Investigation of the impact of prebiotics and blueberry (source of flavonoids) on the production of metabolites by gut microbiota and related impacts on cognitive function

By

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To my son, Cinar Ali

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Abstract:

The gut-brain axis (GBA) is emerging as a complex communication that can impact on emotional and cognitive centres of the brain. The aim of this work was to investigate the effect of prebiotics such as inulin and blueberry flavonoids on the gut microbiota composition and associated neuroactive metabolite production. In order to assess the mechanism of how gut microbiota might impact on cognitive functions, a series of *in-vitro* studies and one human trial was performed. A combination of *in vitro* models was initially used to access if the microbiota could produce molecules related to neurological pathways in the absence of human cells and then to determine if supplementation with blueberries or prebiotics could result in enhanced levels of neuroactive metabolites along with growth of beneficial gut bacteria. Data supported the positive shift of gut bacteria and the production of metabolites, therefore a more complex gut model system was used and indicated increased potential for these interventions to support the gut brain axis. Finally, a pilot intervention study was carried out to explore links of the *in vitro* findings with the *in vivo* situation with children aged 7-10 years old, who would be undergoing rapid cognitive changes. Following intervention a significant increase in the % abundance of *Faecalibacterium prausnitzii* for the inulin group was observed. Moreover, both inulin and berry groups experienced significantly higher accuracy in an attention network task and significant increases the memory tasks. These results are promising as to show a potential for inulin to have direct effects on GBA through microbiota, whilst the cognitive changes observed of berries might be by a different pathway. Although larger trials are necessary this thesis indicates that the GBA can be altered through diet and both prebiotics and berries may be relevant foods to support these changes.

Key words: probiotics; microbiota; beneficial bacteria; psychobiotics; gut-brain axis; cognitive function

LIST OF ABBREVIATIONS:

GBA: Gut Brain Axis

CNS: Central Nervous System

ENS: Enteric Nervous System

HPA: The hypothalamic-pituitary-adrenal axis

GABA: Gamma-Amino Butyric Acid

SCFAs: Short Chain Fatty Acids

WBB: Wild Blueberry

MANT: Modified Attention Network Task

ADHD: Attention Deficit Hyperactivity Disorder

ASD: Autism Spectrum Disorder

FISH-FCM: Fluorescence *In Situ* Hybridization with Flow Cytometry

LC-MS: Liquid Chromatography-Mass Spectrometry

CHAPTER 1 (GENERAL INTRODUCTION):

The gut brain axis and the potential role of prebiotics and flavonoid-rich blueberry supplements

Abstract

Current research has pointed to a relationship between intestinal microbiota dysfunctions and brain disorders. The gut-brain bidirectional interaction has brought out a complex communication system including immune mechanisms, the vagus nerve and microbial neurometabolite production.

These communications are involved in the classification of "gut-brain axis (GBA)". Recent studies have provided strong evidence of an important role of gut microbiota in the regulation of cognitive state through the GBA. The role of GBA is to monitor and incorporate gut functions to relate emotional and cognitive centres of the brain with peripheral intestinal functions and mechanisms such as immune activation, intestinal permeability, and entero-endocrine signalling. Dysbiosis of gut microbial function has been related with behavioural and cognitive diseases, as such, research primarily focused on developing novel dietary approaches by targeting the regulation of gut microbiota. A number of factors may impact on the gut microbiota composition such as health status, mode of birth delivery and genetics, but diet is accounted the most considerable factor on the human gut microbiota. In this manner, dietary interventions may have the potential to regulate the brain functions via the GBA. Further *in-vitro* studies are also needed to better figure out the core mechanisms of the connections between nutrition, gut microbiota, and control of cognitive and behaviour health. Moreover, the impact of flavonoid-rich food such as blueberries, on cognitive function has been the focus of a number of recent studies. Certain epidemiological, preclinical, and clinical trials have exposed positive cognitive benefits from flavonoid interventions. Data obtained from these studies include cognitive benefits that might be associated with the delayed memory and executive function especially in children. These studies should be expanded in the future considering with the dose used, cognitive tasks, and age and gender groups (Hein et al., 2019).

In this review, evidence for the impact of the prebiotics and flavonoids on gut microbiota and the impacts of the gut microbiota on brain functions has been considered. Therefore, leading to the consideration of whether the gut could be used as a functional tool for improving cognition.

Key Words: probiotics; microbiota; beneficial bacteria; psychobiotics; gut-brain axis; cognitive functions

1. INTRODUCTION:

There is growing evidence suggesting a link between gastrointestinal function and the central nervous system (CNS). Results in animal studies have indicated a significant role of intestinal microbes in gut-brain communication (Bercik et al., 2012). Research into gut-brain crosstalk has revealed a complex communication system where the maintenance of gastrointestinal homeostasis is likely to impact on the brain, motivation, and cognitive function. The complex mechanism of these interactions encompasses in the definition of "gut-brain axis (GBA)" (Carabotti et al., 2015). The GBA comprises of bidirectional interaction mainly between the central and the enteric nervous systems, linking emotional and cognitive centres of the brain with intestinal function (Mayer et al., 2015; O'Mahony et al., 2015). Both clinical and experimental research indicates that enteric microbiota has an important impact on GBA, interacting with intestinal cells and enteric nervous system (ENS), and also directly with central nervous system (CNS) through neuroendocrine and metabolic pathways. Data has indicated that both brain-gut and gut-brain dysfunctions occurring in the GBA could be related to brain and cognitive diseases (Dinan & Cryan, 2017). Therefore, alterations in gut microbiota might modulate the peripheral and central nervous systems resulting in stimulated brain and cognitive functioning which highlights the importance of a microbiota gut-brain axis. Diet can also change the profile of gut microbiota and, therefore impact on the brain. The composition of the gut microbiota might impact on the production of molecules such as neurotransmitters, e.g., gamma amino butyric acid, serotonin and catecholamines and products of fermentation, e.g., short chain fatty acids butyrate, propionate, and acetate, that are also implicated in GBA. As such, dietary interventions might have the significant effects to modulate psychiatric symptoms related with gut/brain axis dysfunction (Marques et al., 2014).

1.1. Gut Brain Axis:

Currently, there is considerable evidence that the microbiota–gut–brain axis plays an important role in mental and cognitive health, human clinical studies have yet to provide clear answers as to how the gut microbiota influences brain development and function. Furthermore, mechanisms of how dietary supplements exert their apparent effects on stress, mood, and cognition is a growing exploratory area (Chakrabarti et al., 2022). As such, focus has been extended to the role of the gut microbiota (referring to the trillions of microorganisms and viruses residing in the gut), creating considerable excitement with findings that suggest specific intestinal microorganisms (the greatest amount of information comes from studies of bacteria) may be associated with memory, learning, stress, and mood, and even neurodevelopmental, and neurodegenerative disorders (Margolis et al., 2021).

Principally, the gut microbial processes occurring in the gastro-intestinal track (GIT) can be modulated by dietary ingredients such as prebiotics (Grimaldi et al., 2018), probiotics (Cerdo et al., 2017) or even flavonoids (Rodriguez-Daza et al., 2020). The microbiota composition in the human intestine effects host physiological functions through metabolic activities. The microbiota produces small bioactive molecules which impact on the host and contribute to the neurohumoral axes connecting the intestine with other parts of the body (Krishnan et al., 2015). The theory of modulation of the brain and cognition within the microbiota-gut-brain axis is based on the specific mechanisms by which the bacteria within the gut microbiome (the gut microbiota) collaborate with the host. Interactions between intestinal microbiota and the brain can occur via the central nervous system (CNS), neuroendocrine and immune systems, the autonomic nervous system (ANS), the enteric nervous system (ENS) **(Figure 1)** and the hypothalamic pituitary adrenal (HPA) axis. These systems are communicating via several pathways which are, neural, endocrine and immune pathways (Mayer et al., 2015). As it can also be seen in **Table 1.,** this bidirectional communication comprises interactions with the gut microbiota releasing immune activating and other signalling molecules, such as short chain fatty acids (SCFAs) and neurotransmitters, such as GABA, serotonin and biologically active forms of catecholamines in the lumen of the gut (Asano et al., 2012) which have a significant impact in regulating cognitive and memory function (Cryan & Dinan, 2012; Dinan & Cryan, 2017; Foster & McVey Neufeld, 2013).

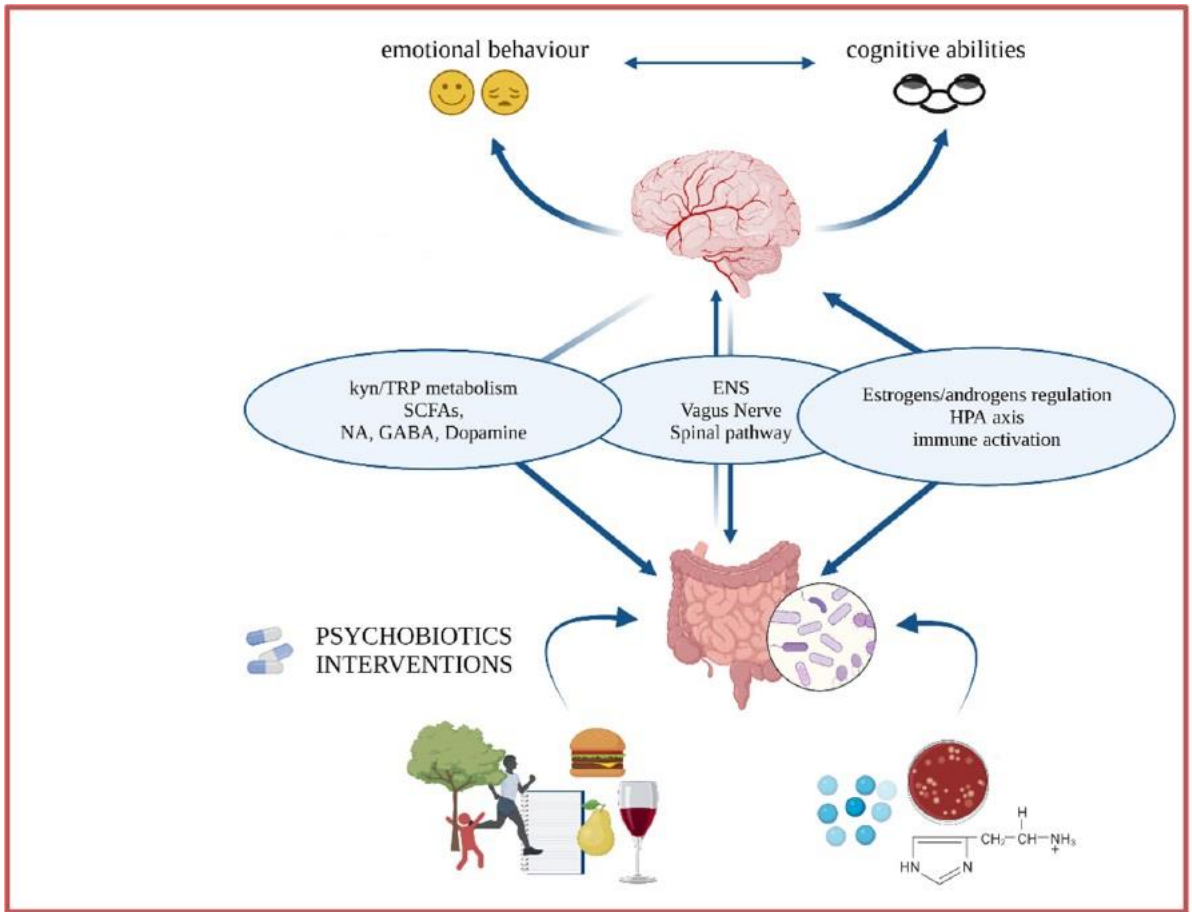

Figure 1. Interaction of the gut microbiome, probiotics and prebiotics interventions on the brain gut axis and their outcomes on cognitive abilities and behaviours (Basso et al., 2022).

Evidence indicates that there is a significant but widely indirect impact of beneficial gut microbiota on the central nervous system (CNS) it is still unknown whether gut bacteria have direct effects on neurotransmitter receptors in the CNS. Modifications of central GABA receptor expression are however associated with cognitive function, memory functions, anxiety and depression which are also present concurrently with functional bowel disorders (Bravo et al., 2011; Wang et al., 2018). As such, the gut microbiota might provide a relevant target for modulation of cognitive health in both clinical and non-clinical populations (Sun et al., 2020). According to a review of Li and Zhou (2016), certain clinical studies showed that gastrointestinal symptoms and compositional changes in the gut microbiota are frequently associated with cerebral disorders in patients with ASD. A disturbance in the gut microbiota, which is usually induced by a bacterial infection or chronic antibiotic exposure, has been implicated as a potential contributor to ASD (Li & Zhou, 2016). Thus, it can be seen in Figure 2, the alterations in the gut microbiota composition have subsequent impacts on neurological, cognitive, and behavioural domains (Davidson et al., 2018).

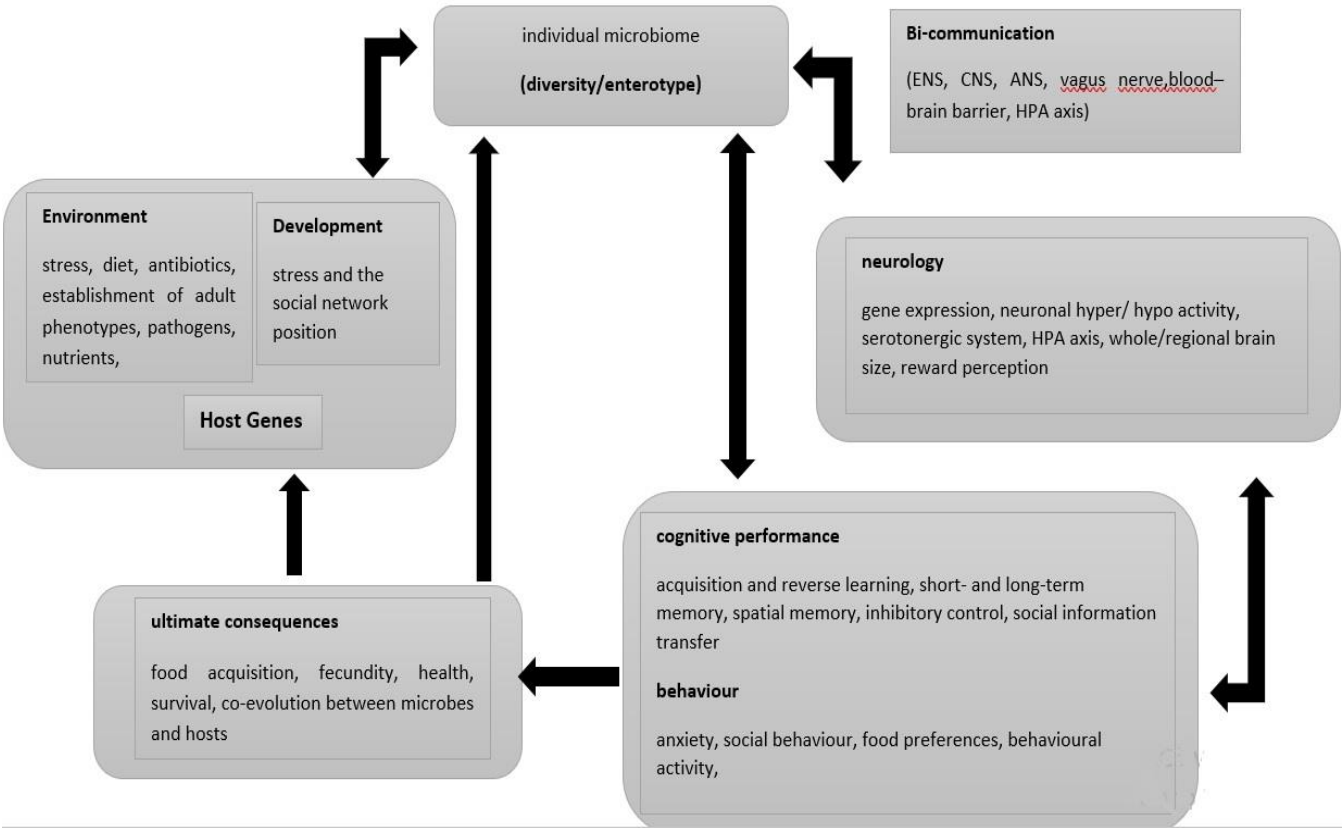

Figure 2. Causes and consequences of individual variation in the microbiome in relation to environmental and developmental effects, and the subsequent impact on neurological, cognitive and behavioural traits. Arrows indicate potential causal directions of relationships and are not exhaustive (for example, development may directly affect cognition independent of microbiome) (Davidson et al., 2018).

Regarding cognitive functioning and mental health, significant alteration of microbial diversity has been associated with behavioural and cognitive abnormalities (Cohen Kadosh et al., 2021). In terms of the childhood period, the individual is likely to undergo significant internal and external environmental changes and demands. Additionally, this is a time window when neuroplasticity is enhanced, allowing brain structures and circuitries to flexibly adapt or maladapt to the environment. Within this scope, gut microbiota might be critical in early developing ages and increased plasticity levels may lead to atypical behaviour patterns and abnormal brain network maturation. As such, childhood could be an appropriate point to target changes in cognitive function to offer initial benefits that may be sustained throughout the life-course (Everson-Rose, 2003).

As a potential functional target for these changes, the gut microbiota acts like a mediator between the environment and the CNS via multiple pathways that include: (1) the vagus nerve and spinal tract, whose action can be either direct or mediated by the ENS; (2) the HPA axis; (3) sex hormones (e.g.,

oestrogens and androgens); (4) microbes' production of proinflammatory compounds, which can lead to systemic inflammation and microglia activation; and (5) microbial metabolites able to cross the BBB (e.g., SCFAs) and to alter the tryptophan/kynurenine pathways. Gut microbiota can be easily manipulated through diet; thus, it could be a promising therapy target in the redirection of neurodevelopmental trajectories (Basso et al., 2022) (Figure 3).

Table 1. Main Principal Mechanisms of the Bidirectional Brain-Gut Microbiota Axis:

From gut microbiota to brain:	**From brain to gut microbiota:**
Production, expression, and turnover of neurotransmitters (i.e., serotonin, GABA) and neurotrophic factor (BDNF)	Alteration in mucus and biofilm production
Protection of intestinal barrier and tight junction integrity	Alteration in motility
Modulation of enteric sensory afferents	Alteration of intestinal permeability
Bacterial metabolites	Alteration in immune function
Mucosal immune regulation	

The vagus nerve is the major nerve of ANS and studies conducted with mice have assessed the impact of probiotics such as *Lactobacillus rhamnosus* and *Bifidobacterium* spp. on stress, depression and anxiety through this path and changes in GABA receptors (Bercik et al., 2012). In a study performed with adult male BALB/c mice (n = 36) chronic treatment with *Lactobacillus rhamnosus* induced region-dependant modulations in GABA receptors mRNA in the brain and additionally, *L. rhamnosus* reduced stress-induced corticosterone and anxiety- and depression-related behaviour. Furthermore, there were no significant neurochemical and behavioural effects found in vagotomised mice, which points that the vagus nerve could be a major regulatory communication pathway between the commensal bacteria to the gut and the brain (Bercik et al., 2012; Bravo et al., 2011). All these findings highlight the important role of bacteria in the bidirectional communication of the gut-brain axis and suggests that certain probiotics may be appropriate to be used as a therapeutic adjunct in stress related disorders (Bravo et al., 2011).

As it can be seen in **Figure 3.,** the gut microbiota is also involved in tryptophan metabolism and kynurenine pathway, HPA, and cytokine production (Golubeva et al., 2017; Kennedy et al., 2017). Additionally, the impact of microbiota composition on CNS may also be altered by the disruption of the luminal/mucosal habitat which can be regulated by dietary intervention including probiotics and prebiotics (Marques et al., 2014).

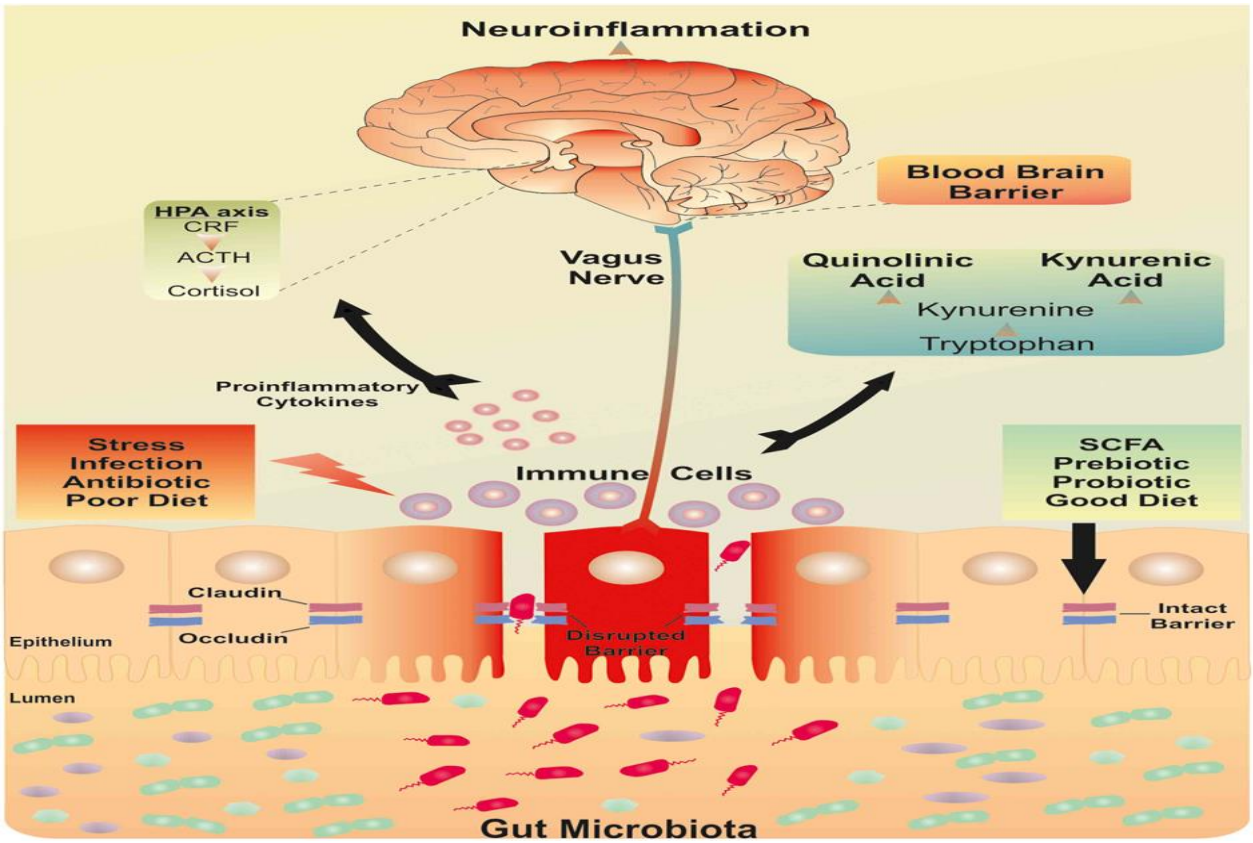

Figure 3. The brain-gut-microbiota axis. Postulated signalling pathways between the gut microbiota, the intestinal barrier, and the brain. A dysfunctional intestinal barrier or "leaky gut" could permit a microbiota-driven pro inflammatory state with implications for neuro inflammation (Kelly et al., 2015).

A major communication mechanism of GBA could be considered to be via modulation of the intestinal barrier, which has significant effects on all underlying parts of the gastrointestinal system of the host (Kelly et al., 2015). This layer separates commensal microorganisms and pathogens from immune cells, this epithelial barrier function provides a defence mechanism against infection and inflammation and is composed of secretory IgA, antimicrobial components and dynamic junctional composites that modulate the permeability between cells. Microbial dysbiosis is linked with a damaged epithelial barrier, bacterial translocation, decreased regulatory T cells in the gut mucosa. Promotion of inflammation, is associated with cognitive impairment (Collins, 2014). As such, certain strains of

probiotics have been observed to improve the epithelial barrier function, resulting in a decrease in permeability (Steenbergen et al., 2015). Stress may have a role in the intestinal barrier and has been related with an increase in gut permeability. Moreover, the effects of stress on intestinal permeability are complicated and contribute to the gut brain axis. Thus, modulation of gut microbiota with dietary intervention might be able to alleviate stress symptoms through impacting on this barrier (Dinan & Cryan, 2013).

1.1.1. Microbial Metabolites Involved in the Gut Brain Axis:

1.1.1.1. Short Chain Fatty Acids (SCFAs):

Short chain fatty acids (SCFAs) are fermentation end products of intestinal bacteria, these have been largely reported to have a positive impact on host metabolism, modulating immune response and epithelial cell growth, and supporting the functioning of the central and peripheral nervous systems (Koh et al., 2016). SCFAs have a chain length between one to six carbon atoms (Dalile et al., 2019) and typically reaching total concentrations of 50–200 mM in the human large intestine (Louis & Flint, 2017). Acetate, propionate and butyrate are the most abundant SCFAs in gastrointestinal tract (den Besten et al., 2013). Acetate and propionate are primarily produced by Bacteroidetes whereas Firmicutes are the primary contributors of butyrate (Koh et al., 2016). Current studies have revealed that acetate is produced by most of the enteric bacteria such as *Lactobacillus* spp., *Bifidobacterium* spp., *Akkermansia muciniphila, Bacteroides* spp., *Prevotella* spp., *Ruminococcus* spp., and *Streptococcus* spp. via Wood-Ljungdahl pathway and acetyl-CoA pathway (Louis & Flint, 2017). Propionate is produced by *Phascolarctobacterium succinatutens, Bacteroides* spp., *Dialister* spp., *Megasphaera elsdenii, Veillonella* spp. *Coprococcus catus, Roseburia inulinivorans, Ruminococcus obeum, Salmonella* spp., via succinate pathway, acrylate pathway, and propanediol pathway (Koh et al., 2016). Butyrate is produced by *Roseburia* spp. *Eubacterium rectale, Clostridium leptum, Eubacterium hallii, Coprococcus eutactus, Faecalibacterium prausnitzii, Eubacterium rectale, Anaerostipes caccae*, and *Coprococcus catus* via the butyryl-CoA:acetate CoA-transferase routes and the phosphotransbutyrylase/butyrate kinase routes (Fernandez-Veledo & Vendrell, 2019). Butyrate is known to have direct effects on the growth, maturation and functioning of gut epithelial cells and on the nervous system, inhibiting calcium-dependant potassium channel (Kunze et al., 2009). Elevated levels of SCFA show a promising impact against certain conditions such as obesity, diabetes, inflammatory (bowel) disease, colorectal cancer, and neurological disorders and are known as a key moderator of host-microbe crosstalk (Bourassa et al., 2016; Stilling et al., 2016).

In the gastrointestinal tract, SCFAs might reduce the luminal pH and enhance the absorption of some nutrients (Macfarlane et al., 2008). Moreover, they are reported to decrease colonic pH; inhibit growth of pathogens; improve intestinal integrity and function of colonic epithelial cells; possess anti-lipolysis effects; increase insulin sensitivity and energy expenditure; inhibit production of proinflammatory cytokines (Reigstad et al., 2015). These roles of SCFAs are especially important in the fight against intestinal diseases such as Crohn's disease, ulcerative colitis, and colorectal cancer (Geirnaert et al., 2017). In addition to this, SCFAs might impact the brain directly via crossing the blood brain barrier, modulating neurotransmission, influencing neurotrophic factor, and promoting serotonin biosynthesis (Dalile et al., 2019). As such, there is a great range of evidence that production of butyrate by the gut microbiota can strongly affect the peripheral immune system which shapes the brain's immune milieu indirectly (Filiano et al., 2015).

Histone acetylation is a post-translational modification by an epigenetic protein, these are proteins that bind to chromatin and influence chromatin structure to change the tendency of the gene to transcribe. Acetylated histones lead the chromatin structure to break up the electrostatic attraction between the histone proteins and the DNA backbone. Reduced histone acetyl transferase activity (HAT) is associated with many neurodegenerative diseases. Thus, histone deacetylase (HDAC) inhibitors are becoming promising therapeutic canditates due to their ability to increase histone acetylation (Bourassa et al., 2016). Acetate and butyrate have been reported to be histone deacetylase (HDAC) inhibitors and function as a ligand for a subset of G protein-coupled receptors and as an energy metabolite. HDACs are a family of proteins which activate the discharge of acetyl groups from lysine remaining within a peptide chain. Butyrate is also associated with promoting histone acetylation as well as inhibiting HDACs and stimulation of gene expression in host cells which led to the use of butyrate as an experimental drug in neurological disorders from depression to neurodegenerative diseases and cognitive impairment (Stilling et al., 2016). The possible role of butyrate in HDAC inhibition could provide the immune system with information about the microbiota composition and metabolic activity in the gut. Butyrate has also been observed to have a profound effect on improving learning and memory, for eample, in mouse models of Alzheimer's disease, histone acetylation is restored and expression of learning-associated genes is increased with butyrate treatment (Kilgore et al., 2010). HDAC inhibitors have an impact on the transcription of several genes related with neurotrophic effects, acetylation plays an important role that affects the enzymatic and metabolic activity of many proteins. As such, this effect also depends on elevating levels of BDNF in specific brain regions, such as the prefrontal cortex, which is likely due to elevating histone acetylation in the BDNF gene (Intlekofer et al., 2013).

Certain psychiatric disorders display important responses to butyrate-activated histone acetylation such as a reduction in depressive-like behaviour in animal models (Sun et al., 2016). This impact also relies on increasing levels of BDNF in prefrontal cortex of the brain, which could be the result of elevating histone acetylation in the BDNF gene (Intlekofer et al., 2013). Production of butyrate by the gut microbiota might have a significant impact on immune system which is also related the brain indirectly (Stilling et al., 2016). Furthermore, butyrate can directly impact serotonin and gut hormone release in the enteric nervous system, and this stimulates the vagus nerve affecting brain function (Filiano et al., 2015; Schroeder et al., 2007).

