fouhy et al., 2019). While initial gut microbiota changes may appear to be fully resolved early in life, it is important to note that during this key developmental period, these disturbances can potentially affect other processes.

Birth by C-section can have a variety of implications during the first years of life. Here, we aimed to examine the long-term consequences of birth by C-section on the gut microbiota. We profiled the gut microbiota of 18- to 24-year-old, healthy male participants to assess whether those C-section-driven microbiota alterations which occur at birth and continue during the first weeks of life progress to adulthood.

Although microbiome changes have been well documented in C-section born individuals (Dominguez-Bello et al., 2010; Hill et al., 2017; Penders et al., 2006), there has been limited investigation on the long-term effects of altered gut microbial profile on psychological and physiological responses to stress. To fill this knowledge gap, adult human volunteers of known birth mode underwent a Trier Social Stress Test (TSST) to investigate the HPA axis, inflammatory and psychological response to acute psychosocial stress (Allen et al., 2014). While no differences were seen in the gut microbiota profile of these participants, long-term psychological vulnerabilities were apparent in young adults born by C-section.

Materials and Methods

Participants and Sample Collection

Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Healthy participants aged 18-24 years were recruited between December 2014 and December 2015 at the Clinical Research Facility, Mercy University Hospital, Cork. An initial screening visit was undertaken involving a MINI neuropsychiatric interview and collection of medical history and demographics. Study participants were matched on the basis of age, years of education, body mass index (BMI) and units of alcohol consumed per week.

Various clinical, physiological, psychological, psychiatric and cognitive data were also collected (see Table 4.1). Half of the participants were initially born by CS and half were delivered vaginally. Faecal samples were collected from participants at two time-points: during a Non-Stress period and during University exam stress. These fresh faecal samples were collected and stored at 4°C and transported in temperature-controlled collection bags to the lab for processing. In order to assess the impact of altered gut microbiota during early life on cognition, endocrine and immune responses, a laboratory based public speaking stress (Trier Social Stress Test), eliciting an acute stress response, was used.

Type	Measure	Baseline	Non-Stress	Exam Stress	TSST (Acute stress)
Clinical	C-section/Non-section	X			
	Medical history	X			
	Family history	X			
	Medication record	X			
	BMI	X			
	Alcohol intake	X			
	Smoking habits	X			
	Birth Info	X			
	Nutrition: Food frequency questionnaire		X	X	
	Exercise levels: MET-min		X	X	
	Sleep quality		X	X	
	Gut health baseline	X			
	GI symptoms during stress v Non-Stress		X	X	
Physiological	Stress, saliva - cortisol levels during acute stress		X		X
	Stress, saliva - cortisol awakening response		X	X	
	Inflammatory profile, bloods -IL-10		X	X	X
	Inflammatory profile, bloods -IL-1b		X	X	X
	Inflammatory profile, bloods -IL-6		X	X	X
	Inflammatory profile, bloods -IL-8		X	X	X
	Inflammatory profile, bloods -TNF-a		X	X	X
	Faecal Microbiota		X	X	

	Urinary metabolomics		X	X	
Psychiatric & Psychological	Traumatic life events - childhood	X			
	Social-cognitive - autism measures	X			
	Social-cognitive - emotional measures	X			
	Empathy measures	X			
	Personality measure	X			
	Depression & Anxiety - self-report 1		X	X	
	Depression & Anxiety - self-report 2		X	X	
	Perceived Stress - self-report		X	X	
	Stress & Mood - self-report 1		X		X
	Stress & Mood - self-report 2		X		X
	Stress & Mood - self-report 3		X		X
Cognitive	Visuospatial memory (Computer based)		X	X	
	Impulse control (Computer based)		X	X	
	Rule acquisition and reversal (Computer based)		X	X	
	Emotion identification (Computer based)		X	X	
	Paper-based emotion identification		X	X	

Table 4.1

Clinical and psychological data collected from the participants at baseline, during a Non-Stress period, during exam stress and during acute stress.

Culture-Dependent Analysis

Fresh faecal samples were weighed and serially diluted in maximum recovery diluent (Fluka, Sigma Aldrich, Ireland) from 10^{-1} to 10^{-8}. Bifidobacteria were enumerated by spread-plating serial dilutions onto de Man, Rogosa, Sharpe (MRS) agar (Difco, Becton-Dickenson Ltd., Ireland), which had been modified by adding 0.05% L-cysteine hydrochloride (Sigma Aldrich, Ireland), 100 ug/ml mupirocin (Sigma Aldrich, Ireland) and 50 units of nystatin (Sigma Aldrich, Ireland). Agar plates were incubated anaerobically for three days at 37°C. *Lactobacillus* selective (LBS) agar (Difco, Becton-Dickenson Ltd., Ireland), supplemented with 50 units of nystatin was used to enumerate lactobacilli. Agar plates were incubated anaerobically for five days at 37°C. Total anaerobic bacteria were enumerated by spread-plating onto Wilkins Chalgren agar (WCA) (Sigma Aldrich, Ireland) supplemented with 50 units of nystatin and 7% defibrinated horse blood (Cruinn Diagnostics Ltd., Ireland). Agar plates were then incubated anaerobically for five days at 37°C. Brain Heart Infusion (BHI) agar supplemented with 50 units of nystatin was used to enumerate total aerobic bacteria. These were also incubated anaerobically for five days at 37°C.

Faecal Water Preparation

Faecal samples were weighed into a sterile 2 ml Eppendorf tube (0.4 g sample in 0.8 ml sterile H_20 (2x wt/vol)). Samples were vortexed continuously for 5-10 mins to produce a slurry and then centrifuged at 16000 x g for 30 mins. The supernatant was removed and transferred to a fresh Eppendorf and centrifuged again for 30 mins at 16000 x g. This was repeated once more (three 30 min centrifugations). Following this, the final supernatant was filtered through a VectaSpin Micro centrifuge filter

(0.2 µm) for 10 mins or as long as needed to fully filter the sample. The faecal water was stored at -20 ℃ until processing.

DNA Extraction

DNA was extracted from adult faecal samples using the RBB method (Yu & Morrison, 2004). A 0.2 g faecal sample was weighed and added to 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm diameter sterile zirconia beads plus a single 2.5 mm diameter bead (BioSpec Products, Bartlesville, USA). To this, 1 ml of lysis buffer was added (500 mM NaCl, 50 mM tris-HCL, pH 8.0, 50 mM EDTA and 4% sodium dodecyl sulphate (SDS)). Each sample was then homogenised using a Mini-Beadbeater™, BioSpec Products, Bartlesville, OK, USA) at maximum speed for 3 min and incubated at 70° C for 15 mins to lyse the cells. Samples were then centrifuged for 5 min at 16,000 x g and the supernatant was transferred to a fresh Eppendorf tube. The bead beating, heating and centrifugation steps were repeated using 300 µl of lysis buffer and the supernatant was pooled. Following this, 260 µl of 7.5 M ammonium acetate was added, and the samples were vortexed and incubated on ice for 5 min.

Isopropanol was added to precipitate the DNA and samples were centrifuged to pellet the nucleic acid. The pellets were then washed with 70% ethanol and allowed to dry before being dissolved in 100 µl TE buffer. The DNA was treated with RNAse and Proteinase K and washed with Qiagen buffers AW1 and AW2 using columns provided in the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK).

The DNA was then eluted in 200 µl Buffer ATE. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland).

16S Compositional Sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

The protocol involved two PCR reactions on the extracted DNA. The DNA was first amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;
Reverse primer 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each reaction contained 2.5 µl genomic DNA, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following program: 95°C x 3mins, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, 72°C x 5mins and held at 4°C. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland).

Following this, a second PCR reaction was carried out on the purified DNA using two indexing primers per sample (Illumina Nextera XT indexing primers, Illumina, Netherlands). Each reaction contained 5 µl purified DNA, 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix and 10µl PCR grade water. PCR amplification was completed using the previous program but

with only 8 amplification cycles instead of 25. PCR products were visualised and purified as described above.

Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Clinical Microbiomics, Denmark using standard Illumina sequencing protocols.

Metabolomics

Faecal water and urine collected from the participants were shipped to the University of Reading, UK for analysis. A total of 111 urine (61 natural-born - VD, and 50 Caesarean-section born - CS) and 140 faecal water samples (76 VD and 64 CS) were acquired on a Bruker AV700 NMR Spectrometer equipped with a 5mm 1H (13C/15 N) inverse Cryoprobe. All samples were analysed at 300K with a standard 1H-1 D NOESY (noesypr) with water signal suppression. For each spectrum, 8 dummy transients were followed by a total of 64 scans, with a relaxation delay (RD) of 5 s and acquisition time of 1 s.

Scans were accumulated in 64k data points with a spectral width of 9803.9 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. All spectra were referenced to the singlet peak of 3-(trimethylsilyl)-2,2′,3,3′- tetradeuteropropionic acid (TSP) at 0.0 ppm, manually phased and automatically baseline corrected applying a Whittaker smoother algorithm in MNova NMR Version 10.0.2 (Mestrelab Research, Spain). Spectra were digitalised and imported in Matlab, where the residual water signal, and urea in the urine samples, were manually deleted. All the spectra were normalised under total area and unit

variance (UV) scaled. Principal Component Analysis (PCA) was performed to detect metabolic group variations and detect possible outliers.

Data were further analysed using orthogonal projection to latent structure-discriminant analysis (OPLS-DA) with 0 or 1 orthogonal components where 1H-NMR spectroscopic profiles were used as a matrix of independent variables (X) and birth delivery modality as response vector (Y). Data were also analysed using PLS regression where independent variables of individual's bacterial taxa counts (X) were used to predict the intensity of the trimethylamine / dimethylamine ratio as response vector (Y). The two values R2Y (goodness of fit: percentage of Y explained by the model) and Q2Y (the goodness of prediction: percentage of Y predicted after 7-fold cross validation) were considered for the OPLS models. Significance of selected models was validated by random permutation tests (500 permutations).

Correlation coefficients plots were constructed from the model outputs by back-scaling transformation of the loading to display the contributions of each metabolite to sample classification. Metabolites were assigned using Chenomx Software (Chenomx Inc.), public metabolic databases (HMDB, http://www.hmdb.ca, BMRB, http://www.bmrb.wisc.edu) and from the literature.

Psychological Measures

TSST Methodology

The TSST procedure began between 13.00-14.30h for each participant, to control for diurnal fluctuations in cortisol levels. Participants were instructed to abstain from alcohol and strenuous physical exercise for 24 hours prior to visits. In addition, they were asked not to consume any caffeine containing products on the day of their Non-Stress study visit, and to consume only water for 2 hours prior to the TSST procedure.

After collection of the first saliva sample for measuring salivary cortisol, participants rested for a 45-minute baseline period. Participants were then given standardised written instructions, introducing the TSST (Figure 4.1) which was carried out as previously described by Allen and colleagues (Allen et al., 2014). Participants were then led to a separate room, equipped with a video camera and microphone and two desks. After reiterating the task instructions, participants were then given a 3-minute speech preparation period after which, participants were required to perform a 5 minute speech outlining their suitability for an ideal job of their choice, followed by a 5 minute mental arithmetic task in which they serially subtract 17 from 2023.

Tasks were performed in front of two committee members (one male and one female), wearing white laboratory coats and introduced as being experts in identifying non-verbal aspects of behaviour. Participants were also informed that their speech would be both audio and video recorded for later behavioural analysis. Saliva samples and self-reported measures of mood and stress were collected at a number of time-points pre- and post- the TSST procedure (see below). Participants were fully debriefed following collection of the last sample.

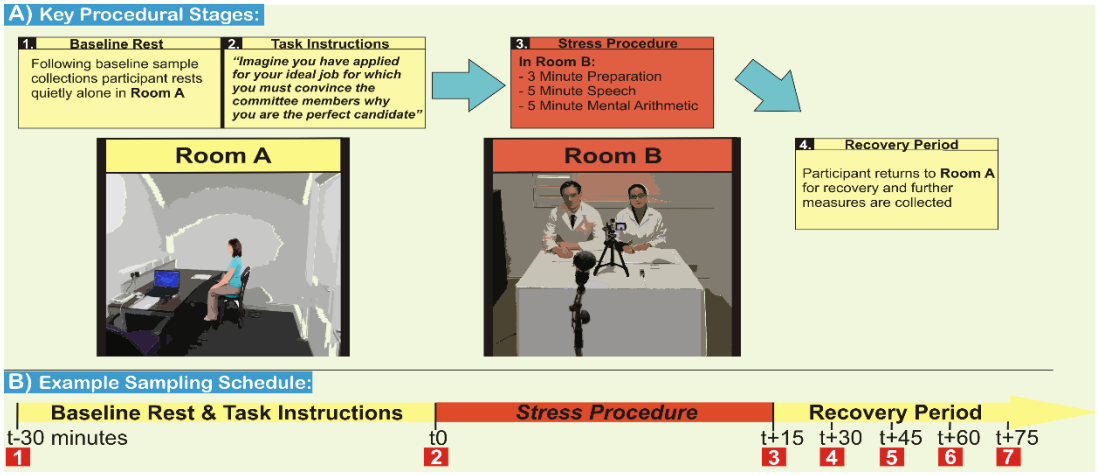


Figure 4.1

Key stages of the Trier Social Stress Test

Self-Report Measures TSST Mood & Stress Measures

Mood was assessed using the Positive and Negative Affect Schedule (PANAS; (Watson et al., 1988), and psychological stress was measured using a visual analogue scale (VAS), ranging from 0 ("not stressed at all") to 100 ("as stressed as I could possibly imagine").

State Trait Anxiety Inventory

The STAI is a self-report measure consisting of two subscales each with 20 items, one measuring trait anxiety and the other measuring state anxiety (Spielberger & Gorsuch, 1983). Participants rate how they feel either right now (state) or generally (trait), in response to each item on a 4-point scale from 'not at all' to 'very much.' The range of scores for each sub-scale is 20-80 with higher scores indicating greater anxiety.

Perceived Stress Scale (PSS)

The PSS is a self-report measure in which participants rate, on a 5-point scale ranging from 0 (never) to 4 (very often), how often they have particular thoughts or feelings described by each of the 10 items (Cohen et al., 1983). Scores range from 0-40 with higher scores indicating greater stress over the previous month.

Beck Depression Inventory (BDI)-II

The BDI-II is a self-report measure consisting of 21 items rated on a 4-point scale from 0 (absence of symptom) to 3 (severe manifestation of symptom) (Beck et al., 1996). Scores range from 0-63. Cut-off scores indicating clinically relevant levels of depression have been determined as 0-13 (minimal); 14-19 (mild); 20-28 (moderate); 29-63 (severe).

Cognitive assessment: Tests from the CANTAB battery

(http://www.cambridgecognition.com/)

CANTAB tests were presented on a high-resolution touch-screen monitor under computer control. Participants interact with the system by touching the touch-screen while a test administrator provides verbal instructions from a standardised script, and specific verbal prompts and encouragement when needed. The test administrator has full control of a keyboard used to start, pause or abort each test. Participants were assessed on the following tests from the battery:

a) IED: Attentional flexibility and reversal learning

The IED is a test of executive function and assesses rule acquisition and reversal, attentional set formation, maintenance and shifting. The outcome measures assessed to determine reversal learning performance were the errors made on stage 2, 5, 7 and

9, and to determine attentional flexibility performance the errors made on stage 6 and 8.

b) Stop Signal Task (SST): Response inhibition

The SST assesses participant's ability to inhibit a prepotent response. The outcome measure assessed was the stop signal reaction time (SSRT; calculated for last 20 sub-blocks)

c) Paired Associates Learning (PAL): Visuospatial memory

The PAL assess conditional learning of pattern-location associations and gives an index of visuospatial memory. The outcome measure assessed to determine visuospatial memory was the PAL total errors adjusted.

Statistical analyses for human data

Independent sample t-tests were used to explore differences in group characteristics (age, BMI, years of education and units of alcohol per week). To allow for repeated measures analysis and to avoid bias that may be introduced by using list-wise deletion of incomplete cases (Graham, 2009), missing data analysis was performed on physiological, psychological and cognitive variables subject to repeated measures analysis.

In total, 5.95% of data were missing and determined to be missing completely at random (MCAR) using Little's MCAR test (Little, 1988); $\chi 2$ (688) = 703.356, p=0.334)). Missing values were imputed by assigning the group mean for that variable except for cytokine and cortisol awakening response data. All analyses were carried out with missing data excluded (data not shown) and missing data imputed, which showed that imputing values using this method did not significantly change the nature of the results. Participants with four or more missing data points for salivary cortisol

during the TSST were excluded from this analysis. If a participant had missing data at all time-points for a given variable (during the TSST or Exam Stress/Non Stress visit) missing values were not imputed and the participant was excluded from this analysis.

Following data imputation, normality checks were performed using the Shapiro-Wilk test and visual inspection of histograms. Outliers were checked using box and whisker plots and only extreme outliers were considered for exclusion from analysis. Salivary cortisol (TSST), IL-10 (TSST), IL 1β (TSST), IL-6 (TSST), IL-8 (TSST), TNF-α (TSST), IL-8 (Non-Stress/Exam Stress), TNF-α (Non-Stress/Exam Stress), PANAS, STAI, BDI and CANTAB PAL (Visuospatial Memory) data were not normally distributed and transformed using a natural log transformation (ln); VAS psychological stress data were transformed using a square-root transformation; IL-10 (Non-Stress/Exam Stress), IL-1β (Non-Stress/Exam Stress), IL-6 (Non-Stress/Exam Stress), CANTAB IED (Attentional Flexibility / Reversal Learning) data were not normally distributed, but no transformations improved normality, so we proceeded with parametric analysis but with caution in interpreting the data.

Salivary cortisol awakening response values at each time-point were converted to area under the curve with respect to ground (AUCg) values (Pruessner et al., 2003). AUCg cortisol data were not normally distributed, and no transformations improved normality, so again we proceeded with parametric analysis but with caution in interpreting the data. PSS and CANTAB SST data (stop signal response time last 20) were normally distributed and no transformations were performed. Following data imputation and transformation (if needed) to improve normality, repeated measures analysis of variance (ANOVA) with group as between-subjects factor and change across time-points due to stress in each variable (salivary cortisol (TSST), IL-10 (TSST), IL-1β (TSST), IL-6 (TSST), IL-8 (TSST), TNF-α (TSST), cortisol awakening

response AUCg ((Non-Stress/Exam Stress), IL-10 (Non-Stress/Exam Stress), IL-1β (Non-Stress/Exam Stress), IL-6 (Non-Stress/Exam Stress), IL-8 (Non-Stress/Exam Stress), TNF-α (Non Stress/Exam Stress), PANAS Positive/Negative, VAS psychological stress, STAI State, BDI, PSS, PAL Total Errors Adj., SSRT last 20, IED Attentional Flexibility, IED Reversal Learning) as the within-subjects factor.

Significant main effects were followed by post-hoc comparisons using a Bonferroni correction for multiple comparisons as appropriate. Where Mauchly's test of sphericity was significant, the Greenhouse-Geisser or Huynh-Feldt correction was applied. Our primary outcome variable was salivary cortisol output in response to the TSST. Based on previous findings from our laboratory, we powered our study to detect between-group differences in the salivary cortisol response with a medium effect size (f= 0.25). At an alpha of 0.05 and obtaining a power of 0.08, a total sample size of 74 was required. This calculation is based on a repeated measures ANOVA, between factors, using G*Power software.

Non transformed data are presented as mean ± standard error of the mean (SEM). Effect sizes are reported as partial Eta squared (ηp^2). All statistical analyses were performed using IBM SPSS Statistics 22.0 for Windows software package.

Microbiome Statistical Analysis

All data were analysed and graphed using either R-3.3.1, Microsoft Excel or GraphPad Prism (Version 5). Data are expressed as SEM. Statistical analysis was carried out using the Kruskal-Wallis test or the Wilcoxon signed-rank test for paired data to identify significant differences between the groups. Differences were considered significant at $p < 0.05$. Correction of *p*-values was performed using the Benjamini-Hochberg method (FDR <0.05). The vegan package was used for Bray-

Curtis based MDS analysis, and the Adonis function in vegan was used for PERMANOVA in beta diversity.

Bioinformatic Analysis

In brief, the 16S rRNA gene amplicon sequences were processed through a bioinformatics pipeline. Resulting 300 bp paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies; (Magoč & Salzberg, 2011)). Further sequence read processing was performed using QIIME (Version 1.8.0.) including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds (QIIME allows analysis of high-throughput community sequencing data). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH (Version 7, 64-bit, search and clustering orders of magnitude faster than BLAST; (Edgar, 2010). OTU sequences were aligned using PyNAST (PyNAST: python nearest alignment space termination; (Caporaso et al., 2009), a flexible tool for aligning sequences to a template alignment) and taxonomy was determined using the SILVA SSURef database release 111 (Quast et al., 2012), at 97% similarity. Alpha diversity estimates were calculated using QIIME. Beta diversity was calculated using Bray-Curtis based multidimensional scaling (MDS) analysis of faecal microbiota.

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Results

Culture Dependent Analysis

Fresh faecal samples were plated on agar plates to enumerate bifidobacteria, lactobacilli, total aerobes and total anaerobes. It was found that there were no differences between either VD or CS participants between Non-Stress and Exam Stress time-points (Figure 4.2). Additionally, there were no differences seen due to mode of delivery at either time-point (Figure 4.3). Samples were compared separately using paired and non-paired statistical analyses to examine both the influence of mode of delivery and stressed state (VD n=30, CS n=30). There were no effects seen on plate counts based on either mode of delivery or time-point.

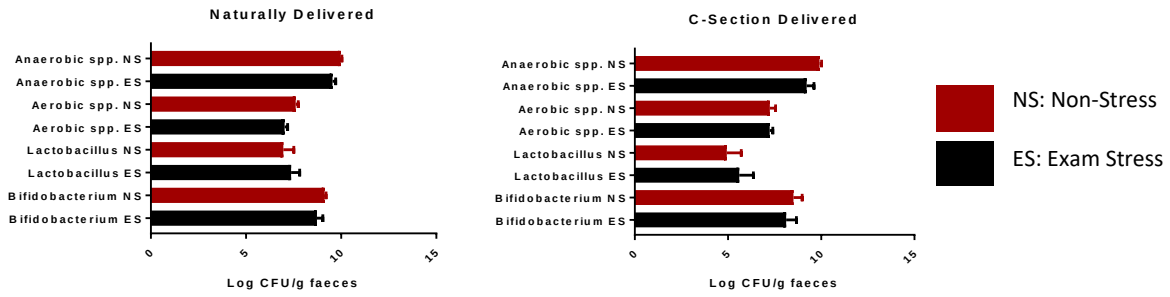

Figure 4.2

Culture dependent analysis examining plate counts to assess if stress time-point influences bacterial counts

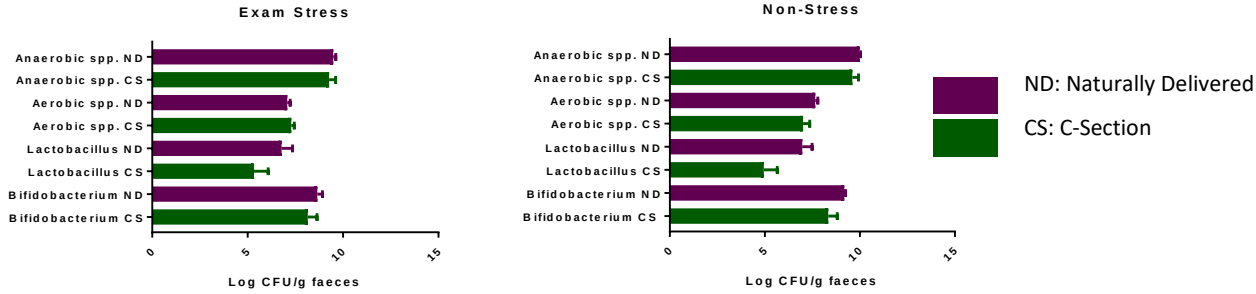

Figure 4.3

Culture dependent analysis examining plate counts to assess if birth mode influences bacterial counts

16S Compositional Sequencing

Following 16 S compositional sequencing, it was found that the gut microbiota composition did not differ between participants born vaginally and those born by C-section. Average relative abundances were similar between the vaginally delivered and the C-section groups (Figure 4.4). Additionally, relative abundances at phylum, family and genus levels did not differ between participants based on stress. No significant differences were seen at any taxonomic levels between the period of Non-Stress and the University exam stress (See Figure 4.4). Examination of alpha diversity between the groups using the following metrics: Chao 1, Observed Species, Phylogenetic Diversity Whole Tree, Simpson index and Shannon index did not show any changes between the groups at either time-point (Figure 4.5). Similarly, when investigating beta diversity using Bray-Curtis based multidimensional scaling (MDS), the samples did not cluster or separate based on mode of delivery or stress time-point (Figure 4.6).

The effect of inflammatory biomarkers Il-10, IL-1B, IL-6, IL-8 and TNF-α on the gut microbiota was analysed. A permutational analysis of variance test was performed using the function Adonis from the vegan package in R. Adonis models the effect of variables on the beta diversity of microbiome samples. At the 5% level of significance, the inflammatory biomarkers were found to have no effect on the microbiota.

Furthermore, the effect of stress hormones on the gut microbiota was tested. The stress hormone levels were represented through the cortisol awakening response

Area Under the Curve metric. This was also tested using the Adonis function. At the 5% level of significance, the cortisol awakening response was found to have no effect on the microbiota (data not shown).

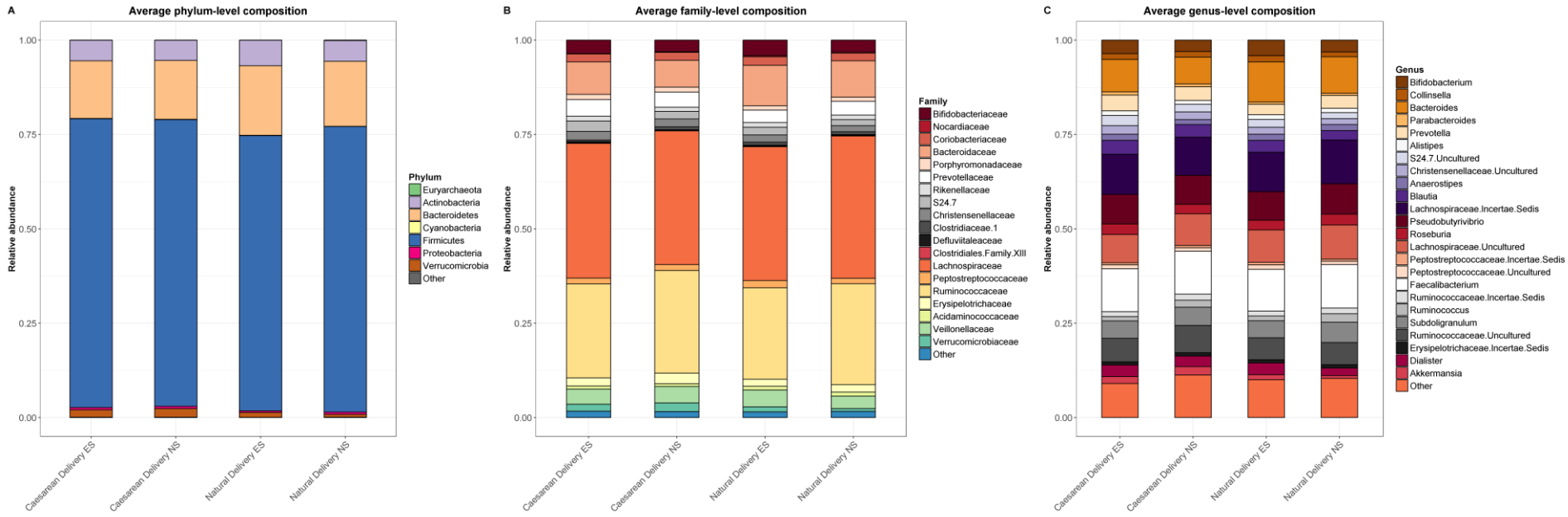

Figure 4.4
Average relative abundances of the gut microbiota at phylum, family and genus levels.