1.1.1.2. Tryptophan Metabolism, Serotonin:

Tryptophan is an essential amino acid and can cross the blood-brain barrier (BBB) and participate in serotonin (5-HT) synthesis in the CNS. Serotonin is mainly located in the gut, but it is synthesised from tryptophan enterochromaffin cells (ECs) of the gastrointestinal tract. As such, ECs have a direct interaction with the external environment including the gut microbiota and microbial metabolites in the gut lumen. Thus, it might be considered that the gut microbiota can have both direct and indirect effects involving tryptophan metabolism and serotonergic signalling within the scope of the gut-brain axis to modulate host brain function (O'Mahony et al., 2015). A study showed that spore-forming bacteria had an effect on serotonin in mice, by producing metabolites directly involved in signalling between gut microbiota and ECs (Yano et al., 2015).

Tryptophan metabolism is thought to be modulated by gut microbiota and microbial regulation with the impact of both on serotonin synthesis and the control of kynurenine pathway metabolism. (Kennedy et al., 2017). In the CNS, 5-HT is related to a range of mood and behavioural and cognitive functions (Cryan & Dinan, 2012; Frohlich et al., 2016; O'Mahony et al., 2015). However, a major metabolite of tryptophan produced by gut bacteria is indole, both of which are kynurenine pathway metabolites and along with circulating 5-HT concentrations are strongly associated with GI 5-HT synthesis. This process seems to be largely driven by microbial metabolites such as SCFAs and tryptophan-derived indole metabolites (Reigstad et al., 2015; Yano et al., 2015). It can be stated that a major direct impact on circulating serotonin availability is likely derived from an adequate dietary supply of tryptophan amino acid and as a result of the bacterial tryptophan utilisation, and the impact of microbial metabolites on host serotonergic production (Kennedy et al., 2017). It is essential to explore which specific members of the gut microbiota are playing the most significant role for this function. Preclinical studies have indicated a strong link between the gut microbiota and the tryptophan availability for kynurenine metabolism and serotonin pathway. As such, manipulating the microbial composition of the gastrontestinal tract modulates plasma concentrations of tryptophan, a

key neurotransmitter within both the enteric and central nervous systems. Within this scope, serotonin has a key role in the gut-brain axis, both at the level of ENS and CNS (Clarke et al., 2014). The mechanism based on the regulation of circulating tryptophan concentrations by the bacteria in our gut is still yet to be clear but might involve degradation of tryptophan along an alternative and physiologically dominant metabolic route, the kynurenine pathway (Clarke et al., 2013). Thus, metabolites produced downstream of kynurenine are neuro-active, such as kynurenic acid and quinolinic acid. The ability of the microbiota to regulate tryptophan and kynurenine likely contributes significantly to its ability to conduct CNS and ENS neurotransmission (Schwarcz & Stone, 2017). Furthermore, it has been shown that circulating tryptophan levels are increased in the absence of a gut microbiota (Kennedy et al., 2017). Kynurenine pathway metabolism and circulating 5-HT concentrations were found to be decreased while circulating tryptophan levels were increased. These findings were consistent with the previous observations that GI 5-HT synthesis modulating circulating levels involves microbial metabolites such as SCFAs and/or tryptophan-derived indole metabolites (Reigstad et al., 2015). Further evidence that the gut microbiota are an important part in serotonin production was gathered on the microbially produced SCFAs acetate and butyrate, these were observed to promote the rate limiting enzyme for mucosal 5-HT synthesis (tryptophan hydroxylase 1) transcription in an enterochromaffin cell model (Reigstad et al., 2015). In another study, butyrate and propionate have also been stated to have a significant role in modulating host 5-HT biosynthesis and regulating both colon and serum levels of 5-HT, proposing that SCFAs might be associated with host 5-HT biosynthesis, regulating both colon and serum levels of 5-HT (Yano et al., 2015).

1.1.1.3. GABA:

Gamma-amino butyric acid (GABA) is an active, non-protein, amino acid and an active biogenic substance synthesised in plants, fungi, vertebrate animals, and bacteria. GABA is present in the central nervous system and indicated in the regulation of sleep-awake cycle, motor activity, vascular tone, memory formation and cognition (Yunes et al., 2016) and is produced by the decarboxylation of glutamic acid (Bowery & Smart, 2006). Certain strains of *Lactobacillus* and *Bifidobacterium* have been reported to produce GABA from its precursor monosodium glutamate (Barrett et al., 2012). DNA of these strains were sequenced; the GAD genes were identified to be widely found in *Bifidobacterium* and *Lactobacillus*. These genes are strongly associated with the GABA production (Yunes et al., 2016). *In-vitro* studies performed with gut bacteria showed that GAD gene is active in acidic environment and GABA/glutamate system plays a protective role against acidic stress (Feehily & Karatzas, 2013). For an appropriate brain development and function, there should be a balance between excitatory (glutamate) and inhibitory (GABA) neurotransmitters. Loss of inhibitory GABA with corresponding

glutamate mediated hyper-excitation is related with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) (Wang et al., 2018). Within this scope, it can be considered that GABA is comprised in the interaction of gut microbiota and the production of GABA by bacterial strains, could be a significant feature in the target of psychobiotics for therapeutic treatments.

Glutamate degradation to GABA is performed under acid stress to modulate intracellular pH homeostasis in a number of gut bacteria (Feehily & Karatzas, 2013). The degradation pathway of GABA is carried out along with succinate degradation via succinate semialdehyde and 4-hydroxybutyrate. Additionally, butyrate production from succinate via this pathway has been demonstrated in *Porphyromonas gingivalis* and *Clostridioides difficile* (Ferreyra et al., 2014). The glutamate decarboxylase (GAD) system for production of GABA is essential to serve as an acid-resistance mechanism for certain species including *Listeria monocytogenes (Feehily et al., 2013)* as well as other species including *E. coli*, *Lactobacillus* spp., and *Bifidobacterium* spp. (Strandwitz et al., 2019). Decarboxylation of glutamate is induced at a low pH and produces GABA, which is then exported from the cell in a protonated form, alkalinizing the cytoplasm (Strandwitz et al., 2019). Indeed, *in-vitro* models have shown that under acidic conditions extracellular glutamate is transported into the cell to convert it to GABA. The glutamate is then decarboxylated to GABA. It is a reaction which utilizes a proton; thus, it helps to prevent acidification of the cytoplasm (Karatzas et al., 2010). On the other hand, the gut environment can change very quickly and significantly, and pH may be close to neutral, particularly in the distal gut. It might be also considered that the role of GABA/glutamate system is not only restricted to a protective role against low pH. It has also been stated that this protective system is also associated with the resistance to oxygen deficiency and other stresses (Bowery & Smart, 2006; Feehily & Karatzas, 2013).

1.1.1.4. Catecholamines (epinephrine, norepinephrine, dopamine):

Catecholamines (e.g., dopamine (DA) and norepinephrine (NE) and epinephrine (EPI)) play vital roles as neurotransmitters or hormones at central and peripheral levels (Arnsten & Pliszka, 2011). DA is a major neurotransmitter associated with the brain's reward system and is a precursor for epinephrine, also known as adrenaline, and norepinephrine, which contributes to arousal and alertness as well as behaviour and cognition. Disorders associated with dopamine deficiency include addiction, schizophrenia, and Parkinson's disease. Research suggests that certain gut bacteria produce or metabolise dopamine (Strandwitz, 2018). NE decreases peripheral circulation, stimulates arteriole contraction, and affects human cardiovascular system. As such, the abnormal concentration of catecholamines might be related to the variety of cognitive diseases including Schizophrenia,

Parkinsonism, Alzheimer, Huntington, severe head trauma, various neuroblastoma, adrenocortical carcinoma, pituitary adenoma, and depression (Del Campo et al., 2011). In terms of norepinephrine, a recent study performed with germ free animals stated that the levels of norepinephrine are significantly lower in the caecal lumen of germ-free mice (Asano et al., 2012). Germ free mice also showed a decreased concentration of dopamine, norepinephrine and serotonin in the brain and this information pointed to a link with the gut microbiota and levels of neuroactive in the brain (Strandwitz, 2018).

1.2. The Gut Microbiota:

The gut microbiota comprises approximately 500-1000 different species of bacteria existing within the gastrointestinal track, most of them belonging to the phyla Firmicutes and Bacteroidetes (Mangiola et al., 2016). Following birth, the infant is exposed to the maternal microbiota not only from vagina, anus, and skin but also from the hospital environment (Marques et al., 2014). The microbiota of the new-born is less complex and stable than the adult microbiota. At the beginning, the gastrointestinal tract is colonised by facultative anaerobes such as *Staphylococcus, Streptococcus* and *Enterobacteria*. Once the bacterial population grows oxygen is used up and a more suitable environment for strict anaerobes such as *Bacteroides* and *Bifidobacterium* is created. In the process of time, facultative anaerobic bacteria cannot outcompete other residents and the alteration of the environment is ultimately exceeded by strict anaerobes (Zhuang et al., 2019). The gut microbiota of an adult consists of approximately 100-trillion organisms, which is approximately 10 times more microbes than human cells (Hills et al., 2019).

The human microbiota derives from an unstable ecosystem during infancy into a more complex and stable ecosystem in adulthood. As diet is one of the main factors regulating the composition of gut microbiota, as a prebiotic source: breast milk provides the best nutritional diet for maturation of the infant gut from birth and, during this developmental stage, if pre and/or pro-biotics are administered during childhood and adolescence, they might optimally act on cognition, anxiety, and stress responses (Cooper et al., 2016). Therefore, interaction with gut bacterial populations could have a significant effect on the host from early life, during this rapid development period. As such, changes in early life nutrition could impact gut microbiota composition and metabolism, resulting in benefits in brain and cognitive functions (Avershina et al., 2016).

1.3. Probiotics, Prebiotics, Microbiota and Cognition:

Prebiotics and probiotics have been widely assessed and have been shown to modulate the intestinal microbiota. As such, they could be a useful novel therapeutic tool in order to alleviate behavioural symptoms and GI discomfort associated with some children with ASD (Doenyas, 2018; Grimaldi et al., 2018).

Probiotics have been defined by the World Health Organization as "live microorganisms which, when administrated in adequate amounts, confer a health benefit to the host" (FAO/WHO 2002). The updated definition of Gibson et al., (2017) has indicated that these microorganisms are usually strains of lactic acid producing bacteria (LAB), in particular members of *Lactobacillus* and *Bifidobacterium* genera. Probiotic bacteria, should provide certain positive effects in order to exert their activity:

1. Surviving the gastrointestinal transit
2. Improvement of tolerance to lactose, as having enzymatic lactase activity
3. Competition with pathogens for colonisation and nutrients
4. Stimulation of the immune system
5. Improvement digestion and gut function
6. Must be regarded as GRAS (Generally Recognized as Safe) which means it should not be pathogenic and should be able to maintain genetic stability (Gibson et al., 2017).

As such, probiotics must remain viable and stable along the gastrointestinal tract and should provide a positive health impact, to be fully effective they may have to compete with an established colonic gut microbiota for nutritional sources (Liu et al., 2016). Probiotic consumption has been observed to regulate CNS function and to help to inhibit the immune-inflammatory cascade (Principi & Esposito, 2016). Probiotics may restore the optimal balance of microflora in the gastrointestinal tract through altering the microbiota profile and modulating intestinal permeability. Thus, gut microbiota may regulate the activity of gut-brain-axis, which has been found to be linked with the pathophysiology of depression (Karakula-Juchnowicz et al., 2019).

The combination of *Lactobacillus helveticus* and *Bifidobacterium longum* showed anxiolytic-like activity and reduced apoptosis in the limbic system in animal models of depression. A strong association between the modulation of gut microbiota and the beneficial effects on brain activity in stress conditions was reported in this study (Ait-Belgnaoui et al., 2014). In another study, a probiotic formulation (PF)*:* A combination of *L. helveticus* R0052 and *B. longum* R0175 was administered to rats daily for 2 weeks, after which rats were tested in a conditioned defensive burying test, a screening model for anti-anxiety agents. Daily administration of PF significantly reduced anxiety-like behaviour (Messaoudi et al., 2011). Secondly, a human trial was performed where healthy volunteers were

administered the same PF for 30 days and assessed with the Hopkins Symptom Checklist (HSCL-90), the Hospital Anxiety and Depression Scale (HADS), the Perceived Stress Scale, the Coping Checklist (CCL) and 24 h urinary free cortisol (UFC). Daily sub chronic administration of PF significantly alleviated psychological distress in volunteers. Additionally, the daily administration of PF for 30 days significantly decreased urinary free cortisol levels in the participants compared to the control group. As such, *L. helveticus* R0052 and *B. longum* R0175 taken in combination might result in anxiolytic-like activity in rats and beneficial psychological effects in healthy human volunteers. Furthermore, in another study performed with participants who were administered prebiotics (B-GOS and FOS) there were significant decreases in the salivary cortisol awakening response compared to placebo group (Schmidt et al., 2015). As such, convincing evidence is accumulating for a role of the gut microbiota composition in the regulation of the stress hormone corticosterone (cortisol in humans).

In a recent systematic review Eastwood et al. (2021) considered the effects of probiotics in cognitive function. From 30 papers identified probiotics demonstrated positive effects on cognitive measure in 21 studies, the authors concluded probiotics might be considered to show the best effects in clinically relevant populations where cognitive dysfunction might be present. To date there are very few papers that focus on the impact of probiotics on cognitive function in children. The focus has been more on infants. For example, Slykerman et al. (2018) assessed the impact of *Lactobacillus rhamnosus* HN001 with *Bifidobacterium animalis* subspecies lactic HN019 or placebo from 36 weeks gestation until 2 years of life. The follow up at 11 years of age did not show the probiotics used to have positive effects on intelligence or executive function. A trial by Firmansyah et al. (2011) observed no positive effects of a probiotic milk treatment on 12-month-old children. Whilst the results in infants have largely been negative it could be worth focusing on the later developmental period.

Prebiotics have been defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Within this scope, prebiotics are also defined as dietary ingredients that reach the large intestine without alteration and have a specific metabolism toward beneficial bacteria rather than harmful bacteria. This would generate a significant alteration in gut microbiota composition, that results in a positive effect on host health (Costabile et al., 2012). To be classified as prebiotic, the following three criteria should be met:

1. The substrate must not be hydrolysed or absorbed in the stomach or small intestine.
2. It must be selective for beneficial gut bacteria in the colon and should be encouraging the growth or activity of these organisms.
3. It will lead to a beneficial effect for the host.

Non-digestible oligosaccharides are dietary substrates assuming a good prebiotic potential, prebiotics are thought to be non-digestible by human or animal digestive enzymes (Grimaldi et al., 2017). Prebiotic supplementation is related with their positive impact on health through support of beneficial microorganisms within the large intestine.

Inulin and fructo-oligosaccharides (FOS) are non-digestible oligosaccharides which can be found in plants, fruits, and vegetables such as bananas, onions, chicory root, garlic, asparagus, artichoke, and leeks. FOS can be obtained by partial enzymatic hydrolysis of inulin by enzymatic synthesis using -fructosidase from *Aspergillus niger*, but the end products have different degree of polymerisation (DP). Orafti NV, have developed an inulin, known as Synergy1, comprising of 1:1 inulin and oligosaccharide. According to the different human intervention studies reported that 5–8 g/d of inulin intake is an adequate dose in order to modulate the gut microbiota (Kolida and Gibson 2007). Galacto-oligosaccharides (GOS) are a chain of galactose with a terminal glucose unit. GOS is made by the enzymatic break down of lactose with the enzyme β-galactosidase (Grimaldi et al., 2017). Differently from FOS, GOS is not a food component, but it is a synthetic product, and is produced usually in syrup or powder form that, in both cases, contains oligosaccharides of different DP, non-reacted lactose, glucose and galactose (Torres et al., 2010).

1.3.1. The Impacts of Prebiotics on Gut Microbiota:

Prebiotics have been demonstrated to affect the gut microbiota and SCFA production within. As such, prebiotics may be considered as to regulate brain function by modulating gut microbiota composition and immune function (Saulnier et al., 2007). Indeed, preliminary studies have shown that prebiotics can regulate emotional attention performance in healthy humans (Schmidt et al., 2015). Recent findings suggest that prebiotic interventions lead to healthy microbiota shifts (Vandeputte et al., 2017) including lactobacilli and bifidobacteria (Rossi et al., 2005). The bifidogenic potential of inulin is also determined by a clinical trial performed with healthy volunteers which showed that the consumption of chicory inulin or Jerusalem artichoke stimulated the growth of bifidobacteria and reduced the amount of *Clostridium* and *Bacteroides* species (Kleessen et al., 2007). Thus, inulin has beneficial effects on healthy individuals, as demonstrated in a human trial published in 2007. According to the study, the consumption of bakery products enriched with inulin stimulated the growth of bifidobacteria, which contributed to dissolution of pathogenic bacteria (Kleessen et al., 2007). Another study on healthy volunteers confirmed the prebiotic effect of fruits and vegetables containing inulin derived from Jerusalem artichoke (Ramnani et al., 2010). A randomized, double-blind, controlled study

compared growth and development of toddlers (12 months) fed milk containing synbiotics (*Bifidobacterium longum, Lactobacillus rhamnosus,* prebiotics (inulin and fructooligosaccharides) for 12 months. Three hundred and ninety-three healthy, 12-month-old toddlers were fed approximately 400 mL/day for 12 months with milk either containing synbiotic or none (control). Significant differences were observed in weight gain in the synbiotic group compared to control group and significant increases in lactobacilli and enterococci counts between 12 months and 16 months in the synbiotic group promoted favorable gut colonization (Firmansyah et al., 2011). Furthermore, a double-blind, randomized, cross-over intervention study showed that inulin can have a beneficial effect by increasing *Bifidobacterium* species and lowering those of *Bilophila* and *Anaerostipes*, thus confirming the positive modulation of the microbiota by inulin (Vandeputte et al., 2017). Inulin stimulates saccharolytic fermentation in colon, which is fermented by bacteria, producing SCFA, known as health promoting metabolites. This statement is sustained also by other studies (Dewulf et al., 2013).

A study conducted with healthy volunteers (average age of 34.4 ±6.7 y) revealed that B-GOS showed bifidogenic effects. In this study, different doses (0 g, 3.6 g, 7 g) of B-GOS were administered to healthy volunteers for 7 days with an equivalent washout period. Significant increases were observed in *Bifidobacterium* group especially in *B. bifidum* and *B. longum* compared to a different form of GOS (Depeint et al., 2008). The effects of B-GOS have also been investigated by Vulevic et al. (2008) in older healthy adults. The aging process is associated with reduced activity of immune system and increased pro-inflammatory cytokines; a number of studies have revealed the negative balance of eldery gut microflora composition compared to younger volunteers. In this study, forty-four volunteers were randomly assigned into 2 groups: placebo (1) and B-GOS (2) (both powder form, 5.5 g/day) for 10 weeks each along with a separated 4 weeks of washout period. The results showed that after 5 weeks, the microbiota of elderly people was more similar to their younger counterparts reducing the growth of putrefactive bacteria, whilst also decreasing inflammation (Vulevic et al., 2008). A recent study conducted with and β-galactooligosaccharides (B-GOS) derived from lactose used fecal slurries from children aged 11 to 24 months old to ferment the substrate during 6 and 24 h. Significant increases in bifidobacterial species and positive modulatory effects on different bacterial groups was observed in the B-GOS group (Marin-Manzano et al., 2020).

1.3.2. The Impacts of Prebiotics on Cognition and Behaviour:

Increasing the number of animal studies show the relation between prebiotics and cognitive effects. As such, a study conducted with rats displayed increased levels of BDNF and decreased anxiety behaviours when administered with prebiotics in early life (Burokas et al., 2017). This was also corroborated by Savignac et al. (2015), who observed increased expression of neurotransmitters and neuromodulators in the hippocampus, after GOS supplementation combined with increased BDNF release in rodent studies (Savignac et al., 2015). Dietary intervention studies performed with GOS have reported GOS intake to have a significant impact in social behaviours (Grimaldi et al., 2018) and in executive function domains in humans (Keefe et al., 2008). Grimaldi et al. (2018) found a significant improvement in social behavior with a restricted diet (casein and/or gluten-free) in male and female children with autism spectrum disorder (ASD) (<12 years of age), following a 6-week supplementation with the prebiotic galactooligosaccharides (1.8 g/day) and greater improvements in social behaviours (Grimaldi et al., 2018).

A systematic review suggested that prebiotic interventions might have a broader effect on overall functioning (domain general), whereas probiotic interventions might be more targeted in their effects (Basso et al., 2022). To date, studies on prebiotics and cognitive function are limited. However, of three studies, two have focused on healthy adults and one on children. Of the adult studies Berding et al., 2021 recruiting 18 healthy adults who consumed either polydextrose (as prebiotic) or maltodextrin for four weeks in a crossover design. In a range of cognitive tests increased positive results, concurrent with increases in *Ruminiclostridium* were observed whilst polydextrose was consumed. The study associated these effects with anti-inflammatory effects supporting the potential role of the GBA through prebiotics in supporting cognitive function (Berding et al., 2021). In the study on children, (Capitao et al., 2020) 35 children consumed either B-GOS or maltodextrin for 12 weeks. An online set of automated tests were used to measure a range of cognitive performance outcomes. However, no influence of cognitive function was recorded, which was considered as a possible effect of only testing pre and post intervention and not during the twelve weeks, thus earlier assessment may be necessary for capturing earlier changes (Capitao et al., 2020). However, in a study on adults Smith et al., (2015) investigated the effects of acute inulin consumption on episodic memory in 47 volunteers. Consumption of inulin over a four-hour period was associated with greater accuracy on a recognition memory task. Thus, there is a potential for prebiotics to support cognitive function, but this requires further investigation.

1.4. Flavonoids, Microbiota and Cognitive Functions:

Flavonoids are formed of two aromatic carbon rings and a benzene ring and divided into six classes: flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanidins. Flavonoids are found in significant numbers and concentrations in vegetables, fruits, fruit juices, chocolate, and certain beverages such as tea, red wine and especially berry fruit. Berries are high in anthocyanins (ACNs) and are regularly consumed for their known antioxidant and health-care characteristics principally to protect against cognitive deficits (Macready et al., 2009). Berries are widely understood to increase cognitive function through their neuroprotective properties and by stimulating neurogenesis (Miller & Shukitt-Hale, 2012). They are stated to have the capacity to cross the blood-brain barrier and they have been confirmed to be present in the specific areas of rat brain related to learning and memory shortly after oral consumption. As such, flavonoids are thought to interact with the neuronal intracellular pathways modulating neurodegeneration and neuroinflammation (Spencer, 2008)

Berries have been reported to prevent age-related neurodegeneration resulting changes in cognitive and motor function (Macready et al., 2009; Whyte et al., 2017). A series of possible mechanisms whereby ACNs can reduce cognitive deficits have been identified, particularly via their ability to interact with neuronal pathways (Macready et al., 2009). ACNs have also been stated to reduce oxidative stress through their capability to scavenge reactive oxygen species (ROS) (Spencer, 2008). The brain is inclined to oxidative damage as a result of its significant amounts of oxygen usage. Oxidative stress develops chronic inflammatory responses which can contribute to increasing neural damage (Miller & Shukitt-Hale, 2012).

In cell models, berry fruits, as a source of ACNs, have been observed to intervene in signalling pathways related to enhancing neuroplasticity, neurotransmission and calcium buffering, all which prompt attenuation of age- and pathology-related deficits in behaviour (Miller & Shukitt-Hale, 2012). Blueberries have been related to a positive effects on a range of health-related disease including metabolic syndrome, cancer, cardiovascular disease and cognitive decline (McAnulty et al., 2017).
The positive cognitive effects of flavonoids among older adults have also been studied. For instance, a grape juice study was performed among 93 older adults suffering from Mild Cognitive Impairment (MCI). The recruited adults consumed grape juice daily (6-9 mL/kg/day) for 12 weeks and they showed improvements in delayed recall in words which means the ability to recall specific information after a distraction from that information in the California Verbal learning test (CVLT) in comparison with the adults given a placebo (Krikorian et al., 2010). In a similar study, older adults with MCI exposed to

blueberry powder for 20 days showed an improvement in memory function, and a better performance relative to placebo controls (Krikorian et al., 2020). A study comprised with blueberry intervention, showed that blueberry extract regulated Ca_2^+ recovery and reduced free radical levels and stress signals. In this study, it was observed that phytochemicals in berry fruits prevented age-related disruption in the brain through protective roles on stress signalling. As such, flavonoids could be considered to prevent age related disruption, whilst improving cognitive function through neuroprotective properties (Spencer, 2010).

Another aspect of the impact mechanism of ACNs is that they are considered to have positive effects on the gut microbiota (Roopchand et al., 2015). Different kinds of fruits that are rich in polyphenols and flavonoids have been revealed to modulate the microbiota in human studies including blueberry powder (lactobacilli, bifidobacteria), prunes (bifidobacteria), kiwi fruit (*Bacteroides, Faecalibacterium prausnitzii*) and raisins (*Ruminococcus, F. prausnitzii*) (Katsirma et al., 2021). The effects of flavonoids and ACNs on age related diseases and cognitive functions may be based on their absorption and the metabolism of flavanols in the gastrointestinal tract by gut bacteria. To date there is limited information regarding the prebiotic potential of flavonoids, previous *in-vitro* studies have stated that proanthocyanins modulate the growth of *Lactobacillus* spp. and *Bifidobacterium* spp.. Furthermore, it has been noted that proanthocyanins can be catabolised by the colonic microbiota to aromatic acids and valerolactones. As such, these compounds might have significant roles on the biological activities on the colonic epithelium (Cires et al., 2016).

An animal study was conducted with mice that were fed a high-fat diet (HFD) administered with 1% concord grape polyphenol (GP). GP was observed to attenuate several effects of HFD, including weight gain, adiposity, serum inflammatory markers and glucose intolerance. Moreover, GP increased intestinal expression of genes involved in barrier function; 16S rRNA gene sequencing and quantitative PCR of fecal samples showed that GP significantly increased the growth of *Akkermansia muciniphila* and decreased the proportion of Firmicutes to Bacteroidetes. The results are parallel with prior studies that similar changes in microbial community structure can be associated with protection from diet-induced obesity and metabolic disease (Roopchand et al., 2015). There is a strong association between the balance of these bacterial species and the health of large intestine and accordingly in general health (Petschow et al., 2013). Furthermore, improved barrier function has been associated with reduction in depression status (Foster & McVey Neufeld, 2013). As such the gut microbiota could be considered a potential pathway by which ACN impact on mental well-being.

Research has indicated that blueberries might selectively increase intestinal bifidobacteria, suggesting a gut related effect. Guglielmetti et al. (2013) observed a significant increase of *Bifidobacterium longum* subsp. *infantis* cell concentration following consumption of a wild blueberry drink. This bifidobacterial group was shown to hold immunomodulatory abilities and to alleviate symptoms of several gastrointestinal disorders. As such, wild blueberry consumption is suggested to have specific bifidogenic effect with demonstrating health-regulating properties (Guglielmetti et al., 2013). Another dietary intervention study also revealed that six-week consumption of a wild blueberry drink versus a placebo drink, positively modulated the intestinal microbiota. As such, following blueberry treatment increased *Bifidobacterium* spp. significantly (Vendrame et al., 2011).

Berries are rich sources of ACNs, which together with flavonoids, phenolic acids, folate, minerals, and fibre are important contributors to the in vivo biological activities of these fruits. It is known that absorption of ACN is limited, therefore most of the compounds can reach the colon, where they can be metabolised by the intestinal microbiota (Paredes-Lopez et al., 2010).

It is therefore possible that the consumption of flavanol rich foods may support gut health via their prebiotic-like effects along with antioxidant, anti-inflammatory features having direct effects on the brain and cognitive functions through containing neuroavailable and neuroactive phytochemicals (Macready et al., 2009). Researchers state that more *in vivo* and *in vitro* studies are required to accomplish a complete picture of the mechanisms of action on neuroavailability for the phytochemicals and their metabolites which occur in berry fruits.

2. HYPOTHESIS, AIMS, LIMITATIONS & CONCLUSION:

To date, studies focussing on the interactions between gut microbiota dysbiosis and cognitive function show common and consistent results. Therefore, modifications in gut microbiota composition might be associated with CNS and consequently on cognition, mood and behaviour. Dietary intervention therapies have been hypothesised to improve these conditions. The potential of prebiotics and flavonoids to modulate the GI microbiota has been widely investigated in healthy individuals but there still is a lack of knowledge about their impact on gut-brain axis and cognition particularly in children.

There are a number of major metabolic, endocrine and neural pathways connecting the gut and the brain. Certainly, the trillions of microbes and microbial metabolites within the gut promote modulation of these pathways. Despite the growing evidence, this area still needs to be progressed. To some extent, there is a lack of knowledge within the scope of which bacterial metabolites can

impact on brain function. This is an area which could be addressed with further progresses in metabolite studies along with the gut microbiota and cognitive function.

2.1. Rationale:

Functional characterisation of microbiota metabolic pathways can be defined through multiple analytical approaches including omics strategies such as metabolomic analysis and genomic analysis, molecular assays and targeting a set of genes. These approaches may provide insights into the mechanisms of host-microbiota interactions and inform for targeted diet in terms of a rational design of prebiotic and probiotic studies (Krishnan et al., 2015)

Data indicates that flavonoids may act in the intestine to optimise the gut microbial community structure resulting in improved metabolic outcomes. As such, the gut microbiota might provide a missing link in the mechanism of action of dietary flavonoids which are poorly absorbed. Both prebiotics and flavonoids may have different effects on cognitive function related to their effects on production of certain neuroactive components. In humans, several factors, most of which act simultaneously, may be important in conditioning gut microbiota regulation. A whole series of pathways occur in the body as such the pathways involved are likely to be complex, furthermore it is likely that precursors are necessary in the production of neurotransmitters, e.g. glutamine and amino acids (Mazzoli & Pessione, 2016). However, further research and more clinical trials in this field are needed for mechanistic evidence for gut microbiota to alter brain and behaviour and positively impact on cognitive function.