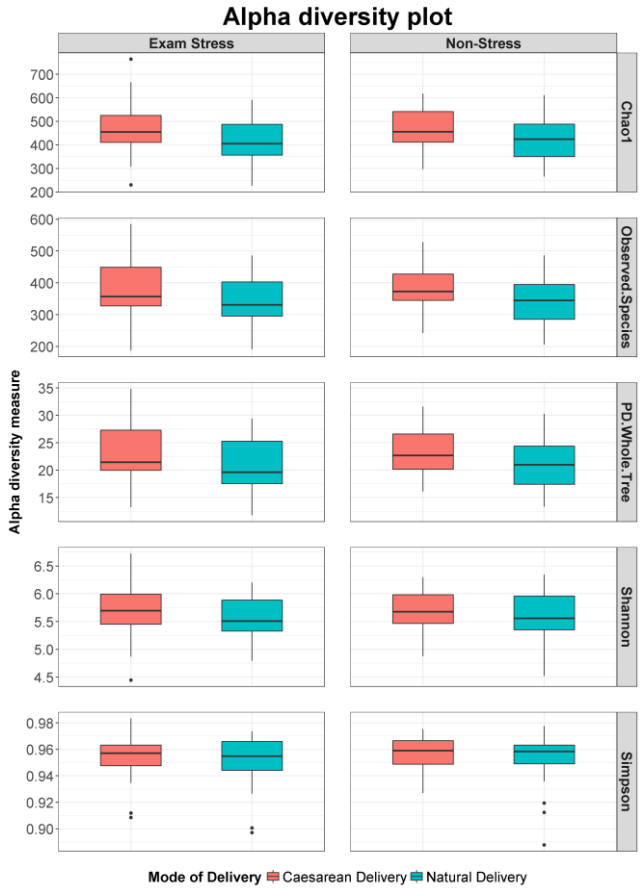

Figure 4.5
Alpha diversity, calculated using Chao 1, Observed Species, Phylogenetic Diversity Whole Tree, Simpson index and Shannon index.

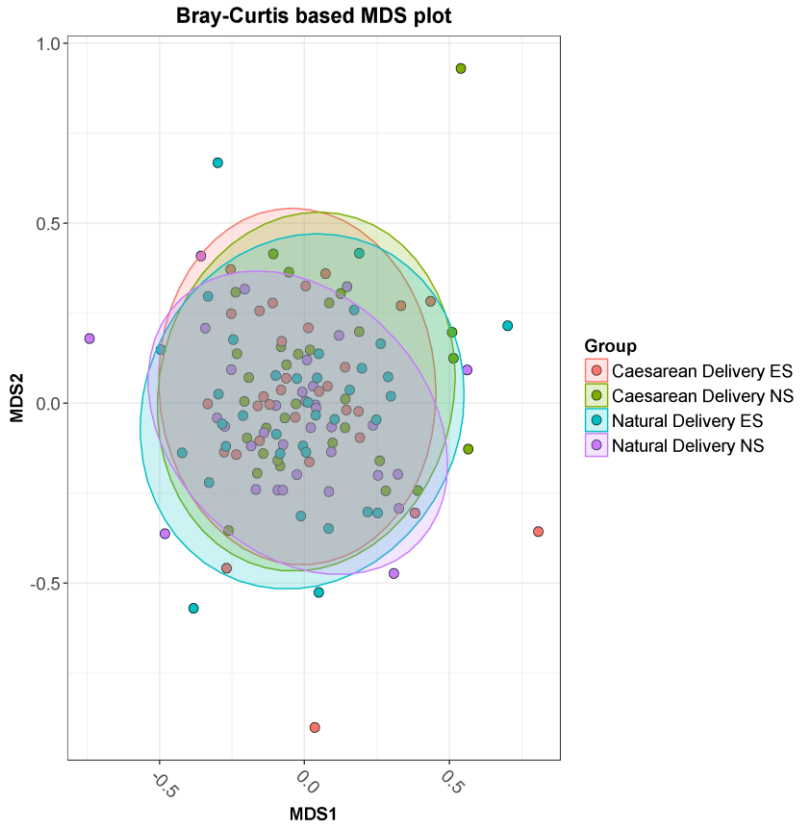

Figure 4.6
Beta diversity, represented by Bray-Curtis based multidimensional scaling (MDS) analysis of faecal microbiota composition in adult male participants. ES=Exam Stress; NS=Non-Stress

Metabolomics

Multivariate statistical analysis was applied to the urine samples collected from male participants, born by either natural vaginal delivery (VD) or C-section (CS). PCAs were applied to identify clustering and outliers. PLS-DA models were built using delivery mode as a response vector to identify a linear correlation between the two groups and their metabolic patterns. PLS-DA models were built using either samples from the Non-Stress (NS) time-point (basal state), samples from the exam stress (ES) time-point or a delta matrix (Z). This delta matrix was obtained by subtracting Non-Stress samples from exam stress samples, in order to identify differences in the metabolic response to stress relative to each individual's basal Non-Stress state.

Analyses undertaken on the Non-Stress samples did not reveal any association between birth mode and metabolic fingerprint, therefore these results are not discussed.

PCA analysis on the exam stress samples showed a tendency for the CS group to cluster and separate from the VD group along the first component, which describes 12% of the metabolic variation (Figure 4.7A). The O-PLS-DA model (Figure 4.7B) computed using the metabolic stressed state profiles and the mode of delivery as a response vector showed a positive Q^2Y value (0.1721) associated with a relatively good goodness of fit (R^2Y=0.61). The OPLS-DA model was validated by 500 random permutations, resulting in a p-value of 0.0160, which indicates the robustness of the model.

Analysis of the loadings plot derived from the validated OPLS-DA model indicated that most metabolites were moderately contributing to the separation between the VD and CS groups. Among these, the strongest correlation values were

trimethylamine (TMA), which was associated with the VD group and dimethylamine, which was associated with the CS group (Figure 4.8).

Trimethylamine (TMA) takes its source directly from the diet and is produced as a result of microbial metabolism from amines such as choline, betaine or carnitine. In the gut, TMA can then be converted to either dimethylamine (DMA) or trimethylamine N-oxide (TMAO) by distinct microbes. Similarly, once TMA is absorbed through the intestinal barrier and reaches the liver, its main fate is to be oxidised by FMO3, although a small minority can be demethylated into DMA but the latter has been shown to be negligible in humans. As a consequence, it can be concluded that DMA is mostly from microbial metabolism origin.

A comparison between the two groups of TMA/DMA ratios can therefore give an indication of the ability of gut microbes to demethylate TMA, which was associated with vaginal delivery. Hence, the association of DMA with CS volunteers indicates that demethylation was increased compared to the VD group. We therefore calculated the relative amounts of TMA/DMA in each group (Figure 4.9A). This indicated that there was a tendency for the VD samples to be associated with higher TMA/DMA, although this difference was not statistically significant with a risk alpha of 5% (t test p-value=0.0627).

We then questioned whether these ratios could be associated with a specific gut microbial profile and performed an OPLS (Figure 4.9B) model computed using taxa counts as X matrix and TMA/DMA ratio values as Y response vector. However, this model had poor predictability and was classed as non significant.

The statistical analysis summarised here allowed to evaluate whether birth delivery modality was associated with different metabolic profiles in stressed state (ES samples). The PCA analysis showed a tendency of CS group to cluster and separate

from the VD groups, which was also supported by a robust OPLS-DA model (p-value= 0.0160; $Q^2Y = 0.1721$; $R^2Y = 0.61$). The analysis of the corresponding loadings plot to identify the metabolic variations driving the model revealed a complex mixture of metabolites. Trimethylamine (TMA) and dimethylamine (DMA) were identified as key metabolites involved in this model, as they were associated with the vaginal-born group and the Caesarean-born group, respectively. TMA conversion to trimethylamine N-oxide (TMAO) or DMA is driven by different microbial metabolic pathways. Differences in the TMA/DMA ratio between the two groups ($p = 0.06$) is therefore indicative of distinct microbiota metabolic activity but a regression using individual's microbial composition and TMA/DMA values as response vector failed to link any specific microbial profile to this ratio.

A similar statistical approach was applied to a Z matrix obtained by subtracting the NS baseline from ES samples. The PCA analysis showed a tendency to separate between the VD and CS groups (Figure 4.10A) along the first components and the O-PLS-DA model (Figure 4.10B) built on the same matrix using the delivery mode as response vector gave a positive Q^2Y value (0.2250) associated with a relatively good value of goodness of fit ($R^2Y = 0.59$). The strength of the model was validated by 500 random permutations test (p-value=0.012). The correlation coefficients plot of the validated OPLS-DA model was computed to identify the metabolic contribution driving the separation. The loading plot resulted in a complex mixture of metabolites of low to medium correlation. An identifiable metabolite was DMA, correlated with the CS group (Figure 4.11). Compared to the ES dataset, the difference of TMA/DMA ratios between the two groups was less pronounced, which was mostly due to a higher variability in the VD group (Figure 4.12A). A PLS regression model failed to identify

any linear relationship between TMA/DMA variations and their gut microbial composition (Figure 4.12 B).

The statistical analysis summarised here allowed to evaluate whether the delivery modality was associated with a different metabolic response between the two investigated groups (VD and CS) in stressful situations. The analysis showed a tendency to cluster VD and CS groups and the O-PLS-DA model (p-value=0.012) built on the same matrix using the delivery mode as response vector gave a positive Q^2Y value (0.2250) associated with a relatively good goodness of fit ($R^2Y = 0.59$), which indicates that post stress urinary metabolic profiles were affected by the method of delivery in some individuals. This is consistent with the results obtained for the analysis of ES samples only. An identifiable metabolite was DMA, correlated with the CS group, which was also consistent with the ES dataset. The OPLS-DA model failed to reveal any linear relation between TMA/DMA ratios in these two groups and their gut microbial composition.

A similar approach to urine samples was applied for the analysis of faecal water samples. PCA and O-PLSDA models were built using either only the samples collected in the Non-stress (NS) condition (basal state), in the Exam stress (ES) condition or a Z matrix obtained by subtracting NS samples from ES samples. PCA analyses did not show any clustering of the two groups and all OPLS-DA models returned negative Q^2Y values, indicating poor model predictability. Overall, multivariate statistical analysis of faecal water samples of male adults did not reveal any linear variation between birth delivery modality and faecal metabolic profiles in any of the investigated conditions.

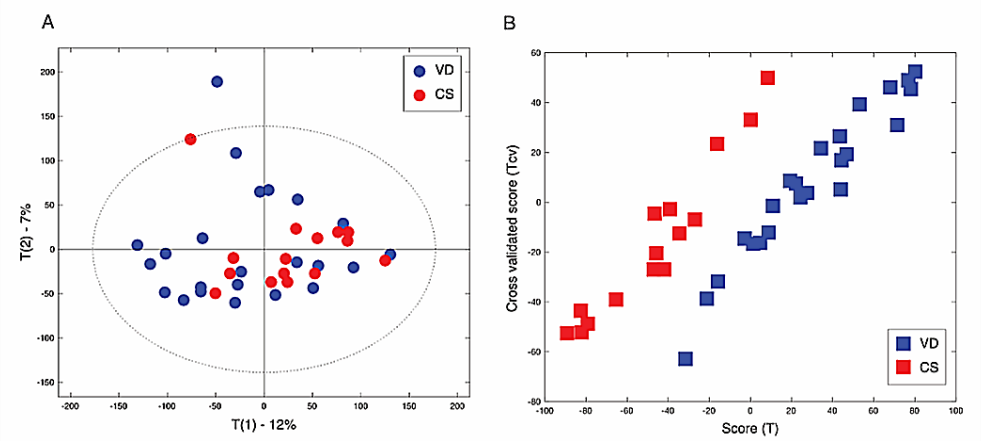

Figure 4.7 Supervised and unsupervised multivariate statistical analysis of urine metabolic profile in stressed state (ES).

A - Principal component analysis (PCA) scores plot; B - Scores plot derived from the O-PLS-DA model using delivery modality as response vector. The calculated scores (x axis) are plotted against the cross-validated scores (y axis); R^2Y = 0.61; Q^2Y = 0.1721; Blue: Vaginal-delivered individuals; Red: Caesarean delivered individuals.

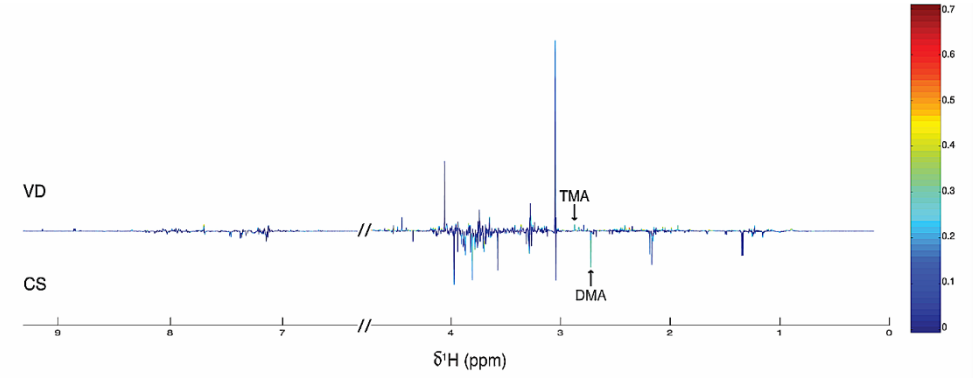

Figure 4.8 Loadings plot derived from OPLS-DA model using samples in stressed state (ES) and delivery modality as response vector. Top: VD; Bottom: CS. Key: TMA, Trimethylamine; DMA, dimethylamine.

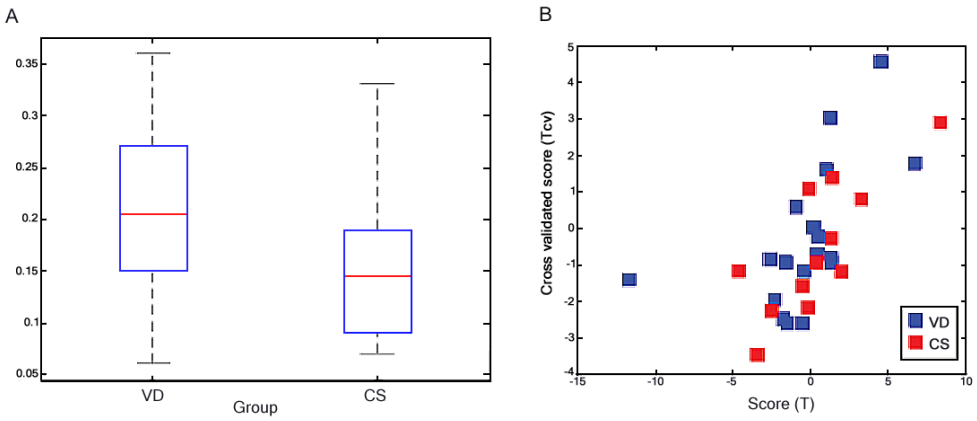

Figure 4.9 TMA/DMA values distribution and association with bacterial taxa counts in a stressed situation. A – Boxplot describing the distribution of TMA/DMA values of VD and CS groups in stressed metabolic state; B - OPLS model using taxa counts as matrix of independent variables and TMA/DMA ratios as response vector. The calculated scores (x axis) are plotted against the cross-validated scores (y axis); R^2Y = 0.5537; Q^2Y = -0.1707; Blue/VD: Vaginal-delivered individuals; Red/CS: Caesarean delivered individuals.

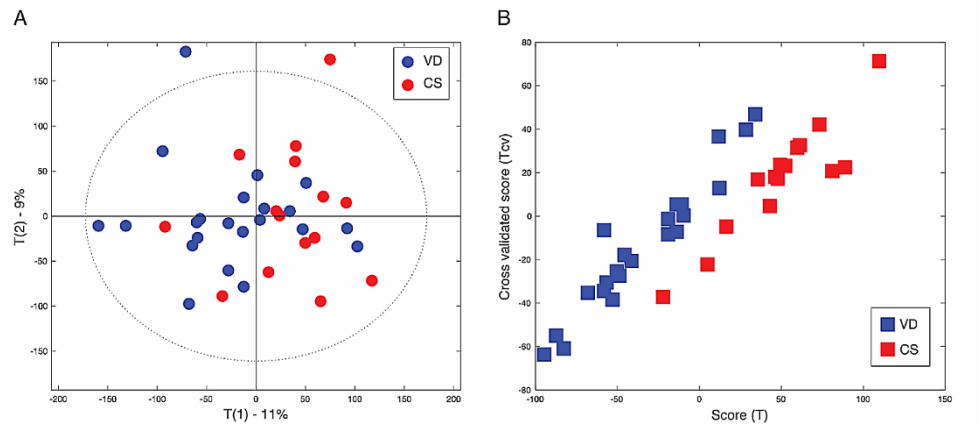

Figure 4.10 Multivariate statistical analysis of urine metabolic profile variation in response to stress. A - Principal component analysis (PCA) scores plot; B - Scores plot derived from the O-PLS-DA model using delivery modality as response vector. The calculated scores (x axis) are plotted against the cross-validated scores (y axis); R^2Y = 0.59; Q^2Y = 0.2250; Blue/VD: Vaginal-delivered individuals; Red/CS: Caesarean delivered individuals.

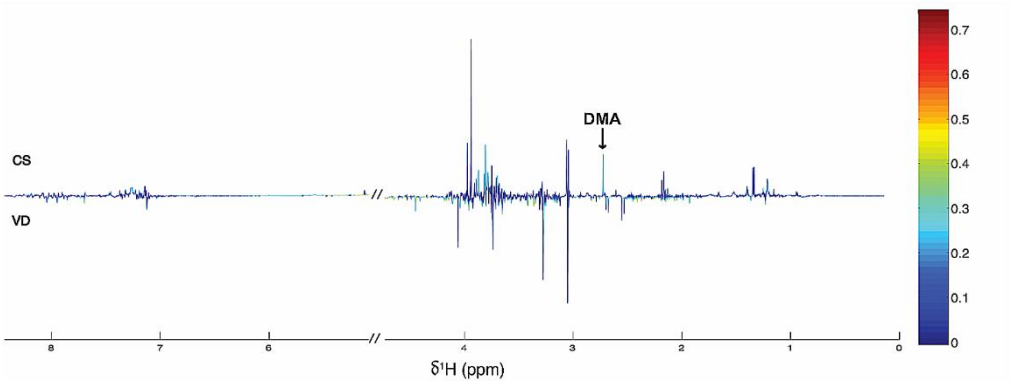

Figure 4.11 Loadings plot derived from OPLS-DA model using a matrix obtained by subtraction of NS basal metabolic profile from the ES stressed profile and delivery modality as response vector. Dimethylamine (DMA) was the metabolite with the strongest association with CS group.

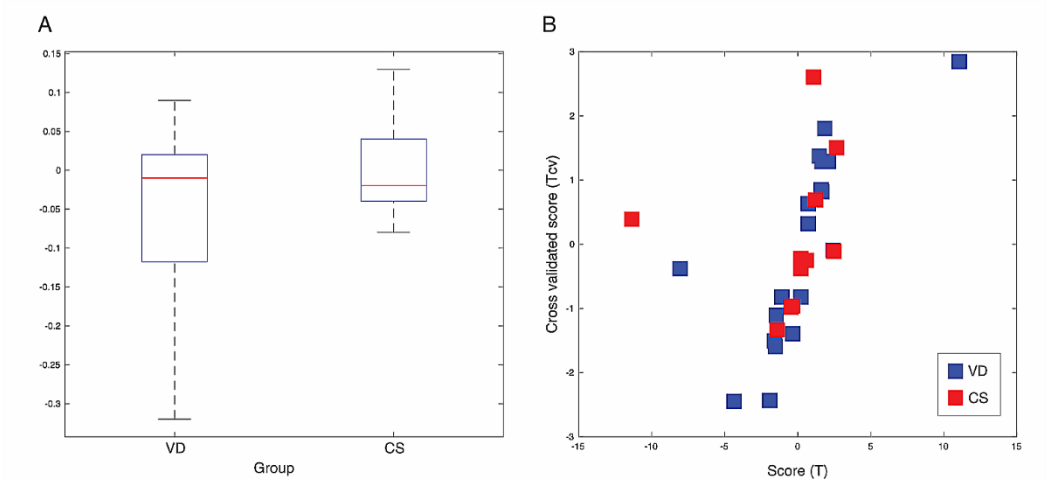

Figure 4.12 TMA/DMA values distribution and association with bacterial taxa counts in response to stress. A - Boxplot describing the distribution of TMA/DMA values in VD and CS groups; B - OPLS-DA model using taxa counts as matrix of independent variables and TMA/DMA ratios as response vector. The calculated scores (x axis) are plotted against the cross-validated scores (y axis); R^2Y (variation of X explained by the model)= 0.7885; Q^2Y (cross-validated predicted percentage of the response Y)= -0.0149; Blue/VD: Vaginal-delivered individuals; Red/CS: Caesarean delivered individuals.

Psychological Measures

In order to assess the impact of altered gut microbiota during early life (due to mode of delivery at birth) on cognition, endocrine and immune response to laboratory based public speaking stress (Trier Social Stress Test), and a 3-week period of naturalistic (university examination) stress, during young adulthood, saliva, blood, urine and stool samples were collected from all participants during the acute stress exposure (Laboratory based) while a subset of participants (38 natural-born and 32 C-section) further completed a visit during a period of naturalistic stress (university exam period).

To determine the HPA axis response to acute psychosocial stress, saliva samples were collected at seven time-points throughout the TSST; t -45, t0, t+20, t+35, t+50, t+65, t+80. C-section participants did not exhibit a differential salivary cortisol response to the TSST (Figure 4.14 A). Plasma levels of IL-7, IL-10, IL-1β, IL-6, IL-8, and TNF-α were assayed in duplicate from blood collected throughout the acute stress protocol (TSST). Concentrations of IL-1β (Figure 4.14 B) and IL-10, but not IL-6, IL-8 or TNF-α (Figure 4.15; A-D), were significantly higher in C-section subjects compared with vaginally-born participants throughout the testing period, although this was due to elevated baseline levels and not acute stress. However, the anti-inflammatory cytokine IL-10 was significantly elevated during the Exam Stress period in C-section participants (Figure 4.16 A). There was no group difference in IL-1β, IL-6 or IL-8 (Figure 4.16; B-D). Marginally elevated levels of TNF- α were observed in the C-section participants during the Non-Stress and Exam Stress periods compared with vaginally-born participants (Figure 4.16 E).

Measures of positive affect were significantly lower throughout the procedure (Figure 4.17 A), although negative affect was not (Figure 4.17 B). Interestingly,

participants born by C-section reported greater psychological stress in response to the TSST when compared to vaginally-born participants (Figure 4.17 C). To further examine the effect of exam stress on HPA axis function, the salivary cortisol awakening response (CAR) was measured at each time-point (Non-Stress and Exam Stress), but this was not significantly different between C-section and vaginally-born participants (Figure 4.17 D).

When comparing psychological distress levels during the Non-Stress and Exam Stress periods, participants born by C-section reported significantly greater levels of trait anxiety, perceived stress, but not depression when compared to vaginally-born participants, during the Exam Stress period but not during the Non-Stress period (Figure 4.19). To determine the effect of stress on cognitive function, tasks from the CANTAB battery of cognitive tests were used at each time-point (Non-Stress and Exam Stress). Interestingly, we identified no difference in cognitive performance on tests of visuospatial memory, response inhibition attentional flexibility or reversal learning between C-section and vaginally-born participants during the Non-Stress or Exam Stress period (Figure 4.19).

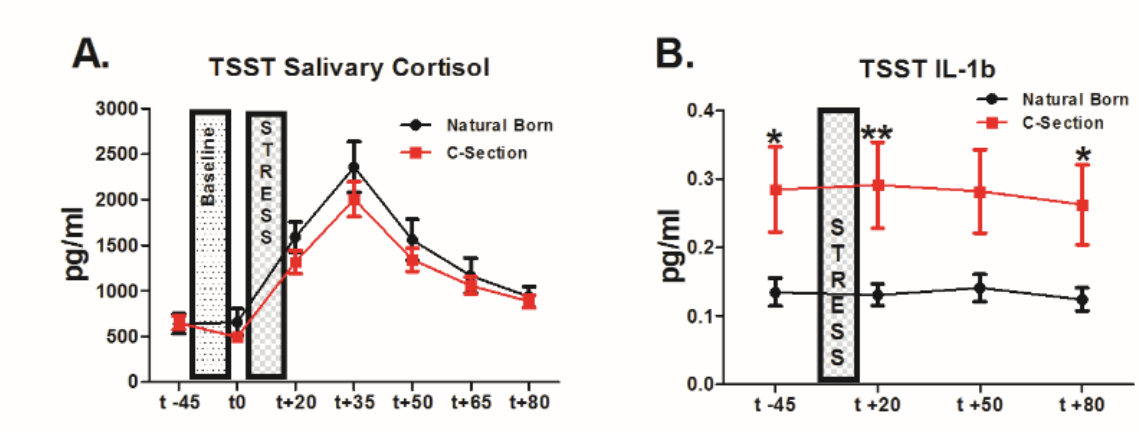

Figure 4.14 No stress differences were seen between participants during the TSST as measured by salivary cortisol; however, elevations in IL-1b were seen in C-section participants during the procedure

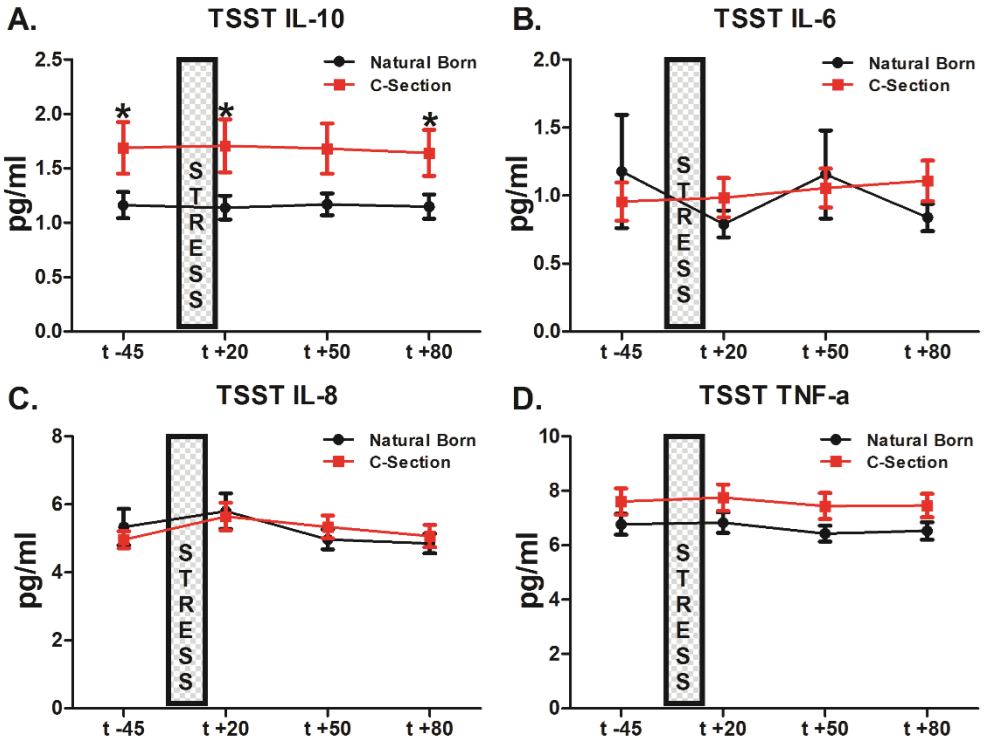

Figure 4.15 The TSST had no differential effect on immune activity in C-section participants in response to the TSST procedure.

A) Levels of the anti-inflammatory cytokine IL-10 are elevated in C-section participants from baseline and across time-points

B) IL-6

C) IL-8

D) TNF-α

(Post hoc comparisons using Bonferroni correction: *p> 0.05; **p< 0.01. Data are presented as mean ± standard error of the mean (SEM)).

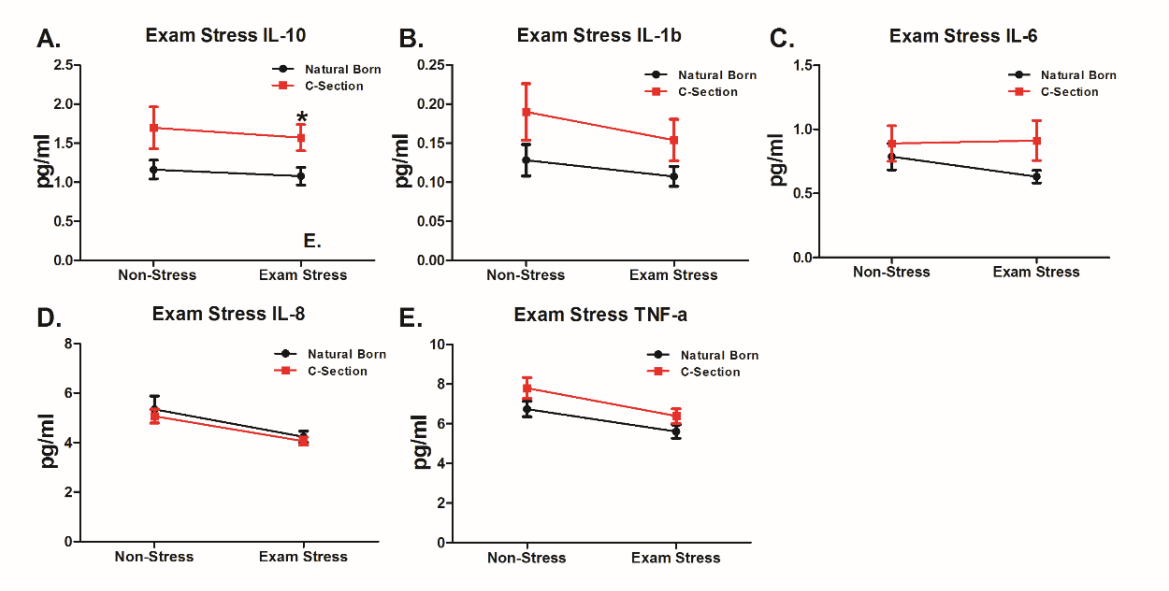

Figure 4.16 Exam stress causes elevated levels of the anti-inflammatory cytokine IL-10 in C-section participants.

A) IL-10

B) IL-1β

C) IL-6

D) IL- 8

E) TNF-α

Data are presented as mean ± standard error of the mean (SEM).

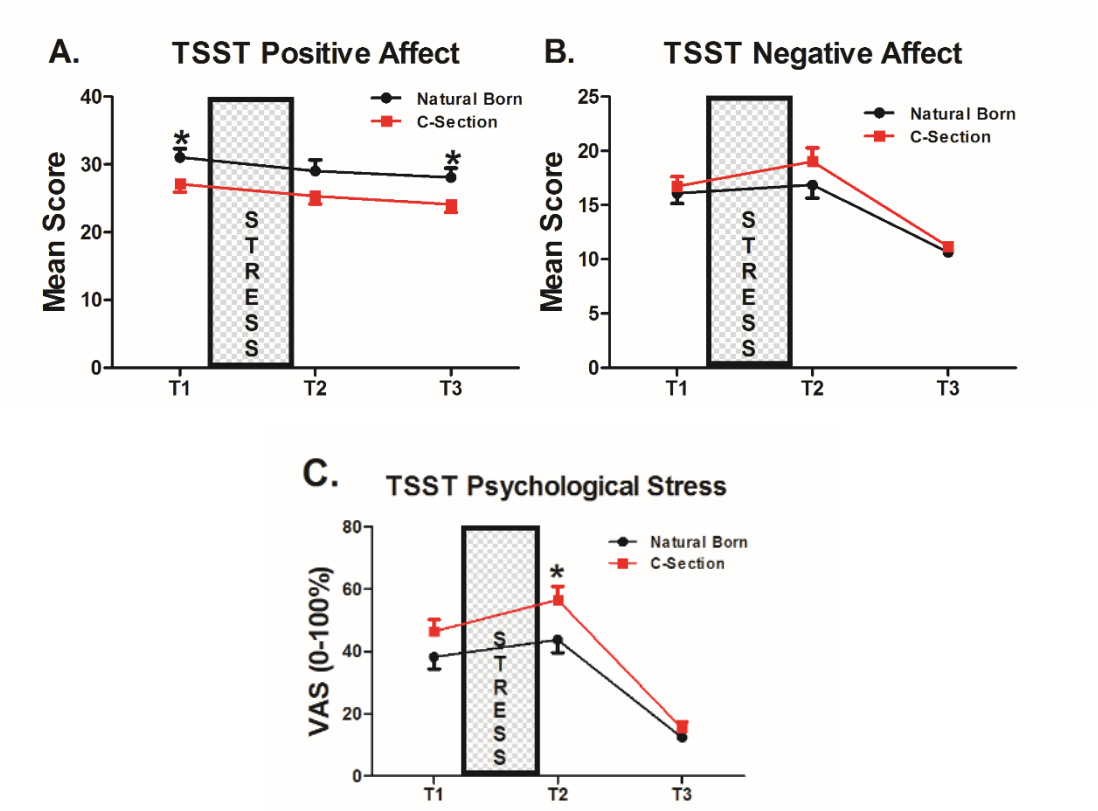

Figure 4.17 Positive affect is lower in C-section participants throughout the TSST procedure. C-section participants also reported increased psychological stress

A) Positive Affect response as measured using the Positive and Negative Affect Schedule

B) Negative Affect response to the TSST procedure as measured using the Positive and Negative Affect Schedule

C) Psychological Stress

Data are presented as mean ± standard error of the mean (SEM).

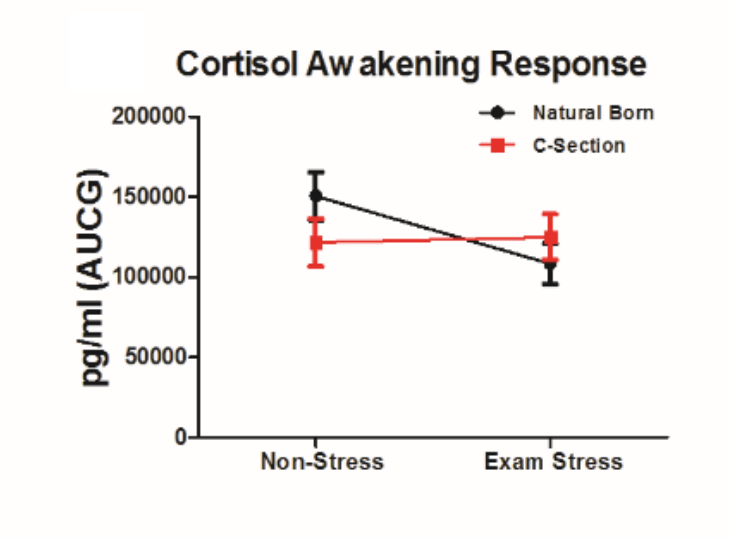

Figure 4.18 Exam stress has no effect on HPA axis function as measured by the cortisol awakening response

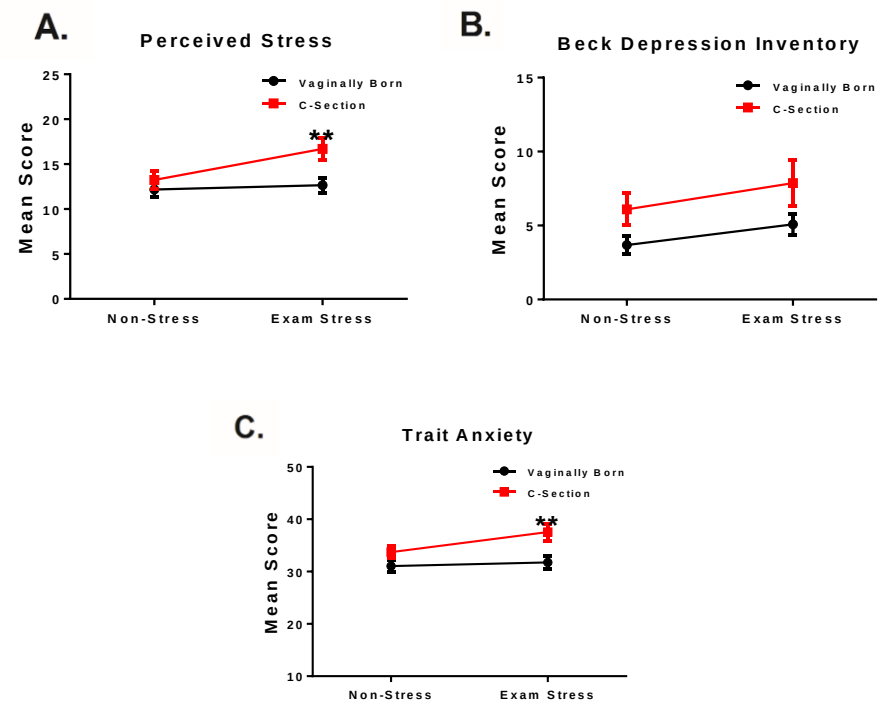

Figure 4.19 Stress response and mood during Non-Stress and Exam Stress periods

A) Psychological stress as measured using the Perceived Stress Scale showing a significant increase in perceived stress levels in the CS delivered participants during exam stress

B) Depression levels as measured using the Beck Depression Inventory-II. No differences were noted between groups at either time-point

C) Trait anxiety levels as measured using the State Trait Anxiety Inventory showing increased trait anxiety level in CS delivered participants during the exam stress period

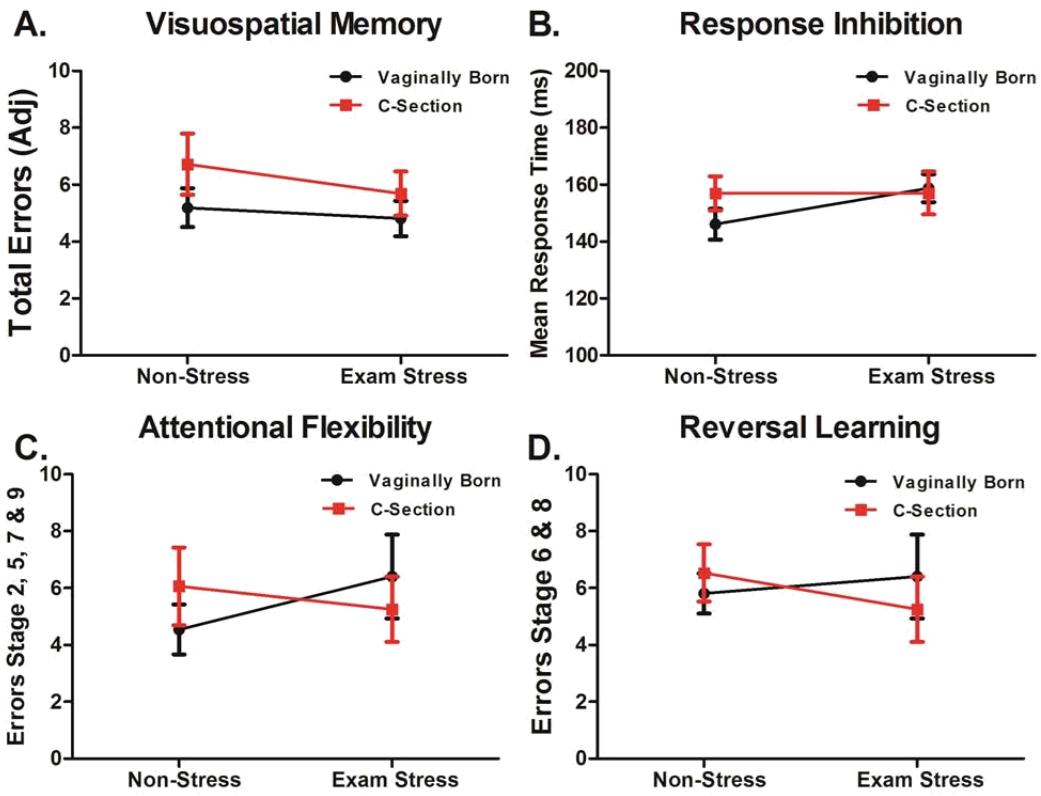

Figure 4.20 Cognitive function did not differ in response to exam stress in C-section participants.

A) Visuospatial memory as measured by the Paired Associates Learning (PAL) test

B) Response inhibition as measured by the Stop Signal Task (SST), stop signal reaction time (SSRT; calculated for last 20 sub-blocks)

C) Attentional Flexibility as measured by the Intra-Extra Dimensional Set Shift (IED)

D) Reversal Learning as measured by the IED

Data are presented as mean ± standard error of the mean (SEM).

Discussion

Mode of delivery is a significant factor affecting the microbiota composition of infants (Brestoff & Artis, 2013; Makino et al., 2013). Disturbances in the appropriate establishment of the microbiota composition at the beginning of life have been implicated in long-term programming of health and in increasing susceptibility of immune and metabolic disorders (Brestoff & Artis, 2013). Here, we report that the known negative outcomes of C-section can include lasting changes in behaviour and stress sensitivity, demonstrated by increased psychological distress and anxiety in healthy young adults born by C-section.

We demonstrate that, by adulthood, all differences in gut microbiota which would have occurred due to differing birth modes (Dominguez-Bello et al., 2010; Hill et al., 2017) are resolved in this adult human population. It was found that the gut microbiota of participants delivered by C-section did not differ from those born vaginally. In terms of bacterial culture counts, there were no differences noted between microbial abundances based on stress or birth mode. This was also true for compositional sequencing and diversity analyses. It is interesting to note that while the faecal metabolic profiles of the participants did not vary based on delivery mode, the urinary metabolic profile indicated that the diversity of metabolic responses to stress was partially influenced by birth mode. As there was no correlation between the metabolic profile and birth mode during the Non-Stress period, this supports the hypothesis that delivery mode has an influence on the individual response to stress. The poor correlation between gut microbial profiles and urinary metabotypes indicates that this distinction may be due to the collegial action of multiple microbes composing the gut ecosystem, or that faecal gut microbial profiles used in the PLS regression

models failed to represent the colonic ecosystem associated with this specific metabolism.

The lack of changes detected in the gut microbiota profiles of these individuals supports previous work describing progression towards normalisation of gut microbiota profile in C-section babies by 24 weeks post-birth and four years (Fouhy et al., 2019; Hill et al., 2017). However, altered microbiome composition at critical periods during early life, at a time during which the central nervous system is in a state of rapid development, has been negatively implicated in a number of behavioural changes in both animals (O'Mahony et al., 2017) and humans (Carlson et al., 2018; Christian et al., 2015).

It is therefore interesting to note, that while the alterations in microbial composition have recovered in the young adult cohort, negative psychological effects of C-section endure. These preliminary data from healthy human volunteers, implies that mode of delivery at birth has a long-lasting effect on immune and stress responses. As previously mentioned, the gut microbiota composition of the participants in both groups is indistinguishable (Figures 4.4-4.6). However, high levels of IL-1β and IL-10 noted in C-section participants support a dysregulation of immune-brain signalling in regulating behaviour, possibly as a consequence of altered immune priming at birth, as certain early microbiome colonisers can influence the maturation of the immune system, including promoting production of certain cytokines (Dominguez-Bello et al., 2010).

Measures of positive affect in the human cohort were significantly lower throughout the acute stress procedure and these individuals reported greater psychological stress in response to the TSST. Perhaps surprisingly, there is a dissociation between self-reported stress measures and cortisol output in the TSST.

However, there are a large number of studies which have not reported any relationship between self-reported stress and cortisol output (Campbell & Ehlert, 2012).

To probe this dysregulation in stress sensitivity we took advantage of a naturalistic stressor, University examination stress, and found that the anti-inflammatory cytokine IL-10 was significantly elevated during the Exam Stress period in C-section participants and levels of TNF-α were increased. Individuals born by C-section also reported significantly greater levels of trait anxiety and perceived stress during the Exam Stress period but not during the Non-Stress period.

Given the importance of mode of delivery in microbiota composition and subsequent immune and HPA axis priming, it is tempting to speculate that it may be causally related to the changes observed. However, the nature of the current study design does not allow us to investigate factors that are responsible for such changes in this cohort as they occurred >20 years prior to testing.

Future studies investigating the role of mode of delivery on the gut microbiome and subsequent long-term psychological effects should also include additional details on participants, for example, rationale behind CS delivery, details of complicated pregnancy, comprehensive maternal information i.e., stress, anxiety, obesity, to allow for various microbiome influences to be considered. Additionally, examining the role of sex and birth order may be interesting to explore in future similar studies.

Together, these findings raise significant concerns regarding the increased use of C-section deliveries due to the long-term implications for psychological vulnerability, immune parameters, and cognition. However, as C-section deliveries are frequently unavoidable lifesaving interventions, the possibility of developing microbiota-targeted therapies to alleviate any long-term negative consequence on stress responses, physiology and behaviour is an attractive prospect.

Acknowledgements

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References

Allen, A. P., Kennedy, P. J., Cryan, J. F., Dinan, T. G., & Clarke, G. (2014). Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. *Neuroscience & Biobehavioral Reviews, 38*, 94-124.

Beck, A. T., Steer, R. A., & Brown, G. (1996). Beck depression inventory–II. *Psychological Assessment*.

Ben-Ari, Y. (2013). Neuropaediatric and neuroarchaeology: understanding development to correct brain disorders. *Acta Paediatrica, 102*(4), 331-334.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in Molecular Medicine, 20*(9), 509-518.

Brestoff, J. R., & Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nature immunology, 14*(7), 676.

Campbell, J., & Ehlert, U. (2012). Acute psychosocial stress: does the emotional stress response correspond with physiological responses? *Psychoneuroendocrinology, 37*(8), 1111-1134.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2009). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics, 26*(2), 266-267.

Carlson, A. L., Xia, K., Azcarate-Peril, M. A., Goldman, B. D., Ahn, M., Styner, M. A.,... Knickmeyer, R. C. (2018). Infant gut microbiome associated with cognitive development. *Biological psychiatry, 83*(2), 148-159.

Christian, L. M., Galley, J. D., Hade, E. M., Schoppe-Sullivan, S., Dush, C. K., & Bailey, M. T. (2015). Gut microbiome composition is associated with temperament during early childhood. *Brain, behavior, and immunity, 45*, 118-127.

Cohen, S., Kamarck, T., & Mermelstein, R. (1983). A global measure of perceived stress. *Journal of health and social behavior*, 385-396.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences, 107*(26), 11971-11975.

Edgar R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England), 26*(19), 2460–2461.

Fouhy, F., Watkins, C., Hill, C. J., O'Shea, C.-A., Nagle, B., Dempsey, E. M.,... Stanton, C. (2019). Perinatal factors affect the gut microbiota up to four years after birth. *Nature communications, 10*(1), 1517.

Graham, J. W. (2009). Missing data analysis: Making it work in the real world. *Annual review of psychology, 60,* 549-576.

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A.,... & Stanton, C. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome, 5*(1), 1-18.

Little, R. J. (1988). A test of missing completely at random for multivariate data with missing values. *Journal of the American statistical Association, 83*(404), 1198-1202.

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963.

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T.,... Knol, J. (2013). Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. *PloS one, 8*(11), e78331.

O'Mahony, S. M., Clarke, G., Dinan, T., & Cryan, J. (2017). Early-life adversity and brain development: Is the microbiome a missing piece of the puzzle? *Neuroscience, 342,* 37-54.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I.,... Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511-521.

Pruessner, J. C., Kirschbaum, C., Meinlschmid, G., & Hellhammer, D. H. (2003). Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology, 28*(7), 916-931.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research, 41*(Database issue), D590–D596.

Spielberger, C. D., & Gorsuch, R. L. (1983). *State-trait anxiety inventory for adults: sampler set: manual, test, scoring key*: Mind Garden.

Watson, D., Clark, L. A., & Tellegen, A. (1988). Development and validation of brief measures of positive and negative affect: the PANAS scales. *Journal of personality and social psychology, 54*(6), 1063.

Yu, Z., & Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques, 36*(5), 808-812.

CHAPTER 5

A PLACEBO-CONTROLLED INTERVENTION STUDY OF BIFIDOBACTERIUM LONGUM 1714™ IN EXAM STRESS

CHAPTER CONTRIBUTIONS:

Amy Murphy

- planned sample quantity and processing methods,
- collected all stool samples,
- carried out DNA extraction,
- planned and prepared 16 S compositional sequencing library,
- planned and carried out culture-based work and isolated specific microbes,
- planned and carried out statistical analyses,
- significantly contributed to manuscript preparation.

Bioinformatic analysis was completed by Dr. Fiona Fouhy

Dr. Caitriona Long-Smith is responsible for study design, psychological measures and analyses

Ms. Daniëlle Dorland conducted psychological analyses and contributed to manuscript preparation

THIS STUDY IS PUBLISHED IN TRENDS IN BRAIN, BEHAVIOUR & IMMUNITY – HEALTH, 2020

Abstract

Communication pathways between the brain and the gut have received significant attention in recent years. Additionally, it has been shown that the microbes residing in the gut can affect the brain and have an impact on several neurological processes. This has led to the emergence of the microbiota-gut-brain axis, and further examination of the mechanisms involved in communication between the gut microbiota and the CNS and implications for brain health.

Indeed, microbiota-gut-brain axis disruption has been implicated in a variety of psychological disorders. Prebiotics and probiotics have gained significant interest in recent years as possible therapeutic strategies to alleviate the effects of microbiota disturbances on neurological processes. The aim of the current study was to examine the impact of treatment with a probiotic, *Bifidobacterium longum* 1714™, on stress, cognition and mood, in a healthy population. A randomised, placebo-controlled, repeated measures, cross-over intervention was used. Measures focusing on stress, mood and cognition were conducted. In addition, stool samples were also collected to examine the gut microbiota composition in response to probiotic treatment.

Mood was found to deteriorate during the naturalistic stressor for both probiotic and placebo groups. However, significant changes in the participants' sleep were noted between the groups. It was found that, during exam stress, while sleep quality deteriorated in those individuals consuming the placebo, it remained stable in participants who had received the probiotic.

Introduction

It has become apparent that the gut microbiota, and consequently, the microbiota-gut-brain axis, plays a significant role in maintaining and influencing brain health (Sampson & Mazmanian, 2015). Several studies have shown the importance of a normal microbiota composition with respect to neurodevelopment and behaviour (Dinan & Cryan, 2016). Indeed, alterations in microbiota composition can have significant effects on physiology and host health (Cryan & Dinan, 2012). As this has become more apparent, the use of probiotics to potentially target the microbiota-gut-brain axis and influence brain function and behaviour has emerged as an interesting therapeutic avenue to explore.

The use of probiotics to alleviate the effects of gut microbiota disruption on psychological processes has increased recently, with most clinical interventions focused on mood, stress and cognition. However, most probiotic studies to date have been conducted animal models, rather than in human subjects. While treatment with probiotic strains in animal models is important to examine the potential effects on gut microbiota composition and on psychological processes, it is important to recognise that many preclinical findings are not translated in human subjects. Promising probiotic interventions in animal models cannot always be corroborated in clinical studies, the use of a psychobiotic, *Lactobacillus rhamnosus* JB-1 which had previously been shown to affect behaviour in a preclinical study, did not modify any related behaviours in a healthy male cohort (Kelly et al., 2017).

This study investigated the use of a probiotic on chronic stress. Exam stress was used as a naturalistic stressor. Utilising the period of exam stress as a representation of chronic stress has been used previously and provides an accurate

representation of chronic stress responses on psychological and immunological parameters (Zunhammer et al., 2013).

This intervention was carried out for between seven and nine weeks leading up to the participants' university exams. Several self-report measures and biological analyses were used to examine stress, mood and cognition during the exam stress periods. Stool samples were also collected to examine the gut microbiota composition. While mood and cognition were not statistically significantly different between probiotic and placebo groups, a difference was noted in the subjects' sleep quality. Additionally, no differences were seen in the gut microbiome composition in response to treatment with *B. longum* 1714™.

Materials and Methods

Participants and Sample Collection

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals and conducted in accordance with the ICH Guidelines on Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all participants at the screening visit, before any study procedures were conducted. Participants were free to withdraw from the study at any time. The main inclusion criteria were; that the participant be between 18 and 30 years of age; be male; be in generally good health as determined by the investigator. Exclusion criteria included being less than 18 and greater than 40 years of age; having a significant acute or chronic illness; having a condition or taking a medication that would interfere with the objectives of the study, pose a safety risk or confound the interpretation of the study results. From the 30 participants who were included, 20 completed the study.

This study was a double-blind, randomised, placebo-controlled, repeated measures, cross-over design. Participants were screened before the beginning of the intervention and asked a variety of standard demographic and health questions. Participants were also screened using the common self-report measures (see Table 5.1 for an overview). Following screening, participants were assigned to two groups. One group of participants were randomly assigned to either the placebo group (maltodextrin) or the probiotic group, while the second group received the opposite during the first interveention period. This was reversed for the second invertention period in a cross-over, double-blind study design. Both intervention periods lasted for 7-9 weeks. The products (placebo

and intervention) were both taken in once a day in capsule form, with the probiotic containing 1 x 10^9 CFU.

The study visits involved collection of various samples and measurements, including saliva, blood, sand samples and several self-report measures examining mood, food intake, stress and sleep quality. The CANTAB was used to examine cognitive performance.