Here in this review, the impact of the gut microbiota on cognitive function has been considered, with a focus on the importance of dietary interventions in terms of prebiotics and flavonoids and their effects on brain function via modulation of the gut microbiota. Long term dietary regimens have been confirmed to contribute to the composition of the human gut microbiota (Marques et al., 2014). The role of nutrition on gut maturation in infants and the use of probiotics, prebiotics, and flavonoids as dietary strategies to modulate the gut microbiome and consequently behavioural and brain functions are also discussed in this review. Moreover, the relationship between the gut microbiota and the Central Nervous System (CNS) through the Gut Brain Axis (GBA) and how diet may impact on host cognitive function and behaviour has been considered.

3. LIMITATIONS & RESEARCH GAPS:

Although there is accumulating evidence for gut bacteria playing an important role in gut-brain axis, our knowledge is limited to identify the key players in this complex bacterial community. Most of the evidence comes from animal experiments where the microbiota is less diverse than in humans and can be controlled experimentally (e.g., gnotobiotic). Currently, significant progression has been achieved by the advancement of molecular techniques, such as deep sequencing. Sequencing technologies facilitated to specify bacteria at the species level without the need for culturing. However, the task to identify a microorganism responsible for effects on the central nervous system is challenging. As such, key bacterium could be missed when scanning for total microbiota composition. Also, results can be influenced by the techniques performed in the analysis of microbiota (454 pyrosequencing, Illumina platform, microarrays (Clifford et al., 2012)).

Moreover, there is also a significant challenge in determining whether neuroactive metabolites are the product of host or microbiota metabolism thus many metabolites can be produced in both mammalian and bacterial cells. As such, the metabolites might be in the intestine as a result of co-metabolism including reactions from multiple organisms involving the host (Krishnan et al., 2015). However, there is a need to perform more studies to understand the communication between the gut and the central nervous system, to determine if prebiotics have effects on cognitive function in a direct manner by producing neurotransmitters and neuromodulators (Barrett et al., 2012; Dinan et al., 2013) or by indirect mechanisms such as activation of the vagus nerve, modulation of gut microbial composition and regulation of circulating levels of cytokines (Cryan & Dinan, 2012).

4. HYPOTHESIS:

The gut microbiota impacts on cognitive function. The activities of prebiotics and berries on cognitive function are driven, in part, by the gut microbiota.

4.1. The aims and objectives of this research project were therefore (Figure 1.4):

- To investigate the impact of certain precursor amino acids (glutamate, tryptophan, and tyrosine) on the growth of the anaerobic faecal bacterial populations and their capability to produce neuroactive metabolites using pH-controlled *in-vitro* batch culture fermentation systems inoculated with faecal samples

- To determine the effect of maltodextrin (non-prebiotic component), inulin and B-GOS (widely known prebiotics) and freeze-dried blueberries (source of anthocyanins) on the gut microbiota composition and metabolites production using pH-controlled *in-vitro* batch culture fermentation systems inoculated with faecal samples from children (7-10 years of age)
- To determine the effect of inulin and freeze-dried blueberries on the gut microbiota composition and metabolite production using a more complex three-stage continuous system models inoculated with faecal samples from children (7-10 years of age)
- To design and perform a human intervention study investigating the effects of inulin and freeze-dried blueberries on the gut microbiota composition and cognition on healthy children (7-10 years of age)
    - The first aim was to investigate the effect of a 4-week inulin and freeze-dried blueberries supplementation on cognitive functions in this target group
    - The second aim was to assess the impact of this treatment period on gut microbiota composition in healthy children

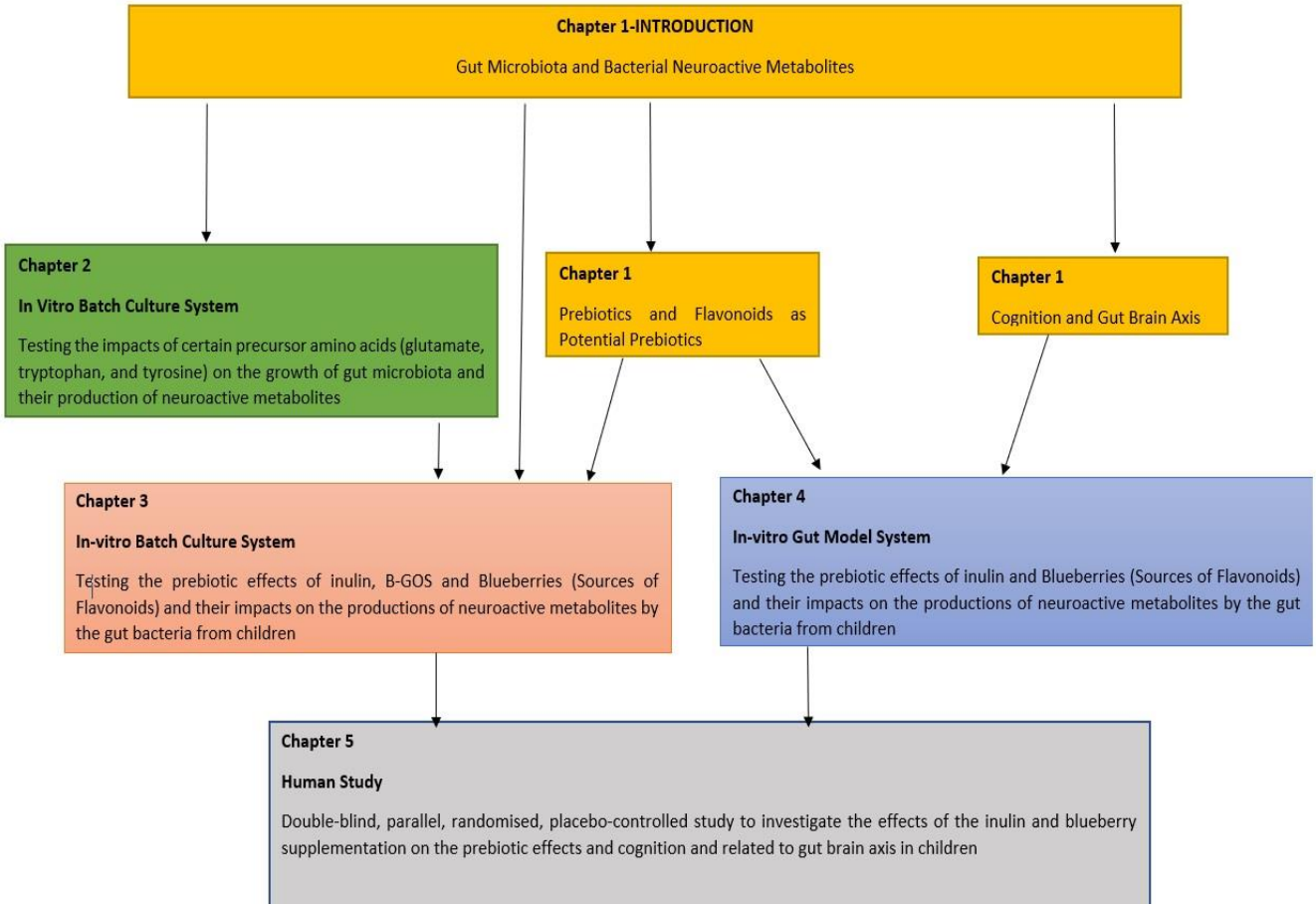

Figure 1.4 Summary of the research strategies and principal aims of the study

In-vitro models can closely imitate the microbial composition and activity in certain regions of the human gut. They provide an overview of different stages of fermentation processes through continuous sampling of different consecutive regions of the human colon. Furthermore, *in-vitro* models mostly provide results that can indicate mechanistically how the microbiota may be involved in GBA. Additionally, there are fewer ethical restrictions with *in-vitro* models, as such, certain compounds such as pathogens, can be used without ethical issues, thus providing information on the microbial component of the GBA in an isolated model. On the other hand, *in-vitro* approaches need to be proven (or disproven) in human clinical trials. Additionally, it has significant importance to create conditions that closely resemble the *in-vivo* situation (Venema & van den Abbeele, 2013). Indeed, dietary interventions to positively impact on the gut/brain axis may have potential as therapeutic strategies for improving cognitive function.

5. CONCLUSION:

There is growing evidence supporting the role played by GBA in cognitive function. As such, exploration of the microbial role in the GBA warrants exploration.

Dietary interventions, including prebiotics and berry interventions can positively regulate microbial composition and function. Thus, a combination of *in-vitro* and metabolomic study and a nutrition intervention combined with human study approach would be more likely to obtain answers regarding the mechanisms of action of the gut bacteria on cognitive function.

Diet-induced gut microbiota modifications may be associated with metabolic changes and subsequently effects on the GBA. The emerging evidence of a microbiota-gut/brain axis dysregulation in certain neuropsychiatric states warrants further clinical and *in-vitro* studies to investigate gut microbiota-targeted interventions as novel therapeutic strategies.

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CHAPTER 2

Investigating whether the faecal microbiota can produce neuro-active molecules within *in vitro* models of the distal colon

Abstract:

It has been revealed that the gut microbiota influences immune activating and other signalling molecules, such as neurotransmitters, which may play a significant role in regulating cognitive function. These neuroactive metabolites consist of γ-aminobutyric acid (GABA), dopamine, epinephrine, norepinephrine, serotonin, and short chain fatty acids (SCFAs).

To assess the neuroactive metabolite production of gut bacteria, pH-controlled faecal batch culture systems were conducted. The batch culture system was inoculated with faeces (1%) and basal media enriched with potato starch (5 g/L) and Tryptone (3 g/L) with either monosodium glutamate (MSG), Tryptophan or Tyrosine as treatments and no treatment as a control. There were no significant effects on the growth of the gut bacteria observed following these treatments, there was however a significant decrease in GABA concentration during the fermentations. A trend of increased levels of *Bifidobacterium* spp. was observed upon fermentation of all substrates. Moreover, significant increases were obtained in acetate, propionate, and butyrate following fermentation of all substrates between the times of 0 and 24 hours. In conclusion, there were no significant differences among all the substrates. A breakdown in GABA was apparent during the fermentations. On the other hand, it can be stated that the gut bacteria are capable of producing SCFAs and low levels of serotonin which are related to the cognitive function. Therefore, it could be assumed that an addition of tryptone: source of amino acid would be sufficient to be involved in the production of metabolites as there were no significant differences between the control and the treatments. Therefore, batch culture fermentation system is considered a very good method to investigate the growth and the metabolite production of gut bacteria in terms of neuroactive molecules.

Key Words: GABA, dopamine epinephrine, norepinephrine, gut-brain axis

1. INTRODUCTION

The brain and the intestine have a close and bidirectional communication called gut-brain axis and this communication includes immune, endocrine, and neural pathways (Dinan & Cryan, 2017; Mayer et al., 2015). Interactions within this communication implicate the gut microbiota releasing immune activating and other signalling molecules and neurotransmitters (Lyte, 2013). The primary neurotransmitters are classified into two subgroups: monoamine and amino acid neurotransmitters. Monoamine neurotransmitters consist of dopamine, serotonin, epinephrine and norepinephrine and amino acid neurotransmitters include glutamic acid, tryptophan, γ-aminobutyric acid (GABA), alanine, aspartic acid, and taurine (Yan et al., 2017). Production of these neurotransmitters could involve the gut microbiota and therefore link the microbiota to cognitive function (Desbonnet et al., 2008).

Protein fermentation of certain amino acids is associated with neuroactive metabolite production, this is because amino acids are known precursors of certain neuroactive metabolites. For example, tyrosine is a catecholamine precursor (dopamine, norepinephrine, and epinephrine) (Figure 1), glutamine is known as GABA precursor (Figure 2) and tryptophan is associated with serotonin and kynurenic acid production (Figure 3) (Agus et al., 2018; Barrett et al., 2012).

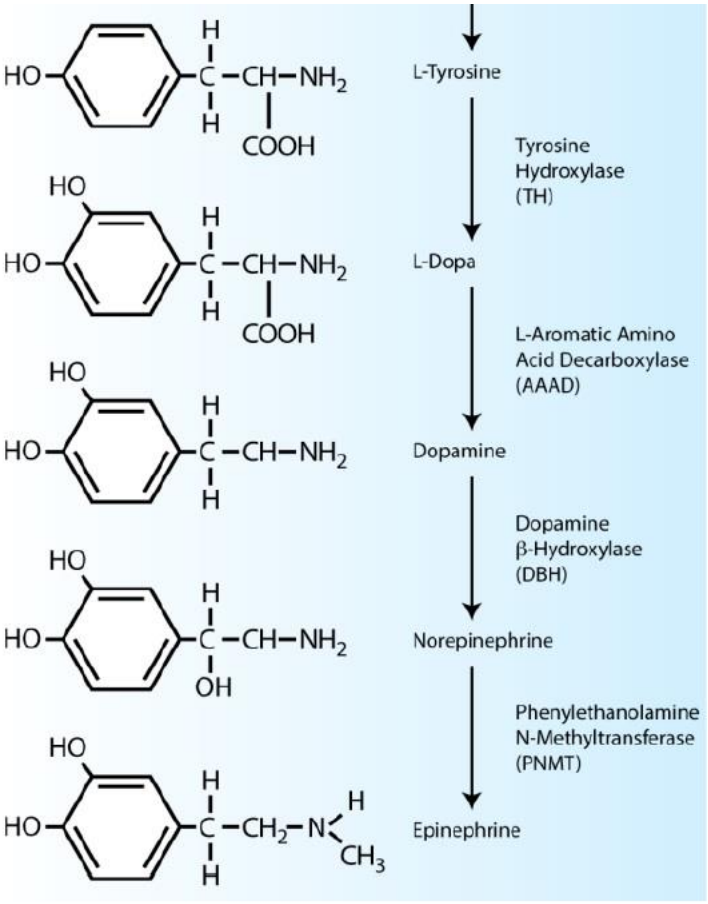

Figure 1. Pathway of catecholamine biosynthesis. Synthesis of epinephrine and norepinephrine is regulated by catecholamine synthesizing enzymes (Vegh et al., 2016).

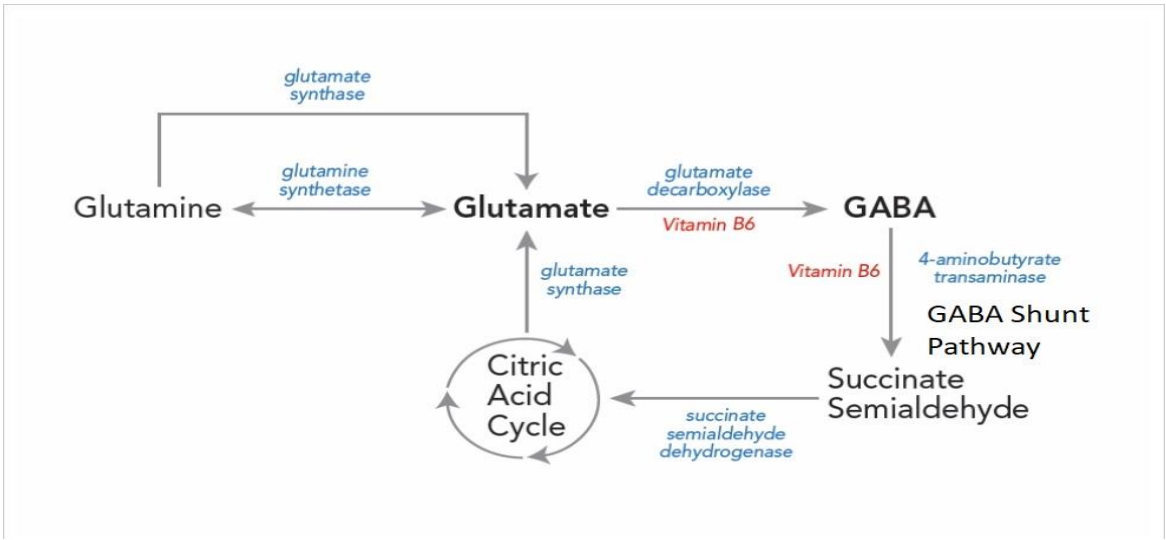

Figure 2. GABA and GABA shunt pathway (Succinate Semialdehyde) Synthesis

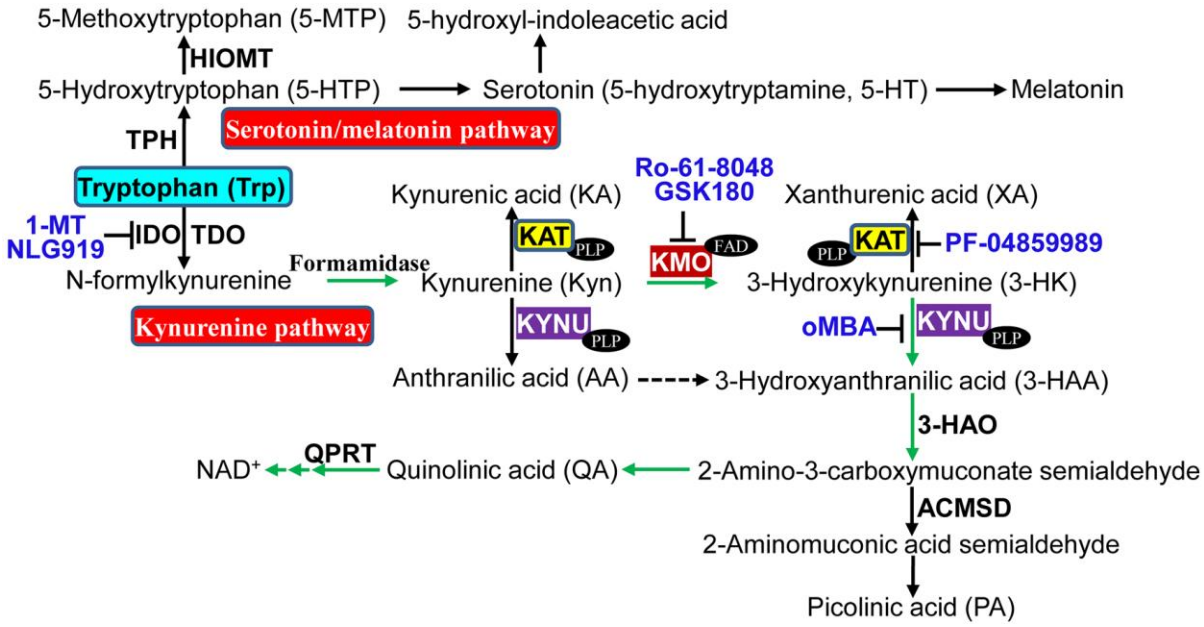

Figure 3. Serotonin and Kynurenine Pathways. Tryptophan is metabolized via two major pathways, kynurenine pathway and serotonin/melatonin pathway. 3-HAO: 3-hydroxyanthranilate 3,4-dioxygenase, ACMSD: 2-amino-3-carboxymuconate semialdehyde decarboxylase, FAD: flavin adenine dinucleotide, HIOMT: hydroxyindole O-methyltransferase, oMBA: o-Methoxybenzoyalanine, QPRT: quinolinate phosphoribosyltransferase, TPH: tryptophan hydroxylase (Song et al., 2017).

It has been determined in many studies that the human intestinal microbiota, especially *Lactobacillus* and *Bifidobacterium* can produce GABA from glutamine, which is in the form of monosodium glutamate (MSG). In a study of Barrett and co-workers, faecal culture strains were grown in specific medium supplemented with MSG and certain species of *Lactobacillus* and *Bifidobacterium* were reported to produce GABA through the conversion of MSG (Barrett et al., 2012). As such, it is likely that in a mixed microbial population, some of the bacteria that might be targeted by prebiotics could also result in increased levels of GABA. In a study, a variety of gut bacteria have been isolated and *Bacteroides* spp. has been found to produce large quantities of GABA with the support of other strains of gut bacteria, e.g., KLE1738 whose name has been proposed as *Evtepia gabavorous*. Reduced levels of *Bacteroides* were associated with reduced levels of GABA levels and depressive-like behaviours in the rats (Hassan et al., 2019).

Furthermore, it has been observed that the production of GABA occurs between the pH range from 2.5 to 7.6 while the highest production range of GABA has been observed when the pH was between 2.5 and 3.5 where the levels approximately reached to 5 mM (Feehily & Karatzas, 2013). As such, the greatest amount of GABA production would be expected at the proximal colon (pH 5.5) as the

transverse and distal colon pH levels are closer to the neutral pH. Indeed, GABA production was only observed at a low pH (≤5.5) by *Bacteroides fragilis* (Strandwitz et al., 2019). Much smaller amounts of GABA production have been observed at more neutral pH levels (Feehily & Karatzas, 2013). On the other hand, the impact of the gut microbiota on GABA production within a host still needs to be determined. Current studies have determined that potential strains of bacteria have been identified to utilize GABA including *Pseudomonas, Acinetobacter,* and *Mycobacterium* genera (Strandwitz et al., 2019). The degradation pathway of GABA is carried out along with succinate degradation via succinate semialdehyde and 4-hydroxybutyrate. Butyrate production from succinate via this pathway has been demonstrated in *Porphyromonas gingivalis* and *Clostridioides difficile* (Ferreyra et al., 2014). As such it is likely that the microbiota can have a great impact on GABA concentration.

Catecholamines (epinephrine, norepinephrine, and dopamine) and serotonin also play a significant role in cognitive function, and they have been stated to be responsible for activating areas of the brain that are needed for concentration. Low levels of these neuroactive compounds have been associated with ADHD (Pelsser et al., 2011). Even though it has not been proved that the human gut microbiota modulates norepinephrine or dopamine *in vivo*, there is expanding evidence indicating that gut microbiota may play a role in host biosynthesis and catabolism reactions. In terms of norepinephrine, a recent study performed with germ free animals stated that the levels of norepinephrine are significantly lower in the caecal lumen of germ-free mice (Asano et al., 2012). Germ free mice also showed a decreased concentration of dopamine, norepinephrine and serotonin in the brain and this information pointed to a link with the gut microbiota and levels of neuroactives in the brain, but whether the bacteria is producing norepinephrine directly or impacting on host production is still yet to be determined (Strandwitz, 2018).

Tryptophan metabolism is host based, in which the gut bacteria promote catabolic transformations of tryptophan, comprising a fermentation of certain bioactive metabolites such as serotonin and kynurenic acid (Figure 3) (Kennedy et al., 2017; Morris et al., 2017; O'Mahony et al., 2015). The profile of the gut microbiota is considered an indirect contributor to the serotonin secreted by the host cells and the degree of serotonin in the brain (O'Mahony et al., 2015). Thus, gut microbiota is related to modulating the production of GABA, noradrenaline, and dopamine (Diaz Heijtz et al., 2011).

Short chain fatty acids (SCFAs) are produced in the gut at levels of approximately 500-600 mmol per day, depending on the fibre content of the diet. Acetate (C2), propionate (C3) and butyrate (C4) are the most abundant SCFAs in the human body and in the human colon (Koh et al., 2016). They are responsible for reducing the colonic pH while progressing from the terminal ileum to the proximal colon. Acetate, propionate, and butyrate are present in the colon in an approximate molar ratio of

60:20:20, respectively. However, the amount and the ratio of each SCFA depend on the substrate, the microbiota composition and gut transit time (Macfarlane & Macfarlane, 2003). Several studies state that brain function and behaviour are influenced by SCFAs, which are the key microbial metabolites. Acetate is a net fermentation product for many gut anaerobes including *Bifidobacterium* and almost achieves the highest concentrations among the other SCFAs in the gut lumen whereas butyrate and propionate are produced in lower concentrations (Louis & Flint, 2017). It has been well investigated that butyrate is a key mediator of host-microbe crosstalk as its effects are mediated by it being an inhibitor of histone deacetylases (HDACs), promoting histone acetylation and stimulation of gene expression in host cells. Moreover, acetate and propionate were also reported as being capable of inhibiting HDACs to a lesser extent (Dalile et al., 2019). This has led to the use of butyrate, acetate, and propionate as experimental drugs in the treatment of neurological disorders including depression and cognitive impairment (Polyviou et al., 2016). It has been hypothesised that butyrate and other volatile SCFAs produced by microbes may be involved in regulating the impact of the microbiome on behaviour including social communication (Stilling et al., 2016).

Accumulating evidence that SCFAs and neurotransmitters: GABA, serotonin, and catecholamines might be involved in the gut brain axis and might impact on cognition, therefore production of these metabolites by the gut microbiota was assessed in this study. To date, GABA is well established to be produced directly by the gut microbiota. 5-HT, dopamine, norepinephrine and epinephrine have been reported to be produced by the gut microflora in several reviews, but direct microbial production data is lacking. As such this experiment was designed to assess neurotransmitter production under physiologically relevant conditions, including the use of amino acids at 66 mg/L (10 mg/150 mL) to represent a concentration found *in vivo* in pigs (Jansman et al., 2010), but in the absence of human cells.

Batch culture basal media was enriched with tryptone (3 g/L) to provide a protein source to aid the growth of proteolytic microorganisms and potato starch (5 g/L) to provide carbohydrate to support a range of microbiota to grow. Additionally, to assess whether addition of neurotransmitter precursor ammino acids could further impact on neurotransmitter production. As such, tryptophan (10 mg/150 mL), monosodium glutamate (MSG) (10 mg/150 mL) and tyrosine (10 mg/150 mL) were added as different pre-cursors in the media. Overall, it will be determined whether, in the absence of human cells, the gut bacteria can produce neurotransmitters serotonin, GABA, catecholamines and SCFA under these *in vitro* conditions. Furthermore, this will enable the use of suitability of *in vitro* batch culture systems for monitoring neurotransmitter and SCFAs production to be determined.

The aim of this study was to investigate whether gut microbiota, under basic nutrient conditions combined with neurotransmitter precursors, could produce products relevant to the GBA in the absence of human cells.

2. MATERIALS and METHODS:

2.1. Chemicals:

All the analytical grade chemicals and formulated basal media components were purchased from Sigma-Aldrich (Merck), UK. The media was supplemented with starch (5 g/L), tryptone (3 g/L), monosodium glutamate (MSG) and essential amino acids (0.067 g/L). Neuroactive standards were obtained from Sigma (Merck), UK. Oligosaccharide probes for Flow-FISH analysis were purchased from Eurofins, UK.

2.2. Faecal inoculation:

Faecal samples were provided from three healthy donors (3 female aged between 18-30) who were free from any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics for 6 months before stool sample donation. All donors were provided a consent form and were asked to fill a standard questionnaire to obtain information about their health status, drug use, clinical illness history and lifestyle factors. This study was conducted according to the guidelines laid down in the Declaration of Helsinki following Good Clinical Practice and approved by The University of Reading Research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJar™ 2.5L; Oxoid Ltd) with an anaerobic gas generating kit (AnaeroGen™; Oxoid). Samples were diluted 1/10 w/w in PBS (0·1 mol/l phosphate buffer solution, pH 7·4) and homogenised (Stomacher 400 Circulator; Seward) for 2 min at 240 paddle beats/min. Samples were inoculated to anaerobic fermenters within 30 min of production.

2.3. *In vitro* batch culture fermentation:

This method was previously described by Gomez et al. (2010). Sterilised vessels were attached to 37 °C water bath with N_2 input and outputs. 135 mL of sterile basal medium were added to each vessel aseptically. The water bath was turned on and adjusted to 37 °C to bring the vessels up 37 °C. N_2 was turned on and the vessels were left to gas overnight to achieve anaerobic conditions. The next day, a calibrated probe was placed into the vessel and the pH adjusted at a range between of 6.7-6.9 to achieve a mean pH of 6.8. The pH was maintained in the range of 6.7–6.9 through pH controllers (Fermac 260; Electrolab, Gloucestershire, UK). During fermentation period, pH was automatically

adjusted by adding 0.5 M NaOH and 0.5 M HCl to the vessels when required. The pH and the temperature imitated the conditions of the distal part of the human large intestine.

2.4. Experiment Design:

Substrates: tryptophan, monosodium glutamate and tyrosine were added to the vessels (10 mg/150 mL) before adding 15 mL of faecal slurry (10 % (w/v). A control, no treatment vessel with no added amino acid was also included. The experiment was performed 3 times with a different faecal donor for each run. Batch culture fermentations were run for 24 h, and the samples (5ml from each vessel) were collected at 0, 6 and 24 h for analysis of bacterial populations and metabolite production**.** The stationary growth phase is considered to have been reached after 24 h of fermentation in most of the gut bacteria (Villegas et al., 2016), as such the time points of 0 h, 6 h and 24 h of fermentation were considered as appropriate times to assess growth and metabolites.

Table 1. Amounts of substrates added to the Basal Media (BM) enriched with Tryptone (3 g/L) and Potato Starch (5 g/L) in 150 mL volume vessels for 24 hours fermentation in pH and volume-controlled batch fermentation experiments.