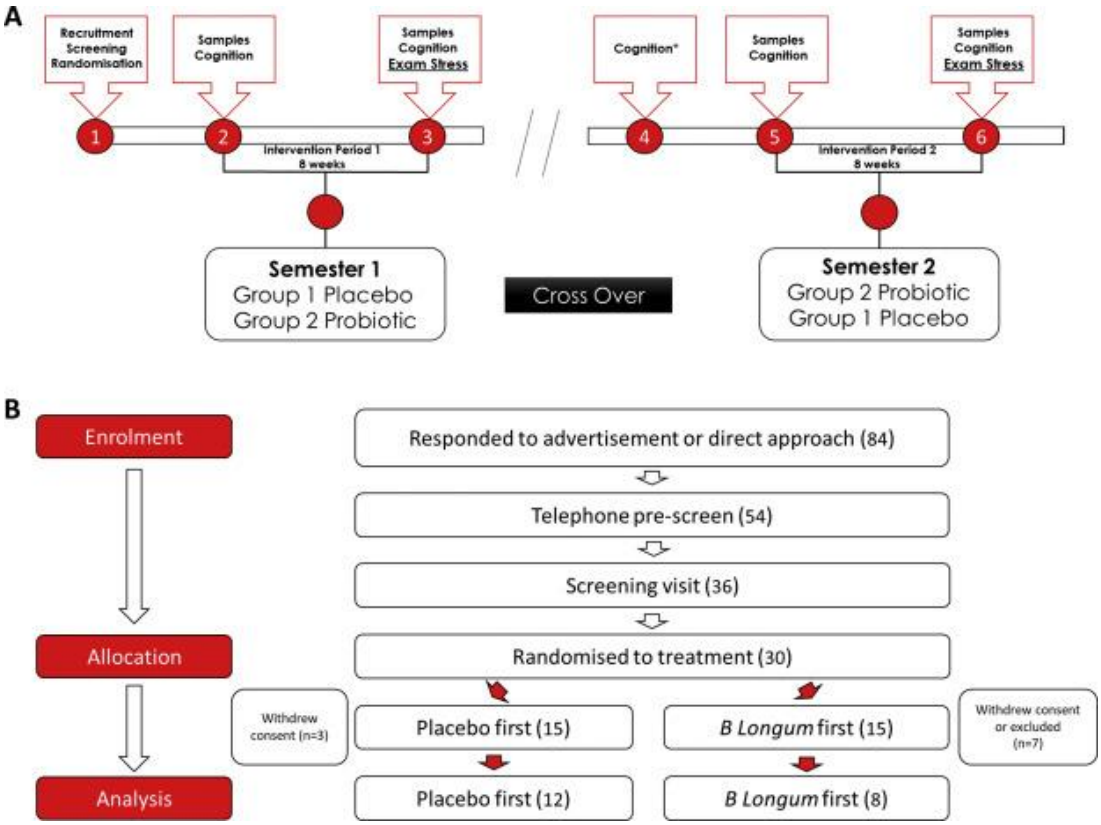

Figure 5.1 Timeline of the intervention

Procedure	Visit 1 Screen	Visit 2 Baseline 1	Intervention Phase 1	Visit 3 Phase 1 End	Phase 1 Follow-up	Visit 4	Visit 5 Baseline 2	Intervention Phase 2	Visit 6 Phase 2 End	Visit 7 Intervention 2 Follow-up
Informed consent	X									
Inclusion/ exclusion	X									
General medical history	X									
Demographic data	X									
MINI International Psychiatric Interview	X									
Self-report scales (Childhood Trauma Questionnaire-Short Form/ TIPI/ ROME III / Cambridge Behaviour Scale/ Interpersonal Reactivity Index/ Autism Quotient / Handedness / WAYS)	X									
Cognitive Assessment (NART)	X									
Physical exam		X		X			X		X	
Heart rate, heart rate variability & galvanic skin response		X		X			X		X	
Self-report scales (Hopkins Symptom Checker / Hospital Anxiety and Depression Scale / Beck Depression Inventory-II/State-Trait Anxiety Inventory/ Perceived Stress Scale / Hassles and Uplifts/ Pittsburgh Sleep Quality Index/International Physical Activity Questionnaire/ Positive and Negative Affect Schedule/ GI symptoms VAS/ Bristol Stool Chart)		X		X	X		X		X	X
Food Frequency Questionnaire	X			X					X	
Cognitive Assessment (CANTAB /Reading the Eyes in The Mind Test)	X (Practice)	X		X		X (Practise)	X		X	
Intervention (probiotic or placebo)			X					X		
Self-Report Scales during Intervention (Perceived Stress Scale, Positive and Negative Affect Schedule, GI symptoms VAS)			X					X		
Exam stress				X					X	
Self-Report Scales During Exam stress (Primary Appraisal Secondary Appraisal/ Positive and Negative Affect Schedule/ Bond-Lader visual analogue mood rating scales)				X					X	
Stool sample		X		X			X		X	
Hair sample		X		X			X		X	
Blood sample (5 x 4ml; 1 x 10ml; droplet)		X		X			X		X	
Urine sample (Metabolomics)		X		X			X		X	
Saliva sample (x8 for cortisol awakening response collected in 2 consecutive mornings (4 samples each morning))		X		X					X	
Adverse events		X	X	X	X		X	X	X	X
Concomitant Medications Record	X	X		X		X	X		X	
Intervention Palatability				X					X	

Table 5.1 Various measures collected from the participants throughout the intervention

Culture-Dependent Analysis

Fresh faecal samples were weighed and serially diluted in maximum recovery diluent (Fluka, Sigma Aldrich, Ireland) from 10^{-1} to 10^{-8}. Bifidobacteria were enumerated by spread-plating serial dilutions onto de Man, Rogosa, Sharpe (MRS) agar (Difco, Becton-Dickenson Ltd., Ireland), which had been modified by adding 0.05% L-cysteine hydrochloride (Sigma Aldrich, Ireland), 100 ug/ml mupirocin (Sigma Aldrich, Ireland) and 50 units of nystatin (Sigma Aldrich, Ireland). Agar plates were incubated anaerobically for three days at 37°C. *Lactobacillus* selective (LBS) agar (Difco, Becton-Dickenson Ltd., Ireland), supplemented with 50 units of nystatin was used to enumerate lactobacilli. Agar plates were incubated anaerobically for five days at 37°C. Total anaerobic bacteria were enumerated by spread-plating onto Wilkins Chalgren agar (WCA) (Sigma Aldrich, Ireland) supplemented with 50 units of nystatin and 7% defibrinated horse blood (Cruinn Diagnostics Ltd., Ireland). Agar plates were then incubated anaerobically for five days at 37°C. Brain Heart Infusion (BHI) agar supplemented with 50 units of nystatin was used to enumerate total aerobic bacteria. These were also incubated anaerobically for five days at 37°C.

DNA Extraction

DNA was extracted from adult faecal samples using the RBB method (Yu & Morrison, 2004). A 0.2 g faecal sample was weighed and added to 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm diameter sterile zirconia beads plus a single 2.5 mm diameter bead (BioSpec Products, Bartlesville, USA). To this, 1 ml of lysis buffer was added (500 mM NaCl, 50 mM tris-HCL, pH 8.0, 50 mM EDTA and 4% sodium dodecyl sulphate (SDS)). Each sample was then homogenised using a Mini-Beadbeater™, BioSpec Products,

Bartlesville, OK, USA) at maximum speed for 3 min and incubated at 70° C for 15 mins to lyse the cells. Samples were then centrifuged for 5 min at 16,000 x g and the supernatant was transferred to a fresh Eppendorf tube. The bead beating, heating and centrifugation steps were repeated using 300 µl of lysis buffer and the supernatant was pooled. Following this, 260 µl of 7.5 M ammonium acetate was added and the samples were vortexed and incubated on ice for 5 min.

Isopropanol was added to precipitate the DNA and samples were centrifuged to pellet the nucleic acid. The pellets were then washed with 70% ethanol and allowed to dry before being dissolved in 100 µl TE buffer. The DNA was treated with RNAse and Proteinase K and washed with Qiagen buffers AW1 and AW2 using columns provided in the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK).

The DNA was then eluted in 200 µl Buffer ATE. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland).

16S Compositional Sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

The protocol involved two PCR reactions on the extracted DNA. The DNA was first amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer

5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

Reverse primer

5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each reaction contained 2.5 µl genomic DNA, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following program: 95°C x 3mins, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, 72°C x 5mins and held at 4°C. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland).

Following this, a second PCR reaction was carried out on the purified DNA using two indexing primers per sample (Illumina Nextera XT indexing primers, Illumina, Netherlands). Each reaction contained 5 µl purified DNA, 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix and 10µl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. PCR products were visualised and purified as described above. Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Teagasc Food Research Centre, Moorepark, Fermoy, using standard Illumina sequencing protocols.

Microbiome Statistical Analysis

All data were analysed and graphed using either R-3.3.1, Microsoft Excel or GraphPad Prism (Version 5). Data are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out using the Kruskal-Wallis test or the Wilcoxon signed-rank test for paired data to identify significant differences between the groups. Differences were considered significant at $p < 0.05$. Correction of *p*-values was performed using the Benjamini-Hochberg method (FDR <0.05). The vegan package was used for Bray-Curtis based MDS analysis, and the Adonis function in vegan was used for PERMANOVA in beta diversity.

Bioinformatic Analysis

In brief, the 16S rRNA gene amplicon sequences were processed through a bioinformatics pipeline. Resulting 300 bp paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies; (Magoč & Salzberg, 2011)). Further sequence read processing was performed using QIIME (Version 1.8.0.) including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds (QIIME allows analysis of high-throughput community sequencing data). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH (Version 7, 64-bit, search and clustering orders of magnitude faster than BLAST; (Edgar, 2010). OTU sequences were aligned using PyNAST (PyNAST: python nearest alignment space termination; (Caporaso et al., 2009), a flexible tool for aligning sequences to a template alignment) and taxonomy was determined using the SILVA SSURef database release 111 (Quast et al., 2012), at 97% similarity. Alpha diversity estimates were calculated using

QIIME. Beta diversity was calculated using Bray-Curtis based multidimensional scaling (MDS) analysis of faecal microbiota.

Psychological Measures

As mentioned, a wide variety of psychological measures were examined throughout this intervention. However, for the purpose of this manuscript, only certain stress response measures have been selected for discussion.

Pittsburgh Sleep Quality Index

The PSQI measures the sleep quality of the participants using questions concerning sleep quality and sleep-related issues. It consists of 19 items referring to the last month (Buysse et al., 1989).

Cortisol Awakening Response

Saliva samples were stored at -80°C before being analysed using a commercially available ELISA kit (Enzo Life Sciences). The saliva samples were diluted 1:3 with assay buffer and pipetted into a 96-well goat anti-mouse IgG microtiter plate. After the addition of cortisol conjugate and mouse monoclonal antibody to cortisol, the plate was incubated on a plate shaker at room temperature for two hours. Following washing with buffer and removal of air bubbles, the plate was incubated at RT for one hour without shaking. Finally, a stop solution was added, and the plate was read using a microplate reader at 405 nm.

Statistics on psychological data

Following data imputation and transformation (if needed) to improve normality, repeated measures analysis of variance (ANOVA) with *Time* and *Treatment* as the within-subject factors for each variable was performed. Significant interaction effects were followed by *post-hoc* comparisons with paired sample t-tests using a Benjamini-Hochberg (BH) correction with a false discovery rate (FDR) of 0.10 for multiple comparisons as appropriate. Non-parametric equivalents, Friedman and Wilcoxon respectively, were used if parametric assumptions were violated. An α of 0.05 was considered significant. GraphPad Prism 5 was used to create graphs.

Results

Participant metadata

As shown in Table 5.2, there was no significant difference between groups on compliance and days on treatment. No effects of time or treatment were found for BMI, GI symptom satisfaction, GI symptom life interference, and the Bristol Stool Chart. There was a significant effect on physical activity ($\chi^2(3)=10.32$, p=0.016), expressed in metabolic equivalent (MET)-minutes per week. The higher the MET-minutes per week, the more physical activity was carried out. The Wilcoxon test showed that there was a significant decrease in total physical activity, expressed in MET-minutes/week, for placebo (Z=-3.024, p=0.002).

	Placebo baseline (N=20)	Placebo Exam (N=20)	P	Probiotic baseline (N=20)	Probiotic Exam (N=20)	P
Compliance	-	102.50	-	-	94.75	ns
Days on treatment	-	52.55	-	-	53.20	ns
BMI	24.14 (0.65)	24.26 (0.72)	ns	24.23 (0.77)	24.1 (0.71)	ns
MET-min/week	5805 (3689)	3408 (2895)	0.002	4529 (2973)	4111 (2903)	ns
GI-VAS						
-Abdominal pain (%)	0	1 (5)		0	0	
- Bloating (%)	0	1 (5)		1 (5)	2 (10)	
- Satisfaction	25.14 (4.83)	17.04 (4.67)	ns	20.35 (5.09)	22.76 (5.52)	ns
-Life interference	11.54 (2.93)	8.56 (2.91)	ns	9.35 (2.88)	9.75 (2.94)	ns
Bristol Stool Chart	**3.33 (1.34)**	**3.88 (1.51)**	**ns**	**3.32 (1.56)**	**3.50 (1.24)**	**ns**

Table 5.2 Participant metadata pre- and post-measurement.

Values are the mean score (SEM) or frequency (%). Physical activity, expressed in MET-minutes per week, decreased significantly for placebo (p=0.002). BMI: Body Mass Index; MET: metabolic equivalent; GI-VAS: Gastrointestinal Visual Analogue Scale.

Pittsburgh Sleep Quality Index (PSQI)

Significant differences were seen between the baseline and exam stress period in the placebo group for subjective sleep quality and sleep latency (see Figure 5.2 A & B). Additionally, a trend was noticed for global PSQI score between baseline and exam stress in the placebo group (see Figure 5.2 F). During the exam period, sleep duration decreased for the placebo group. However, participants taking the probiotic had improved sleep duration (Figure 5.3 B). There were no effects for the subscales habitual sleep efficiency, sleep disturbances or daytime dysfunction (see Figure 5.2 A-C).

Perceived Stress Scale

There was no effect on the PSS, see Figure 5.4. Total scores (range: 0-40) that are ≤13 are considered as a low stress level, 14-26 is moderate, and ≥27 is high.

Beck's Depression Inventory (BDI-II)

There was a significant increase for both groups on the BDI-II ($\chi^2(3)=16.120$, p=0.001). A Wilcoxon test subsequently showed that the depression score increased significantly for both probiotic (Z=- 2.769, p=0.006) and placebo (Z=-3.220, p=0.001), see Figure 5.5.

Cortisol awakening response (CAR)

There was no effect on cortisol concentrations ($\chi^2(3)=2.116$, p=0.549; N=19 due to missing samples), see Figure 5.5.

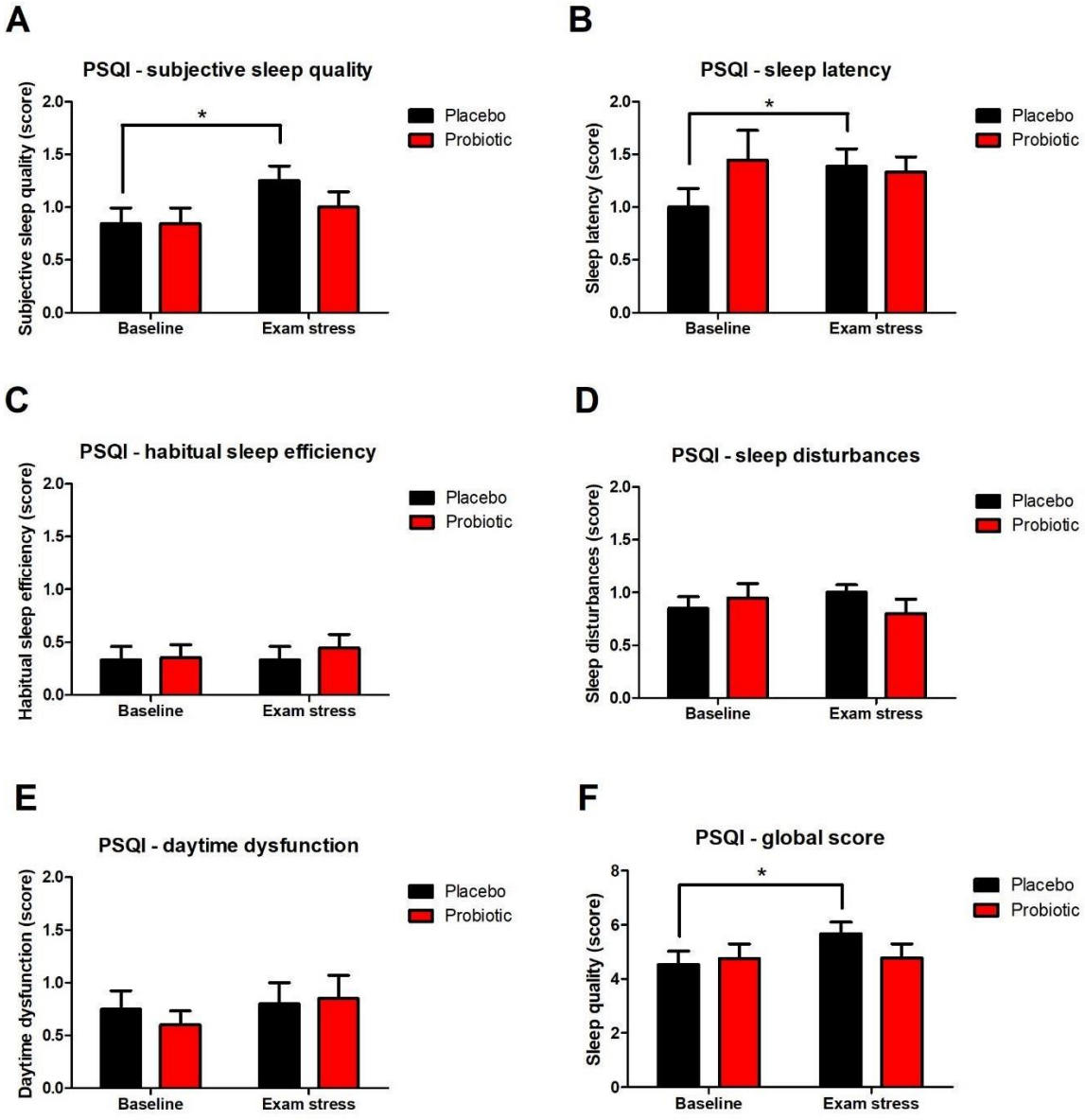

Figure 5.2 Pittsburgh sleep quality index (PSQI).

There was a difference over time for placebo on subjective sleep quality (A; p=0.021), sleep latency (B; 0.036), and the global score (F;0.023) of the PSQI. Data is presented as mean ± SEM. * p<0.05.

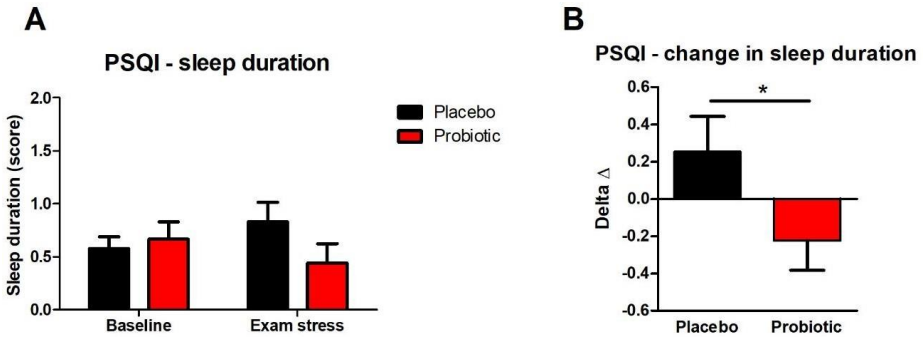

Figure 5.3. Pittsburgh sleep quality index (PSQI) – sleep duration.

Change from baseline in sleep duration (B) differed significantly between groups (p=0.039). Data is presented as mean ± SEM. * p<0.05.

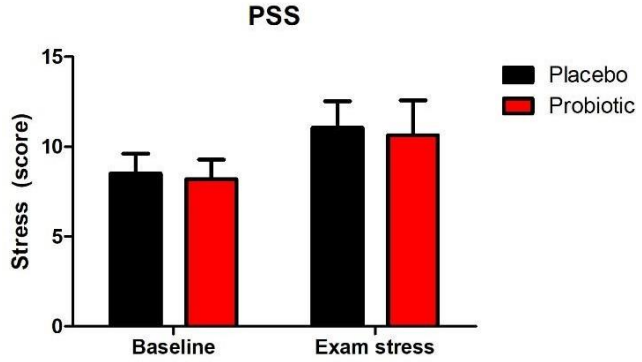

Figure 5.4 Perceived Stress Scale (PSS).

There were no effects on the PSS. Data is presented as mean ± SEM.

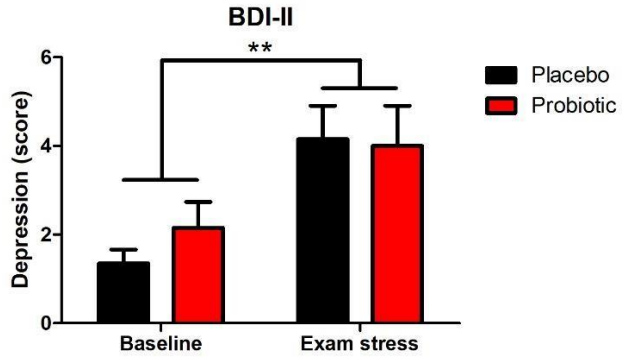

Figure 5.5 Beck's Depression Inventory (BDI-II).

Depression score increased over time for both probiotic (p=0.006) and placebo (p=0.001). There were no differences between groups. Data is presented as mean ± SEM. ** p<0.01

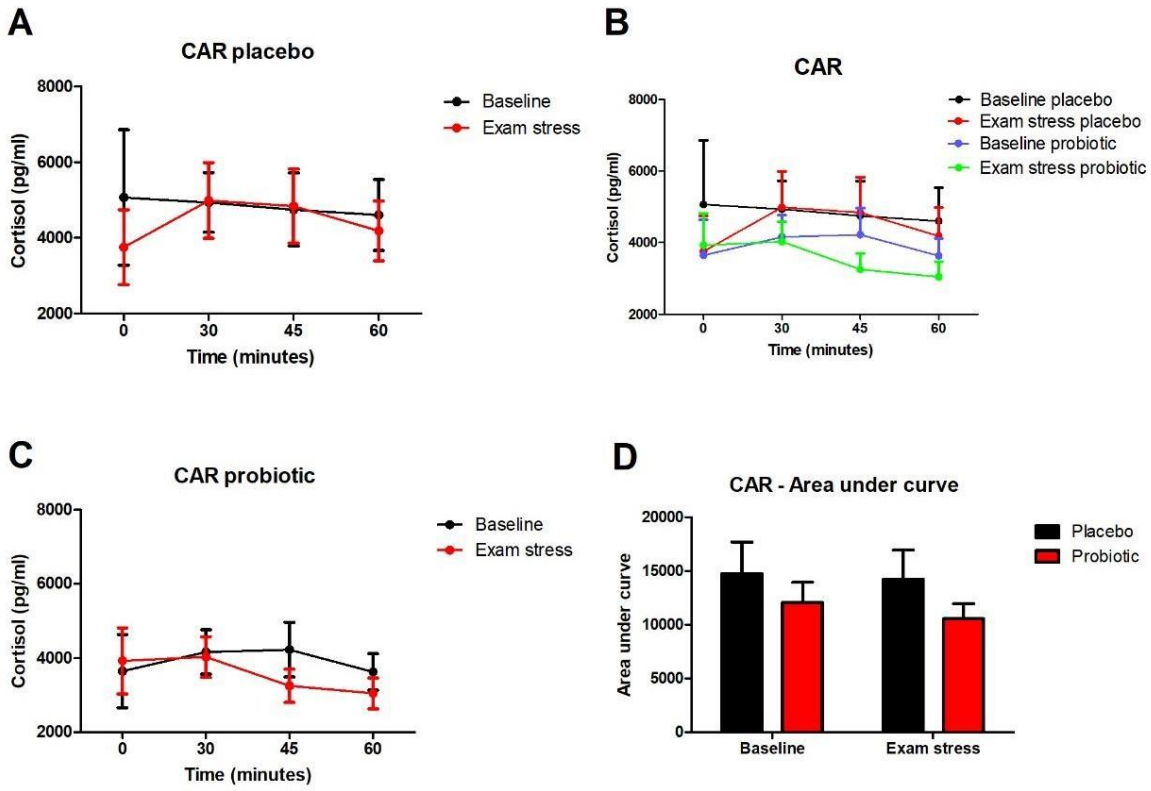

Figure 5.6 Cortisol awakening response (CAR).

There was no effect on cortisol concentrations. Data is presented as mean ± SEM.

Microbiome Results

Culture Dependent Analysis

Stool samples from participants at all time-points were diluted in MRD and plated onto four different types of media. Following incubation, plates were removed, and colonies were counted. Media used were selective for bifidobacteria, Lactobacilli, total number of aerobes and total number of anaerobes. Following enumeration and analysis, it was found that there were no differences in bacterial culture numbers between the groups at any time-point, see Figure 5.7.

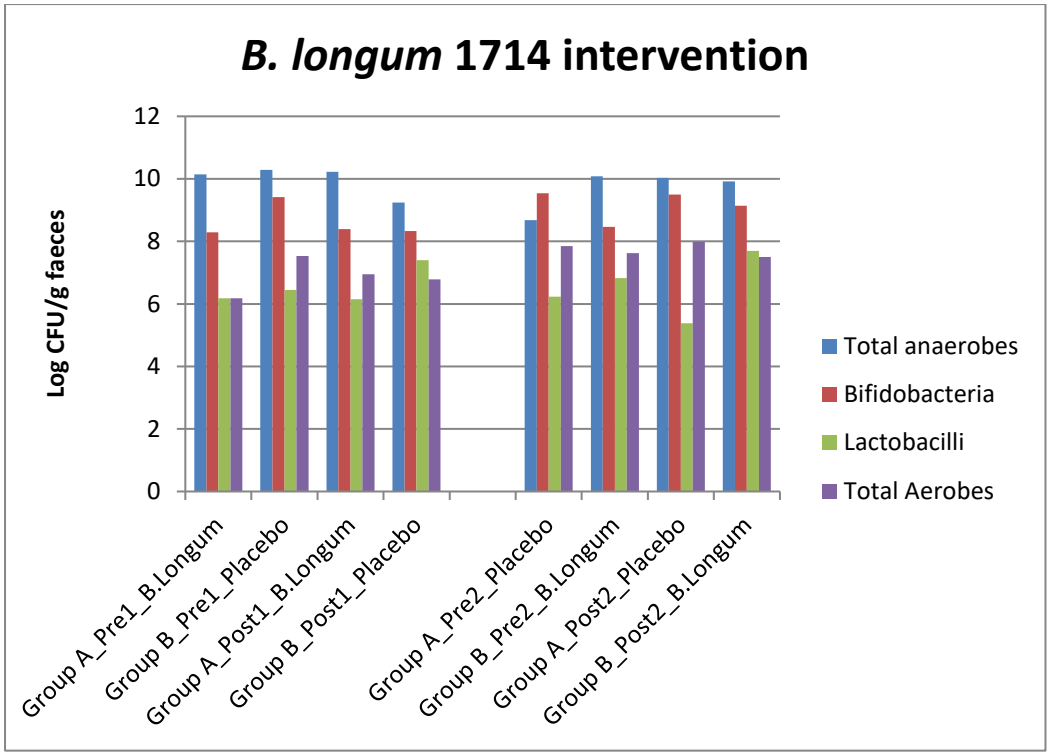

Figure 5.7 Culture dependent analysis examining bacterial counts for both placebo and probiotic groups, before and after interventions. No differences were seen.

16S Compositional Sequencing

Following compositional sequencing, no differences were noted in relative abundance levels between the probiotic and placebo groups for any taxonomic levels (Figures 5.8-5.10). Additionally, when examining the alpha diversity, there were no probiotic-driven differences noted within samples in terms of species richness, evenness, diversity or observed species (Figure 5.11). When investigating the relatedness of samples, it was found that the samples did not cluster based on probiotic or placebo intervention, during either intervention period (Figure 5.12).