Vessel	Treatment	Amount
1	Control (BM+Tryptone (3 g/L) +Potato Starch (5 g/L))	
2	MSG (BM+Tryptone (3 g/L) +Potato Starch (5 g/L) +MSG)	MSG (0.067 g/L)
3	Tryptophan (BM+Tryptone (3 g/L) +Potato Starch (5 g/L) +Tryptophan)	Tryptophan (0.067 g/L)
4	Tyrosine (BM+Tryptone (3 g/L) +Potato Starch (5 g/L) +Tyrosine)	Tyrosine (0.067 g/L)

BM: Basal Media

2.5. Bacterial Enumerations by FISH-FCM (Fluorescence in Situ Hybridization with Flow Cytometry):

Bacterial composition from the batch culture models was examined by fluorescence in situ hybridisation (FISH) coupled to flow cytometry ((BD Accuri TM C6 Plus, Basingstoke, UK), detecting at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software. Samples were removed from storage at -20 C°. Oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesised and labelled at the 5' end with the fluorescent dye Cy3 (Eurofins, Wolverhampton, UK) as reported in **Table 2**. To perform the FISH analysis, 750 µL of samples were centrifuged at 11,200 g for 5 min. Pellets were resuspended in 375 µL of filtered PBS (using a 0.22-µm PVDF membrane) and fixed in 1125 µL of 4% (v/v) paraformaldehyde. After 4h of incubation at 4◦C, fixed samples were washed three times with 1 mL of PBS then resuspended in 300 µL PBS-

ethanol (1:1, v/v) and stored at –20°C until analysis. To prepare the samples for the flow cytometer, a permeabilization step was performed as a first step. 500 µL of PBS were added to 75 µL of the fixed samples and centrifuged at 11,200 g for 3 min. Pellets were resuspended using 100 µL of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H2O, 0.22 µm PVDF membrane) containing lysozyme (1 mg/mL of 50 000 U/mg protein) and incubated for 10 min at room temperature. After the incubation, the samples were then vortexed and centrifuged at 11,200 g for 3 min and the supernatant part was discarded. Pellets were washed with 500 µL of PBS and centrifuged again 11.200 g, 3 min). Once the permeabilisation step was completed, hybridisation was performed by resuspending the pellets in 150 µL of hybridisation buffer (900 mM NaCl, 20 mM Tris/HCl pH 8, 30% formamide, ddH2O, 10% SDS). The resuspended pellets were vortexed and centrifuged (11.200 g), 3 min). Pellets were then resuspended in 1 mL of hybridisation buffer. The samples were distributed into 50 µL aliquots into Eppendorf tubes labelled with the appropriate name of the sample and the probe used. The probes used (Eurofins Genomics, Wolverhampton, UK) are reported in **Table 2**. NON EUB338 and EUB338 I-II-III linked at their 5' end either to Alexa488 or Alexa647. Group-specific probes were linked with Alexa647 at their 5' end. Four microlitres of each probe and 4 µL of Eub338 I-II-III (linked to Alexa488) were added to the working solution and incubated overnight at 36◦C in a heating block. After 12 h of incubation, an aliquot of 150 µL hybridisation buffer was added to the working solution, vortexed and centrifuged (11,200 g, 3 min). One hundred and fifty microlitres of supernatant was removed from each sample and the remaining volume was centrifuged (11,200 g 3min). The pellets were washed with 200 µL of washing buffer (900 mM NaCl, 20mM Tris/HCl pH 8, 5 mM EDTA pH 8, ddH2O, 10% SDS), homogenised by vortexing and incubated for 20 min at 38◦C in a heating block. Afterwards the samples were centrifuged (11,200 g, 3 min) and supernatants were removed. Samples were stored at 4◦C until determinations. Numbers of specific and total bacteria were determined considering dilution factor, calculated from different volumes used in samples preparation steps, and events/µL obtained from NON EUB338 and EUB338 I-II-III probes analysed by FCM (Grimaldi et al., 2017).

Taxonomically, bacteria are classified according to phyla, classes, orders, families, genera, and species. The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Averina & Danilenko, 2017). The Firmicutes phylum is composed of more than 200 different genera including *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminicoccus*. *Clostridium* genera represent 95% of the Firmicutes phyla. Bacteroidetes consists of predominant genera such as *Bacteroides* and *Prevotella* (Barroso et al., 2017). These groups are dominant and functionally active as such were considered appropriate for investigating possible impact of intervention. Examples of dominant gut microbiota probes are shown in Table 2.

Table 2. Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concentration of 50 ng/µL.

Probe Name	Sequence (5' TO 3')	Target Group	Reference
Non Eub	ACTCCTACGGGAGGCAGC		(Wallner et al., 1993)
Eub338 I +	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus, Enterococcus,Melisococcus, Tetragenococcus, Catellicoccus, Pediococcus* and *Paralactobacillus* spp,	(Harmsen et al., 2002)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1995)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium clusters* XIVa and XIVb)	(Manz et al., 1995)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	(Franks et al., 1998)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (Clostridium clusters I and II)	(Franks et al., 1998)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and E*ggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2002)
Prop853	ATT GCG TTA ACT CCG GCAC	*Clostridial cluster* IX	(Walker et al., 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)

DSV687	TAC GGA TTT CAC TCC T	Most *Desulfovibrionales* (excluding *Lawsonia*) and many *Desulfuromonales*	(Purdy et al., 2003)

2.6. SCFA analysis:

Production of SCFAs was determined using GC as previously described (Richardson et al., 1989). Individual solution standards at 5 mM were prepared for acetate, iso-butyrate, butyrate, propionate, valerate, iso-valerate and lactate. The external standard solution contained acetate (30 mM), iso-butyrate (5 mM), n-butyrate (20 mM), propionate (20 mM), n-valerate (5 mM), iso-valerate (5 mM) and lactate (10 mM). 1 mL of each sample was vortexed and transferred into a flat-bottomed glass tube (100 mm x 16 mm, Fisher Scientific UK Ltd., Loughborough, UK) with 0.5 mL concentrated HCl, 50 µL of 2-ethylbutyric acid (0.1 M internal standard solution, Sigma, Poole, UK) and 2 mL diethyl ether. Samples were vortexed for 1 min at 1500 rpm and then centrifuged (2000 g, 10 min, 4 C°, SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK). 2 mL of diethyl ether top layer and 50 µL of N- (tert-butyldimethylsi lyl)-N-methyltrifluoroacetamide (MTBSTFA; Sigma-Aldrich, Poole, UK) were added into a GC screw-cap vial. Samples were kept at room temperature for 72 h to enable complete derivatisation prior to GC analysis. A GC Agilent 7890B gas chromatographer (Agilent, Cheshire, UK) using an HP-5 MS column (L x I.D. 30 m x 0.25 mm, 0.25 µm film thickness) coating of crosslinked (5%-phenyl)-methylpolysiloxane (Hewlett Packard, Reading, UK) was used for SCFA detection. 1 µL of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275 °C and the column temperature programmed from 63 °C to 190 °C and held at 190 °C for 30 min. Helium was the carrier gas (flow rate, 1.7 mL/min, head pressure, 133 KPa). Peaks were integrated using Agilent Chem Station software (Agilent Technologies, Basingstoke, UK), and SCFA content was quantified by single-point internal standard method (Liu et al., 2017). Peak identity and internal response factors were determined using single standards and internal standard solution.

2.7. Neurotransmitter Analysis:

The concentration of neuroactive metabolites were determined by Liquid Chromatography Mass Spectrometry (LCMS) following the method of Zhai et al. (2015) with further modifications. Samples taken from the batch culture at the time points of 0, 6 and 24 hours were centrifuged at 11,200 g (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) for 3 min and 400 µL supernatant was taken and centrifuged again at 11,200 g for 3 min in filtered centrifuge tubes (Corning Costar Centrifuge Tube Filter Acetate membrane-0.22 µm) and the resulting supernatant was used. 200 µL of HPLC water

(Blank), calibration standard samples and processed batch culture samples were placed in 96-well plates. The remaining supernatants were stored at -20°C for future analysis.

2.7.1. Reagents and Chemicals:

HPLC Plus grade acetonitrile (99.9%) was purchased from Sigma-Aldrich (Kent, UK). Formic acid (99% LC/MS grade, HiPerSolv CHROMANORM®) was purchased from VWR, Leicestershire, UK. Centrifuge tube filters (Corning ® Costar ® Spin-X ®, 0.22 µm Pore CA Membrane, Sterile, 96/Case, Polypropylene) was purchased from Sigma-Aldrich, these were used to filter batch culture and gut model fluid samples. Analytical standards powder which was LC-MS grade dopamine hydrochloride (99%), and L (-)-Epinephrine (99%) were purchased from Alfa Aesar (Lancashire, UK). L-Noradrenaline (98%), Gamma-Aminobutyric acid (99%)

2.7.2. Stock Solutions, Calibration Standards and Sample Preparation:

Separate standard stock solutions (10000 ng/mL) of 5 analytes, including Serotonin (5-HT), Dopamine (DA), Gamma-Aminobutyric acid (GABA), Norepinephrine (NE) and Epinephrine (EPI) were individually prepared in HPLC water. A 1000 ng/mL mixed standard solution containing the 5 analytes was made by acquiring aliquots of each separate stock solution. The mixed standard solution was appropriately diluted with HPLC water to prepare a calibration series. A calibration series of spiked standard samples was prepared including 9 levels: 1, 10, 50, 100, 250, 500, 750, 1000 and 2500 ng/mL.

2.7.3. Liquid Chromatography (LC) Conditions and Mass Spectrometry (MS) Conditions:

The chromatographic separation was performed on an Agilent C18 column (250 × 4.6 mm, 5 µm; Agilent Co. Ltd, USA) with a protected C18 column (4.6 × 12.5 mm cartridge, 5 µm; Agilent Company, USA). The column temperature was kept at 25°C. The injection volume was 10 µL. The mobile phase consisted of A (0.1% formic acid + HPLC water) and B (0.1% formic acid + Acetonitrile (ACN)) with gradient elution as follows: 0–2 min, 100%; 2-5 min, 75% A; 11–15 min, 65% A; 15-20 min, 5% A and then in 0.1 min returned to initial 100% A. This was followed by the equilibration period of 5 min prior to the injection of each sample. The flow rate was set at 0.25 mL/min.

The LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using the polarity-switching electrospray ionisation (ESI) mode. The optimal conditions were as follows: dry gas temperature was 300 ◦C, dry gas flow rate of 10.0 L/min. 4 µL samples were injected. Samples were measured as the target compounds based on MRM. For the analysis of primary

metabolites 5-HT, DA, GABA, NE and EPI, LC/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used.

2.7.4. Quantification of Samples

A linear calibration curve was generated for each compound from the range of standards. Good linearity with R^2 greater than 0.98 was obtained across the set calibration in the range from 1 ng/mL to 1000 ng/mL for each of the analytes, with accuracy within 100% ± 20%. Quantification of samples was determined by calibration with 5 analytes including 5-HT, DA, EPI, GABA and NE. Samples were measured using online Nexera LC System coupled to LCMS-8050 triple quadrupole (QQQ) mass spectrometry (Shimadzu Corporation, Kyoto, Japan). Data were processed using LabSolutions LCMS version 5.65 software.

2.8. Statistical Analysis:

Data from LC-MS, GC and FMC-FISH were analysed using SPSS (SPSS Statistics version 25). Student paired t-test was used to compare the three time points of each substrate (0 h, 6 h and 24 h) and to compare the substrates and the control at the same time point for analysing data from LCMS, GC, and FMC-FISH. Results were considered significant when they possessed a P value lower than 0.05.

3. RESULTS

3.1. Bacterial Enumeration:

Bacterial population ($\log_{10}$ CFU/mL) were shown in Table 2. The growth of *Bifidobacterium* spp. significantly increased in tryptophan and tyrosine added vessels between 0 h and 6 h whilst significant increases were also seen in MSG and Tyrosine added vessels between 0 h and 24 h. Moreover, a significant increase was seen in *Clostridium clusters* XIVa and XIVb in the Tyrosine added vessels between the time points of 0 h and 24 h. However, there was an increasing trend in the growth of *Bifidobacterium* spp., no significant increase was observed in the control vessel between the time of 0 h and 24 h. This might be due to higher level of standard deviation. There were no significant differences between substrates and the control vessels at any specific time points. As such, no significant substrate effects were observed in this study.

Table 2. Bacterial groups detected by fluorescence in situ hybridisation in the pH-controlled and volume-controlled batch cultures at 0, 6 and 24 h of fermentation (Mean of the data of three experiments and standard deviations: n 3)

Bacterial Population (log 10 CFU/mL)									
Probes	Time Point (h)	Control		MSG		TRYPTOPHAN		TYROSINE	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Eub 338 I-II-III	0	7.83	0.37	7.80	0.46	7.86	0.26	7.90	0.35
	6	8.14	0.40	8.19	0.29	8.34	0.28	8.34	0.09
	24	8.28	0.28	8.43	0.16	8.35	0.31	8.48	0.24
Bif 164	0	6.82	0.30	6.70	0.44	6.76	0.36	6.82	0.34
	6	7.65	0.60	6.84	0.46	7.37[a]	0.41	7.44[a]	0.55
	24	7.40	0.27	7.22[b]	0.49	7.10	0.77	7.71[b]	0.44
Lab 158	0	5.47	0.34	5.62	0.31	5.88	0.17	5.52	0.75
	6	5.70	0.53	5.32	0.82	5.71	0.59	5.67	0.66
	24	5.97	0.41	6.20	0.41	5.81	0.43	6.22	0.07
Bac 303	0	6.53	0.11	6.54	0.25	6.77	0.31	6.77	0.29
	6	6.07	0.51	6.51	0.48	6.67	0.63	6.48	0.24
	24	6.71	0.78	6.99	0.47	6.32	0.82	6.74	0.51
Erec 482	0	7.31	0.20	7.32	0.25	7.31	0.16	7.36	0.15
	6	7.49	0.35	7.44	0.64	7.33	0.50	7.82	0.35
	24	7.37	0.66	7.49	0.48	7.16	0.61	7.75[b]	0.25
Rrec 584	0	5.95	0.38	5.91	0.52	6.23	0.13	5.97	0.56
	6	6.01	0.35	6.05	0.51	6.04	0.63	6.02	0.37
	24	6.07	0.37	6.31	0.21	5.51	0.50	6.16	0.46
Ato 291	0	5.64	0.08	5.53	0.09	5.71	0.25	5.82	0.54
	6	5.84	0.80	5.50	0.52	5.93	1.08	5.73	0.87
	24	6.32	0.59	6.01	0.48	5.85	0.32	6.70	0.38
Prop 853	0	6.12	0.73	6.07	0.83	6.08	0.78	6.08	0.90
	6	6.36	0.38	6.51	0.78	6.41	0.44	6.36	0.10
	24	6.49	0.66	7.15	0.17	6.93	0.48	6.84	0.58
Fprau	0	6.80	0.22	6.85	0.51	6.81	0.35	6.91	0.23
	6	6.63	0.43	6.72	0.73	6.90	0.12	7.01	0.06
	24	6.89	0.18	7.01	0.30	6.90	0.25	6.95	0.51
DSV	0	5.39	0.71	5.33	1.11	5.99	0.29	5.51	0.93
	6	5.69	0.66	5.63	0.76	5.81	0.60	5.64	0.61
	24	6.06	0.50	5.89	0.27	5.39	0.99	6.70	0.83
Chis 150	0	5.88	0.54	5.92	0.57	5.99	0.16	5.67	0.55
	6	6.20	0.46	6.15	0.34	6.53	0.19	6.46	0.28
	24	6.46	0.23	6.47	0.33	6.56	0.24	6.33	0.21

Results are reported as means ($\log_{10}$ CFU/mL) of the data (n=3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P≤0.05; In italics type: Significant differences with P≤0.01. Probes: total bacteria (Eub338 I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most *Bacteroidaceae* and *Prevotellaceae* (Bac303), *Clostridium coccoides–Eubacterium rectale* group (Erec482), *Roseburia*

subcluster (Rrec584), *Atopobium* spp. (Ato291), *Clostridial* cluster IX (Prop 853), *F. prausnitzii* (Fprau655), Most *Desulfovibrionales* (DSV 687), Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II) (Chis 150). SD: Standard Deviation

3.2. Short Chain Fatty Acids (SCFA):

The concentrations are shown in **Table 3** and **Figure 2.** Data showed acetate as the dominant SCFA produced following fermentation of all substrates. Significant increases in the production of acetate were observed between 0 and 24 h of fermentation upon the fermentation of MSG and tryptophan compared to the control vessels (P< 0·05). A significant increase in the production of butyrate was also achieved upon the fermentation of tryptophan between 6 h-24 h of time (P<0.05). Significant increases in propionate concentration throughout fermentation were observed in tyrosine added vessel and the control vessel between 0 and 24 h of fermentation whilst a significant increase was obtained in MSG added vessels between 6 and 24 h of fermentation (P< 0·05). On the other hand, no significant differences were observed in SCFAs between any treatment and the control at any time point and there were no significant differences in any timepoint among treatments compared to the control.

Table 3. SCFA Production by Gas Chromatography in pH-controlled and volume-controlled faecal batch cultures at 0, 6 and 24 h of fermentation (Mean of the data of three treatments and standard deviations).

		Acetic Acid (mM)		Butyric Acid (mM)		Propionic Acid (mM)	
	Time	Mean	SD	Mean	SD	Mean	SD
NEGATIVE CONTROL	0	20.18	7.61	6.48	0.98	4.77	2.47
	6	64.33	31.48	10.92	8.09	15.51	17.20
	24	129.05	77.42	11.24	2.69	32.88[b]	6.63
MSG	0	16.40	3.66	5.67	2.00	3.20	1.65
	6	76.16	61.27	17.99	19.25	20.70	21.34
	24	165.34[b]	10.64	39.95	23.31	40.13[c]	21.91
TRYPTOPHAN	0	22.79	7.88	6.23	1.48	5.78	2.95
	6	83.42	33.85	17.34	11.64	18.75	12.54
	24	153.71[b]	22.02	35.61[b]	15.54	52.47	14.96
TYROSINE	0	23.54	8.24	6.21	1.70	6.11	3.21
	6	115.42	39.06	26.81	11.55	34.88	14.98
	24	158.34	57.06	45.01	21.35	56.13[b]	11.97

[a] Significant differences between 0 h-6 h with P<0.05; [b] Significant differences between 0 h-24 h with P<0.05; [c] Significant differences between 6h-24h with P<0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P<0.05; In italics type: Significant differences with P<0.01.

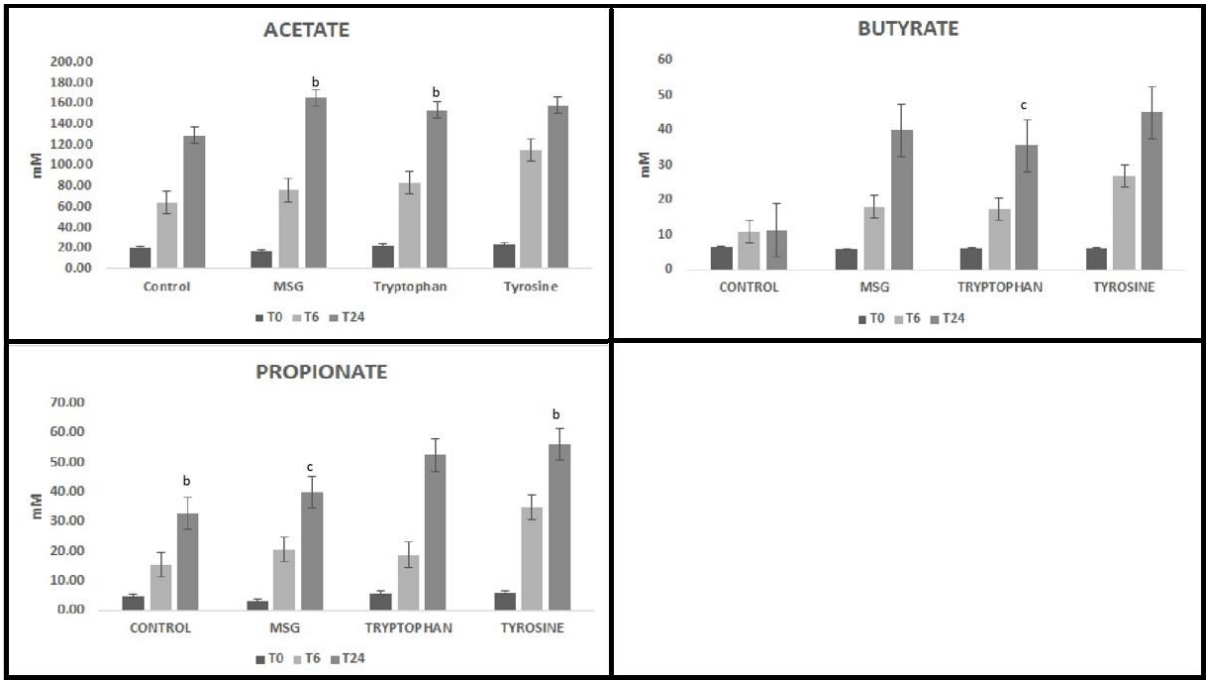

Figure 2. GC Analysis. Acetate, Butyrate and Propionate concentrations in basal media recovered from three different treatments addition with control of pH-controlled and volume-controlled batch faecal culture systems at the time slots of 0, 6 and 24 h. Results are reported as means (mM) of the data (n=3). [a]Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P<0.05; [c] Significant differences between 6h-24h with P<0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P<0.05; In italics type: Significant differences with P<0.01.

3.3. Neurotransmitter Production:

Neurotransmitter concentrations are reported with the changes illustrated in **Figure 3.** As it can be clearly seen, GABA concentration significantly decreased from 0 h to 24 h fermentation in all the vessels except control vessel. Moreover, significant decreases in GABA were also seen between 0 h-6 h in MSG and tryptophan added vessels. There were no significant changes in serotonin, norepinephrine, epinephrine, and dopamine levels throughout the fermentation. Moreover, there were no significant difference in any specific time point in each treatment compared to control. Furthermore, there were no significant differences in any timepoint among treatments compared to control. Epinephrine and dopamine results were not displayed as their quantities were too low to measure accurately.

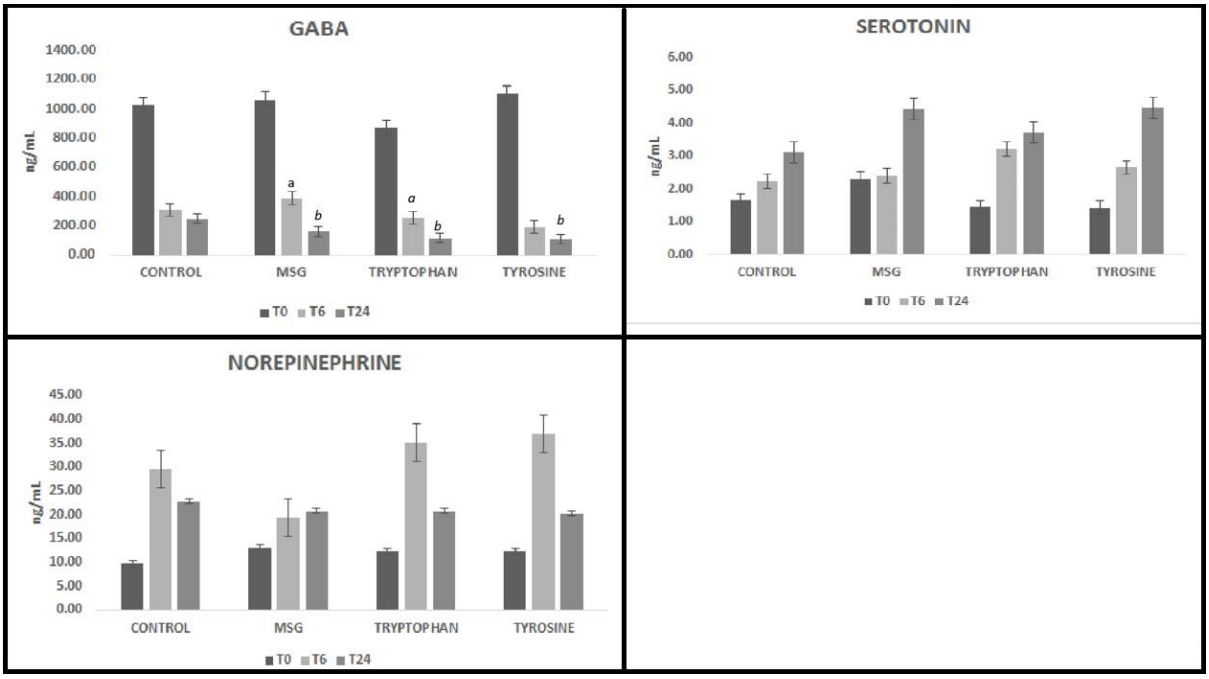

Figure 3. LC-MS analysis. GABA, Serotonin and Norepinephrine concentrations in pH-controlled and volume-controlled faecal batch culture from vessels at 0, 6 and 24 hours of time points from three treatments (MSG, tryptophan, tyrosine) and negative control. [a]Significant differences between 0 h-6 h with P≤0.05; [b]Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d]Significant differences between each substrate compared to control at 0 h, 6 h and 24 h with P≤0.05; In italics type: differences with P≤0.01. 1000 ng/mL of GABA is approximately equivalent to 0.01 mM of GABA and when the amounts of serotonin and noradrenaline are converted from ng/mL to mM, approximately 10^{-5}-10^{-6} mM are obtained which are too small to give accurate readings.

4. DISCUSSION:

This study has focused on the role of neurotransmitter precursors as fermentable substrates on the faecal microbiota and their role in neurotransmitter and SCFAs production in a defined batch culture medium, with temperature, pH and fermentation time controlled in a simple simulation of distal colon physicochemical conditions. *In vitro* batch cultures were conducted to determine if the microbiota can produce neurotransmitters under physiologically relevant conditions. Secondly this experiment explored whether fermentation of amino acid precursors can enhance neurotransmitter production by faecal bacteria.

In this current study, MSG was expected to promote GABA production as being the precursor of GABA. However, MSG did not promote the GABA production. Conversely, GABA breakdown was observed upon fermentation of all substrates. There are several factors affecting GABA synthesis by microbial fermentation, including pH, temperature, fermentation time and different media additives (Dhakal et al., 2012). These fermentation factors can be optimised based on the biochemical characteristics of

GAD activity of the fermenting microorganisms. In addition, nitrogen and carbon sources can affect the amount of GABA production (Yunes et al., 2016).

The results observed were in accordance with the study of Barrett et al. (2012) where GABA was detected in human faecal samples used to inoculate fermentations, with concentrations of less than 2 µg/mL decreasing throughout the 24 h fermentation. The amounts of GABA in the current study were at similar concentrations and decreased in a similar way, where GABA concentrations were below 1 µg/mL. As such the breakdown of GABA was observed in both the current study and in the work of Barrett (Barrett et al., 2012). This could be due to intestinally derived bacteria utilising GABA as well as producing it. GABA can be used as carbon and nitrogen sources and is metabolized through the GABA shunt pathway by certain species including *Escherichia coli* (Kurihara et al., 2010). In another study, *Listeria monocytogenes* was observed to metabolize GABA through the GABA shunt pathway (Feehily et al., 2013). As such this may help to explain the decrease of GABA concentration in this experiment conducted with gut microbiota fermented in basal media.

Looking at the data, MSG led to a positive microbial shift, with the potential for SCFAs producing bacteria by stimulating *Bifidobacterium* growth. However, tyrosine also stimulates the growth of *Bifidobacterium* spp., whilst also promoting the growth of *Clostiridium* clusters XIVa and XIVb. Moreover, tryptophan also increased the production of acetate and butyrate significantly and specifically in butyrate production, as such extra addition of amino acid might help modulate fermentation. However, it is worth noting there were no significant changes noted as compared to the negative control, so the changes observed where just significant over time.

Although MSG, Tryptophan and Tyrosine led to significant increases in the production of SCFAs, control vessels which are including tryptone (provides the aminoacid sources for the growth of the bacteria) were also led to trend of increases in the SCFAs production (Clarke et al., 2014). Therefore, it might be difficult to consider these specific amino acids are significantly effective for the production of neuroactive metabolites and for stimulating the positive bacterial shift. It is still needs to be determined further with more donors with a negative control. Additionally, the dose of pre-cursor amino acids was based on findings of the colon of pigs, however, it is likely that much higher levels of MSG could be present in the gut due to the diet (Jansman et al., 2010). Therefore, perhaps a higher dose could have had a greater impact on microbiota, SCFA and neurotransmitters.

Butyrate is stated to stimulate memory and synaptic plasticity by inhibiting histone deacetylases (Stilling et al., 2016). Research has also indicated that propionate intervention protects the blood–brain barrier (BBB) from oxidative stress (Louis & Flint, 2017). Additionally, SCFAs can impact on neuroinflammation through regulating the production of immune cells such as inflammatory

cytokines and neutrophils (Dalile et al., 2019). In this current study, it has been estimated that MSG, tryptophan, and tyrosine administration positively affect the production of acetate, butyrate and propionate whilst without any positive effect on neurotransmitters. However, this is still promising in regulating the impact on microbial metabolic activity in the distal colon to improve gut health, mood and cognition status. As such, future explorations could be based around whether significant production might be possible with additional substrates such as prebiotics.

It has been suggested that GABA-producing intestinal bacteria, convert glutamate to GABA and that this may have a potentially positive impact on health (Barrett et al., 2012; Lyte, 2011). In the Barrett study, in addition to investigating GABA production of the gut microbiota, the production of GABA by *L. brevis* DPC6108 was also investigated through both inoculating the *L. brevis* to the pH-controlled faecal fermentations and to the synthetic laboratory media to investigate the GABA production in non-pH controlled and pH-controlled (pH 6.8) systems. Besides, *L. brevis* were also observed to significantly increase the production of GABA in both non-pH controlled and pH-controlled systems. On the other hand, the GABA levels which were produced in the pH-controlled faecal fermentations were significantly lower than in non-pH-controlled screening synthetic media. In this current experiment at pH 6.8 it is therefore likely that the conditions were not optimal for GABA production, and a more acidic pH, as would have resulted in the non-pH-controlled culture would have been preferred for GABA production.

With this current experiment it was possible to observe the levels of serotonin and norepinephrine *in vitro*. However, it must be noted that the levels of these neurotransmitters were low and likely to be not physiologically relevant (Watts et al., 2012). These results indicate that the gut bacteria may need a host cell to enhance production of these catecholamines and might involve the production pathways of these neurotransmitters by the host (Hata et al., 2017). On the other hand, although the neurotransmitter production levels were very low, additional dietary prebiotic substrates such as inulin and B-GOS might have effects on the growth of the gut bacteria population which are implicated in neurotransmitter production, thus this further enhance neurotransmitter levels (Flint et al., 2012; Oriach et al., 2016; Verbeke et al., 2015). Overall, these results showed that the extra addition of amino acids might have positive impact on the production of metabolites by the gut bacteria. However, tryptone addition might be enough for the gut bacteria to produce SCFAs, extra addition of amino acid source might shift the production.

In conclusion, the gut bacteria are considered to have the capability to grow up whilst producing a great amount of SCFAs and can also produce a small number of neuroactive metabolites within the presence of basic nutrients with certain essential amino acids at physiological pH level (pH 6.8).

Therefore, according to the current study, the addition of extra amino acids as a precursor of the neurotransmitter metabolites had no significant impact on the promoting of the production of GABA, serotonin and catecholamines. However, there was an increasing trend of serotonin production. This study provides a basic knowledge for the investigation of the production of metabolites by the gut bacteria within the basic nutrient environments and will lead future studies to assess the further impacts on the enhancement of metabolite production by the gut bacteria within a more complex media.

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CHAPTER 3

Batch culture fermentations assessing the impact of maltodextrin, inulin, galactooligosaccharides (B-GOS) and freeze-dried blueberry treatments on the faecal microbiota community

Abstract:

In recent years, attention has been turning to the microbiota and its metabolite production and the potential for neurotransmitter production. In the current study an *in vitro* approach was used to access whether faecal microbial fermentation of maltodextrin (MD), inulin, galactooligosaccharides (B-GOS) and freeze-dried blueberries (flavonoids) can impact on neurotransmitter production and alter the gut microbiota composition. Initially, an upper gut *in vitro* digestion was performed on the freeze-dried blueberry and maltodextrin preparation, to obtain the non-digestible portion, which would reach the colon. *In-vitro* fermenters containing basal media enriched with tryptone (as a protein source) were inoculated with faeces from healthy 7-10-year-old children and maltodextrin, inulin, B-GOS, blueberries or a no treatment were used to investigate the metabolite outputs of the fermentation process. The models were sampled at 0, 6 and 24 hours and gut microbiota composition was analysed using flow cytometry combined with fluorescence *in situ* hybridisation, metabolite production was assessed by GC and LC-MS.

Bifidobacterium composition increased significantly with the addition of B-GOS and there was a trend of an increase in *Bifidobacterium* composition with the addition of inulin. No change in *Bifidobacterium* spp. levels were observed upon fermentation of freeze-dried blueberries and inulin. There was a significant increase in GABA production following fermentation of B-GOS between the 6 and 24 hours of fermentation ($p \leq 0.05$), whilst fermentation of inulin led to a trend of increased GABA ($p \leq 0.10$). Furthermore, fermentation of both inulin and the freeze-dried blueberries led to a trend for increased serotonin levels ($p \leq 0.10$). Furthermore, particularly, the addition of prebiotics inulin and B-GOS significantly altered short-chain fatty acid (SCFA) production whilst fermentation of maltodextrin and freeze-dried blueberries led to a trend of increased SCFA levels.

In conclusion, the production of neurotransmitters: GABA and serotonin and SCFAs increased whilst positively impacting on the microbiota especially with the addition of prebiotic inulin and B-GOS. As such, dietary intervention with prebiotics particularly B-GOS impacts the gut microbiota, influencing the production of neurotransmitters, which could lead to a positive impact on cognitive function. However, flavonoid-rich foods such as blueberries did not exert any prebiotic action on the gut bacteria and on the neurotransmitters and SCFA production in this study, further *in-vitro* studies might be to confirm the potential prebiotic effects of the blueberries which are a source of anthocyanins. As such, trends of increasing were obtained in the probiotic flora with the addition of blueberries in this study and that might mean it needs to be studied with more donors and with a more diverse range of nutrient present at the different pH levels representing different stages of the colon.

Key words: gut microbiota, prebiotics, inulin, galactooligosaccharides, flavonoids,

1. INTRODUCTION

Current studies indicate that the microbiota profile is influenced in infancy by certain factors such as method of delivery, feeding regime and the diet during childhood (Cerdó et al., 2016). Furthermore, the gut microbiota has been associated with neurodevelopment. As such providing a beneficial microenvironment in infancy could help to support cognitive development. Certain neurodevelopment diseases including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD) might be related with the alterations of the gut microbiota. For example, differences in dietary intake could impact on the microbiota and gut permeability and the resulting metabolites might be involved in cognitive health (De Sande et al., 2014; Noble et al., 2017). Focussing on children could be an ideal stage of life to have a long-term impact on cognitive health. At this age a targeted improvement of gut microbiota composition could support neurological health of children at a critical stage of life, when brain development is key (Iglesias-Vazquez et al., 2020).

A prebiotic can be termed as "a nondigestible food ingredient that beneficially impacts the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health" (Gibson et al., 2017). As such, prebiotics can be regarded as dietary ingredients able to reach the large intestine and support the growth beneficial bacteria therein. As such, this provides an opportunity to alter the gut microbiota composition, resulting in a positive effect on host health (Costabile et al., 2012). A prebiotic approach is considered a way to target positive bacterial growth in general and related with the cognitive health. Although, prebiotic effects are not well documented in terms of cognitive diseases, especially in children, they are stated to have a positive impact on the faecal microbiota composition and metabolic profile using *in-vitro* fermentation systems imitating the conditions of distal colonic environment (Grimaldi et al., 2017). Prebiotics are undigested in the small intestine and are fermented by the gut microbiota in the large bowel resulting in the production of the main end products, short chain fatty acids (SCFAs), which may impact certain effects including the modulation of enteroendocrine serotonin secretion (Rios-Covian et al., 2016). Furthermore, the gut microbiota may produce neuroactive metabolites directly, or be involved in their production pathways, such as GABA, serotonin and catecholamines (Yunes et al., 2016).

Many positive effects on the gut, immune and cognitive system have been related with prebiotic use. Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are soluble fibres largely used as prebiotics that are traditionally associated with the promotion of beneficial bacteria such as bifidobacteria and lactobacilli*,* among other gut bacteria (Kapiki et al., 2007). FOS are b-D-fructans with degrees of polymerization (DP) varying between 2 and 60 (inulin) and 2 and 20 (oligofructose).

Bifidogenic effects of both inulin and oligofructose are well establishing in human studies. The consumption of oligofructose as a compensation for sucrose in diet has been observed to result in a significant increase in bifidobacteria concurrent with a decrease in *Fusobacterium* and *Clostridium* (Saulnier et al., 2007). Candidate prebiotics may include non-digestible foods and oligosaccharides which cannot be digested, except for by certain bacterial species (Gibson et al., 2004). A study conducted with healthy human subjects showed that GOS could supress the neuroendocrine stress response (Schmidt et al., 2015). Moreover, administration of FOS and GOS has been investigated in terms of impact on anxiety and depression-like behaviour, cognition, and social behaviour. It was observed that prebiotic treatment induced the alterations in behaviour, HPA axis, immune system through regulating the gut microbiota and accordingly modulating the secretion of certain neurotransmitters such as GABA, serotonin and catecholamines either from the bacteria or from the host (Burokas et al., 2017).

There is increasing evidence that dietary anthocyanins have potential health benefits through their ability to modulate the gut microbiota and that anthocyanins may be able to function as prebiotics (Parkar et al., 2013). Several *in-vitro* and *in-vivo* studies observed growth of beneficial bacteria including *Lactobacillus* spp. and *Bifidobacterium* spp. after application of anthocyanin-rich products (Bialonska et al., 2010; Hidalgo et al., 2012). As such, suggesting the modulation the growth of bifidobacteria and lactobacilli might lead to promote the production of certain metabolites such as short chain fatty acids and neurotransmitters which are associated with cognitive function (Hidalgo et al., 2012; Mao et al., 2015).

It is indicated that there is a cooperation between the bifidogenic effects and an enhancement of colon butyrate production in the human colon. Butyrate is utilised by the colon epithelial cells for energy, it also contributes the modulation of the gut barrier functions and has immunomodulatory and anti-inflammatory properties. It has been stated that the butyrogenic effects of the prebiotics such as inulin might be a result of the cross-feeding interaction with the butyrate producing bacteria such as *Faecalibacterium prausnitzii* and *Roseburium* spp. and bifidobacteria species in the gastrointestinal tract. Furthermore, butyrate is known to improve brain health through having properties of histone deacetylase (HDAC) inhibitor functioning as a ligand for a subset of G-protein coupled receptors. These multifunctional properties are associated with neurological disorders. It has also been reviewed that a high fibre diet that promotes butyrate production might alter gene expression in the brain and prevent neurodegeneration (Bourassa et al., 2016). Thus, inclusion of foods in the diet that lead to increased levels of butyrate could offer help in the fight against neurodegeneration.

It has also been shown that anthocyanins may also work with SCFAs and ferric iron to provide indirect regulation of gut microbiota. Anthocyanins have been reported to increase the SCFAs levels which can impact on immune function (Bialonska et al., 2010).

Inulin and GOS are widely known as prebiotics that modulating the gut microbiota in favour of health (Grimaldi et al., 2017; Vandeputte et al., 2017). As such, inulin, (GOS) and blueberry could be considered as a viable alternative approach to impact cognitive functions to prevent and treat cognitive diseases.

This aim of this chapter was to investigate the impact of inulin, beta-galactooligosaccharides (B-GOS) and blueberry flavonoids (Freeze-Dried Blueberries (FB)) on the composition of the gut microbiota and on the production of fermentation end metabolites. These end metabolites are suggested to be implicated in cognitive function. The prebiotic potential of freeze-dried blueberry through its effects on the gut microbiota in this *in vitro* environment will also be determined.

2. MATERIALS and METHODS:

2.1. Chemicals and Supplements:

All the analytical grade chemicals and formulated basal media supplemented with tryptone (3 g/L), were purchased from Sigma-Aldrich (Merck), UK. Neuroactive standards were obtained from Sigma (Merck), UK. The maltodextrin and the enriched chicory inulin powder products, supplied by Beneo-ORAFTI Ltd, the B-GOS product, supplied by Clasado BioSciences Ltd, and the freeze-dried blueberry powder product, supplied by Wild Blueberry Association of North America. Polyphenol analyses in freeze-dried blueberry powder were conducted by FutureCeuticals, Illinois, USA and vitamin, sugar and dietary fibre analyses conducted by RSSL, Reading, UK (Table 1). Oligosaccharide probes for Flow-FISH analysis were purchased from Eurofins, Wolverhampton, UK.

Table 1. Characteristics of Freeze-Dried Blueberry Product

Compound	Composition
Total polyphenols	2900 mg/100g
Anthocyanins	1900 mg/100g
Procyanidins	Not quantified
Vitamin C	335 mg/100g
Total sugars	70 g/100g
Fructose	36 g/100g
Glucose	34 g/100g
Dietary fibre	16 g/100g
Insoluble	Not quantified
Soluble	Not quantified

2.2. Pre-Digestion:

An upper gut *in vitro* digestion was performed on the maltodextrin and freeze-dried blue berry preparation, to yield the non-digestible portion. The pre-digestion is a step that mimics the phases where the substrates would confront on the way to the digestion tract, the oral phase, the gastric phase, and the small intestinal phase. Supplements (Maltodextrin and freeze-dried blueberries) were prepared for pre-digestion by combining each substrate **(12 g)** with 30 mL of distilled water according to the method of Mills et al. (2008) with slight modifications. Both solutions were stomached (Stomacher 400 circulator, Seward) for 5 min at 240 paddle beats/min. The oral phase was mimicked by combining the stomached substrate solution with α-amylase (A 4551, Sigma; **4 mg**) dissolved in $CaCl_2$ (0.001 mol L^{-1}, pH 7, **1.25 mL**). Substrates were incubated at 37 °C on a shaker (Gyratory shaker, New Brunswick) for 30 min. For the gastric phase vessels were reduced to pH 2 using HCl (6 mol L^{-1}). This was then imitated by combining the stomached substrate solutions with pepsin (P 7000, Sigma; **0.54 g**) dissolved in HCl (0.1 mol L^{-1}, **5 mL**). Vessels were incubated at 37°C on a shaker (Gyratory shaker, New Brunswick) for 2 h. The small intestinal phase was imitated by combining the stomached substrates solutions with porcine pancreatin (P 8096, Sigma; **112m**g) and bile dissolved in $NaHCO_3$ (0.5 mol L^{-1}, **25 mL**). Vessels were adjusted to pH 7 with either HCL (6 mol L^{-1}) or NaOH (6 mol L^{-1}) and incubated at 37°C on a shaker (Gyratory shaker, New Brunswick) for 3 h. The solution was placed into a 500 Da molecular weight cut off regenerated cellulose tubing membrane (Spectra/Por® 6, Spectrum Europe, Netherlands) and dialysed against NaCl (0.01 mol L^{-1}) for 15 hours using. The dialysed product was then freeze dried for five days and the resultant powder was then ready to be used for in vitro fermentation (Mills et al., 2008).

2.3. Faecal inoculation:

Faecal samples were obtained from three healthy children (aged between 7-10) who were free from any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics for 6 months before stool sample donation. None of the children followed any specific or restricted diet. All parents were provided a consent form for use of their children's faeces in the study. This study was approved by The University of Reading research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJar™ 2.5 L; Oxoid Ltd) with an anaerobic gas generating kit (AnaeroGen™; Oxoid). Samples were diluted 1/10 w/w in PBS (0·1 mol/l phosphate buffer solution, pH 7·4) and homogenised (Stomacher 400 Circulator; Seward) for 2 min at 230 rpm. After which anaerobic, pH-controlled batch culture systems were inoculated with faeces (1%) with basal media enriched with tryptone as a protein source. Faecal samples were not pooled, and a sample from a different donor was used for each replication of the experiment. Prebiotic inulin,

B-GOS and freeze-dried blueberries (flavonoids) were used within the vessels to investigate if these substrates better promote neurotransmitter production. Maltodextrin was also tested as a supplement to assess how a non-prebiotic carbohydrate source effects gut microbiota composition and thus their production of metabolites. Gut microbiota composition was analysed using flow cytometry combined with fluorescence *in-situ* hybridisation and metabolite production by GC and LC-MS.

2.4. In vitro batch culture fermentation:

This method was previously described by Gomez et al. (2010) and has been explained in detail in the previous chapter (Chapter 2). Substrates maltodextrin (1.5 g of pre-digested maltodextrin/135 mL of basal media), inulin (1.5 g of pre-digested inulin/135 g of basal media), B-GOS (1.5 mL of B-GOS syrup/135 mL of basal media) and freeze-dried blueberries (1.5 g of pre-digested freeze-dried blueberries/135 mL of basal media) were added before adding 15 mL of faecal slurry (10 % (w/v). An extra vessel with no added extra substrate was also included as a control. Batch culture fermentations were run for 24 h, and samples (5ml from each vessel) were collected at 0, 6 and 24 h for analysis of bacterial populations and metabolite production.

2.5. Bacterial Enumerations by FISH-FCM (Fluoresence In Situ Hybridization with Flow Cytometry):

Bacterial composition from the batch culture models were examined by fluorescence *in situ* hybridisation (FISH) with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesised and labelled at the 5' end with the fluorescent dye Cy3 (Eurofins Genomics, UK) as reported in **Table 1**. The method has been described comprehensively in the previous chapter (Chapter 2).

Table 1. Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concentration of 50 ng/µL.

Probe Name	Sequence (5' TO 3')	Target Group	Reference
Non Eub	ACTCCTACGGGAGGCAGC		(Wallner et al., 1993)
Eub338 I +	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)

Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus, Enterococcus,Melisococcus, Tetragenococcus, Catellicoccus, Pediococcus* and *Paralactobacillus* spp,	(Harmsen et al., 2002)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1995)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium clusters* XIVa and XIVb)	(Manz et al., 1995)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	(Franks et al., 1998)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (Clostridium clusters I and II)	(Franks et al., 1998)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and E*ggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2002)
Prop853	ATT GCG TTA ACT CCG GCAC	*Clostridial cluster* IX	(Walker et al., 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)
DSV687	TAC GGA TTT CAC TCC T	Most *Desulfovibrionales* (excluding *Lawsonia*) and many *Desulfuromonales*	(Purdy et al., 2003)

2.6. SCFA analysis:

Production of SCFA was determinate using GC as previously described (Richardson et al., 1989) and the method has been described in detail in the previous chapter (Chapter 2).

2.7. Neurotransmitter Analysis:

Production of neurotransmitter substances were determined as detailed in Chapter 2.

2.8. Statistical analysis:

Data from LC-MS, GC and FMC-FISH were analysed using SPSS (SPSS Statistics version 25). Student paired t-test was used to compare the three time points of each substrate (0 h, 6 h and 24 h) and to compare substrates with the control at specific time point. Results were considered significant when they possessed a P value lower than 0.05.

3. RESULTS:

3.1. Bacterial Enumeration:

Changes in bacterial composition and the mean and standard deviation of bacterial growth (log $_{10}$ cells/mL) in the batch culture system are reported in **Table 2.** Significant increases were observed in the growth of total bacteria between 0 h and 6 h upon the fermentation of FB ($P<0.05$) whilst a trend of increase was observed in the B-GOS added vessels at the time of 0 h and 24 h ($P<0.1$). The growth of *Bifidobacterium* spp. was significantly higher in the B-GOS added vessels at 24 h compared to the control vessels and also the growth of *Bifidobacterium* spp. increased significantly upon the fermentation of B-GOS at the times between 0 h - 6 h, 0 h -24 h, and 6 h -24 h and upon the fermentation of FB between 0 h and 6 h ($P<0.05$). However, no significant differences were observed in other substrates trends of increases were observed following the fermentation of inulin and FB at the time of 0 h and 24 h ($P<0.1$). There was no significant difference in the growth of *Lactobacillus* spp. in any of the vessels. Significant increases were achieved in Bacteroidaceae at 0 h-6 h upon the fermentation of inulin. Furthermore, a significant increase was observed for *Clostridium* clusters XIVa and XIVb between the time of 6 h and 24 h in the B-GOS added vessels, with, a trend of a decrease between 0 h-6 h. The growth of *Roseburia* subcluster was significantly higher in the inulin added vessels compared to the control vessels at the time of 24 h whilst the growth decreased significantly in the control vessel at the time of 0 h and 6 h ($P<0.05$). Following the fermentation of inulin there was a significant increase in the growth of *Atopobium* spp. between 0 h and 6 h ($P<0.05$). Significant increases in the growth of *Faecalibacterium prausnitzii* were observed upon the fermentation of MD and Inulin between 0 h and 6 h. Moreover, a significant increase was also observed in *C. histolyticum* group (*Clostridium* clusters I and II) following the fermentation of MD between the 0 h and the 24 h of time ($P<0.05$) **(Figure 1** and **Figure 2).**

Table 2. Bacterial groups detected by fluorescence in situ hybridisation in the pH-controlled and volume-controlled batch cultures at 0, 6 and 24 h of fermentation with four different substrates (MD (Maltodextrin), Inulin, B-GOS (Bimuno-galactooligosaccharides, FB (Freeze-dried Blueberries)). Mean of the data of three experiments and standard deviations: n 3)

Bacterial Population (log 10 cells/mL)											
	Time Point (h)	Control		Maltodextrin		Inulin		B-GOS		Freeze-Dried Blueberries (FB)	
Probes		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	0	7.63	0.45	7.56	0.42	7.57	0.47	7.55	0.45	7.02	0.55
	6	7.39	0.90	8.31	0.23	8.50	0.26	8.38	0.16	7.72[a]	0.36
Eub 338 I-II-III	24	7.26	0.82	8.21	0.46	8.52	0.16	8.60	0.18	7.61	0.19
	0	6.01	0.41	6.00	0.36	5.97	0.53	6.02	0.54	5.75	0.36
	6	6.30	0.22	7.72	0.46	7.49	0.51	7.87[a]	0.33	6.97[a]	0.48
Bif 164	24	6.03	1.10	7.83	0.61	7.82	0.37	8.43[b,c,d]	0.32	7.10	0.28
	0	5.81	0.36	5.57	0.17	5.61	0.29	5.07	0.60	5.08	0.59
	6	5.19	0.94	6.02	0.33	6.30	0.26	5.87	0.43	5.71	1.43
Lab 158	24	5.36	0.68	6.10	0.42	5.52	0.64	6.23	0.25	5.45	0.45
	0	6.26	0.39	6.24	0.67	6.16	0.74	6.20	0.54	5.83	0.47
	6	5.62	1.37	7.14	0.36	7.31[a]	0.71	7.39	0.36	6.58	1.29
Bac 303	24	5.34	1.13	6.40	0.57	6.63	0.50	6.81	0.85	6.67	0.19
	0	7.19	0.35	7.03	0.57	7.20	0.44	7.18	0.44	6.66	0.48
	6	6.73	0.61	7.42	0.49	7.65	0.55	6.99	0.28	5.51	0.99
Erec 482	24	6.64	0.98	7.56	0.25	7.97	0.29	7.48[c]	0.28	6.73	0.46
	0	6.28	0.18	6.38	0.47	6.35	0.31	6.26	0.11	5.72	0.19
	6	5.41[a]	0.28	6.76	0.61	7.02	0.82	6.01	0.50	5.56	0.68
Rrec 584	24	5.47	0.49	6.36	0.44	7.42[d]	0.77	6.14	0.57	5.34	0.35
	0	5.57	0.46	5.87	0.71	5.77	0.61	5.20	0.88	5.35	0.54
	6	5.84	0.97	6.43	1.29	6.70[a]	0.87	6.41	1.08	6.29	0.43
Ato 291	24	5.99	1.05	6.61	1.01	6.69	0.90	6.85	1.13	6.04	0.67
	0	5.94	0.63	5.97	0.64	5.99	0.65	5.73	0.90	5.45	0.83
	6	6.08	1.10	6.61	0.84	6.79	0.70	6.40	0.17	5.49	0.46
Prop 853	24	6.31	0.91	6.73	0.74	6.66	0.34	6.56	0.33	5.82	0.39
	0	6.55	0.49	6.47	0.54	6.55	0.65	6.51	0.54	5.90	0.42
	6	5.93	0.64	7.06[a]	0.66	7.16[a]	0.54	6.59	0.45	5.25	0.66
Fprau 685	24	5.87	1.03	6.80	0.76	7.25	0.36	6.59	0.54	6.17	0.09
	0	5.65	0.56	5.30	0.23	5.62	0.23	5.33	0.99	4.51	0.82
	6	5.33	0.42	5.56	0.63	6.15	0.87	5.98	0.69	5.49	0.57
DSV 687	24	5.45	0.99	6.07	0.38	5.47	0.55	5.60	0.10	5.80	0.22
	0	5.75	0.08	5.23	0.42	5.37	0.58	5.83	0.82	5.15	0.88
	6	5.58	1.03	6.28	1.52	6.48	1.27	6.49	1.42	5.02	0.88
Chis 150	24	5.50	0.61	6.23[b]	0.48	6.15	1.71	6.36	1.10	5.34	0.08

Results are reported as log $_{10}$ cells/mL of the data (n=3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P≤0.05; In italics type: Significant differences with P≤0.01. Probes: total bacteria (Eub338 I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most *Bacteroidaceae* and *Prevotellaceae* (Bac303), *Clostridium coccoides–Eubacterium rectale* group (Erec482), *Roseburia* subcluster (Rrec584), *Atopobium* spp. (Ato291), *Clostridial* cluster IX (Prop 853), *F. prausnitzii* (Fprau655), Most *Desulfovibrionales* (DSV 687), Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II) (Chis 150)

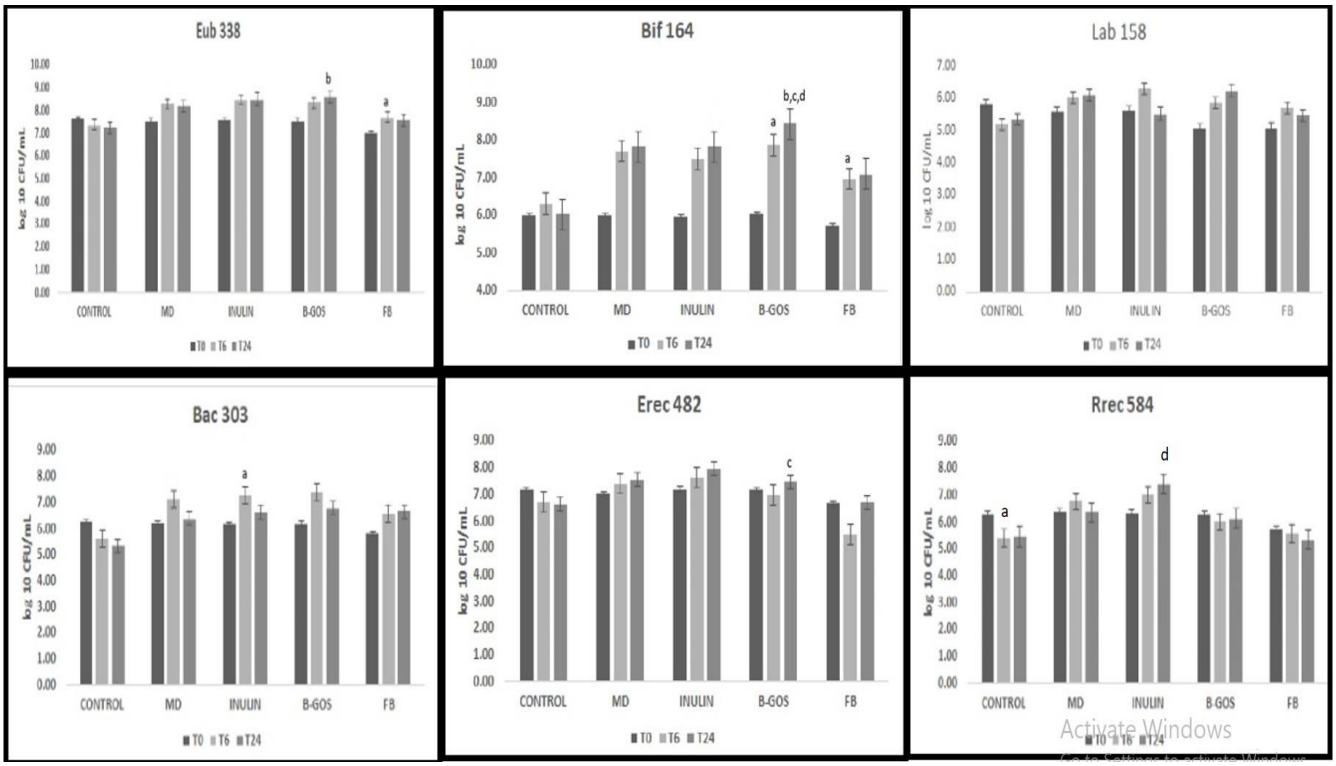

Figure 1. Bacterial groups detected by FISH-FCM (Log10 cells/ml) in the pH-controlled and volume-controlled batch cultures at 0, 6 and 24 h of fermentation with four different substrates (MD (Maltodextrin), Inulin, B-GOS (Bimuno-galactooligosaccharides, FB (Freeze-dried Blueberries)). Mean of the data of three experiments and standard deviations: n 3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P≤0.05; In italics type: Significant differences with P≤0.01. Probes: total bacteria (Eub338 I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most *Bacteroidaceae* and *Prevotellaceae* (Bac303), *Clostridium coccoides–Eubacterium rectale* group (Erec482), *Roseburia* subcluster (Rrec584)

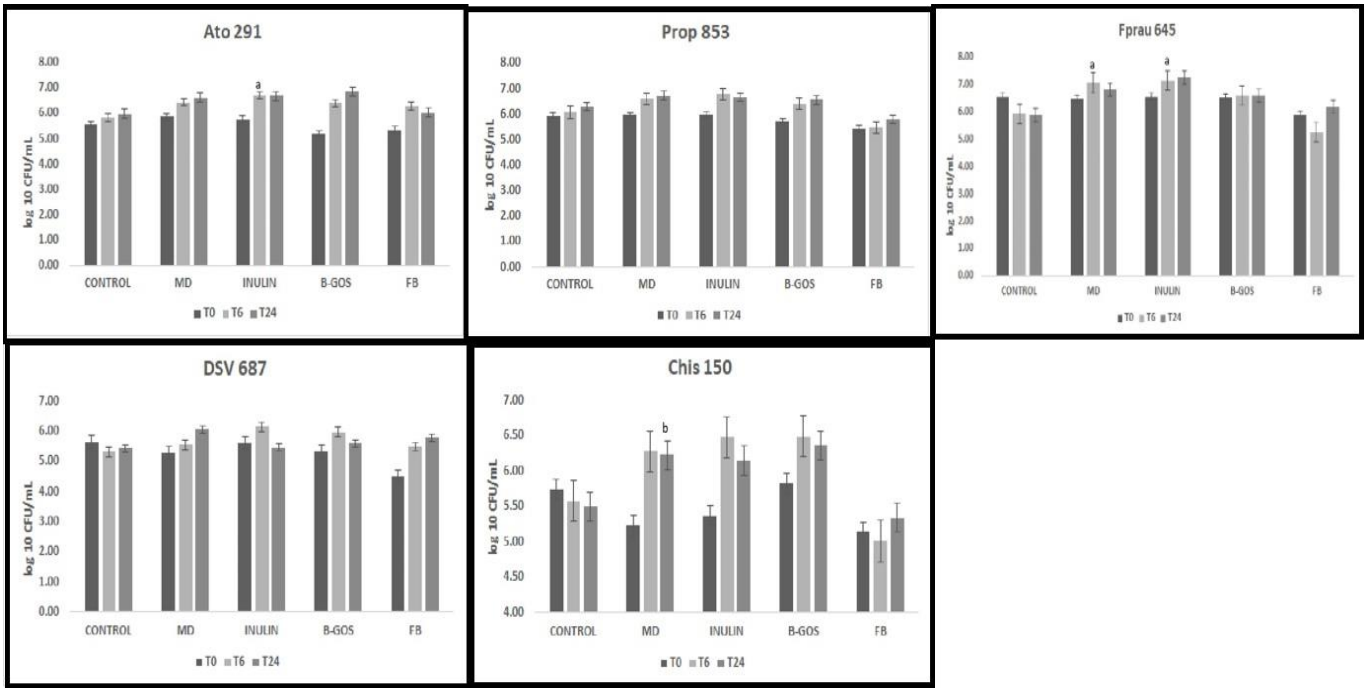

Figure 2. Bacterial groups detected by FISH-FCM (Log10 cells/ml) in the pH-controlled and volume-controlled batch cultures at 0, 6 and 24 h of fermentation with four different substrates (MD (Maltodextrin), Inulin, B-GOS (Bimuno-galactooligosaccharides, FB (Freeze-dried Blueberries)). Mean of the data of three experiments and standard deviations: n 3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 0 h, 6 h, and 24 h with P≤0.05; In italics type: Significant differences with P≤0.01. Probes: *Atopobium* spp. (Ato291), *Clostridial* cluster IX (Prop 853), *F. prausnitzii* (Fprau655), Most *Desulfovibrionales* (DSV 687), Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II) (Chis 150)

3.2. Short Chain Fatty Acids (SCFA):

SCFA concentrations are shown in **Figure 3.** Our data demonstrated significant differences in acetate concentrations when compared to the control following fermentation of all substrates: MD, inulin, B-GOS, and FB at the 24 h (p≤0.05). Significant increases were obtained in acetate upon the fermentation of all substrates: MD, inulin, B-GOS and FB B-GOS at the time points of 0 h and 24 h (p≤0.05). Additionally, at time points 0 h and 6 h, significant increases were obtained in MD, B-GOS and FB added vessels (p≤0.05) whereas a significant increase was observed following the fermentation of FB between 6 and 24 hours (P ≤ 0.01). Significant increases were obtained in butyrate levels following inulin fermentation both the times at 0 to 24 h and 6 to 24 h (p≤0.05). Furthermore, butyrate levels were significantly higher in the inulin added vessels compared to control vessels at the time of 24 h

(p≤0.01). However, there no significant differences observed in other vessels. Significant increases in propionate levels were observed following fermentation of MD, inulin, B-GOS and FB at the time of 0 h- 24 h with significantly higher amounts of propionate in all vessels compared to the control vessel at 24 h (P≤0.05). Moreover, significant increases in propionate were achieved upon the fermentation of both maltodextrin and B-GOS at 6 h-24 h as compared to 0 h (P < 0.05).