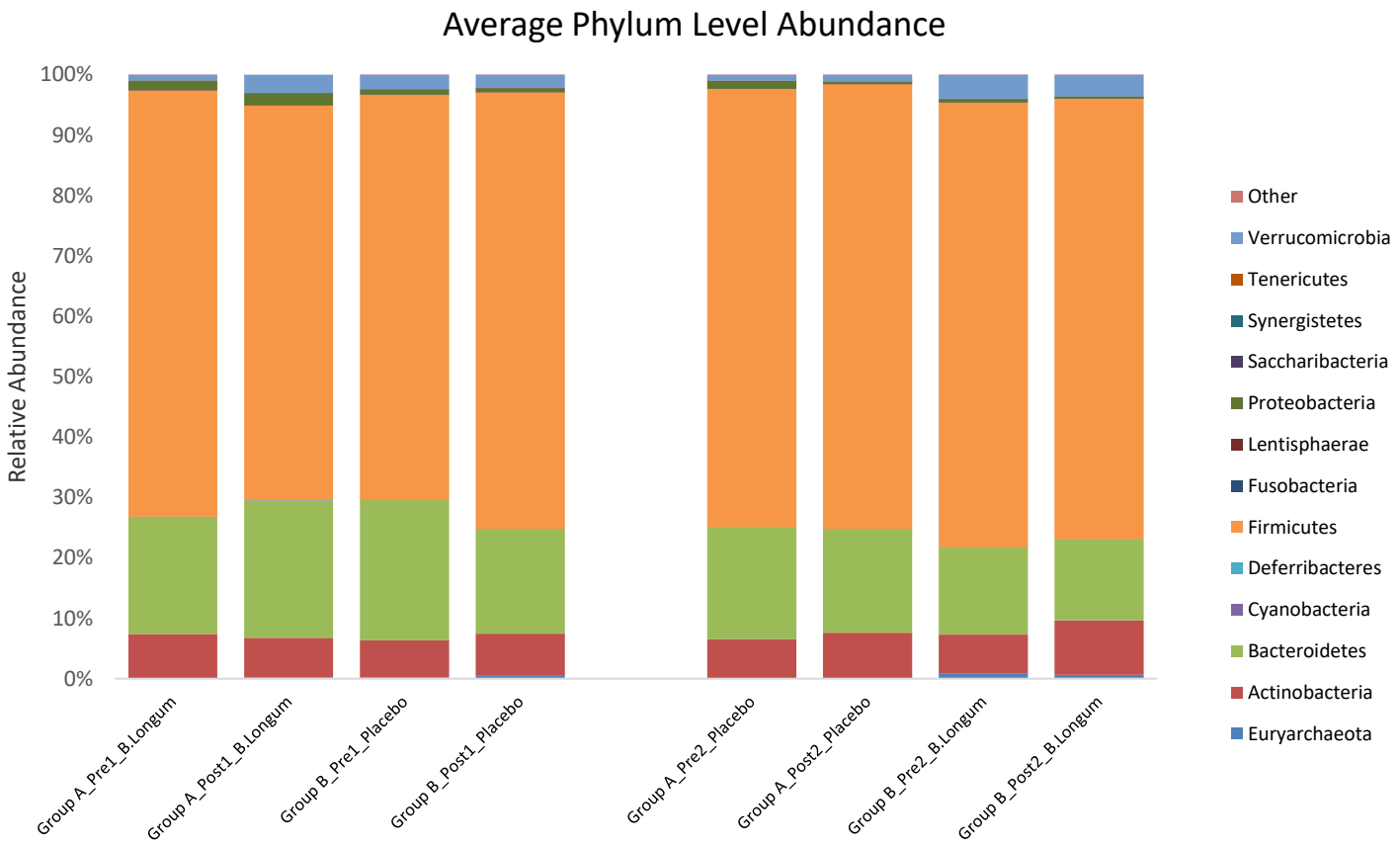

Figure 5.8 Phylum level relative abundances between probiotic and placebo groups during intervention

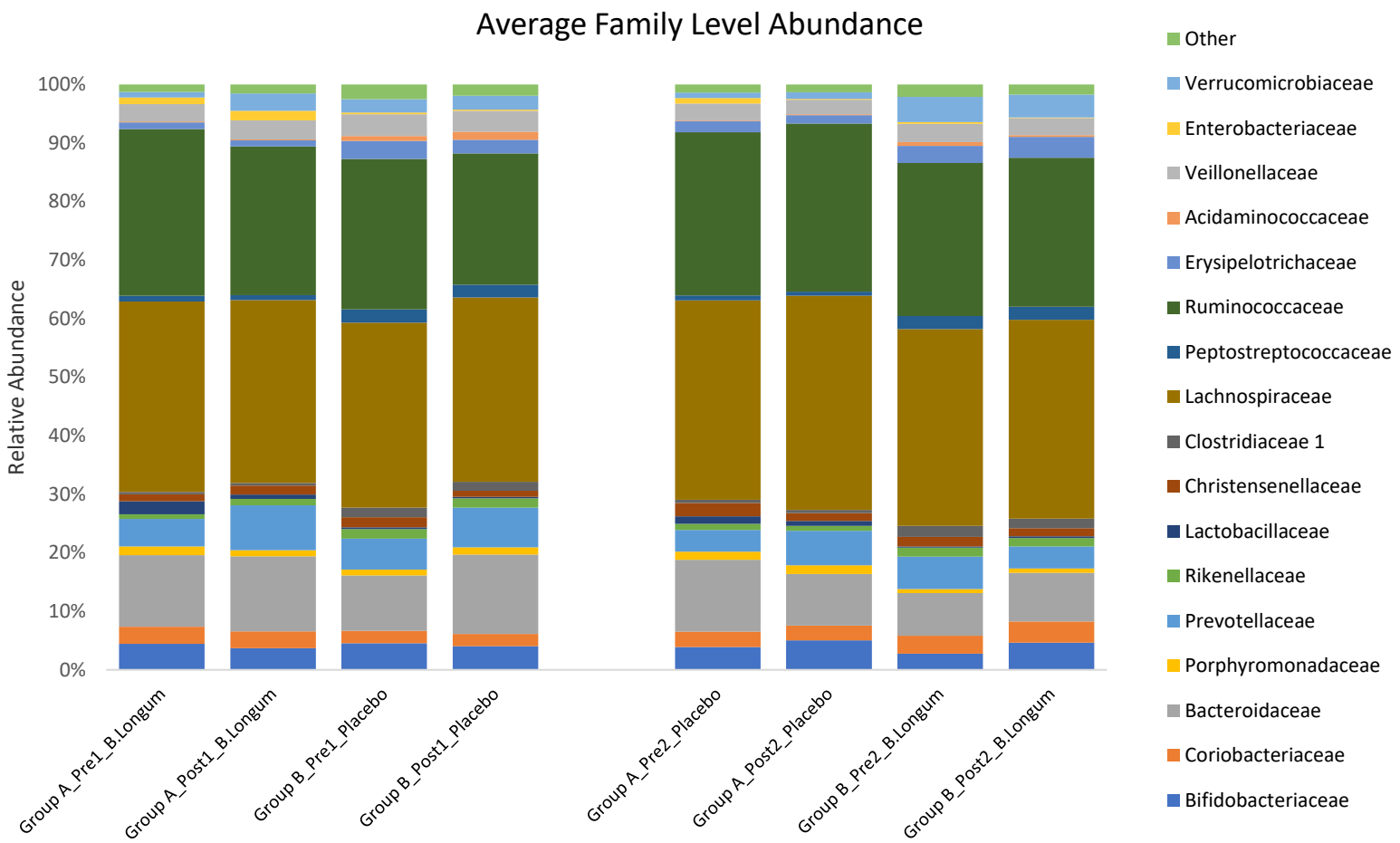

Figure 5.9 Family level relative abundances between probiotic and placebo groups during intervention

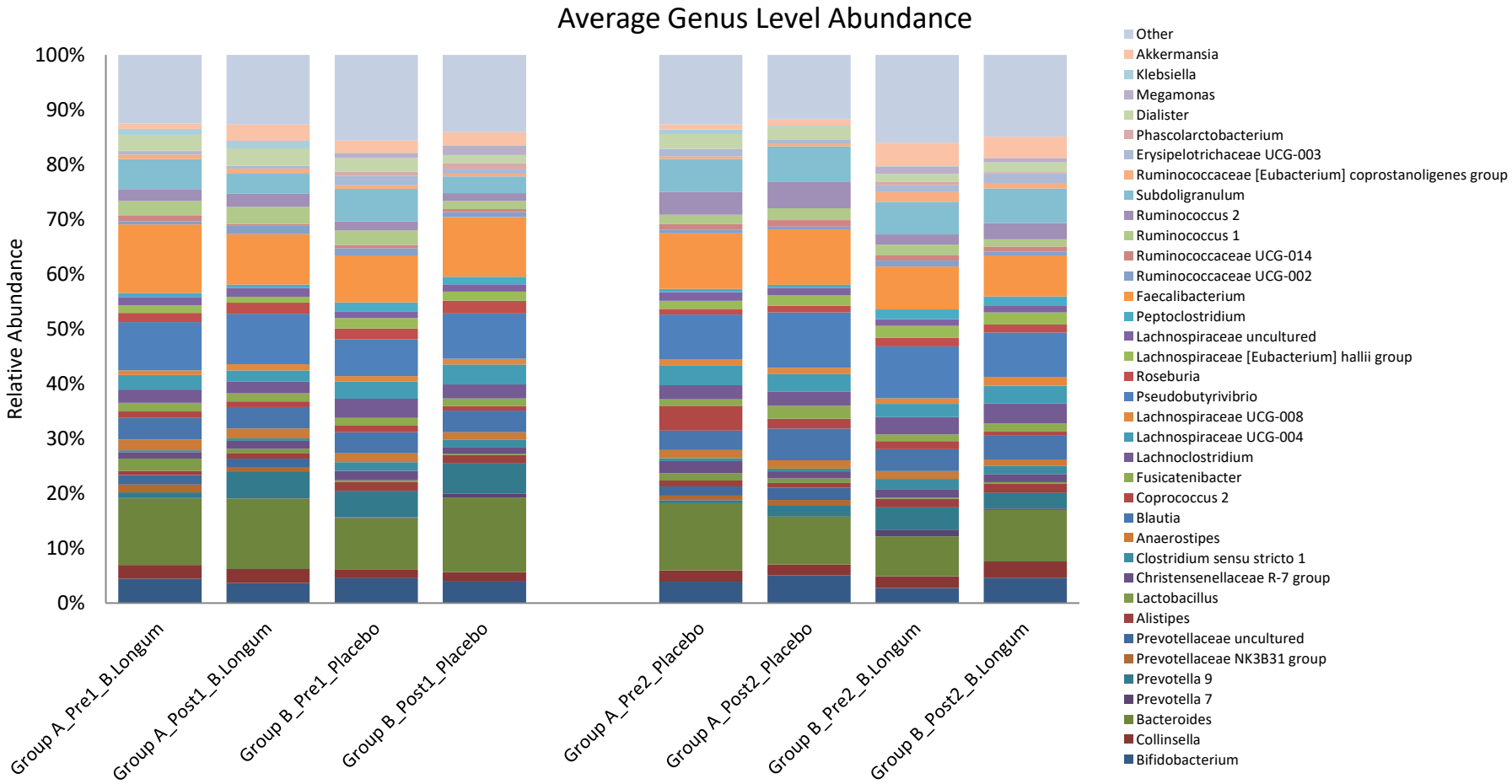

Figure 5.10 Genus level relative abundances between probiotic and placebo groups during intervention

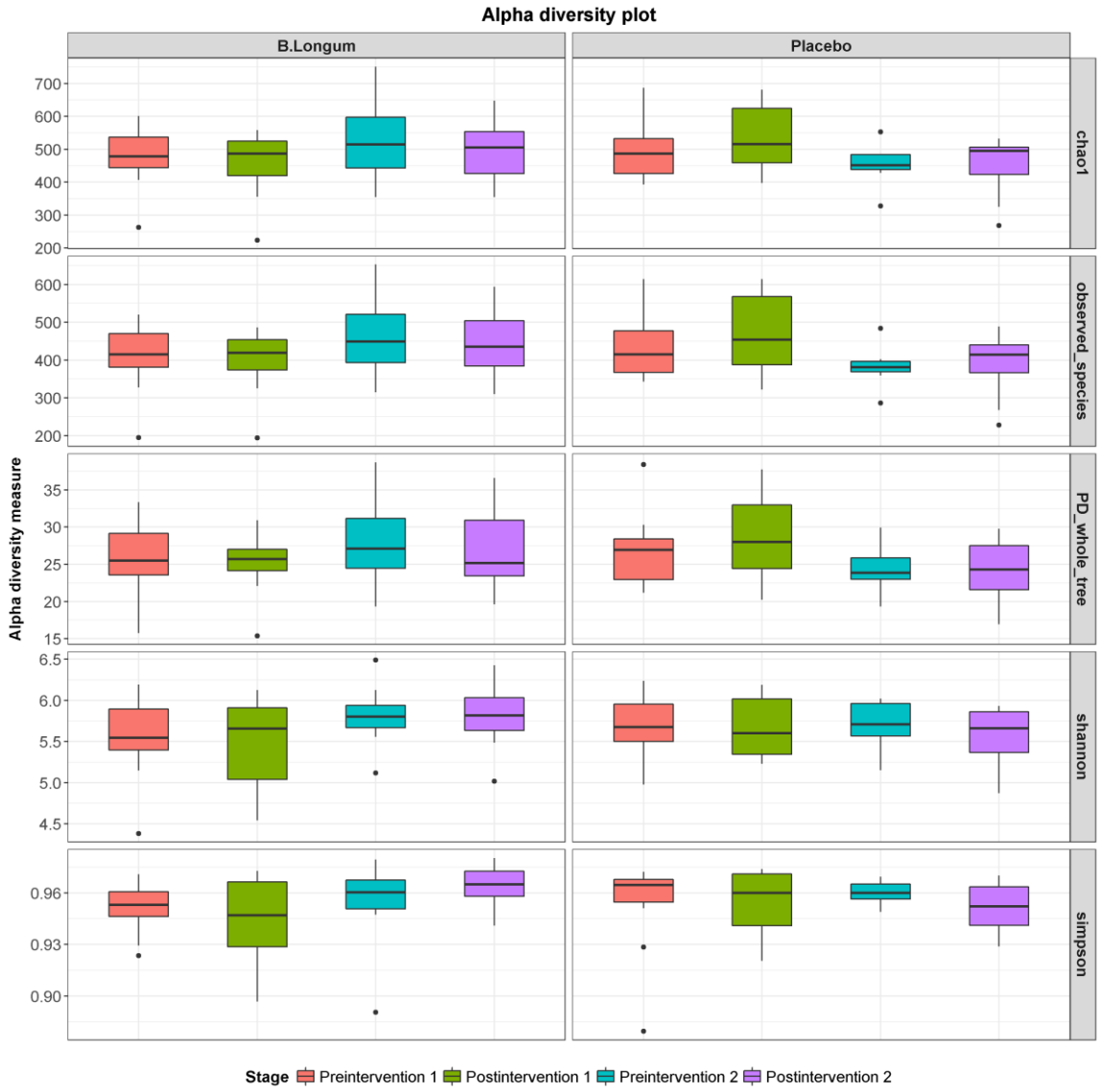

Figure 5.11 Alpha diversity between probiotic and placebo groups at both intervention periods

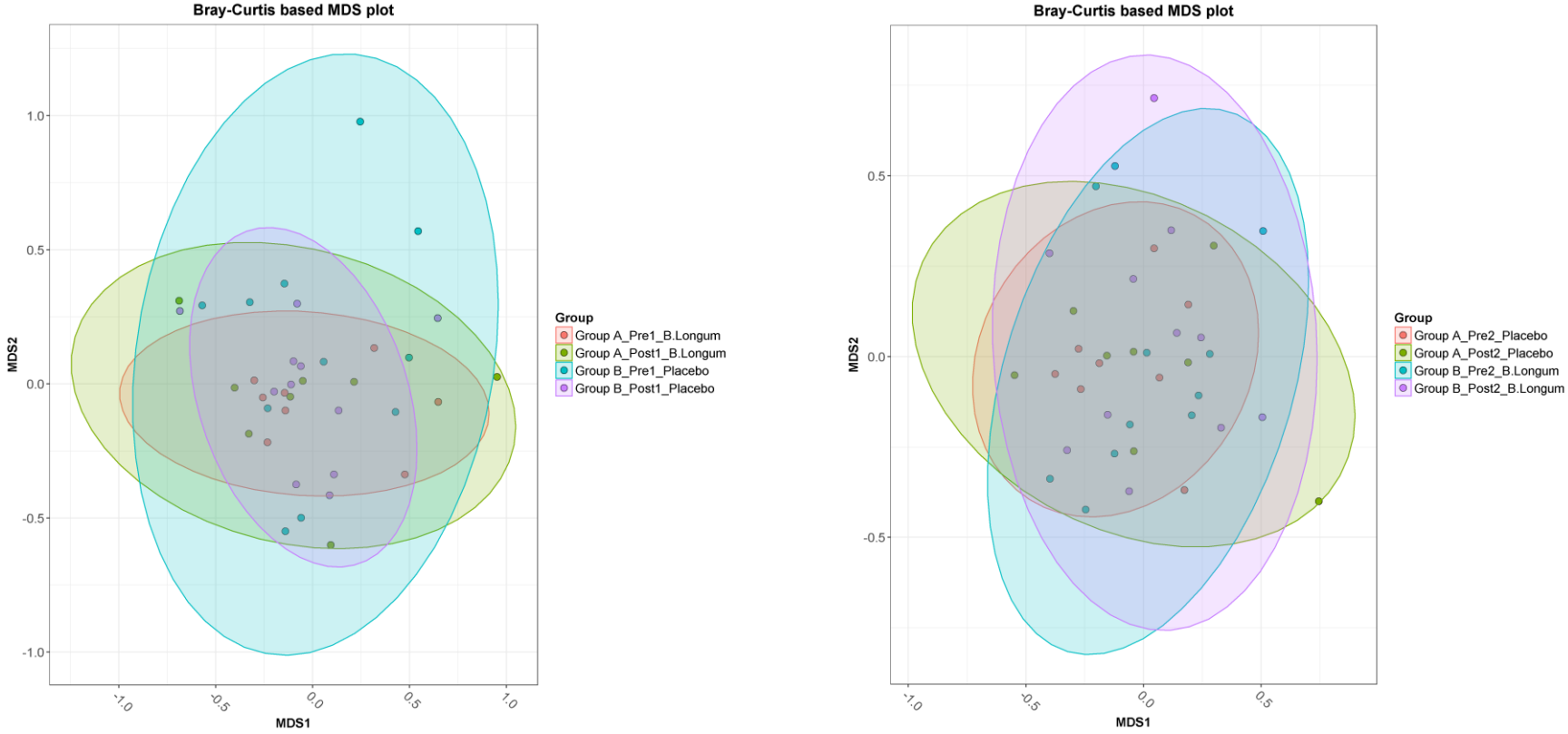

Figure 5.12 Beta diversity between probiotic and placebo groups at both intervention points

Discussion

The use of probiotics has gained significant attention in recent years, however, many of the studies that have been completed to date have been in preclinical animal models. Many examples of interventions in rodent models have shown promise, including the use of *Bifidobacterium longum* NCC3001 over a 2 to 3-week period, which showed a reduction in anxiety-like behaviour in a mouse model of colitis (Bercik et al., 2011). Additionally, intervention with *Lactobacillus rhamnosus* JB-1 has been shown to reduce stress-induced anxiety (Bharwani et al., 2017). Previously, two *Bifidobacterium* strains have shown to reduce stress, anxiety and depression-related behaviours in BALB/C mice, suggesting that some *Bifidobacterium* strains may have significant psychobiotic potential (Savignac et al., 2014; Savignac et al., 2015).

Of the limited number of human probiotic interventions conducted to date, several strains have shown promise, including *L. casei* strain Shirota, which has been shown to reduce symptoms of anxiety and stress in cohorts experiencing exam stress and chronic fatigue (Rao et al., 2009; Takada et al., 2016). *Bifidobacterium longum* NCC3001 has been shown to reduce anxiety and depression in IBS suffers (Pinto-Sanchez et al., 2017), while depression scores have also been reduced after intervention with a cocktail of *L. acidophilus*, *L. casei*, and *B. bifidum* in a cohort of individuals suffering from MDD (Akkasheh et al., 2016). For a comprehensive list of probiotic interventions in preclinical models and clinical cohorts, see an extensive current review by Cryan and colleagues (Cryan et al., 2019).

This study aimed to examine the effect of *Bifidobacterium longum* 1714 supplementation on stress, mood and cognitive performance in healthy participants during an exam stress period. *Bifidobacterium longum* 1714 has previously been

identified as a modifier of stress and cognition in preclinical studies (Savignac et al., 2014; Savignac et al., 2015). Following this, it was investigated whether these promising findings could be translated to a human cohort. In a repeated measures, placebo-controlled design, 22 healthy male participants were given placebo for four weeks followed by 1 x 10^9 CFU/day for four weeks (Allen et al., 2016). The socially evaluated cold pressor test (SECPT) was used to elicit an acute stress response in the volunteers. Intervention with the probiotic was found to result in a reduction in daily stress and state anxiety, accompanied by a reduction in cortisol output in response to the cold pressor test.

Our current study examined the use of this promising psychobiotic in a double-blind, randomised, placebo-controlled, repeated measures, crossover study design. Each intervention period lasted for 8 weeks, twice as long as the previous clinical study. Stool samples were collected from participants before and after each intervention period. It was found that the probiotic was well tolerated by the participants, with no GI issues reported. When examining the gut microbiota profile of the participants, it was seen that no differences were present between the placebo and treatment groups for culture-based analysis. Additionally, following 16 S compositional sequencing, the probiotic was not seen to affect the gut microbiota profile. There were no differences seen in relative abundances at any taxonomic levels. Additionally, no changes were observed within samples with respect to sample diversity or evenness. Furthermore, samples did not cluster based on probiotic or placebo treatment. This is unsurprising, given the length of the intervention. However, with longer intervention periods, reduced compliance is more common. Additionally, a lack of major changes in the gut microbiota composition after administration with a single bacterial strain is expected. While there were no significant shifts in the gut

microbiota composition after the probiotic intervention at relative abundances higher than 1%, it may be interesting to examine any subtle microbial changes or trends toward reduced or increased abundance in genera present at lower abundance levels. Indeed, many subtle probiotic-driven changes that have been reported in clinical studies have been minimal and required further investigation than what has been completed here to notice associations between specific OTUs and probiotic administration (Bagga et al., 2018). Furthermore, studies that have reported such minimal changes have used a cocktail of 8 or 9 different bacterial strains.

Additionally, the lack of gut microbiota changes is consistent with the lack of probiotic effects seen on stress and depression in this study. This absence of influence can possibly be explained by the cohort examined. These healthy male individuals are not stressed at baseline and have low baseline BDI scores. Additionally, the participants' responses to the Primary Appraisal Secondary Appraisal (PASA), suggest that this cohort are particularly capable of dealing with stress. They perceive their exams as somewhat more of a challenge, rather than a stressor that they have no control over. Furthermore, while the naturalistic exam stressor did increase depression scores in both groups, it did not have an effect on salivary cortisol in these participants. In contrast, the acute stressor used in the previous clincial study conducted by Allen and colleagues did increase salivary cortisol and allowed for the influence of the probiotic to be measured (Allen et al., 2016). It is not unreasonable to hypothesise that this cohort, who are quite capable of coping with exam stress, may be affected by hypothalamic-pituitary-adrenal axis habituation across repeated exposure, with a reduction in stress responses over successive exam periods, and thus, this stressor may not be the most suitable for this particular cohort. Perhaps if, in addition to naturalistic exam stress, an acute stress test such as the TSST or SECPT was employed, the

efficacy of this probiotic could be examined more comprehensively. Additionally, it is important to note the sample size used in this study may not be sufficient.

While there were no influences of the probiotic on stress or depression noted in this cohort, an effect on sleep quality was found. Differences were seen between the baseline and exam stress period in the group consuming placebo for subjective sleep quality and sleep latency. Sleep duration was also seen to decrease during exam stress in the placebo group, while those participants taking the probiotic had improved sleep duration. This suggests that the probiotic may be beneficial during stress to maintain sleep quality.

A study investigated the ability of this *Bifidobacterium longum* 1714 strain to modulate brain function in a human cohort (Wang et al., 2019). The study was conducted with 40 volunteers using a randomised, double-blinded, placebo-controlled design. Participants received either placebo or 1 x 10^9 CFU/day of the probiotic for four weeks. Brain activity was measured using magnetoencephalography. The effects on neural responses to social stress were examined. This was conducted using the "Cyberball Game" (CBG), a social stressor. The CBG is a ball tossing game in which the participant has to toss the ball to two other virtual players who are programmed by the tester. The participant believes that these two players are real and are playing the game, just the same as they are. This induces social stress through periods of inclusion and exclusion, where the two other players just throw the ball to each other and leave the participant out. All participants experienced social stress, however, neuroimaging showed that the neural activities of the participants consuming the probiotic were significantly altered after the four-week intervention, both at rest and during social stress. Neural activity was measured, examining frequency band power in various regions of the brain. The authors noted that an increase in beta band power is

associated with mental fatigue, while a reduction in beta band power is linked to alertness and reduced anxiety. They concluded that consumption of the probiotic led to neural alterations in a manner associated with increased vitality and reduced mental fatigue. While both groups responded to social stress, only the participants consuming the probiotic showed changes in neural processing, which suggests that *Bifidobacterium longum* 1714 may play a role in how stress is processed in the brain and could possibly manage stress by modulating certain neural processes.

While the potential for interventions using *Bifidobacterium longum* 1714 and other psychobiotics targeting stress and anxiety is an attractive one, further investigations are necessary to examine the mechanisms by which stress can be modulated. While *Bifidobacterium longum* 1714 in the above study did not reduce levels of social stress when compared with placebo, the authors noticed that the neural changes in the probiotic group were also present at baseline and not in the placebo group, suggesting that this probiotic might be involved in priming of the individuals in anticipation of the stressor and subsequently modifying neural processes to modulate stress responses. This may be interesting to note in further study designs investigating the effect of *Bifidobacterium longum* 1714 during acute stress. Perhaps for an effect to be noted, the stressor should be conducted at the end of the intervention period. Furthermore, in light of this study examining *B. longum* induced neural changes, it is not unreasonable to assume that perhaps in our current study, this probiotic did have an effect on brain function which led to the improvements noted in the sleep quality.

The use of *Bifidobacterium longum* 1714 in this current study had an effect on sleep quality and duration during exam stress when compared with placebo, which suggests that this probiotic may be beneficial during chronic stress periods. However,

as this cohort was uniquely adapted to stress, there were no influences noted on mood or stress responses. Additionally, it has been previously reported that probiotics exhibit the maximum influence if they are given to a population that requires an intervention, i.e., Benton and colleagues showed that after probiotic consumption, effects were noted in participants who were experiencing more depressive symptoms than those whose mood was generally higher (Benton et al., 2007).

It is tempting to speculate that this probiotic may be more beneficial and show greater effects during acute stress periods. Further investigations should be carried out in different populations, instead of healthy volunteers, for example, in those who are experiencing clinical anxiety or depression. This would allow for examination of the impact of *Bifidobacterium longum* 1714 in a cohort whose baseline levels are lower than the normal population, to assess the ability of the probiotic as a possible restorative treatment back to baseline levels seen in a healthy population.

While many positive effects on stress, cognition and behaviour have been noted after probiotic administration, additional robust clinical trials are necessary, accompanied by the analysis of wider biological parameters, particularly focusing on the mechanisms involved in probiotic modifications. The continued research into the use of probiotics targeting mood, stress responses and cognition promises to be an important avenue of investigation however, much additional work is needed before psychobiotics can be used as an alternative to, or in combination with conventional therapies.

References

Akkasheh, G., Kashani-Poor, Z., Tajabadi-Ebrahimi, M., Jafari, P., Akbari, H., Taghizadeh, M.,... Esmaillzadeh, A. (2016). Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomised, double-blind, placebo-controlled trial. *Nutrition, 32*(3), 315-320.

Allen, A. P., Hutch, W., Borre, Y. E., Kennedy, P. J., Temko, A., Boylan, G.,... Clarke, G. (2016). Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. *Translational psychiatry, 6*(11), e939.

Bagga, D., Reichert, J. L., Koschutnig, K., Aigner, C. S., Holzer, P., Koskinen, K.,... Schöpf, V. (2018). Probiotics drive gut microbiome triggering emotional brain signatures. *Gut microbes, 9*(6), 486-496.

Benton, D., Williams, C., & Brown, A. (2007). Impact of consuming a milk drink containing a probiotic on mood and cognition. *European journal of clinical nutrition, 61*(3), 355.

Bercik, P., Park, A., Sinclair, D., Khoshdel, A., Lu, J., Huang, X.,... Moine, D. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterology & Motility, 23*(12), 1132-1139.

Bharwani, A., Mian, M. F., Surette, M. G., Bienenstock, J., & Forsythe, P. (2017). Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. *BMC medicine, 15*(1), 7.

Buysse, D. J., Reynolds III, C. F., Monk, T. H., Berman, S. R., & Kupfer, D. J. (1989). The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry research, 28*(2), 193-213.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2009). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics, 26*(2), 266-267.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience, 13*(10), 701.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M.,... Golubeva, A. V. (2019). The Microbiota-Gut-Brain Axis. *Physiological reviews, 99*(4), 1877-2013.

Dinan, T. G., & Cryan, J. F. (2016). Mood by microbe: towards clinical translation. *Genome medicine, 8*(1), 36.

Edgar R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England), 26*(19), 2460–2461.

Kelly, J. R., Allen, A. P., Temko, A., Hutch, W., Kennedy, P. J., Farid, N.,... Cryan, J. F. (2017). Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects. *Brain, behavior, and immunity, 61*, 50-59.

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963.

Pinto-Sanchez, M. I., Hall, G. B., Ghajar, K., Nardelli, A., Bolino, C., Lau, J. T.,... Rieder, A. (2017). Probiotic Bifidobacterium longum NCC3001 reduces depression scores and alters brain activity: a pilot study in patients with irritable bowel syndrome. *Gastroenterology, 153*(2), 448-459. e448.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research, 41*(Database issue), D590–D596.

Rao, A. V., Bested, A. C., Beaulne, T. M., Katzman, M. A., Iorio, C., Berardi, J. M., & Logan, A. C. (2009). A randomised, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut pathogens, 1*(1), 6.

Sampson, T. R., & Mazmanian, S. K. (2015). Control of brain development, function, and behaviour by the microbiome. *Cell host & microbe, 17*(5), 565-576.

Savignac, H., Kiely, B., Dinan, T., & Cryan, J. (2014). Bifidobacteria exert strain-specific effects on stress-related behaviour and physiology in BALB/c mice. *Neurogastroenterology & Motility, 26*(11), 1615-1627.