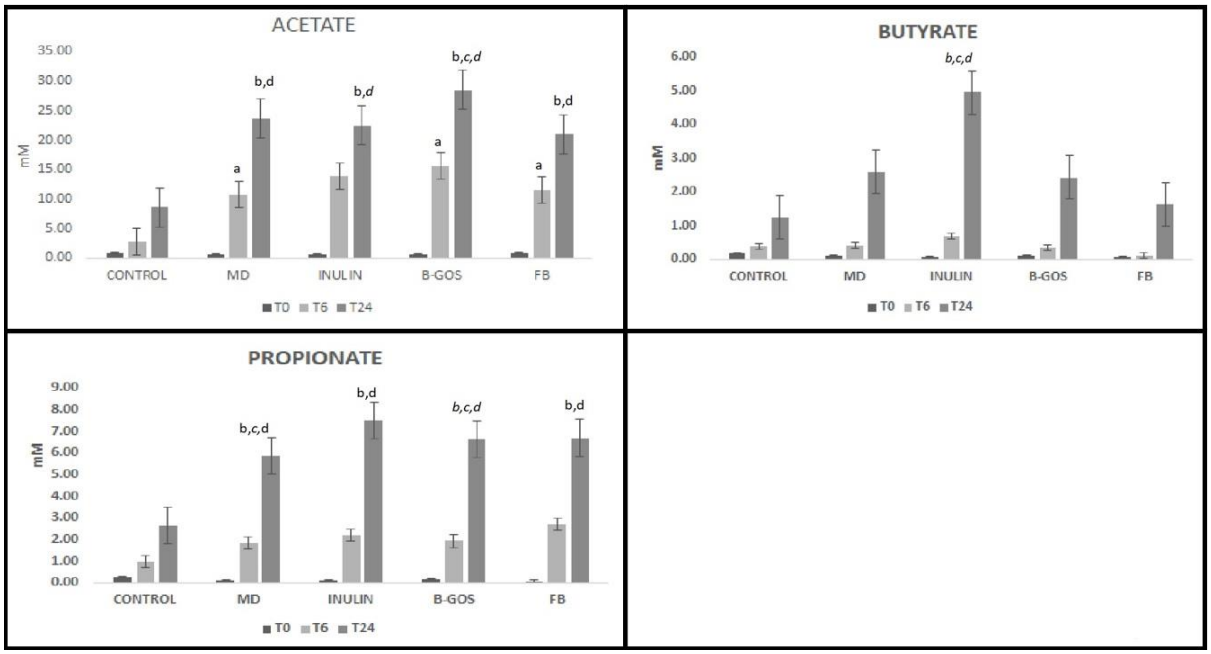

Figure 3. GC Analysis. Acetate, Butyrate and Propionate concentrations in basal media recovered from four different treatments (maltodextrin (MD), inulin, Bimuno-galactooligosaccharides (B-GOS) and freeze-dried blueberries (FB)) addition with negative control of pH-controlled stirred, anaerobic batch culture systems at the times of 0, 6 and 24 h. Results are reported as means (mM) of the data (n=3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 24 h with P≤0.05; In italics type: Significant differences with P≤0.01.

3.3. Neurotransmitter Production:

Neurotransmitter levels are illustrated in **Figure 4.** A significant increase was observed in GABA production between the 0 h and 24 h following fermentation of MD (P<0.01), Inulin and B-GOS (P≤0.05) and of B-GOS between 6 and 24 hours of fermentation (P<0.05). Furthermore, fermentation of MD, inulin and B-GOS led to significantly increased serotonin levels between 0-24 h whilst significant increases were observed in serotonin production upon the fermentation of inulin, B-GOS and FB between 6 h and 24 h (P<0.05). Additionally, there was a significant difference in the amount of serotonin at 24 h in B-GOS added vessels compared to control (P<0.05). Lastly, FB fermentation

resulted in a significant increase in the production of epinephrine between 0 h and 24 h of fermentation (P<0.01).

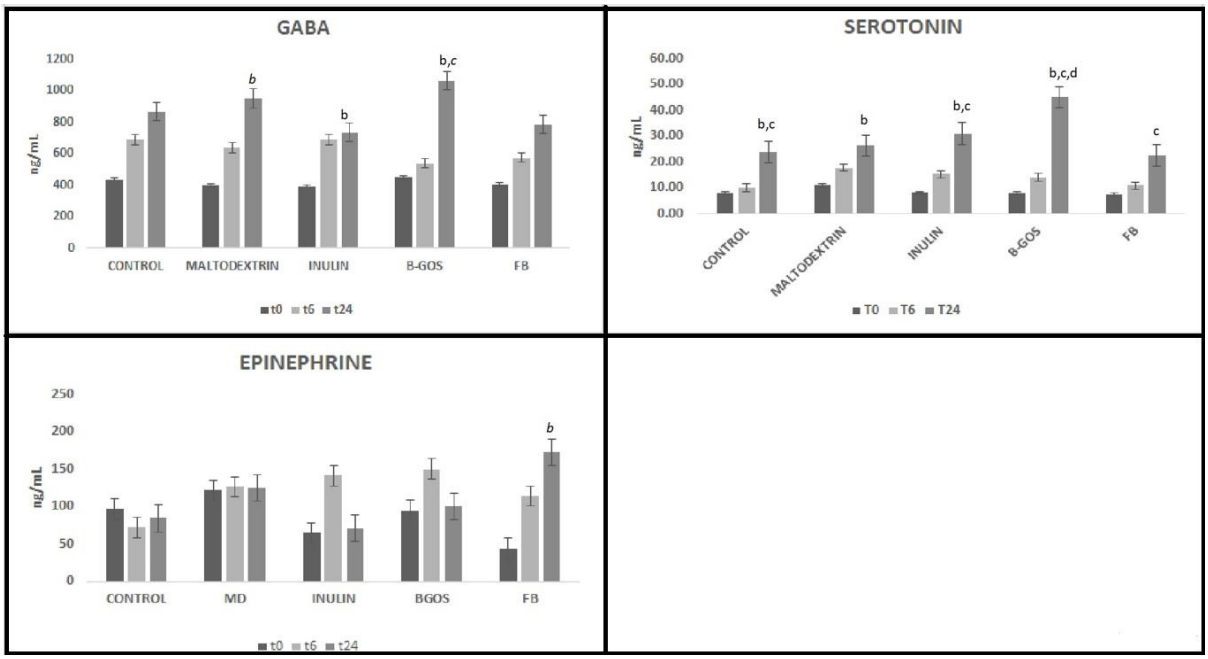

Figure 4. GABA, Serotonin and Epinephrine (EPI) production by LC-MS in the pH-controlled and volume-controlled batch culture from vessels at 0, 6 and 24 hours of time points from four treatments (maltodextrin (MD), inulin, Bimuno- galactooligosaccharides (B-GOS) and Freeze-Dried Blueberries (FB)) and negative control. Results are reported as means (mM) of the data (n=3). [a] Significant differences between 0 h-6 h with P≤0.05; [b] Significant differences between 0 h-24 h with P≤0.05; [c] Significant differences between 6h-24h with P≤0.05; [d] Significant differences between each substrate compared to control at 24 h with P≤0.05; In italics type: Significant differences with P≤0.01.

4. DISCUSSION:

The gut microbiota is increasingly being linked to the brain, as such modulation of this microbial community might impact on neurological functions. *In-vitro* faecal batch cultures supplemented with prebiotic or blueberry substrates were conducted to determine the impact of these treatments on the faecal microbiota and to ascertain whether blueberry may positively modulate the microbiota and whether prebiotic and blueberry fermentation metabolites may be relevant to cognitive function.

Current human volunteer studies have shown that there is a significant increase the butyrate-producing bacteria *Faecalibacterium prausnitzii, Roseburia* subclusters along with *Bifidobacterium* and *Lactobacillus* after the administration of inulin (Ramirez-Farias et al., 2009). Moreover, an *in-vitro* study about the utilization of inulin and FOS by gut bacteria also resulted in significant increases in butyrate (Rossi et al., 2005). This is also the case in our study as inulin promoted the production of

butyrate significantly during the 24 hours of fermentation. Inulin fermentation led to a significant increase of *F. prausnitzii* and *Roseburia* subclusters which is also in accordance with the literature, this is of relevance as through cross-feeding *F. prausnitzii* and *Roseburia* subclusters might be involved in the production of butyrate (Flint et al., 2015). This confirms that this butyrate-producing group may attribute to the butyrogenic effect that works through a cross-feeding mechanism that can be seen after inulin consumption (Gibson et al., 2004).

Gibson et al. (2001) performed a study to compare the fermentation properties and the prebiotic activity of GOS, FOS as compared to MD in mixed faecal culture. FOS, GOS and MD have been reported to increase in *Bifidobacterium*, whereas GOS gave the largest increase in this genera, which is also in accordance with the results of the current study, where GOS fermentation resulted in a significant increase in *Bifidobacterium* spp. as compared to the control vessel, while inulin and freeze-dried blueberries promoted no significant growth of this group but led to a trend of increase (Rycroft et al., 2001). Additionally, Rycroft et al. (2001) found that fermentation of GOS and maltodextrin led to highest levels of SCFA, with the lowest levels of gas upon fermentation of GOS. Gas production is associated with the growth of clostridial population (Rycroft et al., 2001). This was also the case for our study as the administration of maltodextrin significantly promoted the growth of *Clostridium histolyticum* group. As such, MD might be utilised by the gut bacteria as an energy source. On the other hand, MD is digested quite rapidly broken down starting from the mouth with alpha-amylase, MD is absorbed quickly before reaching the colon (Hofman D. L. et al., 2014). In this current study as MD was able to stimulate the growth of *C. histolyticum*, a group that contains potential pathogens, MD can be considered to not be selective, thus did not demonstrate prebiotic potential.

Furthermore, a significant increase in propionate production was found upon the fermentation of MD, inulin, B-GOS and FB which is also in accordance with other research findings (Rossi et al., 2005; Rycroft et al., 2001). Propionate has been reported to have certain protecting effects including activities against colorectal cancer and promotion of satiety and reduction in cholesterol (Louis & Flint, 2017; Polyviou et al., 2016).

All fermentations led to increases in GABA, but these seemed more enhanced in the B-GOS vessel. Fermentation of B-GOS led to a significant increase in *Bifidobacterium* spp. numbers along with an increase in GABA production. It is possible that bifidobacteria were producing GABA, indeed, it was observed by Yunes et al. (2016) that certain *Bifidobacterium* and *Lactobacillus* species have the GAD gene responsible for GABA, this was found in in single strains belonging to 27 different species *(L. reuteri, L. oris, L. helveticus, L. zymae, L. sakei, L. amylovorus* and others). It was stated that the GAD genes possess the ability to produce GABA, and this is widely distributed among strains of the

following species: *B, angulatum, B. dentium* and *B. adolescentis*, *B. stercoris, B. moukalabense, B. subtile, B. ruminantium, B. catenulatum*, and *B. merycicum* species with *Bifidobacterium* strains were found to be most effective among other strains in the production of GABA (Yunes et al., 2016). In the current study this is further supported by the fact that the inulin fermentation also resulted in a trend for more bifidobacteria at the same time as increased levels of GABA. On the other hand, in Barret's study (2012), production of GABA by human derived strains were identified and they investigated that the most efficient GABA producers are *Lactobacillus* spp. In the Barrett et al. (2012) study, strains of *Lactobacillus* and *Bifidobacterium* were grown in medium containing monosodium glutamate (MSG). The cultured strains were administered to a simple pH-controlled (pH 6.8) anaerobic faecal fermentation supplemented with 30 mg/mL of MSG to measure the conversion of MSG to GABA. One *Lactobacillus* strain and four strains of *Bifidobacterium* were found to produce GABA and the addition of *Lactobacillus brevis* DPC6108 to a faecal-based fermentation was found to significantly increase GABA concentration ($P < 0{\cdot}001$) which suggests the concept that this biosynthesis could also occur at this physiological pH (Barrett et al., 2012). This explains that the gut bacteria are capable of producing GABA at this more neutral pH, which is also in accordance with the results of the current study, with GABA production in the distal colon environment (pH 6.8). Furthermore, while GAD genes have been found to be active mostly when the pH is around 2-5, certain *Bifidobacterium* and *Lactobacillus* species contain mutations in GAD genes that are also active in neutral pH (pH 6-7) (Yu et al., 2012). This could also help explain how GABA production could also happen in the distal colon environment around neutral pH (pH 6.8). Furthermore, it could be possible that the role of GABA/glutamate system is not only restricted to a defence mechanism against low pH but also a protective role against other types of stress (Feehily & Karatzas, 2013).

In contrast with the significant increase in the production of GABA, no significant increase in *Lactobacillus* was observed after the administration of the prebiotics (both inulin and B-GOS) and the freeze-dried blueberries. A possible explanation of not stimulating the growth of *Lactobacillus* upon the fermentation of prebiotics might be due to the competition of the *Bifidobacterium* strains with the *Lactobacillus* strains during the fermentation (Rossi et al., 2005; Tuohy et al., 2001). Therefore, it is possible that bifidobacteria were producing GABA, indeed, it has been observed by Rycroft et al. (2001) and is further supported by the fact that the inulin and B-GOS fermentation also resulted in a trend for more bifidobacteria at the same time as increased levels of GABA (Rycroft et al., 2001).

FB do comprise large amounts of anthocyanins, anthocyanins are considered to have an impact on cognitive function and there are certain studies also state that anthocyanins might have prebiotic properties. According to our study, FB showed a potential to stimulate the gut microbiota positively, with increases in bifidobacteria seen over time. Furthermore, FB also led to significant increases in

GABA, serotonin, and epinephrine and in SCFAs except for butyrate. Hidalgo et al. (2012) tested the impact of anthocyanin on gut bacteria composition using similar faecal fermentation systems and found anthocyanins could promote the growth of *Bifidobacterium* spp. and *Lactobacillus−Enterococcus* spp. (Hidalgo et al., 2012). Also, a dietary intervention study showed significant increases in the growth of *Lactobacillus acidophilus* and *Bifidobacterium* spp. after six weeks consumption of a wild blueberry drink (Vendrame et al., 2011). These results showed that anthocyanins might have promising effects on the growth of gut microbiota. This could also suggest that not all anthocyanins work in the same way, or there are other ingredients present in the food matrix impacting on the microbial community. Interestingly, FB was the only substrate that led to a significant increase in levels of EPI, this was a surprising result as little is known on the ability of the microbiota to promote levels of EPI, although murine studies have indicated a microbial involvement in the production of catecholamines (Asano et al., 2012). This is an exciting result that warrants more research on the neuro-active gut brain connection of blueberries. FB used in this study consists of 16 g of fibres/100g. Fibre transit through the digestive tract to the colon where they are fermented by gut microbes with the production of SCFAs, hydrogen, carbon dioxide and biomass (Rodriguez-Daza et al., 2020). As such, the fibre might have also additional positive impacts on modulating the growth of the gut bacteria and regulating the production of SCFAs. A study conducted with a fibre polydextrose (PDX) showed that PD resulted in modest improvements in cognitive function along with the stimulating the gut bacteria composition once utilised for 4 weeks (Berding et al., 2021). This fibre presence could have also influence neurotransmitter production.

In the current study, the production of neurotransmitters: GABA increased whilst as the same time the substrates positively impacted on the microbiota. As such, dietary intervention with B-GOS may have a significant impact on gut microbiota and the production of GABA, which could lead to a positive impact on cognitive function. However, bacteria probably need a host to produce serotonin (5-HT), but gut bacteria might have effects through SCFAs on the production of HT-5 by the host. The majority of 5-HT in the human body is produced by enterochromaffin (EC) cells of the gut and it is synthesized through the rate-limiting enzyme tryptophan hydroxylase (TPH) (Reigstad et al., 2015). A research study conducted with gnotobiotic mice defined that free catecholamines can be produced by the deconjugation of conjugated catecholamines which can be hypothesized to work by deconjugation via bacterial enzymes that might be involved in the production of free serotonin in the gut lumen by the gut bacteria. The results from this study showed that the gut microbiota had a significant impact on promoting the production of biologically active, free 5-HT (Hata et al., 2017).

Moreover, SCFAs were also noted to induce the release of 5-HT from EC cells (Reigstad et al., 2015). Serotonin levels in the peripheral blood are thought to reflect serotonin release from the

gastrointestinal tract. Although there is lack of data regarding that the gut bacteria might produce serotonin without any host cells, in our findings there were a 20-50 ng/mL range of serotonin production which is low compared to the normal range of serotonin levels found in the blood (100-300 ng/mL) (Scarpa et al., 2013). As such, it is possible that although the gut microbiota can produce serotonin it requires host cells to induce the production of serotonin at physiologically relevant amounts. However, research with different dietary ingredients could help to confirm if other nutrients need to be present to bring about direct neurotransmitter production. Based on all these findings it is possible that these metabolites primarily GABA and the SCFAs produced by the gut bacteria might be an important feature in the gut brain axis.

In short, the results displayed a preliminary comparison of faecal fermentation of maltodextrin, inulin, B-GOS and the freeze-dried blueberries. B-GOS showed both bifidogenic effects significantly as increasing the growth of *Bifidobacterium* spp. and increase the acetate levels significantly compared to the control samples. On the other hand, B-GOS did not increase the butyrate levels significantly but showed a trend of increase whilst inulin showed a significant increase in butyrate levels. As such, both known prebiotics exerted potentially positive effects but in different ways.

To our knowledge, the findings in this current study for the production of GABA, serotonin and catecholamines by the gut bacteria in a batch culture fermentation system with the addition of prebiotics and flavonoids are novel. As such, these findings might provide promising evidence for the further studies supporting the gut brain bidirectional communication. Future studies might help to investigate these properties such as the use of a 3-stage continuous fermentation system with a more complex nutrients media to provide a more realistic representation of a large intestinal system with different pH levels and additionally a human intervention study would be necessary to investigate such changes in gut microbiota composition and the microbial metabolites associated with cognitive functions. In conclusion, this *in vitro* study showed promising results in that supplementing the microbiota of children with prebiotics may manipulate the gut bacterial population and metabolic activity to targeted health benefits for the host.

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CHAPTER 4

The impact of inulin and freeze-dried blueberries on the microbiota in a continuous culture system modelling the large intestine

Abstract

Current evidence supports that the gut microbiota produces immune activating and other signalling molecules and neurotransmitters, which may play a significant role in regulating cognitive function. These neurotransmitters consist of γ-aminobutyric acid (GABA), serotonin, along with short chain fatty acids (SCFAs) that are key to several immunological and neurotransmitter pathways. Within this context, flavonoids and prebiotics were used as nutritional supplements to investigate their impact on gut microbiota composition and neurotransmitter production. To investigate the influence of inulin and the flavonoid containing freeze-dried blueberries on gut microbial ecology and metabolite production, faecal samples from healthy children were inoculated into a fermentation system. Anaerobic, pH-controlled, three-stage gut model systems, mimicking the proximal, transverse, and distal large intestine, were inoculated with faeces (20%) with gut model media. Prebiotic inulin and freeze-dried blueberries (flavonoids) were used as substrates to investigate if these could modulate neurotransmitter production. Gut microbiota composition was analysed using flow cytometry combined with fluorescence *in situ* hybridisation and metabolite production, including neurotransmitters, by LC-MS and short chain fatty acids (SCFAs) by GC. Results were assessed before the treatment (steady state 1 (SS1)) and after the treatment (steady state 2 (SS2)). Consistent with previous studies, *Bifidobacterium* composition increased significantly with the addition of inulin in V1 and V3. Additionally, fermentation of freeze-dried blueberries led to enhanced levels of *Bifidobacterium* in V1. Moreover, *Lactobacillus* composition increased significantly upon fermentation of both inulin and freeze-dried blueberries in V1. *Roseburia* spp. increased significantly in V3 of the inulin model whereas, there were significant decreases in *Desulfovibrionales* spp. and *Clostridium histolyticum* in the inulin added V1. Significant increases were observed in acetate production upon fermentation of inulin in all vessels while a significant increase was seen in butyrate following the fermentation of inulin in V1 and of blueberries in V3. Additionally, there were significant increases in the production of GABA and serotonin following fermentation of inulin in V1 and V2 ($p \leq 0.05$), whilst there were no significant changes in GABA or in serotonin upon fermentation of the freeze-dried blueberry. Both the administration of prebiotic inulin and freeze-dried blueberries led to significant changes in the SCFAs. In conclusion, prebiotic inulin increased the production of SCFAs and neurotransmitters: GABA and serotonin whilst positively impacting on the microbiota and freeze-dried blueberries had an impact on the gut microbiota composition and SCFAs. As such, dietary intervention with prebiotics and blueberries may impact on gut microbiota, influencing the production of neuroactive metabolites, especially butyrate: a marker component of prebiotic potential which could lead to a positive impact on cognitive function. In this manner, dietary interventions may have the potential to regulate the brain functions, related to GABA and SCFAs.

Key words: gut microbiota, SCFAs, GABA, serotonin, neurotransmitters, neuroactive metabolites, in-vitro fermentation, inulin, blueberries

1. INTRODUCTION:

Growing evidence indicates that gut bacteria are able to produce neuroactive compounds that have a significant impact on gut physiology and cognitive function. The balance among gut microbiota has been associated with many effects including the gut barrier function, stimulating the host immune system, preventing diarrhoea and allergies, activating, and modulating of provitamins and lipid metabolism (Hidalgo et al., 2012). As such, to modulate the balance among the gut bacteria at early stages of life is essential.

Up until recently, the gut microbiota colonization of children was assessed with culture-based studies. These studies revealed that the infantile gut microbiota is less complex and has a higher proportion of facultative bacteria than the adult gut flora (Adlerberth & Wold, 2009). In adulthood, the intestinal microbiota comprises several hundreds of bacterial species which of are mostly anaerobes. This complex ecosystem is shaped via different internal and external factors such as the mode of delivery, the method of feeding, lifestyle, genetic factors (Cerdó et al., 2016). Research states that the gut bacterial composition during early childhood might influence gut-related diseases. So, it is important to influence immune function by modulating the gut bacteria at young ages.

Anthocyanins are secondary metabolites that give pigmentation to flowers, fruits, seeds, and leaves. They can be found in generous amounts particularly in red and blueberries (Passamonti et al., 2003). They are strong antioxidants as a result of having a phenolic structure belonging to the class of flavonoids. As such, they are likely to protect cells against oxygen radical-related damage including atherosclerosis, cancers and diabetes (Cardona et al., 2013; Spencer, 2010).

Current studies have stated that there is a potential prebiotic effect of polyphenols from fruits and vegetables via promoting the growth of the beneficial bacteria in the gut (Gomez et al., 2010). To determine a products prebiotic potential, it is useful to estimate the impact on the gut microbiota composition (Gibson et al., 2017). Anthocyanins have been reported to increase members of *Lactobacillus* spp. and *Bifidobacterium* spp. genera while decreasing the *Clostridium histolyticum* group. Moreover, gallic acid, one of the most important metabolites of anthocyanins has been observed to reduce the growth of a *C. histolyticum* group without negative effect on beneficial bacteria (Hidalgo et al., 2012). These investigations indicate that anthocyanins might be potential nutrient sources that promote a positive gut bacteria balance, and may therefore promote health (Hidalgo et al., 2012). Further studies are needed to estimate *in vivo* the metabolism of anthocyanins and the potential prebiotic role.

Inulin and FOS are considered as effective prebiotics providing a substrate for the beneficial gut microbiota primarily *Bifidobacterium* spp. and *Lactobacillus* spp. (Costabile et al., 2010). These microorganisms are increasingly being linked to cognitive function via several pathways such as producing and/or modulating host neurotransmitters, such as GABA and serotonin (Dinan et al., 2013; Leke et al., 2011).

A further anaerobic fermentation gut microbial end product, short chain fatty acids (SCFAs) are produced by the gut microbiota and exert positive effects on health, including in brain functions. SCFAs have a significant impact on the gut mucosa and host physiology as an energy source, as regulators of gene expression and as signalling molecules by binding to specific receptors (Louis & Flint, 2017; Polyviou et al., 2016). Higher production of butyrate compared with a control has also been considered as an important indicator of substrate's potential prebiotic effect as butyrate is linked to many positive effects on host health (Gibson et al., 2017). Butyrate is the primary energy source for colonocytes and plays an important role in maintaining both the integrity of the intestinal epithelium and stability of the gut microbiota (Wu et al., 2018) and acting as a histone deacetylation (HDAC) inhibitor which supports cognitive functions (Bourassa et al., 2016). Similarly, Costa et al. (2019) observed that a great amount of butyric acid can be produced by butyrate producing bacteria when grape seed extract was used as a carbon source. It might be a cross-feeding effect between butyrate producing bacteria and *Lactobacillu*s spp. via favouring lactobacilli growth and lactate then being used to enhance butyrate production (Costa et al., 2019; Flint et al., 2015; Gibson et al., 2004). SCFAs are also stated to regulate tyrosine hydroxylase (TH) gene expression through a cAMP-dependent signalling pathway. A study conducted with nuclear run-on assays showed that butyrate increases endogenous TH gene transcription. According to this data, butyrate might principally modulate brain catecholaminergic system by modulating TH gene expression, dopaminergic levels and corresponding cognitive function and behaviour (Shah et al., 2006). Acetate was the major product of gut microbiota and a significant increase in the amount of acetate production was also the case upon the fermentation of inulin which is in accordance with the other *in-vitro* studies.

Gut model system can be a useful tool to assess the gut microbiota composition and the production of metabolites under physiologically relevant conditions, including human large intestine nutrients, temperature, and pH, without any requirement for human participants. This continuous gut model system provides a more realistic mimic of the large colon than batch culture system comprising more complex nutrients with different pH levels and increasing size modelling each stage of the colon (proximal colon; pH 5.5, transverse colon; pH 6.2, distal colon; pH 6.8). The use of the media feed means that the nutrients available decrease from the proximal region to the distal region as they are used by the gut microbiota. The continuous culture design means that media is continually fed into

the model at a retention time appropriate to that of the human body. Within the gut model system after the start of the experiment the bacteria are allowed to equilibrate in the presence of the initial media. Once equilibration has occurred a substrate can be added to the system until the next equilibrium is reached. By this experimental design the two equilibriums can be compared to determine the impact of the substrate on the microbial community, fermentation end products and site of fermentation. The use of faeces from children can help to determine whether these interventions could be of benefit to this population group.

The aim of this experiment was to use a more complex experimental design to determine whether fermentation of inulin or blueberry would have a positive impact on the microbiota and its metabolites when considering cognitive function in children.

2. MATERIALS AND METHODS:

2.1. Chemicals and Supplements:

All the analytical grade chemicals and gut model media components were purchased from Sigma-Aldrich (Merck), Dorset, UK. Neuroactive standards were obtained from Sigma (Merck), UK. Enriched chicory inulin powder products, supplied by Beneo-ORAFTI Ltd, and the freeze-dried blueberry powder product, supplied by Wild Blueberry Association of North America. Polyphenol analyses in freeze-dried blueberry powder was conducted by FutureCeuticals, Illinois, USA and vitamin, sugar and dietary fibre analyses conducted by RSSL, Reading, UK (Table 1.). Oligosaccharide probes for Flow-FISH analysis were purchased from Eurofins, UK.

Table 1. Characteristics of Freeze-Dried Blueberry Product

Compound	Composition
Total polyphenols	2900 mg/100g
Anthocyanins	1900 mg/100g
Procyanidins	Not quantified
Vitamin C	335 mg/100g
Total sugars	70 g/100g
Fructose	36 g/100g
Glucose	34 g/100g
Dietary fibre	16 g/100g
Insoluble	Not quantified
Soluble	Not quantified

2.2. Pre-Digestion:

An upper gut *in vitro* digestion was performed on the freeze-dried blueberry (FB) preparation, to yield the non-digestible portion. Test substrate (FB) was prepared for pre-digestion by combining with distilled of water according to the method of Mills et al. (2008). 12 g of each sample were weighed, and 30 mL distilled water added to obtain the pre-digestion solution. Both solutions were stomached (Stomacher 400 circulator, Seward) for 5 minutes at 240 paddle beats/min. The oral phase was imitated by combining the stomached substrate solution with α-amylase (A 4551, Sigma; 4 mg) dissolved in $CaCl_2$ (0.001 mol L^{-1}, pH 7; 1.25 mL). Substrates were incubated at 37 °C on a shaker (Gyratory shaker, New Brunswick) for 30 minutes. For the gastric phase vessels were reduced to pH 2 using HCl (6 mol L^{-1}). This was then imitated by combining the stomached substrate solutions with pepsin (P 7000, Sigma; 0.54 g) dissolved in HCl (0.1 mol L^{-1}; 5 mL). Vessels were incubated at 37°C on a shaker (Gyratory shaker, New Brunswick) for 2 hours. The small intestinal phase was imitated by combining the stomached substrates solutions with porcine pancreatin (P 8096, Sigma; 112mg) and bile (B 8631, Sigma; 0.7g) dissolved in NaHCO3 (0.5 mol L^{-1}; 25 mL). Vessels were adjusted to pH 7 using NaOH (6 mol L^{-1}) and incubated at 37 °C on a shaker (Gyratory shaker, New Brunswick) for three hours. The solution was dialysed against NaCl (0.01 mol L^{-1}) for 15 hours using 500 Da molecular weight cut off regenerated cellulose tubing (Spectra/Por® 6, Spectrum Europe, Netherlands). The dialysed product was then freeze dried for five days and the resultant powder was then ready to be used for *in vitro* fermentation (Mills et al., 2008).