Savignac, H., Tramullas, M., Kiely, B., Dinan, T., & Cryan, J. (2015). Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behavioural brain research, 287*, 59-72.

Takada, M., Nishida, K., Kataoka-Kato, A., Gondo, Y., Ishikawa, H., Suda, K.,... Igarashi, T. (2016). Probiotic Lactobacillus casei strain Shirota relieves stress-associated symptoms by modulating the gut-brain interaction in human and animal models. *Neurogastroenterology & Motility, 28*(7), 1027-1036.

Wang, H., Braun, C., Murphy, E. F., & Enck, P. (2019). Bifidobacterium longum 1714™ strain modulates brain activity of healthy volunteers during social stress. *The American journal of gastroenterology, 114*(7), 1152.

Yu, Z., & Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and faecal samples. *Biotechniques, 36*(5), 808-812.

Zunhammer, M., Eberle, H., Eichhammer, P., & Busch, V. (2013). Somatic symptoms evoked by exam stress in university students: the role of alexithymia, neuroticism, anxiety and depression. *PloS one*, *8*(12), e84911.

CHAPTER 6

REVISITING METCHNIKOFF: AGE-RELATED ALTERATIONS IN MICROBIOTA-GUT-BRAIN AXIS IN THE MOUSE

CHAPTER CONTRIBUTIONS:
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- planned sample quantity and processing methods,
- carried out DNA extraction,
- conducted short chain fatty acid analysis,
- planned and prepared 16 S compositional sequencing library,
- planned and carried out statistical analyses,
- significantly contributed to manuscript preparation.

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Abstract

Over the last decade, there has been increased interest in the role of the gut microbiome in health including brain health. This is by no means a new theory; Elie Metchnikoff proposed over a century ago that targeting the gut by consuming lactic acid bacteria such as those in yogurt, could improve or delay the onset of cognitive decline associated with ageing. However, there is limited information characterising the relationship between the behavioural and physiological sequelae of ageing and alterations in the gut microbiome.

To this end, we assessed the behavioural, physiological and caecal microbiota profile of aged male mice. Older mice (20-21 months old) exhibited deficits in spatial memory and increases in anxiety-like behaviours compared to younger mice (2-3 months old). They also exhibited increased gut permeability, which was directly correlated with elevations in peripheral pro-inflammatory cytokines. Furthermore, stress exacerbated the gut permeability of aged mice. Examination of the caecal microbiota revealed significant increases in phylum TM7, family Porphyromonadaceae and genus *Odoribacter* of aged mice. This represents a shift of aged microbiota towards a profile previously associated with inflammatory disease, particularly gastrointestinal and liver disorders. Furthermore, Porphyromonadaceae, which has also been associated with cognitive decline and affective disorders, was directly correlated with anxiety-like behaviour in aged mice.

These changes suggest that changes in the gut microbiota and associated increases in gut permeability and peripheral inflammation may be important mediators of the impairments in behavioural, affective and cognitive functions seen in ageing.

Introduction

Over 100 years ago, Nobel Prize winner Elie Metchnikoff hypothesised that beneficial bacteria contained in fermented foods could influence health and delay cognitive decline by manipulating the intestinal environment (Cryan & Dinan, 2015; Mackowiak, 2013). Although popular at the time, the theory was largely ignored by the medical community until a recent resurgence in interest over the past 20 years (Mackowiak, 2013). The gut microbiome is now recognised to play a critical role in health and disease, and this is especially true at the extremes of life (Borre et al., 2014; Brüssow, 2013; Candela et al., 2014; Heijtz, 2016; Dinan & Cryan, 2016). Indeed, changes in microbial composition have been shown to have a distinct impact on health outcomes in infants and in the elderly and also is thought to play a key role in modulating gut-brain axis function, influencing brain and behaviour (Borre et al., 2014; Jeffery & O'Toole, 2013). However, there is limited information on how ageing regulates this axis. With advances in healthcare, longevity has markedly increased; currently it is estimated that within 50 years, approximately 20% of the world population will be classified as elderly (Ellison et al., 2015). Ageing is associated with a number of behavioural and physiological changes, including physical decline, altered mood and cognitive impairments (Ellison et al., 2015; Eshkoor et al., 2015; Joyce & Reich, 2015). Although we cannot stop the march of time, the manner in which one ages can vary greatly; thus there is significant research interest in understanding the biological basis of healthy ageing (Li & Schmiedek, 2002).

Gastrointestinal (GI) disorders are frequently reported in elderly individuals, with 35-40% of patients reporting at least one GI complaint (Tran & Greenwood-Van

Meerveld, 2013). These include malnutrition as a result of poor nutrient absorption, reduced gut motility, associated with constipation, diverticulosis from structural weakening of the GI tract, and increased susceptibility to colon cancer (Langille et al., 2014). Ageing in humans and laboratory animals is also related to increased intestinal permeability and increased colonic cytokine expression resulting in chronic systemic inflammation which has been termed "inflamm-ageing," (Deleidi et al., 2015; Peterson et al., 2015; Tran & Greenwood-Van Meerveld, 2013).

The mechanisms underlying the increased intestinal permeability and peripheral inflammation are still unclear, but the gut microbiota is thought to play a critical role. It is now widely recognised that the gut microbiota is a key modulator of homeostasis, and perturbation of its composition can result in gut dysfunction. Furthermore, the composition of the gut microbiota in both humans and laboratory animals varies across the lifespan (Biagi et al., 2013; Jeffery & O'Toole, 2013; Langille et al., 2014; Lynch et al., 2015; Rehman, 2012). In general, as an individual ages, the microbiota shifts in numerous ways that may predispose one to inflammation.

In addition to the physical deterioration of the body, such as increased risk of disease and frailty, there is great interest in the effects of ageing on the central nervous system (Prenderville et al., 2015). Psychiatric conditions, most notably anxiety, depression, and social withdrawal are frequently reported in the elderly (Kastenschmidt & Kennedy, 2011; Prenderville et al., 2015). It is also well established that peripheral inflammation can directly affect neuroimmune processes in the central nervous system (CNS) resulting in impaired cognitive function (Block et al., 2007; Perry, 2010; Zheng et al., 2015). Moreover, circulating inflammatory markers are often elevated in individuals with mood disorders (Block et al., 2007; Kelly et al., 2016; Perry, 2010; Young et al., 2014; Zheng et al., 2015). It is therefore likely that

age-related alterations in intestinal microbiota and function contribute to chronic systemic inflammation. This may, in turn, lead to central inflammation, manifesting in cognitive impairment.

Taken together, it is clear that the microbiota-gut-brain axis plays a critical role in health, and perturbations in this axis have been implicated in numerous pathological conditions. However, there are a limited number of studies focused on its impact on ageing-induced behavioural and neurobiological outcomes, notwithstanding Metchnikoff's theory that microbes may be the underpinnings of longevity (Cryan & Dinan, 2015). To this end, we hypothesised that alterations in the gut microbiota of young and aged mice will correlate with changes in intestinal permeability, inflammation and neurobehavioural outcomes.

Materials and Methods

Animals

All animal protocols were approved by the Animal Experimentation Ethics Committee at University College Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland, in accordance with EU Directive 2010/63/EU. Twelve young (2 month old) and 10 aged (18 month old) male C57BL/6 J mice were obtained from Charles River (France) and housed 2-4 per cage. All mice were housed in the same temperature and humidity-controlled animal room with a 12 h light:dark cycle and were maintained on *ad libitum* standard chow and water unless otherwise noted. Mice were given 4 weeks to recover from transportation and acclimate to the UCC animal facility.

Behavioural testing

All mice were exposed to a battery of well-validated behavioural tests designed to assess cognitive function as well as depressive- and anxiety-like behaviours over a period of 5 weeks. Young mice were approximately 2-3 months old and aged mice were approximately 20-21 months old at the time of behavioural testing. Behavioural tests were performed in what we considered to be increasing severity to lessen the likelihood of the prior testing influencing future tests. Tests were performed in the following order: 1) Object location, 2) Y-maze, 3) 3 chamber, 4) elevated plus maze, 5) open field, 6) forced swim test and 7) gut permeability tests. A washout period of 2 days was observed between behavioural tests 1-6. A washout period of 2 weeks followed the forced swim test, and 4 weeks followed gut permeability testing and prior to cull.

Forced swim test

The forced swim test (FST) was used to assess anti-depressant-sensitive behaviours and is based on the premise that immobility, performing the minimum amount of movement to keep one's head above water, is a marker of depressive-like or despair-like behaviour (Cryan & Mombereau, 2004; Porsolt, 1979; Porsolt et al., 1978). FST was performed under full light conditions (1000 lux). The tank was a glass cylinder measuring 21 cm in diameter and filled to a depth of 15 cm with 24°C tap water. The test was videorecorded from above for 6 min, and afterwards the mouse was removed from the tank, dried with a towel and placed into an individual cage for recovery. Ninety minutes post-test, mice were returned to their home cages. Water was changed between each test. Immobility was assessed for the last 4 min of the FST and was defined as the animal not actively swimming or moving and performing the minimal amount of activity necessary to keep the head above water.

Spontaneous alteration in the Y-maze

The Y-maze was performed as a test of spontaneous alternation, used to assess exploration, cognition and hippocampal-dependent memory (Hughes, 2004; Senechal et al., 2007). It is based on the premise that mice will alternate between arms visited when exploring a new environment. The test was performed as previously described with minor adaptation (Senechal et al., 2007). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The Y-maze was constructed from black plastic and each arm measured 16 x 6.5. Lighting within the Y-maze was 25 lux. The mouse was placed at the end of the first arm facing the wall. Behaviour was videorecorded from above for 5 min after placement within the maze. The Y-maze was wiped down with 70% ethanol between tests. An entry into an arm was noted when all 4 paws crossed into the area. Alternation was noted as the number

of consecutive entries into the three maze arms. This was calculated as the number of alternations divided by the total number of arm entries during the 5 min test period.

Novel Object/Object Displacement test

The combined Novel Object/Object Displacement test (NOR) was used to assess cognitive function, including and spatial working memory and is based on the premise that mice will spend more time interacting with a novel or displaced object in comparison with a familiar object in a familiar location (Antunes & Biala, 2012). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The test arena was a grey plastic box, measuring 32 x 40 cm. Lighting within the test arena was approximately 60 lux. The arena was wiped with ethanol after each test. Day one consisted of habituation to the empty test arena; the mouse was placed in the centre and allowed to explore for 10 min, while behaviour was videorecorded from above. After this period, the mouse was returned to its home cage. The acquisition phase of the study was performed on day 2. Again, mice were given at least 30 min to habituate to the testing room. During the acquisition phase, the mouse was placed in the centre of the test arena where 2 identical objects were placed. The mouse was allowed to explore for 10 min, after which it was returned to its home cage. After a 3 h period, the mouse was returned to the test arena with the same objects, however one of the objects was now in a different location. The mouse was allowed 5 min to explore and time spent interacting with the 2 objects was videorecorded, after which it was returned to its home cage. This comprised the Object Displacement phase of the study. Following a 5 min break, the mouse was returned to the test arena where the displaced "familiar" object was replaced with a novel one. Exploration of an object was defined as orientation of the mouse with its nose 2 cm or closer to the object.

Open field test

The open field (OF) test was used to assess locomotor activity and anxiety-like behaviour and is based on the premise that mice will avoid open areas (the centre of the test arena), which are anxiety-provoking and will spend more time near the walls (thigmotaxis) (Gould, 2009; Hall & Ballachey, 1932). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The OF arena was a white plastic box measuring 32 x 40 cm and the test was performed under full light conditions (1000 lux). Mice were placed in the centre of the arena and behaviour was recorded from above for 10 min. Mice were returned to their home cage following the test and the arena was wiped down with ethanol. Distance travelled and entries into centre of the arena was analysed using Ethovision (Noldus, Waeginingen, Netherlands).

Elevated Plus Maze

The elevated plus maze (EPM) was used to assess anxiety-like behaviour and is based on the premise that mice prefer enclosed areas when exposed to a novel environment and will spend less time in open areas, which are anxiety-inducing (Pellow et al., 1985). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The room was lit by one red light and the lighting in the centre of the EPM measured 9 lux. The elevated plus maze was constructed of black plastic and had 4 arms measuring 50 x 5 cm. Walls on the 2 closed arms were 15 cm high whereas the 2 open arms had no walls. The plus maze rested on a platform 1 m high. For testing, the mouse was placed in the centre of the EPM facing an open arm. Behaviour was videorecorded from above for 5 min, after which the mouse was returned to its home cage. The EPM was wiped down with ethanol solution between each test. Entry into each arm was noted when all 4 paws crossed into the

arm. The number of entries and percentage of time spent in open and closed arms were calculated.

3-chamber test of social behaviour

Sociability was assessed using the 3-chamber social interaction test, in which time spent interacting with a novel conspecific is compared to time spent with a novel object or familiar conspecific. It is based on the premise that mice will spend more time interacting with a novel conspecific than novel object, and that they will prefer a novel conspecific to a familiar one. Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The 3-chamber social interaction test was performed as previously described (Desbonnet et al., 2014; O'Tuathaigh et al., 2007). The test arena consisted of 3 chambers; the left and right chambers measured 13.5 x 20 x 20 cm and the centre chamber was 9 x 20 x 20 cm. A solid partition separated the chambers, which could be replaced with partitions with a small hole enabling access to the other chambers. There were 3 phases of the test: habituation, sociability, and social novelty preference. All phases of the test were 10 min in duration, performed sequentially and videorecorded from above for later analysis. During Phase 1, the habituation phase, the mouse was placed into the centre chamber and then allowed access to the empty left and right chambers for 10 min. The mouse was then returned to the centre chamber and a novel mouse was placed in a mesh cage in one of the side chambers, whereas a novel object (a small rubber duck) was placed in a mesh cage in the other side chamber for Phase 2. Location of the novel mouse and novel objects were randomised between animals to eliminate side preferences. The mouse was then allowed to explore these chambers for 10 min, after which it was returned to the centre chamber. For the 3rd phase, a new, novel mouse was placed in the mesh cage that had previously housed the novel object. The mouse

was then allowed to explore the chambers, which held the familiar mouse (from Phase 2) and the novel mouse, for 10 min. The 3-chamber apparatus was cleaned with ethanol between animals. The number of entries and time spent in each chamber were then measured.

Intestinal permeability (FITC-D)

Intestinal barrier function was assessed using fluorescein isothiocyanate-labelled dextran (FITC-D) (FD4, Sigma Aldrich, Ireland). FITC-D (MW= 4 kDa) was dissolved in phosphate buffered saline (pH 7.4) to make a solution of 80 mg/ml. Mice were fasted overnight, prior to the study, and in the morning (9.00), they were gavaged with FITC-D (600 mg/kg). Two hours following gavage, mice were placed in ventilated plastic restrainers and a basal blood sample was taken. Briefly, a scalpel blade was used to remove the very tip (<1 mm) of the tail. Blood was then collected using a heparinised capillary tube and transferred to a microcentrifuge tube. To assess the impact of acute stress on gut permeability, a small piece of gauze was used to gently remove the clot at the tip of the tail and another blood sample was collected 1 h post-restraint. Mice were then returned to their home cages. Blood samples were kept on ice and then centrifuged at 2500 x g. Plasma was collected and stored at -20°C for later analysis. To assess FITC-D, samples were analysed using a spectrometer (Victor Spectrometer, excitation max= 490 nm, emission max= 520 nm). Serial dilutions of FITC-D in PBS were used to generate a standard curve. A separate aliquot of plasma was collected at each time-point to assess basal and stressed corticosterone levels.

Corticosterone response to acute stress

Plasma corticosterone (CORT) levels prior to, and following exposure to forced swim and restraint stress were used to assess hypothalamic-pituitary-adrenal (HPA) axis activity. On the day of the forced swim test, each mouse was removed from its home cage and moved to a testing room where a basal blood sample was taken. Blood samples were also taken 15, 45, and 90 min following the onset of the FST to assess peak and recovery CORT levels. Blood was processed as previously described, and stored at -20°C for later analysis. Blood samples were also collected to assess HPA axis response to restraint stress (in conjunction with the FITC-D test of intestinal permeability). Plasma CORT was assessed by ELISA, following vendor instructions (Enzo Corticosterone ELISA, ADI-900-097, Enzo Life Sciences, Exeter, UK).

Plasma cytokines

At the end of the study, trunk blood was collected and processed as described above for collection of plasma. Samples were analysed using the MSD V-Plex Custom Mouse Cytokine kit (MesoScaleDiscovery, Brinny, Ireland) as per vendor instructions. The lower limits of detection for the kit ranged from 0.11 pg/ml (IL-1β) to 0.95 pg/ml (IL-10).

Caecal microbiota analysis

DNA extraction

Caecal contents were snap frozen at the end of the study and stored at -80°C until the samples could be processed. DNA was extracted from caecum using the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK) according to manufacturer's instructions with the addition of a 3 min vortex step using 2 ml screw-cap tubes

(Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm diameter sterile zirconia beads plus a single 2.5 mm diameter bead (BioSpec Products, Bartlesville, USA). Briefly, 200 mg of each caecal sample was added to a screw-cap tube containing beads with 1 ml of Qiagen InhibitEX® Buffer and vortexed for 3 min. Samples were then incubated at 70°C for 5 min to lyse cells. Samples were centrifuged and the DNA was pelleted and treated with proteinase K. The DNA was then washed with buffers AW1 and AW2 and eluted in 200 µl Buffer ATE. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland).

PCR and 16S compositional sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

The protocol involved two PCR reactions on the extracted DNA. The DNA was first amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer

5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

reverse primer

5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each reaction contained 2.5 µl genomic DNA, 5 µl forward

primer (1 μM), 5 μl reverse primer (1 μM) and 12.5 μl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following program: 95°C x 3 min, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, 72°C x 5 min and held at 4°C. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland). Following this, a second PCR reaction was carried out on the purified DNA using two indexing primers per sample (Illumina Nextera XT indexing primers, Illumina, Netherlands). Each reaction contained 5 μl purified DNA, 5 μl index 1 primer (N7xx), 5 μl index 2 primer (S5xx), 25 μl 2x Kapa HiFi Hot Start Ready mix and 10 μl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. PCR products were visualised and purified as described above. Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Clinical Microbiomics, Denmark using standard Illumina sequencing protocols.

Bioinformatic analysis

Paired-end reads were assembled using FLASH. Raw sequence reads were quality trimmed using the QIIME suite of tools (Version 1.8.0). This included the filtering of reads which failed to reach a quality score of > 25 and the removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection and operational taxonomic unit (OTU) grouping at 97% similarity were performed in QIIME using USEARCH v7. OTU sequences were aligned using PyNAST and the SILVA SSURef database release 111 was used to determine taxonomy. The vegan R package (v. 2.4-1) was used to for statistical calculations of

alpha and beta diversity. Alpha diversity was calculated using the Shannon index, Mann Whitney U test was performed to assess significance between groups. Principal coordinate analysis (PCoA) plots were used to visualise beta diversity between groups based on Bray-Curtis distance matrices; significance of beta diversity between groups was computed by performing an ADONIS PERMANOVA test.

Statistical analyses

All data, with the exception of microbiota data, are presented as mean +/- SEM. Data were excluded from analyses if greater than 2 standard deviations from the mean. Two-way ANOVAs were used to assess percentage of time interacting for object location, social interaction, plasma FITC-D and CORT. When a main effect was detected, a Bonferroni post-hoc test of multiple comparisons was used. Unpaired t-tests were used to analyse behaviour in the OF, EPM, FST and plasma cytokines. Correlations were performed using Spearman correlation coefficient (r). Mann Whitney U analysis was used to assess statistical differences in microbiota compositions and p-values were corrected for multiple comparisons using the Benjamini-Hochberg (BH) correction (FDR<0.05). Microbiota were analysed by subject rather than cage.

Results

Aged mice do not differ in spontaneous alternation behaviour, but exhibit impaired displaced object recognition.

Ageing is associated with cognitive decline, including impaired memory. Therefore, we performed the Y-maze test of spontaneous alternation and a novel object/object location test to assess memory in aged and young mice. Aged mice performed similarly to young mice in the spontaneous alternation test (Figure 6.1A). Whereas young mice spent a significantly greater percentage of time interacting with a displaced familiar object ($p<0.01$), aged males did not (Figure 6.1B).

Aged mice exhibit behaviours associated with anxiety but not depression.

Because ageing is often associated with changes in mood, we also investigated anxiety- and depressive- like behaviours using the open field, elevated plus maze, and forced swim tests. Aged mice spent less time in the centre arena and made fewer entries into this area during the open field test ($p<0.01$) (Figure 6.1C and 6.1 D). Aged animals also spent a significantly lower percentage of time in the open arms of the plus maze ($p<0.05$) (Figure 6.1E), and together, these findings suggest increased anxiety. Immobility, used as a marker of depressive- or despair- like behaviour did not differ between young and aged mice in response to the forced swim test (Figure 6.1F).

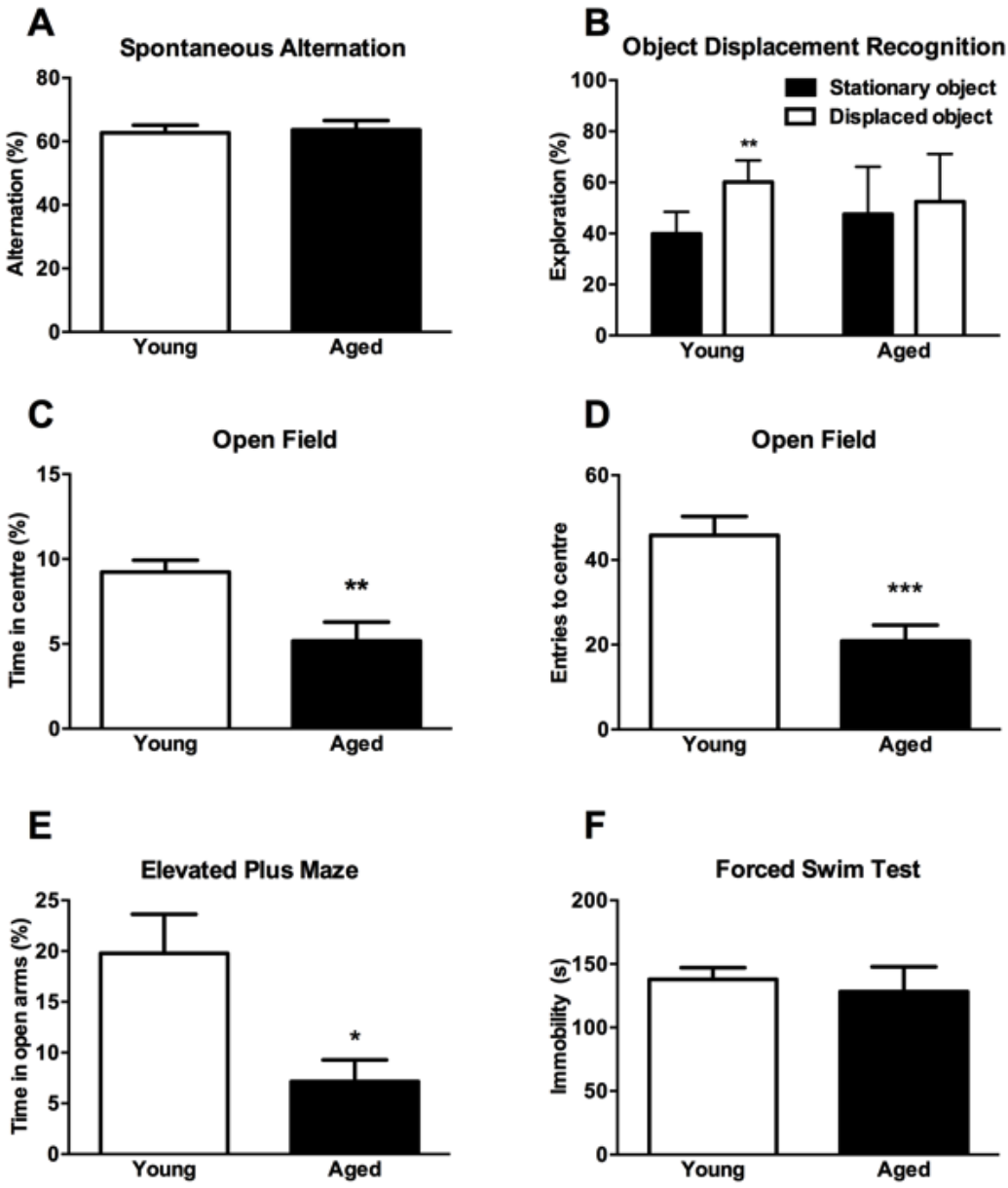

Figure 6.1. Behavioural tests of cognitive function, anxiety-, and depressive-like behaviours in young (n=9-12) and aged (n=8-10) mice. (A) No differences in spontaneous alternation were observed. (B) Young mice spent a significantly greater percentage of time interacting with a displaced, familiar object compared to a stationary, familiar object. (C) Aged mice spent significantly lower percentage of time in the centre of the open field. (D) Aged mice made fewer entries into the centre of the open field. E. Aged mice spent a significantly lesser percentage of time in the open arms of the EPM. F No differences in time spent immobile in the FST.

*p<0.05,**p<0.01, ***p<0.001

Aged mice exhibit behaviours associated with reduced social recognition and/or preference for social novelty.

As ageing is also associated with social withdrawal, social preference and social recognition were assessed using the 3-chamber social interaction test. Both aged and young mice spent more time investigating a novel mouse as opposed to a novel object, suggesting that there were no differences in preference for a conspecific ($p<0.0001$)(Figure 6.2A). However, whereas younger mice spent significantly more time interacting with a novel mouse than a familiar mouse ($p<0.0001$), aged mice spent similar amount of time investigating the novel and familiar mice, suggesting an impairment in social recognition or a reduced preference for social novelty (Figure 6.2B).

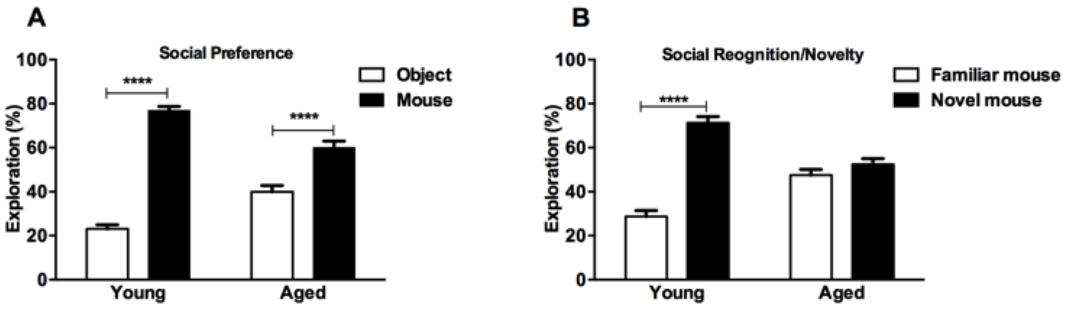

Figure 6.2. Behaviours of young (n=9) and aged (n=9) mice in the 3-chamber test. (A) Aged and young mice spent significantly more time investigating a novel mouse than a novel object. (B) Young mice spent significantly more time investigating a novel mouse than a familiar mouse. Aged mice exhibited no preference for the novel mouse.

****$p<0.0001$

Aged mice exhibit increased basal gut permeability, which is further exacerbated by acute stress exposure.

Gut permeability was assessed in young and aged mice prior to and following 1 h of restraint stress. Aged mice had significantly greater basal intestinal permeability than young mice ($p<0.05$). Whereas gut permeability of young mice was unaffected by restraint stress, permeability was further enhanced in aged mice (Aged basal v. aged-stressed, $p<0.01$, Young-stressed v. aged-stressed, $p<0.001$) (Figure 6.3).