2.3. Three-stage continuous culture gut model system:

Physicochemical conditions in the colon were replicated in a continuous culture system, comprised of three connected fermentation vessels (V) representing the proximal (V1, 80 mL, pH = 5.5), transverse (V2, 100 mL, pH = 6.2) and distal colon (V3, 120 mL, pH = 6.8) (Figure 1). A small-scale version of the validated system described by Macfarlane et al. (1998) was used in this study.

The systems were inoculated with 20% (wt:v) faecal homogenate from healthy children volunteers in a growth medium. The growth medium was prepared in distilled water and consisted of per L: 5 g starch, 5 g peptone water, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 4 g mucin (porcine gastric type III), 3 g casein, 2 g pectin (citrus), 2 g xylan (oatspelt), 2 g arabinogalactan (larch wood), 1.5 g $NaHCO_3$, 1.25 g $MgSO_4.7H_2O$, 1 g guar gum, 1 g inulin, 0.8 g cysteine, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts No. 3, 0.15 g $CaCl_2.6H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.05 g hemin, 10 ml Vitamin K and 1 ml Tween 80. Following inoculation, the colonic model was run as a batch culture for 24 h and pH in each

vessel was maintained using a pH pump (Electrolab, UK) with 1 M NaOH and 1 M HCl solutions as appropriate. This batch phase was in order to stabilise bacterial populations prior to the initiation of media flow. After 24 h (T0), the media flow was initiated. An anaerobic environment was maintained by continuous sparging oxygen-free nitrogen supply (15 mL/min).

Faecal samples were obtained from three healthy children's donors (aged 7–10 years old) who were free of any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics 6 months before faecal sample donation. None of the children followed any specific or restricted diet. All parents provided written informed consent for use of their children's faeces in the study. This study was approved by The University of Reading research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJarTM 2.5 L, Oxoid Ltd) including a gas-generating kit (AnaeroGenTM, Oxoid). An aliquot of 20 g of an individual sample was diluted in 100 mL anaerobic PBS (0.1mol/L phosphate buffer solution, pH 7.4, w/w) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 min at 240 paddle beats per min. The sample was then added to anaerobic fermenters within 2 hours of producing. This process was repeated on three times, using a different sample each time. Following inoculation, the system was run for 24 h to allow the bacteria to multiply within the vessels. Then, the flow was started with a retention time of 36 hours with 8-9 mL/h of flow rate when considering the operating volume (300 mL) which is appropriate to children at the age of 7-10 (Hedsund et al., 2013). The system was run for at least 8 full volume turnovers to allow for the gut bacteria and the metabolites to reach equilibrium. When the composition of the gut bacteria reached equilibrium, steady state 1 was achieved (SS1). To confirm SS short-chain fatty acid (SCFA) profiles were assessed as stable (±10%) before starting prebiotic inulin and pre-digested freeze-dried blueberries administration. Taking into account the operating volume (300 mL) and the retention time (36 h, flow rate 8-9 mL/h) of the colonic model system, after reaching SS1 (12 days) prebiotic inulin (containing 98% of inulin and 2% of fructooligosaccharides) (0.6 g/daily) inulin was added daily into V1 (In total 5-8 g of inulin considering the whole gut model system which is appropriate for recommended daily intake of inulin) (Smith et al., 2015) or pre-digested freeze-dried blueberries (FB) (1 g /daily) (In total 12 g of FB considering predigestion and the whole gut model system which is equivalent to approximately 240 g fresh wild blueberries; 253 mg of anthocyanins) (Barfoot et al., 2021) was added daily into V1. The substrates were added to the system for at least a further 8 volume turnovers upon which a second equilibrium was achieved (steady state 2 (SS2)) (12 days). Aliquots of 5 mL were removed at SS1 and SS2. This colonic model system is comprised SS1 and SS2 and took 24 days to complete.

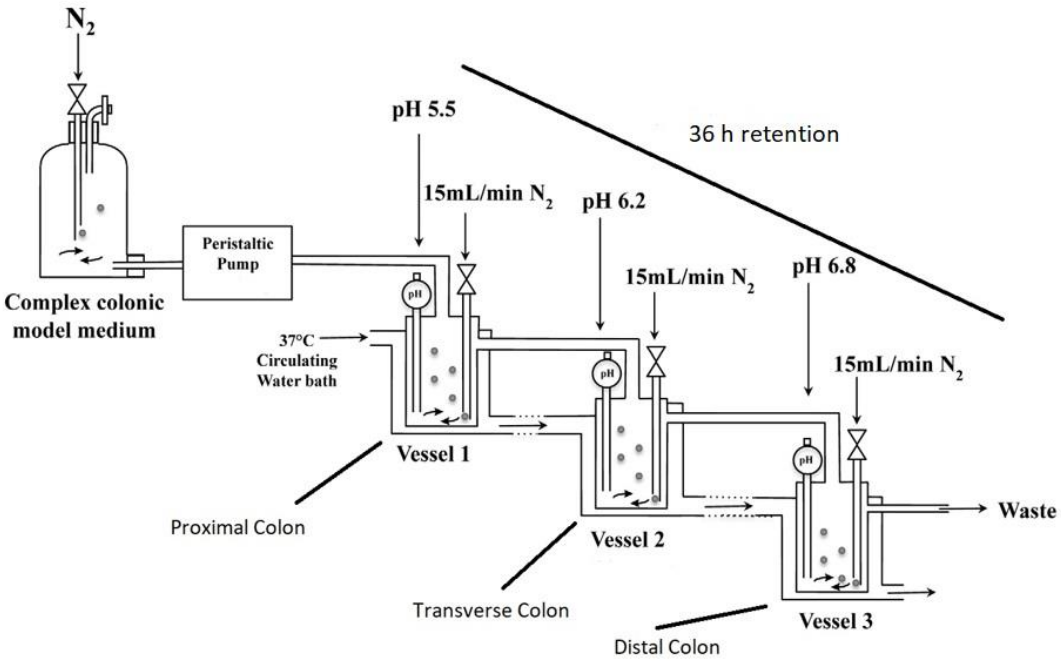


Figure 1. Schematic of gut model system indicating the retention time and the relation of the vessels to the large intestine (Sannasiddappa et al., 2011).

2.4. Bacterial Enumerations by FISH-FCM (Fluorescence In Situ Hybridization with Flow Cytometry):

To examine bacterial composition from the gut model by fluorescence *in situ* hybridisation (FISH), oligonucleotide probes were used which are designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesised and labelled at the 5' end with the fluorescent dye Cy3 (Eurofins Genomics, UK) as reported in **Table 1**. 750 µL of sample was taken from the gut model and centrifuged at 11,200 g for 5 min. Pellets were resuspended in 375 µL of filtered PBS (using a 0.22-µm PVDF membrane) and fixed in 1125 µL of 4% (v/v) paraformaldehyde. After 4h of incubation at 4 ◦C, fixed samples were washed three times with 1 mL of PBS then resuspended in 300 µL PBS-ethanol (1:1, v/v) and stored at –20◦C until the samples were needed for analysis. To prepare the samples for the flow cytometer, permeabilization step was performed as a first step. 500 µL of PBS was added to 75 µL of the fixed samples and centrifuged at 11,200 g for 3 min. Pellets were resuspended using 100 µL of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O, 0.22 µm PVDF membrane)

containing lysozyme (1 mg/mL of 50 000 U/mg protein) and incubated for 10 min at room temperature. After incubation, the samples were then vortexed and centrifuged at 11,200 g for 3 min and the supernatant discarded. Pellets were washed with 500 µL of PBS and centrifuged again (11,200 g, 3 min). Once the permeabilization step was completed, hybridisation was performed by resuspending the pellets in 150 µL of hybridisation buffer (900 mM NaCl, 20 mM Tris/HCl pH 8, 30% formamide, ddH_2O, 10% SDS). The resuspended pellets were vortexed and centrifuged (11,200 g, 3 min). Pellets were then resuspended in 1 mL of hybridisation buffer. The samples were distributed into 50 µL aliquot to the Eppendorf tubes labelled with the appropriate name of the sample and the probe used. The probes used (Eurofins Genomics, UK) are reported in **Table 1** NON EUB338 and EUB338 I-II-III linked at their 5' end either to Alexa488 or Alexa647. Group-specific probes were linked with Alexa647 at their 5' end. 4 µL of each probe and 4 µL of Eub338 I-II-III (linked to Alexa488) were added to the working solution and incubated overnight at 36◦C in a heating block. After 12 h of incubation, an aliquot of 150 µL hybridisation buffer was added to the working solution, vortexed and centrifuged (11,200 g, 3 min). 150 µL of supernatant were removed from each sample and the remaining volume was centrifuged (11,200 g, 3min). The pellets were washed with 200 µL of washing buffer (900 mM NaCl, 20 mM Tris/HCl pH 8, 5 mM EDTA pH 8, ddH2O, 10% SDS), homogenised by vortexing and incubated for 20 min at 38◦C in a heating block. Afterwards the samples were centrifuged (11,200 g, 3 min) and supernatants were removed. Numbers of specific and total bacteria were determined considering dilution factor, calculated from different volumes used in samples preparation steps, and events/µL obtained from NON EUB338 and EUB338 I-II-III probes analysed by FCM (Grimaldi et al., 2017).

Fluorescence in situ hybridization (FISH) is a useful tool for the rapid initial identification of gut bacteria composition. Binding of short fluorescence-labelled oligonucleotide probes with bacterial DNA from the 16s rRNA gene followed by consecutive analysis by fluorescence microscopy or, as in this case, flow cytometry, allows identification and quantification of bacteria at a group level. FISH analysis also provides quick determination and quantification of target gut bacteria group (Frickmann et al., 2017). On the other hand, 16S rRNA gene sequencing results are based on relative percentages rather than accurate quantifications, but give a look at everything present in the sample, rather than just what is looked for, but results might change depending upon the choice of reference database and extraction technique used (Jo et al., 2016). In the FISH procedure, by using a range of probes for functionally important groups of bacteria quantitative data on these bacteria can be generated.

Table 1. Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concentration of 50 ng/µL.

Probe Name	Sequence (5' TO 3')	Target Group	Reference
Non Eub	ACTCCTACGGGAGGCAGC		(Wallner et al., 1993)
Eub338 I +	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus, Enterococcus, Melisococcus, Tetragenococcus, Catellicoccus, Pediococcus* and *Paralactobacillus* spp.	(Harmsen et al., 2002)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1995)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	(Manz et al., 1995)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	(Franks et al., 1998)
Chis150	TTATGCGGTATTAATCTYCCTT T	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	(Franks et al., 1998)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and E*ggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2002)
Prop853	ATT GCG TTA ACT CCG GCAC	*Clostridial* cluster IX	(Walker et al., 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)

DSV687	TAC GGA TTT CAC TCC T	Most *Desulfovibrionales* (excluding *Lawsonia*) and many *Desulfuromonales*	(Purdy et al., 2003)

2.5. SCFA Analysis:

Production of SCFAs was determined using GC as previously described (Richardson et al., 1989). Individual solution standards at 5 mM were prepared for acetate, iso-butyrate, butyrate, propionate, valerate, iso-valerate and lactate. The external standard solution contained acetate (30 mM), iso-butyrate (5 mM), n-butyrate (20 mM), propionate (20 mM), n-valerate (5 mM), iso-valerate (5 mM) and lactate (10 mM). 1 mL of each sample was vortexed and transferred into a flat-bottomed glass tube (100 mm x 16 mm, Fisher Scientific UK Ltd., Loughborough, UK) with 0.5 mL concentrated HCl, 50 µL of 2-ethylbutyric acid (0.1 M internal standard solution, Sigma, Poole, UK) and 2 mL diethyl ether. Samples were taken at SS1 and SS2 time points from the gut model system and were vortexed for 1 min at 1500 rpm and then centrifuged (2000 g, 10 min, 4 C°, SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK). 2 mL of diethyl ether top layer and 50 µL of N- (tert-butyldimethylsi lyl)-N-methyltrifluoroacetamide (MTBSTFA; Sigma-Aldrich, Poole, UK) were added into a GC screw-cap vial. Samples were kept at room temperature for 72 h to enable complete derivatisation prior to GC analysis. A GC Agilent 7890B gas chromatograph (Agilent, Cheshire, UK) using an HP-5 ms (L x I.D. 30 m x 0.25 mm, 0.25 µm film thickness) coating of crosslinked (5%-phenyl)-methylpolysiloxane (Hewlett Packard, UK) was used for SCFA detection. 1 µL of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275 C° and the column temperature programmed from 63 C° to 190 C° and held at 190 C° for 30 min. Helium was the carrier gas (flow rate, 1.7 mL/min, head pressure, 133 KPa). Peaks were integrated using Agilent Chem Station software (Agilent Technologies, Basingstoke, UK), and SCFA content was quantified by single-point internal standard method (Liu et al., 2017). Peak identity and internal response factors were determined using a 20-mM calibration cocktail including acetic, propionic, and butyric acids.

2.6. Neurotransmitter Analysis:

The concentration of neuroactive metabolites were determined by Liquid Chromatography Mass Spectrometry (LCMS) following the method of Zhai et al. (2015) with further modifications. Samples were taken from the gut model system at SS1 and SS2 time points from proximal (V1), transverse (V2) and distal (V3) vessels and were centrifuged at 11,200 g (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) for 3 min and were taken 400 µL from the supernatant and were centrifuged again at 11,200 g for 3 min with filtered centrifuge tubes (Corning Costar Centrifuge Tube Filter Acetate membrane-

0.22 µm). 200 µL of HPLC water (Blank), calibration standard samples and batch culture samples were placed in 96-well plates. The remaining supernatant were stored at -20°C for the future analysis.

2.6.1. Reagents and Chemicals:

HPLC Plus grade acetonitrile (99.9%) was purchased from Sigma-Aldrich (Kent, UK). Formic acid (99% LC/MS grade, HiPerSolv CHROMANORM®) was purchased from VWR. Centrifuge tube filter (Corning ® Costar ® Spin-X ®, 0.22 µm Pore CA Membrane, Sterile, 96/Case, Polypropylene) was purchased from Sigma-Aldrich, which was used to filter batch culture and gut model fluid samples. Analytical standards powder which are LC-MS grade dopamine hydrochloride (99%), and L (-)-Epinephrine (99%) L-Noradrenaline (98%), Gamma-Aminobutyric acid (99%) were purchased from Alfa Aesar (Lancashire, UK).

2.6.2. Stock Solutions, Calibration Standards and Sample Preparation:

Separate standard stock solutions (10000 ng/mL) of 5 analytes, including Serotonin (5-HT), Dopamine (DA), Gamma-Aminobutyric acid (GABA), Norepinephrine (NE) and Epinephrine (EPI) were individually prepared in HPLC water. A 1000 ng/mL mixed standard solution containing the 5 analytes was made by acquiring aliquots of each separate stock solution. The mixed standard solution was appropriately diluted with HPLC water to prepare a calibration series. A calibration series of spiked standard samples was prepared including 9 levels: 1, 10, 50, 100, 250, 500, 750, 1000 and 2500 ng/mL.

2.6.3. Liquid Chromatography (LC) Conditions and Mass Spectrometry (MS) Conditions:

The chromatographic separation was performed on an Agilent C18 column (250 × 4.6 mm, 5 µm; Agilent Co. Ltd, USA) with a protected C18 column cartridge (4.6 × 12.5 mm cartridge, 5 µm; Agilent Company, USA). The column temperature was kept at 25°C. The injection volume was 10 µL. The mobile phase consisted of A (0.1% formic acid + HPLC water) and B (0.1% formic acid + Acetonitrile (ACN)) with gradient elution as follows: 0–2 min, 100%; 2-5 min, 75% A; 11–15 min, 65% A; 15-20 min, 5% A and then in 0.1 min returned to initial 100% A. This was followed by the equilibration period of 5 min prior to the injection of each sample. The flow rate was set at 0.25 mL/min.

The LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using the polarity-switching electrospray ionisation (ESI) mode. The optimal conditions were as follows: dry gas temperature was 300 °C, dry gas flow rate of 10.0 L/min. 4 µL samples were injected. Samples were measured as the target compounds based on MRM. For the analysis of primary

metabolites 5-HT, DA, GABA, NE and EPI, LC/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used.

2.6.4. Quantification of Samples:

A linear calibration curve was generated based on the detected signal proportional to the concentration of the analyte. Good linearity with R^2 greater than 0.98 was obtained across the set calibration in the range from 1 ng/mL to 1000 ng/mL for each of the analytes, with accuracy within 100% ± 20%. Quantification of samples was determined by calibration with 5 analytes including 5-HT, DA, EPI, GABA and NE. Samples were measured using online Nexera LC System coupled to LCMS-8050 triple quadrupole (QQQ) mass spectrometry (Shimadzu Corporation, Kyoto, Japan). Data were processed using LabSolutions LCMS version 5.65 software.

2.7. Statistical analysis

Data from LC-MS, GC and FMC-FISH were analysed using SPSS (SPSS Statistics version 25). Student paired t-test was used to compare the two time points of each substrate SS1 and SS2, before and after treatment and to compare substrate effects. Results were considered significant when they possessed a P value lower than 0.05.

3. RESULTS

3.1. Bacterial enumeration:

Changes in bacterial composition following gut model fermentation are reported in **Figure 2.** Significant increases in *Bifidobacterium* spp. following administration of inulin were observed in V1 and V3 and of FB in the V1. At SS2, the growth of *Bifidobacterium* spp. was significantly higher upon the fermentation of inulin in V1 and V3 compared to FB in V1 and V3 ($P<0.01$). Moreover, in SS2, the growth if *Bifidobacterium* spp. was significantly higher in V1 compared to V3 in the FB model ($P<0.01$). Significant increases in the growth of *Lactobacillus* spp. were observed following fermentation of both inulin ($P<0.01$) and the blueberries in V1 ($P < 0.05$). The growth of *Lactobacillus* spp. increased in the other vessels, but this was not significant. Additionally, at SS2, the growth of *Lactobacillus* spp. was significantly higher in V1 in the FB model compared to the inulin model in V1 ($P<0.05$). There was an increase in the *Roseburia* spp. following fermentation of inulin in V3 ($P<0.01$) and a significant increase was observed in FB model in V1 ($P<0.05$). Additionally, at SS2, the growth of *Roseburia* spp. was significantly higher in the proximal region (V1, pH 5.5) compared to the distal region (V3, pH 6.8) in

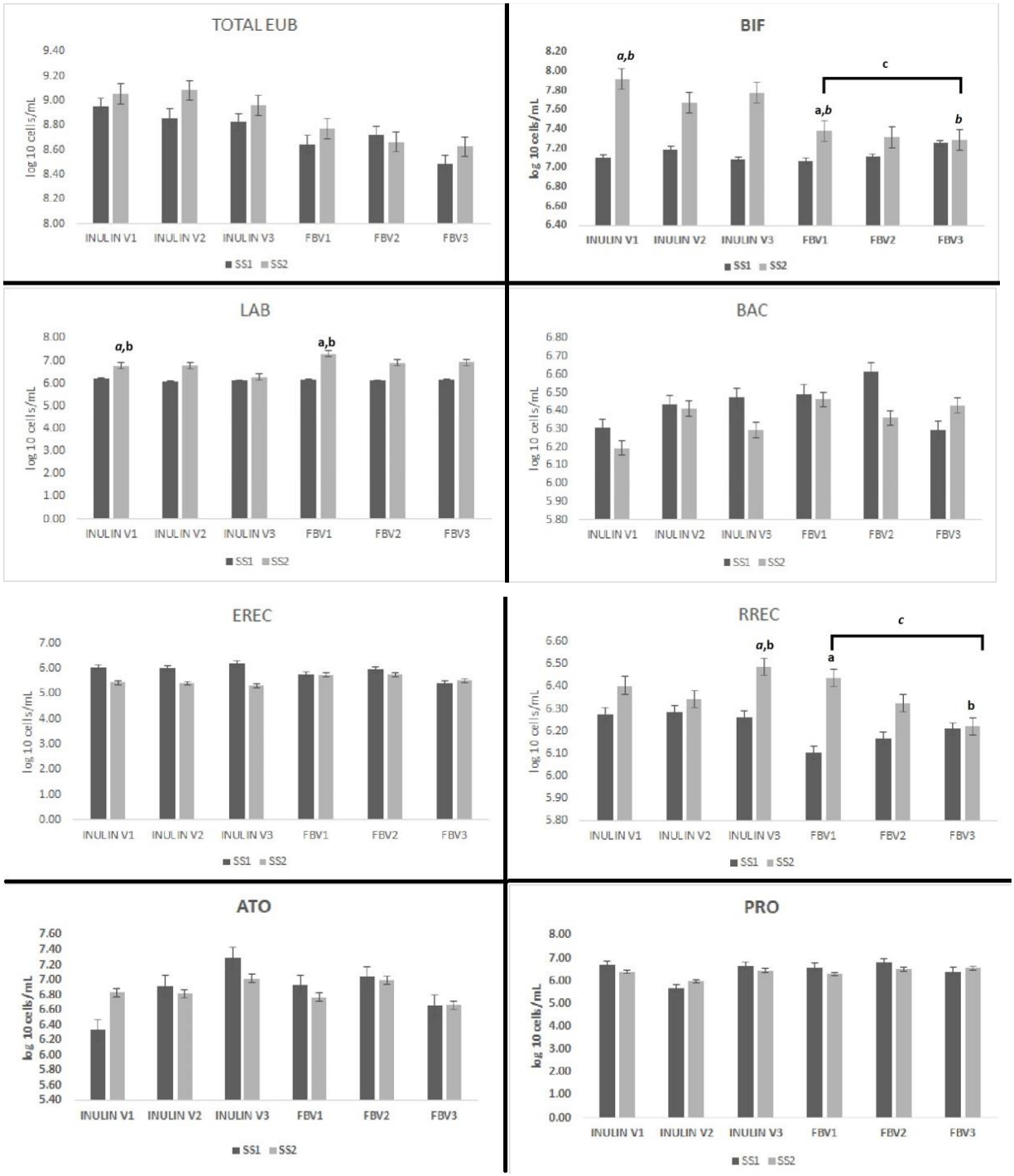
the FB model whilst the growth was significantly higher in V3 in the inulin model compared to the FB model in V3 ($P<0.05$). Moreover, there were significant decreases in the growth of *Desulfovibrionales* spp. and the *Clostridium histolyticum* group following the fermentation of inulin in V1 ($P<0.005$).

3.2. Short Chain Fatty Acids (SCFA):

SCFA concentrations are illustrated in **Figure 3.** Significant increases were observed in acetate after the administration (SS1-SS2) of inulin in all vessels (V1, V2, and V3) ($P<0.01$) whereas no significant differences were observed after the administration of FB. Supplementation of inulin led to significant increases in butyrate concentration in the vessels modelling proximal (V1), transverse (V2) and distal (V3) regions, whilst FB led to a significant increase in butyrate concentration in the vessels modelling distal (V3) colonic region ($P<0.05$). There were significant increases in propionate concentration following the fermentation of FB in the proximal (V1) and distal regions (V3) ($P<0.05$) while no significant effect was seen upon the fermentation of inulin. Acetate and butyrate were the main end products after the administration of both inulin and the blueberries. Additionally, acetate and butyrate production were significantly higher upon the fermentation of inulin compared to FB in the proximal region (V1) and distal region (V3) respectively. However, no significant differences were observed among the vessels (V1, V2, and V3) within each model (Inulin and FB).

3.3. Neurotransmitter Concentration:

Neurotransmitter concentrations are shown in **Figure 4.** Significant increases were observed in both GABA production following the fermentation of inulin between SS1 and SS2 time points in the proximal (V1) and the transverse regions (V2) ($p<0.05$). Furthermore, the production of GABA was significantly higher in inulin model compared to FB model in the distal region (V3) ($P<0.01$). The amount of GABA was significantly higher in the proximal region (V1, pH 5.5) and in transverse region (V2, pH 6.2) compared to distal region (V3, pH 6.8). This was also the case for FB model. However, no significant differences were observed in GABA production between proximal (V1, pH 5.5) and transverse (V2, pH 6.2) regions, the production of GABA was found significantly higher in proximal region compared to distal region (V3, pH 6.8) upon the fermentation of FB ($P<0.05$). A significant increase in serotonin production was observed following inulin fermentation in the proximal regions (V1) ($P<0.01$) and transverse regions (V2) ($P<0.01$). Moreover, the production of serotonin was found significantly higher in the inulin model in the V1 and V2 compared to FB model in the V1 and V2 ($P<0.05$).


TOTAL EUB
log 10 cells/mL
9.40
9.20
9.00
8.80
8.60
8.40
8.20
8.00
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
BIF
log 10 cells/mL
8.20
8.00
7.80
7.60
7.40
7.20
7.00
6.80
6.60
6.40
a,b
c
a,b
b
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
LAB
log 10 cells/mL
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a,b
a,b
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
BAC
log 10 cells/mL
6.80
6.70
6.60
6.50
6.40
6.30
6.20
6.10
6.00
5.90
5.80
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
EREC
log 10 cells/mL
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
RREC
log 10 cells/mL
6.60
6.50
6.40
6.30
6.20
6.10
6.00
5.90
5.80
a,b
c
a
b
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
ATO
log 10 cells/mL
7.60
7.40
7.20
7.00
6.80
6.60
6.40
6.20
6.00
5.80
5.60
5.40
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2
PRO
log 10 cells/mL
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
INULIN V1
INULIN V2
INULIN V3
FBV1
FBV2
FBV3
SS1
SS2

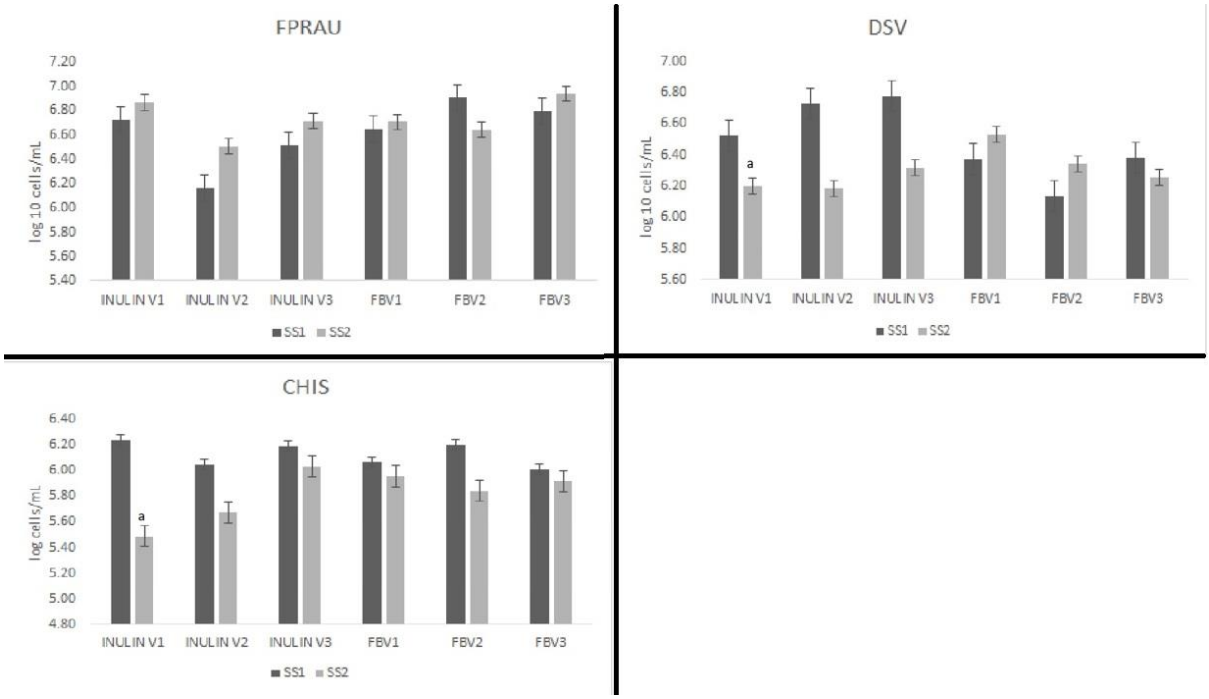


Figure 2. Bacterial groups detected by FISH-FCM (Log10 cells/ml) in gut model media enumerated from each vessel (V1, V2 and V3) of a colonic model before (SS1) and after (SS2) the daily administration of inulin (0.6 g/d) and of FB (1 g/d). Significant difference after the treatment: [a]P<0.05; [a]P<0.01. Significant difference between substrates after treatment:[b] P<0.05; [b]P<0.01. Significant difference among vessel within substrate in SS2: [c] P<0.05; [c] P<0.01. Probes: total bacteria (Eub338I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most Bacteroidaceae and Prevotellaceae (Bac303), *Clostridium coccoides–Eubacterium rectale* group (Erec482), *Roseburia* subcluster (Rrec584), *Atopobium* spp. (Ato291), Clostridial cluster IX (Prop 853), *F. prausnitzii* (Fprau655), Most *Desulfovibrionales* (DSV 687), Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II) (Chis 150). (Mean of the data of three experiments and standard deviations: n 3)

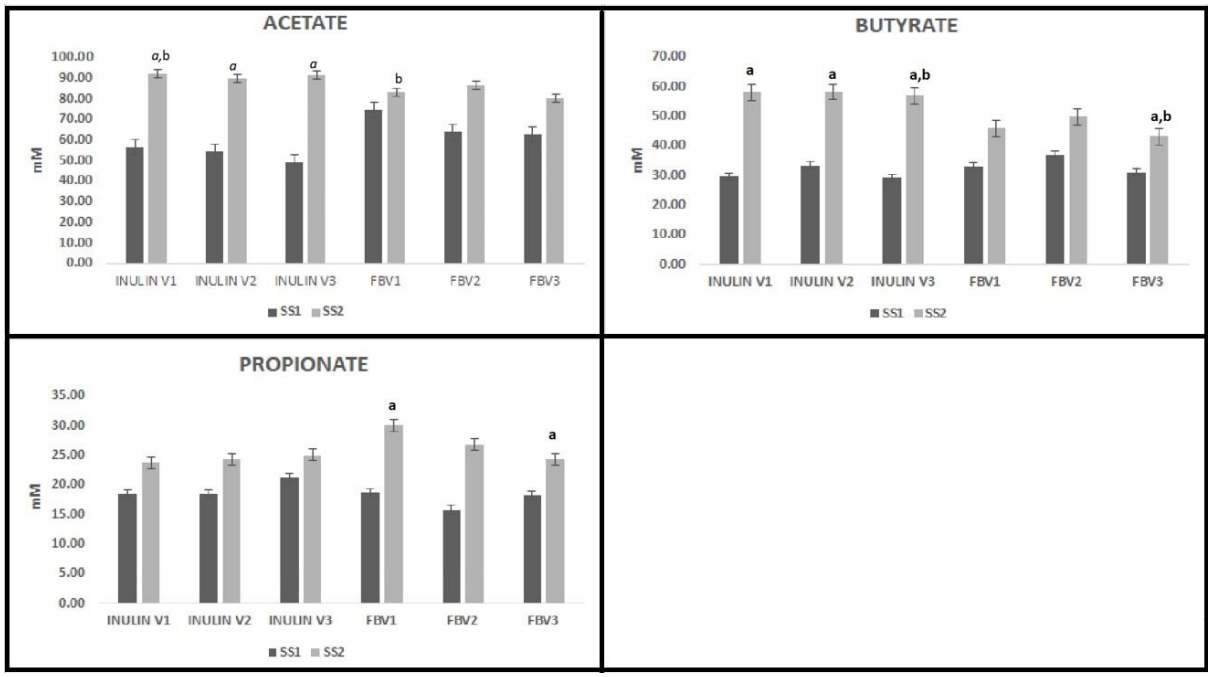


Figure 3. GC analysis. Acetate, propionate, and butyrate concentrations in gut model media from vessels (V1, V2 and V3) of in vitro gut model systems before (SS1) and after (SS2) administration of inulin (INU, 0.6 g/d) and freeze-dried blueberries (FB, 1 g/d). Results are reported as means (mM) of the data (n = 3): healthy children. Significant difference after the treatment: [a] P < 0.05; [a] P < 0.01. Significant difference between substrates after treatment: [b] P < 0.05; [b] P < 0.01. Significant difference among vessel within substrate in SS2: [c] P<0.05; [c] P<0.01.