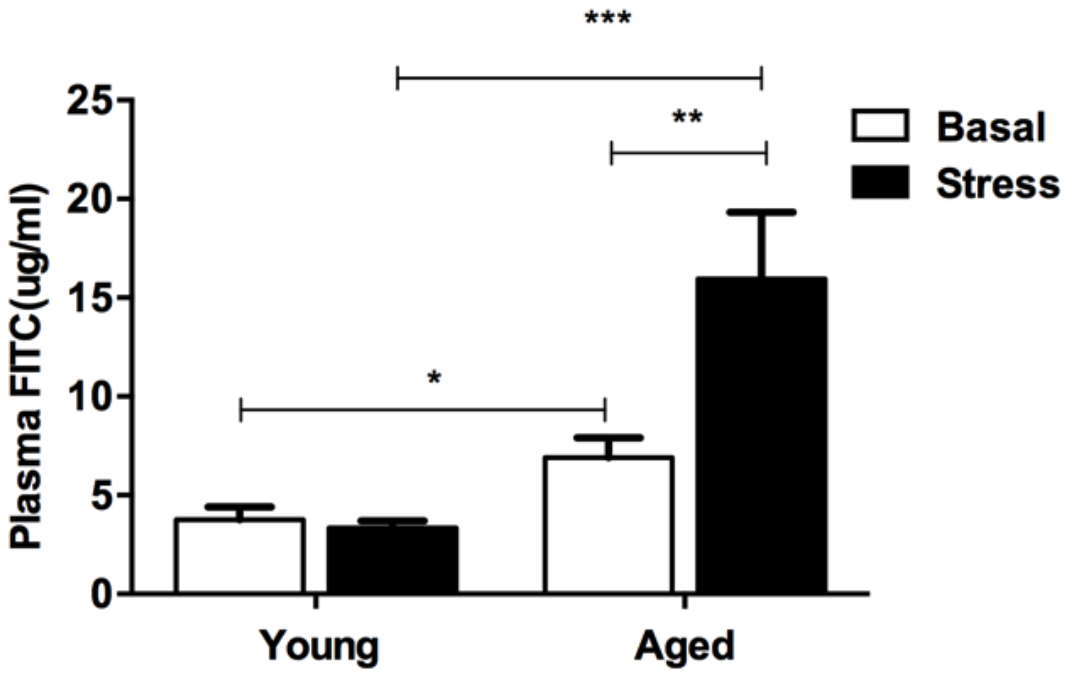

Figure 6.3. Basal and stress-induced gut permeability in young (n=12) and aged (n=10) mice. Basal gut permeability of aged mice was significantly greater than that of young mice and was significantly increased in response to acute restraint. Gut permeability of young mice was not increased in response to acute restraint stress.

*p<0.05 **p<0.01 ***p<0.001

Aged mice exhibit alterations in corticosterone response to acute stress exposure.

Despite seeing no behavioural effect, aged mice exhibited altered plasma CORT responses to the FST. Although basal CORT levels did not differ between groups, aged mice had significantly lower plasma CORT levels 15 min following the

onset of the test ($p<0.001$) (Figure 6.4A). Plasma CORT did not differ 45 and 90 min post FST; however, the trajectory of the CORT response differed. Although levels were dropping at these time-points in young mice, suggesting recovery, they were rising in aged mice, suggesting prolonged CORT release. CORT was also measured pre- and post- acute restraint, which was employed during the FITC-D test of intestinal permeability. Similar to our findings in the FST, basal CORT levels did not differ between young and aged mice. However, while plasma CORT was significantly increased in young mice following 1 h of restraint ($p<0.001$), CORT was unchanged in aged mice (young-stressed v. aged-stressed, $p<0.001$) (Figure 6.4B).

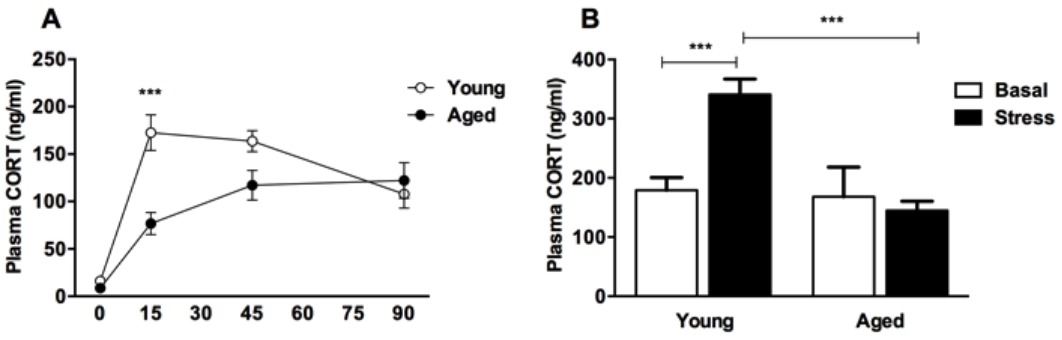

Figure 6.4. Corticosterone response to acute stress exposure in young (n= 12) and aged (n=10) mice. (A) Young mice had a significantly greater peak CORT response 15 min after the onset of FST (B) Young mice had significantly higher plasma CORT following 1 h of restraint in comparison with basal levels and significantly higher plasma CORT than aged mice exposed to restraint.

***$p<0.001$

Circulating plasma cytokines are elevated in aged mice and correlate with gut FITC-D permeability

Plasma cytokines were assessed as markers of peripheral inflammation at the end of the study. A number of pro-inflammatory cytokines were elevated in aged mice, including IL-1β (Figure 6.5A, $p<0.01$), TNF-α (Figure 6.5B, $p<0.0001$) and IL-2

(Figure 6.5C, $p<0.01$). There was a trend towards increased IL-6 expression in aged mice (Figure 6.5E, $p=0.07$). Anti-inflammatory cytokine IL-10 was unchanged in aged mice (Figure 6.5F). Plasma cytokines assessed at the end of the experiment were also plotted against plasma FITC-D levels assessed prior to and following restraint stress. In the basal state, IL-6 and TNF-α were significantly and positively correlated with plasma FITC-D levels ($p<0.05$), while there was a trend towards positive correlation between IL-1β ($p=0.07$), (Figure 6.6A). In the stressed state, all 3 of these cytokines were significantly and positively correlated with plasma FITC-D levels ($p<0.05$) (Figure 6.6B).

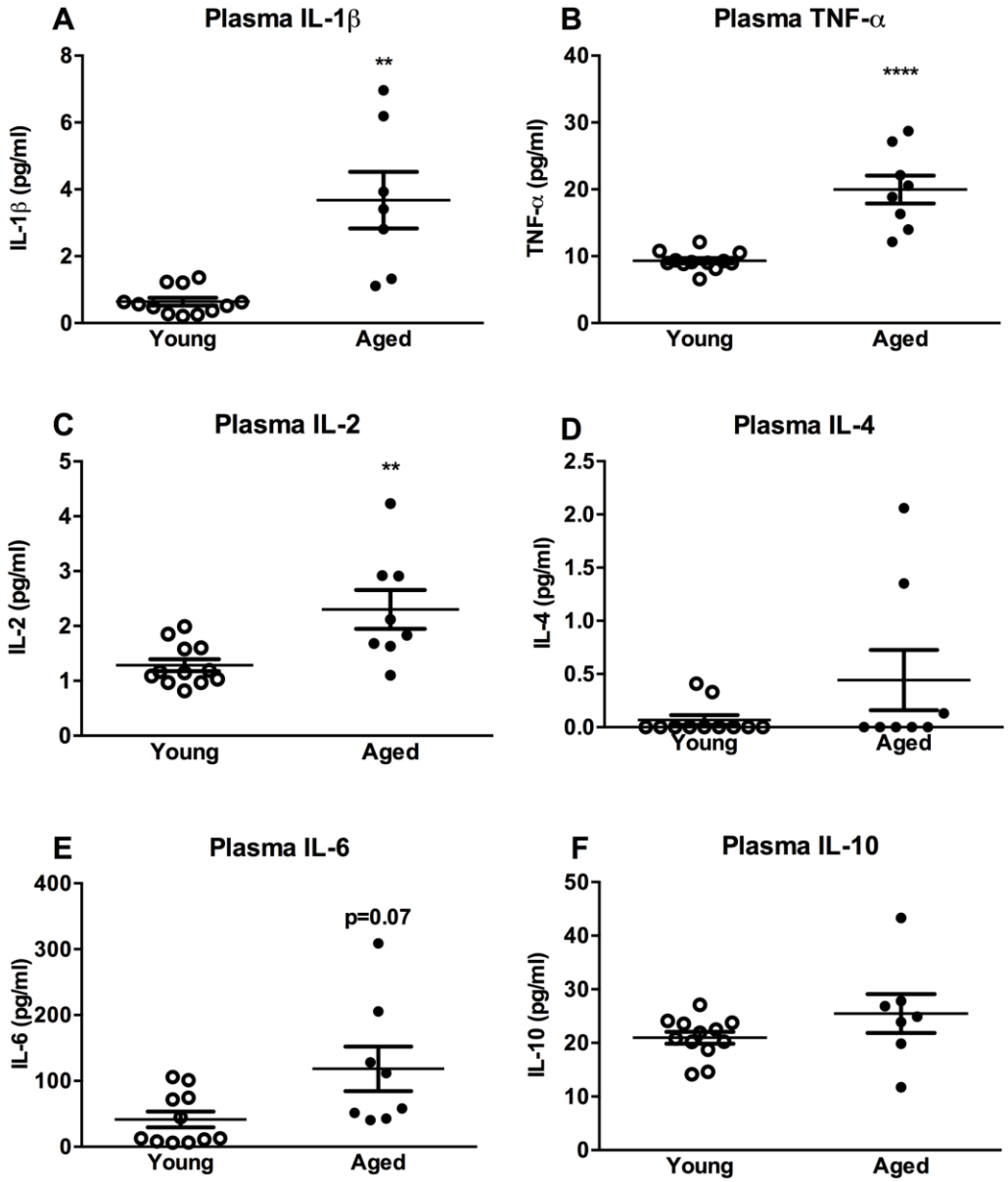

Figure 6.5. Plasma cytokines in young (n= 11-12) and aged (n=7-8) mice. (A) Plasma IL-1β was significantly elevated in aged mice. (B) Plasma TNF-α was significantly elevated in aged mice. (C) Plasma IL-2 was significantly elevated in aged mice. (D) There was no difference in plasma IL-4. (E) There was a trend towards increased plasma IL-6 levels in aged mice (p=0.07). (F) Plasma IL-10 did not differ between young and aged mice.

**p<0.01 ***p<0.001

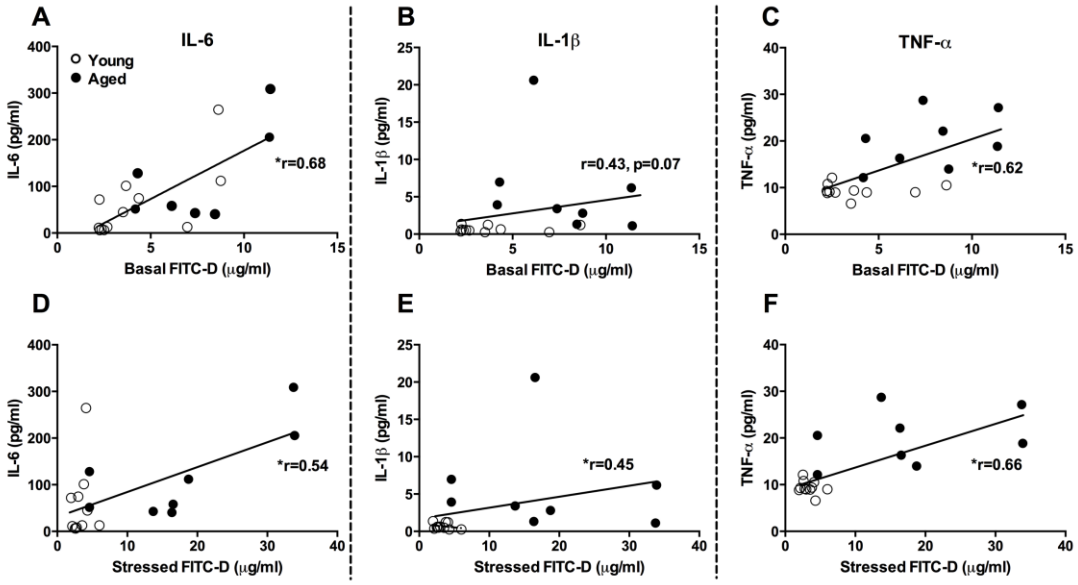

Figure 6.6. Correlation between plasma cytokines and gut permeability in young (n= 11-12) and aged (n= 8) mice. A positive correlation was found between plasma cytokines and basal (unstressed) gut permeability and in response to acute restraint stress (D-F). Open circles represent young and shaded circles represent aged mice.

*p< 0.05

The caecal microbiota of mice is significantly altered with ageing

MiSeq sequencing yielded a total of 8,339,660 raw reads, ranging from 108,541 to 339,456 reads per sample. Following quality filtering, reads were clustered into 1033 OTUs, which were assigned to taxa from phylum to genus level. Due to the variation in read number, the OTU table was rarefied to 108,000 reads, to allow for comparison between samples. The microbiota of aged mice differed significantly from that of young mice. Interestingly, only significant increases in bacterial taxa were seen in aged mice compared to young (Table 6.1).

At phylum level the most prevalent microbial taxa in both sample groups were Bacteroidetes and Firmicutes accounting for a combined relative abundance of >95% in the young and aged samples (Figure 6.7). Other bacterial phyla had relative

abundances of <2% in both groups. The large abundance of S24-7 and Rikenellaceae at family level was reflected at genus level as *S24-7 uncultured*, *Alistipes*, and *RC9 gut group*. Together, these highly abundant genera account for approximately 50% relative abundance at the genus level in both aged and young mice.

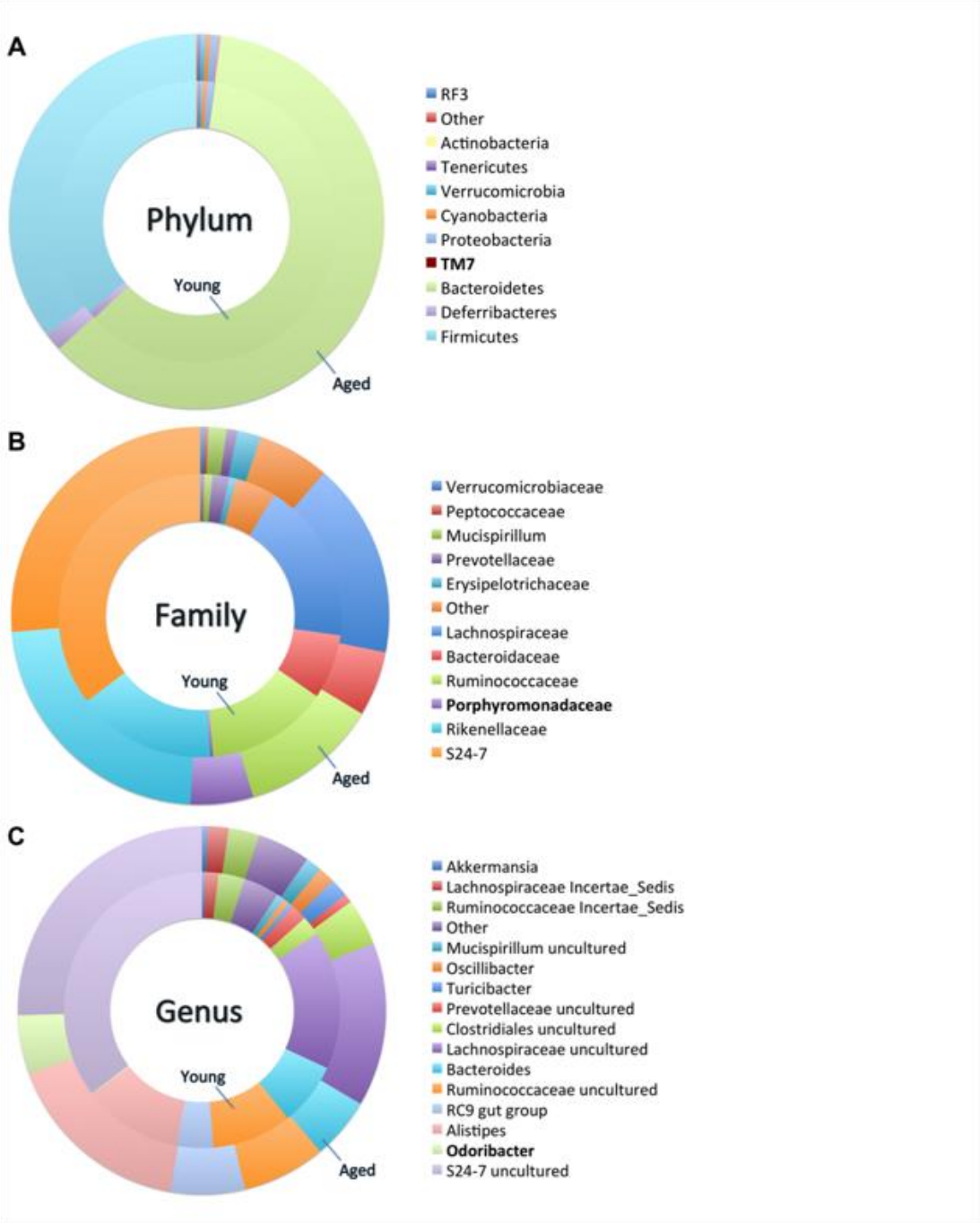

Figure 6.7. Visual representation of relative abundance of caecal bacterial changes on the order of (A) Phylum, (B) Family and (C) Genus of young (n=10) and aged (n=7) mice. Significant changes are denoted by bold type.

Several statistically significant changes in bacterial groups were observed in aged mice (Table 6.1). At the phylum level, TM7 was significantly higher in aged mice ($p<0.01$). At family level abundance less than 1%, significant increases in Porphyromondaceae ($p<0.01$), TM7 uncultured ($p<0.01$) Clostridiaceae ($p<0.05$), Thermoanaerobacteraceae ($p<0.05$), Desulfovibrionaceae ($p<0.05$) and Oxalobacteraceae ($p<0.05$) were seen in aged mice compared to young. Genera *Odoribacter* was much higher in the aged group (5.14%) than in the young mice (0.16%) ($p<0.01$). In addition, aged mice had significant increases in other bacterial genera, including *Butyricimonas* ($p<0.01$), *TM7 uncultured* ($p<0.01$), *Gelria* ($p<0.05$), *Anaerosporobacter* ($p<0.05$), *Clostridium* ($p<0.05$), *Oxalobacter* ($p<0.05$).

Mann-Whitney	Phylum	Class	Order	Family	Genus
p-value < 0.01	Bacteroidetes	Bacteroidia	Bacteroidales	↑ **Porphyromonadaceae**	↑ ***Butyricimonas***
	Bacteroidetes	Bacteroidia	Bacteroidales	↑ **Porphyromonadaceae**	↑ ***Odoribacter***
	↑ **TM7**	Uncultured	Uncultured	↑ **TM7 uncultured**	↑ ***TM7 uncultured***
p-value < 0.05	Firmicutes	Clostridia	Thermoanaerobacterales	↑ **Thermoanaerobacteraceae**	↑ ***Gelria***
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	↑ ***Anaerosporobacter***
	Firmicutes	Clostridia	Clostridiales	↑ **Clostridiaceae**	↑ ***Clostridium***
	Proteobacteria	Betaproteobacteria	Burkholderiales	↑ **Oxalobacteraceae**	↑ ***Oxalobacter***
	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	↑ **Desulfovibrionaceae**	N/A

↑ Increase, ↓ Decrease in Aged relative to Young

Significant at taxonomic level

Table 6.1. Differences in caecal microbiota between young (n=10) and aged (n=7) mice. Arrows in bold type denote significant changes within aged mice.

The Chao 1 index (which estimates species richness) was significantly higher in the aged group ($p=0.01$). In addition, the number of observed species and the phylogenetic diversity were also higher in the aged mice ($p=0.01$, $p=0.03$). The Shannon index (alpha diversity) was significantly higher in aged mice ($p<0.0001$,

Figure 6.8A). Beta diversity was significantly different between aged and young groups (p=0.001, Figure 6.8B).

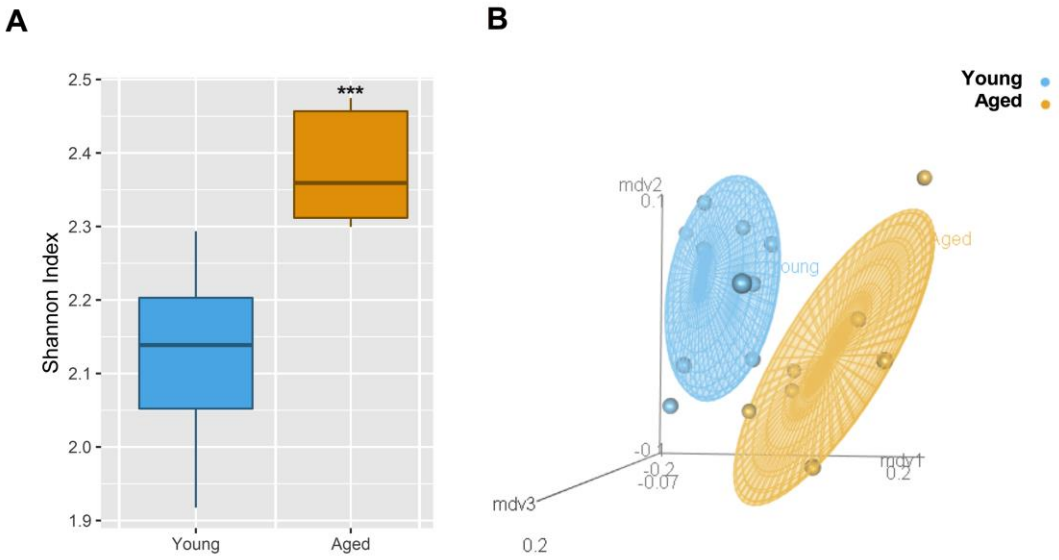

Figure 6.8. Alpha diversity, quantified by the Shannon index (A) and beta diversity, represented by principal coordinate analysis (B) of caecal bacteria of young (n=10) and aged (n=7) mice.

The relative abundance of Porphyromonadaceae correlates with anxiety-like behaviour in aged mice

Because some of the microbiota changes observed have been associated with cognitive dysfunction, we compared relative abundance of 3 taxa that were significantly altered in aged mice, Porphyromonadaceae, Odoribacter and TM7, with behavioural data. Interestingly, in aged mice, the relative abundance of members of the family Porphyromonadaceae was significantly and negatively correlated with the number of entries and time spent in the open arms of the elevated plus maze (Figure 6.9A and 6.9B, $p<0.05$). That is, as Porphyromonadaceae increased in caecal contents, so did anxiety-like behaviour in aged mice. No correlations with anxiety-like behaviours were found in young mice, as presence of taxa from the

Porphyromonadaceae family was so low. We found no other correlations between behaviours and the relative abundance of Porphyromonadaceae, Odoribacter or TM7 bacterial groups.

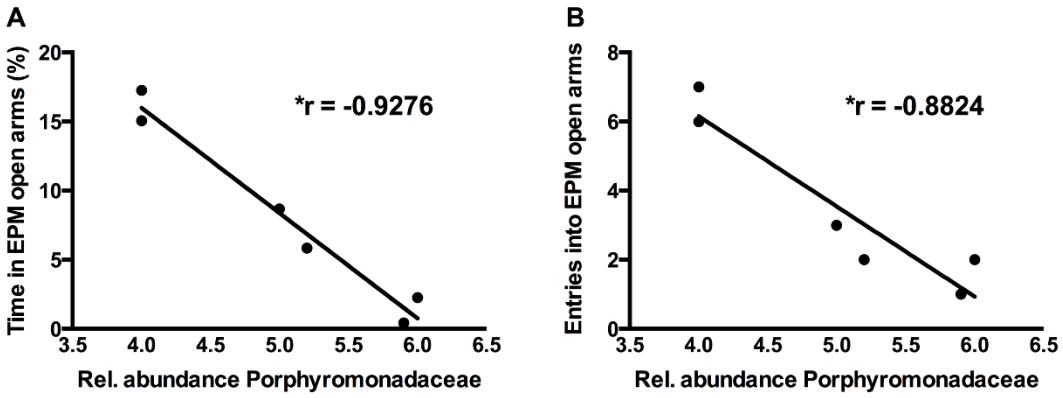

Figure 6.9. Correlation between behaviours and relative abundance of Porphyromonadaceae. Porphyromonadaceae and (A) time spent in the EPM and (B) entries into open arms of the EPM were negatively correlated for aged (n=6) mice.

*p<0.05

Discussion

Although the microbiome has been implicated in the ageing process, there have been few studies investigating the age-related changes in microbiota concomitant with gut permeability, stress response, peripheral inflammation and behaviour. Here we show that ageing is associated with marked changes in microbiota composition coupled with behavioural and physiological alterations.

Some of the most troubling features associated with ageing are negative affect and cognitive decline (Eshkoor et al., 2015; Prenderville et al., 2015). In these studies, we found that aged mice exhibited several behavioural changes that are thought to reflect these symptoms. The most robust of these were increases in anxiety-like behaviours, as assessed by thigmotaxis during the open field test and less time spent in the open arms of the elevated plus maze. It should be noted that aged mice exhibit reductions in overall locomotor activity; therefore, all data were expressed as percentages to avoid this as a confounding variable. However, we cannot rule out the possibility that reduced activity influenced the results. Anxiety is often reported in the elderly, as are anxiety-like behaviours in aged laboratory animals (Andreescu & Varon, 2015; Brouwer-Brolsma et al., 2014; Creighton et al., 2016; Ellison et al., 2015; Kastenschmidt & Kennedy, 2011). Importantly, epidemiological studies suggest that anxiety disorders in the elderly predispose or exacerbate the onset of cognitive impairments and other conditions, including cardiovascular disease (Andreescu & Varon, 2015).

Impaired performance of aged mice in the object displacement task is in line with other studies suggesting age-associated deficits in hippocampal-dependent spatial memory (Klencklen et al., 2012; Maasberg et al., 2012; Wimmer et al., 2012).

Whereas both young and aged mice demonstrated a preference for a novel conspecific over a novel object, aged mice did not exhibit a preference for novel mice when presented with the choice between novel and familiar mice. However, it is unclear whether these findings reflect a true impairment in social recognition or a lack of preference for a novel conspecific.

In addition to alterations in behaviour, aged mice exhibited several physiological changes. It is well established that many aspects of gut function, including intestinal barrier function, are impaired with age, and we observed a similar impairment in aged mice (Man et al., 2014; Saffrey, 2014; Tran & Greenwood-Van Meerveld, 2013). Interestingly, acute restraint stress exacerbated gut permeability in aged animals, despite a lack of rise in plasma glucocorticoid levels; in our studies, aged mice actually had a blunted and slower HPA axis response to acute stress, although it appears that they may also experience prolonged corticosterone release. The reason for this disconnect between plasma glucocorticoids and increased gut permeability is unclear, as the general consensus is that elevations in glucocorticoids contribute to increased gut permeability (Bhatia & Tandon, 2005; Meddings & Swain, 2000). However, other studies suggest that glucocorticoids may actually serve to protect the gastrointestinal tract (Filaretova, 2007). Furthermore, Crohn's disease, marked by increased inflammation and gut permeability has been associated with hypoactivity of the HPA axis; this may arise because glucocorticoids have anti-inflammatory effects, and insufficient corticosteroid levels may result in excessive inflammation (Stasi & Orlandelli, 2008). Although ageing is often associated with basal hyperactivity of the hypothalamic-pituitary-adrenal axis, blunted and delayed activation and recovery of the HPA axis has also been previously reported (Buechel et al., 2014; Veldhuis et al., 2013). Furthermore, it has been previously demonstrated

that the intestinal microbiota is sensitive to stress exposure, and this may in turn, modulate immune responses (Bailey, 2014; Mackos et al., 2016). In the current study we employed acute stressors, but future studies will incorporate chronic stress, which is particularly relevant to unhealthy ageing.

Numerous factors are thought to contribute to age-associated impairments in gut function. These include histological changes within the structure of the gut, including decreased expression of tight junction proteins and altered morphology of intestinal villi (Ren et al., 2014; Tran & Greenwood-Van Meerveld, 2013). Mucous and bicarbonate secretion by the gut and short chain fatty acid (SCFA) production by the gut microbiota, which serve to protect the intestinal epithelia, also decrease with ageing (Rehman, 2012; Saffrey, 2014; Woodmansey, 2007). Moreover, gut motility is also often reported to decrease in the elderly and in aged laboratory animals, likely due to changes in the smooth muscle structure, gut innervation and disrupted signalling.

Previous studies have demonstrated that the gut microbiota also influences gut permeability and behaviour (Saffrey, 2014; Yarandi et al., 2016). The age-related changes in microbiota that we observed have been previously implicated in inflammation and cognitive decline. The most significant increases that we observed are seen in phylum TM7, family Porphyromonadaceae and genus *Odoribacter*. These bacteria have been previously associated with inflammatory diseases such as cirrhosis and inflammatory bowel disease (IBD) (Collins et al., 2012; Giannelli et al., 2014). Interestingly, our studies also revealed changes in bacteria previously associated with cognitive decline. In a study of encephalopathic patients with cirrhosis, increases in Porphyromonadaceae positively correlated with increased cognitive dysfunction (Bajaj et al., 2012; Collins et al., 2012). A previous study also found that poor

cognitive performance in the elderly was associated with increases in Porphyromonadaceae irrespective of cirrhosis (Bajaj et al., 2016). Caecal microbiota analysis in our studies revealed bacterial changes that have been previously observed in studies of depression and exposure to psychological stressors (Bangsgaard Bendtsen et al., 2012; Desbonnet et al., 2015; Watanabe et al., 2016). We found that relative abundance of Porphyromonadaceae was directly correlated with anxiety-like behaviours in aged mice. Increases in bacterial taxa from this family have also been observed in faecal samples from individuals with major depressive disorder (Jiang et al., 2015).

While we did not observe behavioural changes in the forced swim test, a common assay of anti-depressant-sensitive behaviours, this may reflect the overall sensitivity of the test. In future studies we may incorporate other methods of assessing depressive-like behaviour across different endophenotypes of the disorder (Kelly et al., 2016; Slattery & Cryan, 2012; Slattery & Cryan, 2014). The increased anxiety that we observed in aged mice may reflect microbiota changes in stress sensitivity, as noted above (Bangsgaard Bendtsen et al., 2012; Desbonnet et al., 2014; Watanabe et al., 2016). Interestingly, the caecal microbiota of aged mice was significantly more rich and diverse than that of young mice. Reductions in microbial diversity are often reported with ageing, but some studies have challenged these findings (Biagi et al., 2012). The reasons for increased diversity in aged mice is unclear; however, decreases in diversity are often reflective of unhealthy ageing and frailty, which we did not subjectively observe in this study (Claesson et al., 2012; Jackson et al., 2016). Another possibility is that with ageing, the stability of the microbiota is reduced, leading to proliferation of opportunistic bacteria (Biagi et al., 2012).

Perturbations in gut structure, function, and microbiota are believed to contribute to an increased risk of infection and inflammation, and markers of inflammation and immune responses have been reported in aged humans and in laboratory animals (Chung et al., 2009; Krabbe et al., 2004; Mabbott, 2015; Man et al., 2014; Tran & Greenwood-Van Meerveld, 2013). Similarly, we observed that plasma levels of several pro-inflammatory cytokines were elevated in aged mice, and IL-6, IL-1β and TNF-α correlated positively with gut permeability. Indeed, chronic systemic inflammation is linked to numerous neurodegenerative disorders and cognitive deficits associated with ageing, including Alzheimer's disease (Chung et al., 2009; Deleidi et al., 2015; Perry, 2010). Moreover, there is a growing literature linking microbiome-based changes with susceptibility to Alzheimer's disease and neurodegenerative disorders (Cattaneo et al., 2016; Fröhlich et al., 2016; Xu & Wang, 2016).

In conclusion, we show, for the first time to our knowledge, that perturbations of the microbiome-gut-brain axis, resultant of normal ageing, may contribute to peripheral inflammation and the development of altered anxiety behaviours and cognitive impairments. Aged male mice exhibited significant shifts in gut microbiota and marked differences in stress responsivity, gut permeability and peripheral inflammation in comparison with young adult mice. In addition, they exhibited behavioural changes associated with cognitive deficits and increased anxiety. These increases in anxiety-like behaviour were directly correlated with abundance of bacteria from the Porphyromonadaceae family; this is in agreement with previous studies finding an association between this family and cognitive dysfunction and mood disorders (Bajaj et al., 2016; Collins et al., 2012; Jiang et al., 2015).

Future studies should focus on the mechanisms that are at play in driving these changes. There is increasing emphasis on understanding pathways of microbiome to brain signalling (including vagus nerve, neuroendocrine pathways, enteric nervous system short chain fatty acids, tryptophan metabolism and the immune system) but there is still a lack of knowledge in this field (Cryan & Dinan, 2012; Galland, 2014; O'Mahony et al., 2015; Rogers et al., 2016). Moreover, the microbiome has been shown to regulate adult hippocampal neurogenesis (Möhle et al., 2016; Ogbonnaya et al., 2015), microglia activation (Erny et al., 2015), blood brain barrier function (Braniste et al., 2014) and neuroinflammation (D'Mello et al., 2015), all of which are altered in ageing. Future studies should also address the relative contribution of these to the alterations in behaviour seen in ageing. These studies suggest that the gut microbiota may prove a worthy target for the development of novel therapies to ameliorate or prevent some of the adverse neurobehavioural consequences of unhealthy ageing.

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Alkermes, and Janssen, and has received research funding from Mead Johnson, Cremo, Suntory Wellness, Nutricia and 4D Pharma. MI and TI are employed by Suntory Wellness.

References

Andreescu, C., & Varon, D. (2015). New research on anxiety disorders in the elderly and an update on evidence-based treatments. *Current psychiatry reports*, *17*(7), 53.

Antunes, M., & Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing*, *13*(2), 93-110.

Bailey, M. T. (2014). Influence of stressor-induced nervous system activation on the intestinal microbiota and the importance for immunomodulation. *Microbial endocrinology: the microbiota-gut-brain axis in health and disease*, 255-276.

Bajaj, J. S., Ahluwalia, V., Steinberg, J. L., Hobgood, S., Boling, P. A., Godschalk, M.,... & Wade, J. B. (2016). Elderly patients have an altered gut-brain axis regardless of the presence of cirrhosis. *Scientific reports*, *6*(1), 1-12.

Bajaj, J. S., Ridlon, J. M., Hylemon, P. B., Thacker, L. R., Heuman, D. M., Smith, S.,... & Gillevet, P. M. (2012). Linkage of gut microbiome with cognition in hepatic encephalopathy. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *302*(1), G168-G175.

Bendtsen, K. M. B., Krych, L., Sørensen, D. B., Pang, W., Nielsen, D. S., Josefsen, K.,... & Hansen, A. K. (2012). Gut microbiota composition is correlated to grid floor induced stress and behaviour in the BALB/c mouse. *PloS one*, *7*(10), e46231.

Bhatia, V., & Tandon, R. K. (2005). Stress and the gastrointestinal tract. *Journal of gastroenterology and hepatology*, *20*(3), 332-339.

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., & Brigidi, P. (2012). Ageing of the human metaorganism: the microbial counterpart. *Age*, *34*(1), 247-267.

Biagi, E., Candela, M., Turroni, S., Garagnani, P., Franceschi, C., & Brigidi, P. (2013). Ageing and gut microbes: perspectives for health maintenance and longevity. *Pharmacological research*, *69*(1), 11-20.

Biddle, A., Stewart, L., Blanchard, J., & Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity*, *5*(3), 627-640.

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature reviews. Neuroscience*, *8*(1), 57–69.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in molecular medicine*, *20*(9), 509-518.

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., & Pettersson, S. (2014). The gut

microbiota influences blood-brain barrier permeability in mice. *Science translational medicine*, *6*(263), 263ra158.

Brouwer-Brolsma, E. M., Schuurman, T., de Groot, L. C. P. M. G., Feskens, E. J. M., Lute, C., Naninck, E. F. G.,... & Steegenga, W. T. (2014). No role for vitamin D or a moderate fat diet in ageing induced cognitive decline and emotional reactivity in C57BL/6 mice. *Behavioural brain research*, *267*, 133-143.

Brüssow, H. (2013). Microbiota and healthy ageing: observational and nutritional intervention studies. *Microbial biotechnology*, *6*(4), 326-334.

Buechel, H. M., Popovic, J., Staggs, K. H., Anderson, K. L., Thibault, O., & Blalock, E. (2014). Aged rats are hypo-responsive to acute restraint: implications for psychosocial stress in ageing. *Frontiers in aging neuroscience*, *6*, 13.

Candela, M., Biagi, E., Brigidi, P., O'Toole, P. W., & De Vos, W. M. (2014). Maintenance of a healthy trajectory of the intestinal microbiome during ageing: a dietary approach. *Mechanisms of ageing and development*, *136*, 70-75.

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C.,... & Group, I. F. (2017). Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiology of aging*, *49*, 60-68.

Chung, H. Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A. Y., Carter, C., Yu, B. P., & Leeuwenburgh, C. (2009). Molecular inflammation: underpinnings of ageing and age-related diseases. *Ageing research reviews*, *8*(1), 18–30.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., Harris, H. M., Coakley, M., Lakshminarayanan, B., O'Sullivan, O., Fitzgerald, G. F., Deane, J., O'Connor, M., Harnedy, N., O'Connor, K., O'Mahony, D., van Sinderen, D., Wallace, M., Brennan, L., Stanton, C., … O'Toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature*, *488*(7410), 178–184.

Collins, S. M., Surette, M., & Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nature reviews. Microbiology*, *10*(11), 735–742.

Creighton, A. S., Davison, T. E., & Kissane, D. W. (2016). The prevalence of anxiety among older adults in nursing homes and other residential aged care facilities: a systematic review. *International journal of geriatric psychiatry*, *31*(6), 555–566.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature reviews. Neuroscience*, *13*(10), 701–712.

Cryan, J. F., & Dinan, T. G. (2015). Gut microbiota: Microbiota and neuroimmune signalling-Metchnikoff to microglia. *Nature reviews. Gastroenterology & hepatology*, *12*(9), 494–496.

Cryan, J. F., & Mombereau, C. (2004). In search of a depressed mouse: utility of models for studying depression-related behaviour in genetically modified mice. *Molecular psychiatry*, *9*(4), 326–357.

D'Mello, C., Ronaghan, N., Zaheer, R., Dicay, M., Le, T., MacNaughton, W. K., Surrette, M. G., & Swain, M. G. (2015). Probiotics Improve Inflammation-Associated Sickness Behaviour by Altering Communication between the Peripheral Immune System and the Brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *35*(30), 10821–10830.

Deleidi, M., Jäggle, M., & Rubino, G. (2015). Immune ageing, dysmetabolism, and inflammation in neurological diseases. *Frontiers in neuroscience*, *9*, 172.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., & Cryan, J. F. (2014). Microbiota is essential for social development in the mouse. *Molecular psychiatry*, *19*(2), 146–148.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D., Cotter, P. D., Dinan, T. G., & Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity*, *48*, 165–173.

Diaz Heijtz R. (2016). Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behaviour. *Seminars in fetal & neonatal medicine*, *21*(6), 410–417.

Dinan, T. G., & Cryan, J. F. (2017). Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *The Journal of physiology*, *595*(2), 489–503.

Duda-Chodak, A., Tarko, T., Satora, P., & Sroka, P. (2015). Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. *European journal of nutrition*, *54*(3), 325-341.

Ellison, D., White, D., & Farrar, F. C. (2015). Ageing population. *The Nursing clinics of North America*, *50*(1), 185–213.

Erny, D., Hrabě 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., & Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nature neuroscience*, *18*(7), 965–977.

Eshkoor, S. A., Hamid, T. A., Mun, C. Y., & Ng, C. K. (2015). Mild cognitive impairment and its management in older people. *Clinical interventions in aging*, *10*, 687–693.

Filaretova L. P. (2007). Activation of the hypothalamo-hypophyseal-adrenocortical system as an important gastroprotective component of the stress reaction. *Neuroscience and behavioral physiology*, *37*(4), 355–362.

Fröhlich, E. E., Farzi, A., Mayerhofer, R., Reichmann, F., Jačan, A., Wagner, B., Zinser, E., Bordag, N., Magnes, C., Fröhlich, E., Kashofer, K., Gorkiewicz, G., & Holzer, P. (2016). Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain, behavior, and immunity*, *56*, 140–155.

Galland L. (2014). The gut microbiome and the brain. *Journal of medicinal food*, *17*(12), 1261–1272.

Giannelli, V., Di Gregorio, V., Iebba, V., Giusto, M., Schippa, S., Merli, M., & Thalheimer, U. (2014). Microbiota and the gut-liver axis: bacterial translocation, inflammation and infection in cirrhosis. *World journal of gastroenterology*, *20*(45), 16795–16810.

Dao, D. T., & Kovacsics, C. E. (2009). *Mood and anxiety related phenotypes in mice: characterization using behavioral tests* (Vol. 2). T. D. Gould (Ed.). New York: Humana Press.

Hall, C., & Ballachey, E. L. (1932). A study of the rat's behaviour in a field. A contribution to method in comparative psychology. *University of California Publications in Psychology*.

Hughes, R. N. (2004). The value of spontaneous alternation behaviour (SAB) as a test of retention in pharmacological investigations of memory. *Neuroscience & Biobehavioral Reviews*, *28*(5), 497-505.

Jackson, M. A., Jeffery, I. B., Beaumont, M., Bell, J. T., Clark, A. G., Ley, R. E.,... & Steves, C. J. (2016). Signatures of early frailty in the gut microbiota. *Genome medicine*, *8*(1), 1-11.

Jeffery, I. B., & O'Toole, P. W. (2013). Diet-microbiota interactions and their implications for healthy living. *Nutrients*, *5*(1), 234-252.

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y.,... & Ruan, B. (2015). Altered faecal microbiota composition in patients with major depressive disorder. *Brain, behavior, and immunity*, *48*, 186-194.

Joyce, M. F., & Reich, J. A. (2015). Critical care issues of the geriatric patient. *Anesthesiology clinics*, *33*(3), 551-561.

Kastenschmidt, E. K., & Kennedy, G. J. (2011). Depression and anxiety in late life: diagnostic insights and therapeutic options. *Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine*, *78*(4), 527-545.

Kelly, J. R., Borre, Y., O'Brien, C., Patterson, E., El Aidy, S., Deane, J.,... & Dinan, T. G. (2016). Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. *Journal of psychiatric research*, *82*, 109-118.

Klencklen, G., Després, O., & Dufour, A. (2012). What do we know about ageing and spatial cognition? Reviews and perspectives. *Ageing research reviews*, *11*(1), 123-135.

Krabbe, K. S., Pedersen, M., & Bruunsgaard, H. (2004). Inflammatory mediators in the elderly. *Experimental gerontology*, *39*(5), 687-699.

Langille, M. G., Meehan, C. J., Koenig, J. E., Dhanani, A. S., Rose, R. A., Howlett, S. E., & Beiko, R. G. (2014). Microbial shifts in the ageing mouse gut. *Microbiome*, *2*(1), 1-12.

Li, S. C., & Schmiedek, F. (2002). Age is not necessarily ageing: Another step towards understanding the 'clocks' that time ageing. *Gerontology*, *48*(1), 5-12.

Lynch, D. B., Jeffery, I. B., & O'Toole, P. W. (2015). The role of the microbiota in ageing: current state and perspectives. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, *7*(3), 131-138.

Maasberg, D. W., Shelley, L. E., & Gilbert, P. E. (2012). Age-related changes in detection of spatial novelty. *Behavioural brain research*, *228*(2), 447-451.

Mabbott, N. A. (2015). A breakdown in communication? Understanding the effects of ageing on the human small intestine epithelium. *Clinical Science*, *129*(7), 529-531.

Mackos, A. R., Varaljay, V. A., Maltz, R., Gur, T. L., & Bailey, M. T. (2016). Role of the intestinal microbiota in host responses to stressor exposure. *International review of neurobiology*, *131*, 1-19.

Mackowiak P. A. (2013). Recycling metchnikoff: probiotics, the intestinal microbiome and the quest for long life. *Frontiers in public health*, *1*, 52.

Man, A. L., Gicheva, N., & Nicoletti, C. (2014). The impact of ageing on the intestinal epithelial barrier and immune system. *Cellular immunology*, *289*(1-2), 112–118.

Meddings, J. B., & Swain, M. G. (2000). Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. *Gastroenterology*, *119*(4), 1019–1028.

Möhle, L., Mattei, D., Heimesaat, M. M., Bereswill, S., Fischer, A., Alutis, M., French, T., Hambardzumyan, D., Matzinger, P., Dunay, I. R., & Wolf, S. A. (2016). Ly6C(hi) Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. *Cell reports*, *15*(9), 1945–1956.

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., & Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural brain research*, *277*, 32–48.

O'Tuathaigh, C. M., Babovic, D., O'Sullivan, G. J., Clifford, J. J., Tighe, O., Croke, D. T., Harvey, R., & Waddington, J. L. (2007). Phenotypic characterisation of spatial

cognition and social behaviour in mice with 'knockout' of the schizophrenia risk gene neuregulin 1. *Neuroscience*, *147*(1), 18–27.

Ogbonnaya, E. S., Clarke, G., Shanahan, F., Dinan, T. G., Cryan, J. F., & O'Leary, O. F. (2015). Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biological psychiatry*, *78*(4), e7–e9.

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open:closed arm entries in an elevated plus maze as a measure of anxiety in the rat. *Journal of neuroscience methods*, *14*(3), 149–167

Perry V. H. (2010). Contribution of systemic inflammation to chronic neurodegeneration. *Acta neuropathologica*, *120*(3), 277–286.

Peterson, C. T., Sharma, V., Elmén, L., & Peterson, S. N. (2015). Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. *Clinical and experimental immunology*, *179*(3), 363–377.

Porsolt R. D. (1979). Animal model of depression. *Biomedicine / [publiee pour l'A.A.I.C.I.G.]*, *30*(3), 139–140.

Porsolt, R. D., Anton, G., Blavet, N., & Jalfre, M. (1978). Behavioural despair in rats: a new model sensitive to anti-depressant treatments. *European journal of pharmacology*, *47*(4), 379–391.

Prenderville, J. A., Kennedy, P. J., Dinan, T. G., & Cryan, J. F. (2015). Adding fuel to the fire: the impact of stress on the ageing brain. *Trends in neurosciences*, *38*(1), 13–25.

Rehman T. (2012). Role of the gut microbiota in age-related chronic inflammation. *Endocrine, metabolic & immune disorders drug targets*, *12*(4), 361–367.

Ren, W. Y., Wu, K. F., Li, X., Luo, M., Liu, H. C., Zhang, S. C., & Hu, Y. (2014). Age-related changes in small intestinal mucosa epithelium architecture and epithelial tight junction in rat models. *Aging clinical and experimental research*, *26*(2), 183–191.

Rogers, G. B., Keating, D. J., Young, R. L., Wong, M. L., Licinio, J., & Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Molecular psychiatry*, *21*(6), 738–748.

Saffrey M. J. (2014). Ageing of the mammalian gastrointestinal tract: a complex organ system. *Age (Dordrecht, Netherlands)*, *36*(3), 9603.

Senechal, Y., Kelly, P. H., Cryan, J. F., Natt, F., & Dev, K. K. (2007). Amyloid precursor protein knockdown by siRNA impairs spontaneous alternation in adult mice. *Journal of neurochemistry*, *102*(6), 1928–1940.

Slattery, D. A., & Cryan, J. F. (2012). Using the rat forced swim test to assess anti-depressant-like activity in rodents. *Nature protocols*, *7*(6), 1009–1014.

Slattery, D. A., & Cryan, J. F. (2014). The ups and downs of modelling mood disorders in rodents. *ILAR journal*, *55*(2), 297–309.

Stasi, C., & Orlandelli, E. (2008). Role of the brain-gut axis in the pathophysiology of Crohn's disease. *Digestive diseases (Basel, Switzerland)*, *26*(2), 156–166.

Tran, L., & Greenwood-Van Meerveld, B. (2013). Age-associated remodelling of the intestinal epithelial barrier. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *68*(9), 1045–1056.

Veldhuis, J. D., Sharma, A., & Roelfsema, F. (2013). Age-dependent and gender-dependent regulation of hypothalamic-adrenocorticotropic-adrenal axis. *Endocrinology and metabolism clinics of North America*, *42*(2), 201–225.

Watanabe, Y., Arase, S., Nagaoka, N., Kawai, M., & Matsumoto, S. (2016). Chronic Psychological Stress Disrupted the Composition of the Murine Colonic Microbiota and Accelerated a Murine Model of Inflammatory Bowel Disease. *PloS one*, *11*(3), e0150559.

Weindruch, R., & Prolla, T. A. (2002). Gene expression profile of the ageing brain. *Archives of neurology*, *59*(11), 1712-1714.

Wimmer, M. E., Hernandez, P. J., Blackwell, J., & Abel, T. (2012). Ageing impairs hippocampus-dependent long-term memory for object location in mice. *Neurobiology of aging*, *33*(9), 2220–2224.

Woodmansey E. J. (2007). Intestinal bacteria and ageing. *Journal of applied microbiology*, *102*(5), 1178–1186.

Xu, R., & Wang, Q. (2016). Towards understanding brain-gut-microbiome connections in Alzheimer's disease. *BMC systems biology*, *10 Suppl 3*(Suppl 3), 63.

Yarandi, S. S., Peterson, D. A., Treisman, G. J., Moran, T. H., & Pasricha, P. J. (2016). Modulatory Effects of Gut Microbiota on the Central Nervous System: How Gut Could Play a Role in Neuropsychiatric Health and Diseases. *Journal of neurogastroenterology and motility*, *22*(2), 201–212.

Young, J. J., Bruno, D., & Pomara, N. (2014). A review of the relationship between pro-inflammatory cytokines and major depressive disorder. *Journal of affective disorders*, *169*, 15–20.

Zheng, X., Zhang, X., Kang, A., Ran, C., Wang, G., & Hao, H. (2015). Thinking outside the brain for cognitive improvement: Is peripheral immunomodulation on the way?. *Neuropharmacology*, *96*(Pt A), 94–104.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

The overall aim of this thesis was to examine the current understanding of the microbial colonisation of the gut from birth and throughout life, as well as the implications of an altered microbiome profile on brain development, stress reponses, immunity and behaviour. In Chapter 1, the progression of a typical healthy microbiome composition was examined from birth to adulthood. Following this, we focused on the factors known to significantly impact this progression (Marques et al., 2010). Beginning during the perinatal period and continuing into adulthood, many influences on the gut microbiota composition throughout life are discussed including diet, stress, infection and age (Milani et al., 2017). We then examined the use of probiotics and prebiotics as a promising avenue for gut microbiota modulation, including viable methods of delivery and key research investigating the effects of probiotics and prebiotics on the gut microbiota composition in both infants and adults. The role of potential probiotics which target the gut-brain axis to confer a mental health benefit (psychobiotics) was then explored (Dinan et al., 2013).

In Chapter 2, we discussed the microbiota-gut-brain axis in more detail, examining signalling pathways and potential future psychobiotic targets. The communication network between the gut microbiota and the brain is explored as well as the role of the gut-brain axis in mood, cognition and mental health (Sampson & Mazmanian, 2015). Potential microbiota-targeted functional foods for brain health are discussed including polyphenols and omega-3 PUFAs. This field has grown significantly in recent years with new strains of bacteria emerging as potential probiotics targeting mental health and psychiatric conditions (Cryan et al., 2019).

Chapter 3 focused on the impact of an altered gut microbiota composition from birth. Faecal samples were collected from infants who were born by C-section and also received antibiotic treatment in the first four days of life. It is widely known that C-section delivery can cause significant disturbances in the infant gut (Bäckhed et al., 2015). The gut microbiota composition of these infants was analysed from birth until 24 weeks. The impact of both antibiotic treament and mode of delivery was seen when the gut microbiota composition was compared to infants who were born vaginally (Hill et al., 2017). Several key microbial communities were absent in the first weeks of life and many alterations at phylum, family and genus levels were still present 24 weeks later. The examination of the impact of both insults (antibiotic exposure and C-section delivery) on the maturing infant gut highlights not only the magnitude of the gut microbiota disturbances, but also the fact that these alterations persist for at least 24 weeks after birth with respect to composition and diversity.

In Chapter 4 we investigated the long-term effects of an altered microbiota composition at birth. We examined the gut microbiota composition and stress responses of a cohort of young adults who had been born by C-section. It was found that the gut microbiota composition did not differ between individuals born by C-section or those born naturally. However, when examining stress responses, it was seen that the participants who were born by C-section reported higher levels of anxiety during an exam stress period and higher psychological stress during an acute stressor. This highlights the negative impact of gut microbiota alterations during infancy on psychological processes into adulthood.

Chapter 5 explored the effects of a potential probiotic, *Bifidobacterium longum* 1714™, on mood, stress, and cognition. A cohort of healthy male participants were given either placebo or the probiotic during an exam stress period in a randomised,

placebo-controlled, repeated measures, cross-over design. It was noted that the probiotic did not affect the gut microbiota in terms of composition or diversity. Additionally, the probiotic did not show a significant effect on measures of mood or stress. However, while the sleep quality of the placebo group decreased during the exam stress period, it remained consistent for the individuals consuming the probiotic. While this probiotic did not directly influence measures of mood and stress, it is known that anxiety and sleep quality are linked and thus, future work on the effects of this probiotic is warranted (Ramsawh et al., 2009; Vandekerckhove & Cluydts, 2010).

While previous chapters have focused on the microbiota-gut-brain axis in young adults, Chapter 6 examined associations between the gut microbiota and the ageing brain and cognitive decline. The caecal microbiota of aged and young male C57BL/6 J mice was analysed along with a variety of behavioural tests designed to examine cognition and mood. It was found that ageing is associated with microbiota alterations accompanied by behvaioural and physiological changes. Aged mice displayed increased anxiety-like behaviour and gut pereability when compared to young mice. Additionally, the microbiota of aged mice demonstrated a shift toward a compositional profile consistant with increased inflammation.

In conclusion, the role of the gut microbiota throughout life, from infancy to adulthood has been shown to be significant. This thesis has highlighted the implications of an altered gut microbiota at birth, resulting in a substantial change in the development and progression of the gut microbiota over the first 24 weeks of life. The disruption in the normal colonisation pattern of the infant gut as it co-matures with the infant brain can be seen in later life psychological vulnerabilities. It is evident that the gut microbiota is dynamic and fluid throughout life, from infancy into adulthood, and again and into old age, with additional aged-related gut microbiota

profile shifts. Many of the factors discussed that affect the gut microbiota disturbances are unavoidable, however, the continued use of probiotics as intervention therapies to restore gut microbiota composition and diversity in infants and adults and the elderly is a growing and promising area of investigation. Continued exploration into the relationship between the gut-microbiota-brain axis and altered microbiota profiles, as well as cognitive, psychological, behavioural and mood disorders is key to providing further insight into potential future therapeutic targets.

References

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P.,... Zhong, H. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell host & microbe, 17*(5), 690-703.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M.,... Golubeva, A. V. (2019). The Microbiota-Gut-Brain Axis. *Physiological reviews, 99*(4), 1877-2013.

Dinan, T. G., Stanton, C., & Cryan, J. F. (2013). Psychobiotics: a novel class of psychotropic. *Biological psychiatry, 74*(10), 720-726.

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A.,... Tuohy, K. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome, 5*(1), 4.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A., & Stanton, C. (2010). Programming infant gut microbiota: influence of dietary and environmental factors. *Current opinion in biotechnology, 21*(2), 149-156.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The First Microbial Colonisers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiology and molecular biology reviews : MMBR, 81*(4), e00036-17.

Ramsawh, H. J., Stein, M. B., Belik, S. L., Jacobi, F., & Sareen, J. (2009). Relationship of anxiety disorders, sleep quality, and functional impairment in a community sample. *Journal of psychiatric research, 43*(10), 926–933.

Sampson, T. R., & Mazmanian, S. K. (2015). Control of brain development, function, and behaviour by the microbiome. *Cell host & microbe, 17*(5), 565-576.

Vandekerckhove, M., & Cluydts, R. (2010). The emotional brain and sleep: An intimate relationship. *Sleep Medicine Reviews, 14*(4), 219-226.