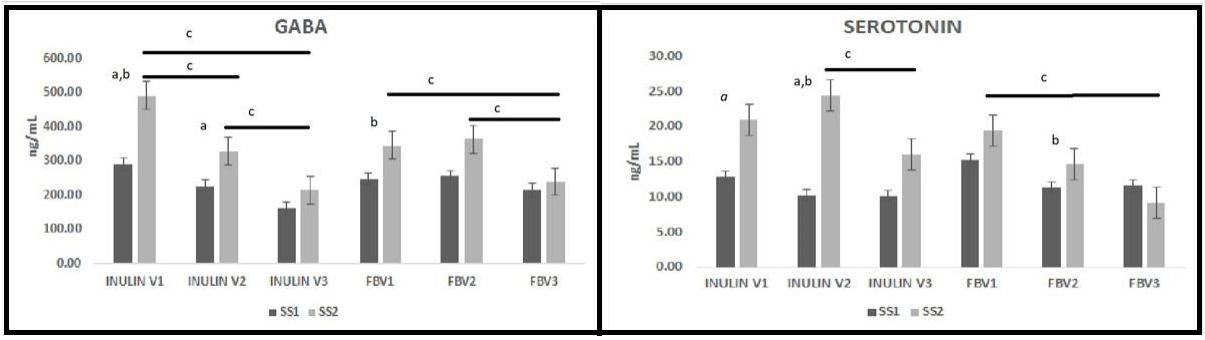


Figure 4. GABA and serotonin concentrations determined by LC-MS in gut model media from vessels (V1, V2 and V3) of in vitro gut model systems before (SS1) and after (SS2) administration of inulin (INU, 0.6 g/d) and freeze-dried blueberries (FB, 1 g/d). Results are reported as means (mM) of the data (n = 3): healthy children. Significant difference after the treatment: [a] P < 0.05; [a] P < 0.01. Significant difference between substrates after treatment: [b] P < 0.05; [b] P < 0.01. Significant difference among vessel within substrate in SS2: [c] P<0.05; [c] P<0.01.

4. DISCUSSION:

The current study was conducted to explore the impact of inulin and blueberries on the faecal microbiota, metabolites and neurotransmitter levels in an *in vitro* gut model system inoculated with faeces from children. This experiment was designed to investigate whether these substrates promoted the growth of the beneficial gut bacteria and the production of SCFAs and neurotransmitters related with the cognitive function. Such research is important for determining possible psychobiotics, foods that impact on the brain via the gut.

In the current study fermentation of blueberries were observed to lead to increases in the levels of *Lactobacillus* and *Bifidobacterium*. This matches well with findings of other researchers who found that berry products could enhance *Lactobacillus* (Andrade et al., 2020) and *Bifidobacterium* (Guglielmetti et al., 2013; Vendrame et al., 2011). Furthermore, another study that investigated flavan-3-ols, a type of polyphenol, reported increased growth of *Lactobacillus* spp. in a similar mixed culture experiment (Cueva et al., 2013). This was also the case for inulin. As such, significant increases were found in *Lactobacillus* and *Bifidobacterium* upon the fermentation of inulin. This is in accordance with other studies who found that the administration of inulin could promote the beneficial gut bacteria including *Bifidobacterium* spp. and *Lactobacillus* spp. (Gibson et al., 2017). On the other hand, the growth of *Bifidobacterium* was significantly higher in the inulin model (V1 and V3) compared to FB model (V1 and V3). Indicating inulin is used more effectively by *Bifidobacterium* than FB. *Bifidobacterium* are major acetate producers among the gut bacteria and in accordance with this, the amount of acetate was found significantly higher in inulin added vessel (V1) compared to FB added vessel (V1). One point to be considered is that fermentation of both FB and inulin led to a significant increase in *Lactobacillus* spp. whilst the growth of *Lactobacillus* was significantly higher in the FB added vessel (V1) compared to the inulin added vessel (V1). This effect happened within V1 (pH 5.5), which could be due to *Lactobacillus* spp. having resistance to the acidic environment of the proximal colon (pH 5.5) which provides an opportunity to grow (Prasad J. et al., 1999). Furthermore, the growth of *Bifidobacterium* spp. and *Roseburia* spp. were significantly higher in the proximal region of the colon (V1) than in the distal region of the colon (V3) in the FB model. As such, the proximal region of the colon (and the model (V1)) has greater nutrient availability, therefore, the proximal colon might have been more optimal in this region for the growth of this genus in the presence of blueberries as a substrate. Upon fermentation of inulin, there was a significant decrease in the bacteria in the *C. histolyticum* group, these is often proteolytic bacterium, that can be associated with negative effects,

including tumour promoting properties and inflammatory bowel disease (Rinninella, Raoul, et al., 2019). As such, the Clostridial group are associated with ASD diseases (De Sande et al., 2014).

The effects of inulin on this microbial group have been observed before (Perillo et al., 2020). Furthermore, in several batch-culture fermentation studies, a significant decrease was observed in *C. histolyticum* group following the fermentation of grape fibre and wine extracts (Sánchez-Patán et al., 2012). Upon fermentation of FB, there was no significant decrease in the *C. histolyticum*, although decrease trend was observed. As such, it is possible that with a higher dose a reduction could be observed. Overall, by increasing potentially positive bacteria (*Bifidobacterium* and *Lactobacillus*), whilst decreasing potentially negative organisms (*C. histolyticum* group) inulin fermentation led to a potentially positive microbiota shift, as would be expected from a prebiotic. As a promising finding in this current study is that the berries had a similar effect with regards to *Lactobacillus, Roseburia* and *Bifidobacterium*. A further observation from the current research was a significant increase of *Roseburia* spp., the butyrate producing bacteria, following the fermentation of inulin and FB. As such, *Roseburia* are likely to have led to increased butyrate production. In parallel with that, there were significant increases in butyrate production both in the FB and inulin-models, with significant increases in the growth of *Roseburia* spp*.* On the other hand, butyrate was significantly higher in V3 in the FB-model whilst *Roseburia* spp. was significantly higher in V1 vessel in both FB and inulin-model. *Roseburia* is a likely butyrate producer, so could explain an increase in this SCFA, furthemore, non-significant increases in *F. prausnitzii* could be contributing to butyrate production in the FB model, vessel 3 (Hao et al., 2019). Moreover, propionate production was also significantly higher in V3 (pH 6.8) in FB-model, although as the producing bacteria are not apparent this change could be due to another strain of bacteria which have not been investigated in this study, for example, *Akkermansia muciniphila. A. muciniphila* is immobile, strictly anaerobic Gram-negative species that can use mucin in the intestine as energy sources for growth. The main metabolites of *A. muciniphila* are SCFAs including butyric acid and propionic acid and this bacterium is considered as potential probiotic (Li et al., 2021). *A. muciniphilia* growth has been seen to grow on polyphenols before. The levels of propionate were significantly greater in the more distal regions, as such, higher colonic pH might have a positive impact on *Akkermansia* abundance and this might help promoting the production of propionate and butyrate production within this region (Van Herreweghen et al., 2018). A study conducted with mice on a high fat diet showed that grape polyphenols (at 1%) significantly increased the *Akkermansia muciniphila*. As such, significant improvements were also observed in the gene expression of the inflammatory markers and the gut barrier integrity along with *A. muciniphila* (Roopchand et al., 2015). These enhanced levels of SCFA upon fermentation of both substrates are of

great interest due to the involvement of these molecules in GBA. For example, through the catecholaminergic system (Shah et al., 2006).

In-vitro studies showed that GABA is produced by many *Lactobacillus* and *Bifidobacterium* strains; GAD genes are found in the gut microbiome and GABA-producing probiotic strains have significant effects on cognitive function (Yunes et al., 2016). According to our results, GABA production is significantly increased upon the fermentation of inulin, which also corresponded with significant increased growth of *Bifidobacterium* spp. and *Lactobacillus* spp. This is a possible explanation that the inulin fermentation leads to the growth of the GABA-producing bacteria which is also appropriate with the findings of significant increased growth of *Bifidobacterium* spp. and *Lactobacillus* spp. upon the fermentation of inulin specifically in V1. These findings might be associated with positive impacts on cognition (Strandwitz et al., 2019). Significant increases in GABA production following the fermentation of inulin only occurred in V1 and V2 where the pH is 5.5 and 6.2 respectively and lower than the distal region of the colon (pH 6.8). Moreover, GABA production was found significantly higher in V1 (pH 5.5) compared to V2 (pH 6.2) and V3 (pH 6.8) upon the fermentation of inulin. GABA production also occured in the distal colon (V3) (pH 6.8), but the increase was not significant both in inulin and FB model. One of the possible reasons for not observing any significant increase in GABA production in V3 could be due to lower nutrients and prebiotic substrates decreasing by the time they reach the distal part of the colon. The other reason, is that GABA production is associated with an acid-resistance mechanism by the bacteria through their GAD genes (Feehily & Karatzas, 2013), thus the more neutral pH is not the optimal environment for GABA production. However, the results indicate that inulin fermentation resulted in enhanced GABA levels, showing a clear link of inulin to GBA.

Around 95% of body's serotonin (5-HT) is mainly synthesised in the enterochromaffin cells (EC) in association with the gut microbiota and through the rate-limiting enzyme tryptophan hydroxylase (TPH). SCFAs are stated to promote the release of 5-HT from the EC (Mayer et al., 2015; Reigstad et al., 2015). However, serotonin has increased significantly upon the fermentation of inulin in the V1 and V2, the amounts were not physiologically relevant (20-50 n/mL). Current studies suggest that the gut bacteria modulate the serotonin production, but host cells are required to produce serotonin to reach the relevant levels found in the blood (100-300 ng/mL) (O'Mahony et al., 2015; Scarpa et al., 2013). Nevertheless, many previous authors have reported that the microbiota can produce serotonin, which is as found here, although, under these physiological conditions the concentrations are unlikely to be sufficient for effects on the host.

In conclusion, the dietary intervention with a well-known prebiotic, inulin promoted positive modulation of the gut microbiota, whilst also increasing the production of neuro active metabolites which have positive impacts on cognitive functions (Smith et al., 2015). Freeze-dried blueberries, a great source of anthocyanins still possess certain potential prebiotic properties through positive effects on *Bifidobacterium* and *Lactobacillus* and the butyrate production (Gibson et al., 2004; Guglielmetti et al., 2013). As such, despite the conflicting results, freeze-dried blueberries still might be considered as a potential prebiotic and further *in-vitro* and *in-vivo* analysis is warranted. Moreover, it is well-known positive cognitive effects might also be related to its prebiotic properties as well as its widely known antioxidant effects. On the other hand, the applicability of such impacts remains to be estimated in *in vivo* human dietary intervention studies.

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CHAPTER 5

A Pilot Study Assessing Chronic Effects of Inulin and Freeze-Dried Blueberry on Cognitive Function and Gut Microbiota Composition in Healthy Children

Abstract:

Flavonoids and prebiotic supplements are widely known to have benefits for cognitive function especially in childhood. In this study, effects of a wild blueberry treatment (WBB) made from freeze-dried blueberry powder containing 264 mg anthocyanins (equivalent to 178 g of fresh weight blueberries, approximately 2 fresh weight portions) and enriched chicory inulin powder product (5 g) were used as substrates versus a matched placebo (5 g of maltodextrin, Beneo Company, UK) (0 mg anthocyanins and 0 mg prebiotic components), used daily for 4 weeks were investigated. The study design was a double-blind, 3-armed, parallel randomised controlled trials (RCT) with a study population consisting of 13 healthy children aged between 7-10 years old. Cognitive function (primary outcome) and microbiota analyses (secondary outcome) were examined at baseline, 4 weeks post-intervention and after a 4 week wash out. To analyse the gut microbiota diversity, faecal samples were taken to assess any composition and quantity changes following treatments WBB, inulin or control. To assess the cognitive function, a cognitive battery was used comprising of 3 tasks measuring different domains of cognitive function. The tasks included Rey Auditory Verbal Learning Test (RAVLT) to measure immediate and delayed episodic memory, Serial subtraction tasks (Serial 3s) to measure working memory, and Modified Attention Network Task (MANT) to measure executive function. In order to perform the data analysis, Linear Mixed Modelling (LMM) analysis were performed.

LMM data revealed that accuracy scores on the modified attention network task (MANT), a measure of executive function, had significant improvements for the WBB and inulin group compared to placebo post consumption. Both inulin and WBB groups maintained significantly higher accuracy on congruent trials compared with placebo participants whilst no significant differences were seen on the harder incongruent trials. Moreover, significant changes were observed for the reaction times (RTs) scores on MANT with quicker RTs were seen on the inulin and WBB groups compared with placebo. No significant inulin and WBB-related differences were observed in Serial 3s subtraction task whereas significant enhancements were observed on the Rey's Auditory Verbal Learning Task (RAVLT), following both inulin and WBB consumption, on the acquisition and delayed recall aspects of the memory task ($p \leq 0.05$). Significant increases were seen in *Faecalibacterium prausnitzii* known as butyrate producer in the inulin group and a trend for an increase was obtained in the WBB group compared to placebo ($p \leq 0.05$) whilst *Blautia* spp. significantly decreased post consumption in the inulin group ($p \leq 0.05$). Significant alterations were also obtained in the phylum level. As such, Bacteroidetes significantly increased whilst Firmicutes significantly decreased post consumption in the WBB group ($p \leq 0.05$).

This study highlights the importance of a dietary intervention studies in order to investigate the potential prebiotic effects and the possible cognitive outcomes of these effects. However, further research with higher numbers of participants is necessary to assess the potential prebiotic effects of inulin and WBB and possible related outcomes of other cognitive domains in children.

Key Words: Gut microbiota, Prebiotic, Inulin, Flavonoid, Anthocyanin, Cognition, Memory, Executive function

1. INTRODUCTION

The gut microbiota of heathy individuals differs throughout the gastrointestinal tract and is influenced by several factors including age, dietary habits, lifestyle, and antibiotic consumption. However, significant changes in gut microbiota composition can be related to diseases (Rinninella, Raoul, et al., 2019). In the human intestinal system, thousands of species of gut bacteria have been identified, these belong to six dominant phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Bacteria from the phyla Firmicutes and Bacteroidetes are the most common, representing 90% of the gut microbiota (Stojanov et al., 2020). The ratio between Firmicutes and Bacteroidetes (F/B) has been associated with maintaining homeostasis (Stojanov et al., 2020). Increased or decreased F/B ratio is associated with dysbiosis, where the increased ratio of F/B is associated with obesity whilst the decreased ratio of F/B is associated with inflammatory bowel disease (IBD). Prebiotics and potential prebiotics such as flavonoids are considered to balance the F/B ratio (Morgan et al., 2012). Furthermore, the phylum Firmicutes includes Gram-positive bacteria from the genera *Bacillus, Clostridium, Enterococcus, Lactobacillus,* and *Ruminicoccus* whereas the phylum Bacteroidetes includes different species of Gram-negative bacteria from the genera *Bacteroides, Alistipes, Parabacteroides,* and *Prevotella* in majority (Rinninella, Cintoni, et al., 2019).

The intestinal tract also acts as an endocrine organ through direct and indirect production of microbial metabolites and neurometabolites such as short chain fatty acids (SCFAs), vitamins and neurotransmitters. These microbial end products have been observed to influence gut/brain interactions and have been shown to impact on several cognitive disorders (Oriach et al., 2016). Cognitive disorders including hyperactivity disorder (ADHD), autism spectrum disorder (ASD), depressive disorder, Alzheimer disease are commonly associated with dysbiosis of the gut microbiota (Sarkar et al., 2018). In particular, deficits in executive function (EF) are apparent, EF defines cognitive processes such as working memory, planning, problem-solving, cognitive flexibility, inhibitory control, and the ability to direct attention and thoughts (Whyte et al., 2017). Frontal areas of the brain are where EF function is derived, develops, and matures during childhood and into early adulthood (Goddings et al., 2021). Any disorders in the frontal region during development could have an impact long-term, therefore it is critical to prevent these cognitive diseases in the early years of life (Best & Miller, 2010). Cognitive disorders that affect young children, could potentially be treated and/or symptoms attenuated through psychoeducation, parent training, child behavioural interventions, and pharmaceuticals. Interestingly a number of studies have reported that certain symptoms and risk factors of cognitive disorders could be influenced by changes to the diet (Pelsser et al., 2017).

Studies using *in vitro* and *in vivo* techniques have been used to explore the role of the microbiota in cognitive illness. Gut modifying dietary intervention foods include prebiotics which are oligosaccharides e.g., inulin and its fructooligosaccharide (FOS) derivatives (Grimaldi et al., 2016). To date, inulin and its FOS derivatives have been related to the ability to improve selectively the growth of *Bifidobacterium* and accordingly modulate the gut microbiota composition, which may confer health benefits to the host (Rycroft et al., 2001). A range of studies showed that diets comprising prebiotics can promote diversification of the microbiota and stimulate the production of SCFAs. The gut microbiota might improve the cognitive function by acting as an endocrine organ through direct and indirect production of microbial metabolites and neurometabolites such as SCFAs and neurotransmitters which have been reported to impact on gut/brain interactions (Oriach et al., 2016). To date there is growing evidence to support the hypothesis that modulating the gut microbiota could positively impact on cognition (Sarkar et al., 2018).

Flavonoids are polyphenolic compounds found in many plant-based foods. There are several dietary intervention studies focusing on the subclass of flavonoids and evidence suggest that flavonoids might be beneficial to several cognitive domains including attention, working memory, and executive function (Bell et al., 2015). Berries contain a wide range of different flavonoid subclasses, but they are especially rich in anthocyanins. The impact of berries on cognitive performance has been investigated by administering a number of interventions including freeze-dried blueberries and fresh whole blueberries (Rodriguez-Daza et al., 2020). Indeed, single-dose freeze-dried blueberry interventions have been observed to have a positive impact on attention, inhibition, visuospatial memory, and executive function between 2-6 h post-consumption (Whyte & Williams, 2015). On the other hand, administration of berry fruits (rich in flavonoids) for 1.5-8 weeks has been linked with improved visuospatial memory and improved long-term memory (Miller & Shukitt-Hale, 2012; Rendeiro et al., 2015). Several different mechanisms of action have been proposed to explain the positive effects of flavonoids on cognition including increases in cerebral blood flow, protecting against neuronal stress via anti-inflammatory and anti-oxidative effects, and positively stimulating neural signalling pathways, such as Extracellular Signal-Regulated Kinase (ERK), Serine/Threonine-specific Protein Kinase (Akt) and Brain-Derived Neurotrophic Factor (BDNF), leading to improved neural signalling (Khalid et al., 2017; Miller & Shukitt-Hale, 2012). Further to this, as flavonoids have been observed to impact on the gut bacteria, a link to the gut microbiota and possible impact on cognitive performance is plausible.

The aim of this study was to investigate the prebiotic potential and cognitive and behavioural effects of chronic supplementation of a blueberry preparation rich in flavonoids and of inulin in healthy children. An age group of 7-10 years was chosen, as this is at a stage of development where there is a significant growth in the frontal lobes of the brain. This process corresponds to a period in the field

that contributes to executive function, including cognitive flexibility, goal setting and information processing (Khalid et al., 2017).

2. MATERIALS and METHODS

The research was reviewed and given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (UREC 15/10, UREC 15/58) and was conducted in accordance with the Declaration of Helsinki and Human Tissue Act 2004.

2.1. Participants:

15 participants aged 7–10 years (M = 8.54 years, SD 1.27) were recruited from local primary schools in the Berkshire area, UK. Two participants withdrew from the study (reason: preferred not to continue) making the total participant number 13 (6 female). At the beginning of this study, it was suggested that 60 participants should be recruited for this study. This number was based on obtaining 20 healthy children for each condition group (Placebo, Inulin, and WBB). A power calculation (Hedwig, Harvard) indicated that based on words learnt in **Rey's Auditory Verbal Learning Task** a total of 20 participants would provide an 80 percent probability that the study would detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments was 1.5 words, based on the standard deviation of, 1.67 words as observed by Barfoot et al., (2020). However, due to COVID-19, this study ended up only 13 participants in total (Inulin group=5, WBB group=4, Placebo group=4). Power analysis with this smaller cohort revealed that 13 participants would result in an 18% chance of seeing a significant difference in number of words learnt based on the same parameters reported above. This should be kept in mind when interpreting results. However, this study was performed as a pilot study to provide information to guide on the potential of prebiotic and flavonoid intervention to impact on cognitive function via the microbiota. Written consent was obtained from parents or legal guardians in advance of the child's participation. On initial recruitment, parents or legal guardians confirmed that the children spoke English as a first language, had not been diagnosed with ADHD or any other cognitive diseases, and had no known fruit or fruit juice intolerance. Participants were asked to maintain their normal diet during the course of the study. Food diary records were kept for 3 consecutive days to ensure volunteers diets did not vary during the study (Appendix 4). Demographic details of the participants are shown in Table 1. No participant had any known health conditions e.g., diabetes, obesity, high blood pressure, thyroid, kidney or liver diseases, or psychological diagnoses e.g., ASD. Participants had not taken any antibiotics for 6 months before

the study. Participants were also advised not to take any flavonoid supplements, prebiotics or probiotics 24 hours before each testing sessions.

Table 1. Demographic data for Inulin, WBB and Placebo participants

	Inulin (n=5)			WBB (n=4)			Placebo (n=4)		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Age	9.20	1.30	7-10	8.00	1.41	7-10	8.50	1.29	7-10
BAS 3[a]	50.40	2.41	48-53	49.75	1.71	48-52	49.25	0.50	49-50
Gender (M: F)	3:2	-	-	3:1	-	-	1:3	-	-

[a]British Ability Scale 3; measured against normal data (Swinson, 2013).

2.2. Supplements:

The freeze-dried blueberry powder product, supplied by Wild Blueberry Association of North America. Anthocyanin content of the blueberry powder was analysed as detailed in Rodriguez-Mateos et al. (2012). Analyses were conducted by RSSL, Reading, UK (Table 2). Oligofructose-enriched inulin named as Orafti® Synergy 1 (Table 3) and maltodextrin (Table 4) powder products, supplied by Beneo-ORAFTI Ltd.

Table 2. Characteristics of Freeze-Dried Blueberry Product

Compound	Composition
Total polyphenols	2900 mg/100g
Anthocyanins	1900 mg/100g
Procyanidins	Not quantified
Vitamin C	335 mg/100g
Total sugars	70 g/100g
Fructose	36 g/100g
Glucose	34 g/100g
Dietary fibre	16 g/100g
Insoluble	Not quantified
Soluble	Not quantified

Table 3. Characteristics of Inulin Powder Product

Standard	**Content**	**Sweatness Level**	**Desciption**
Orafti®Synergy1	~92%	N/A	Dedicated combination of longer and shorter chain Inulin to achieve specific physiological effects

Table 4. Nutritional Values of Maltodextrin Powder Product (in 100 g of product)

Substance	Content (in 100 g)
Energy	1620 kJ
Fat	0 g
thereof saturated fatty acids	0 g
Carbohydrates	95 g
thereof sugars	9 g
Dietary fibers	0 g
Protein	0 g
Salt	<0.1 g
Bread Units	<Approx 7.9 g

2.3. Treatment and Study Design:

Participants were randomly assigned to receive either WBB (N=4), inulin (N=5), or a matched placebo (N=4) according to a randomised, double-blind, parallel-groups design over 4 weeks (chronic period). The randomisation was performed through an online tool named sealedenvelope.com by creating a simple randomisation list. All interventions were weighed, packaged (sachet), and stored at – 18 °C by a researcher in the University of Reading School of Psychology and Clinical Language Science Nutritional Psychology lab. In this pilot study, children received a daily dose of either a 13.3 g WBB drink (253 mg anthocyanins; equivalent to 240 g fresh blueberries) or a prebiotic supplement (5 g of oligofructose-enriched chicory inulin product, BENEO Orafti®Synergy1) or a placebo-matched control (5 g of maltodextrin) administered in 170 mL of water and 30 mL of a flavonoid-free orange squash concentrate (Rocks Orange Squash, Rocks Drinks Limited, UK). A double-blind design was performed by giving codes representing each treatment of each sachet by the researcher's supervisors. The powders of each substrate were placed inside jiffy bags. The researcher only saw the codes (A, B and C) on these jiffy bags comprising the sachets inside. The participants' parents were distributed to participants as 28 daily sachets (inside jiffy bags), alongside a 500 mL bottle of Rock's Orange Squash, there was a 30 mL measurer, an opaque shaker flask with preparation instructions. The drinks were prepared by the participants' parents. The participants were only allowed to see the drinks in the opaque shaker cups when the drinks were ready to consume to maintain participant blinding procedures.

Participants were asked to perform cognitive tests and to collect a stool sample at the beginning of the study (baseline period), after 4 weeks (chronic period). After the chronic period (post

consumption) participants were informed not to use any supplements for 4 weeks and were asked to perform the cognitive tests again and to collect a stool sample after these 4 weeks. Measures of cognition were the primary outcomes and were recorded at baseline, 4 weeks (28 days) for chronic and 8 weeks (56 days) following the wash-out period. Measures of gut microbiota composition were the secondary outcome and were taken at baseline, 4 weeks and 8 weeks and the samples were collected each testing sessions (baseline, chronic, wash-out) (See Figure 1). Testing took place outside of school hours in to minimise organisational demands and to maximise cognitive demand on participants. All chronic interventions were prepared daily at the participants' house by the participants' parent(s) or guardian(s). To measure compliance parent(s) or guardian(s) were required to fill food diaries and drink consumption logs (Appendix 4) stating the daily time of drink consumption. After 4-week session (chronic session), completed food diaries and drink consumption logs were retrieved from parents.

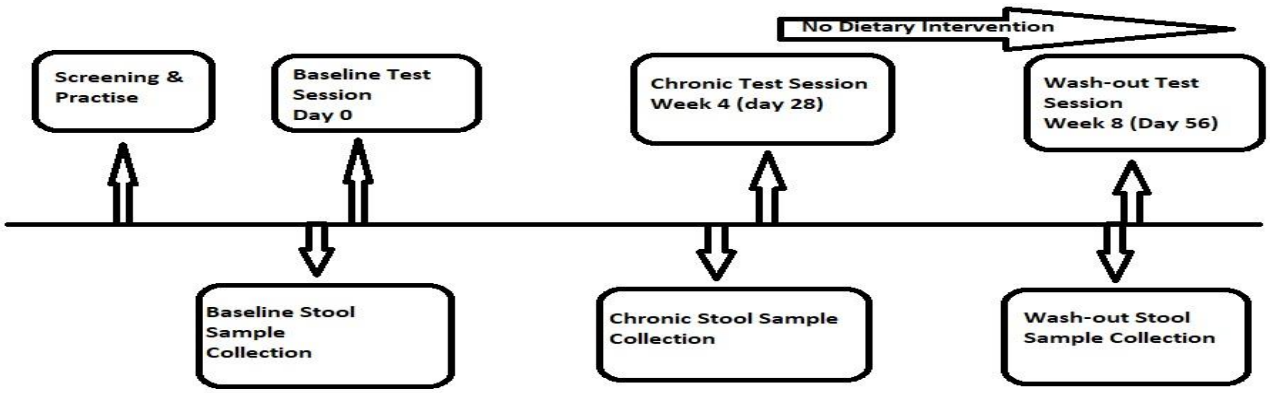

Figure. 1 Study design comprising of cognitive and stool data collection at baseline, chronic (4 weeks) and wash-out (8 weeks)

2.4. Cognitive Tests:

Cognitive tests formed the primary outcome of this trial. Verbal memory (assessed using Rey's Auditory Verbal Learning Task; RAVLT), working memory (measured using the Serial 3s task) and executive function (assessed using the Modified Attention Network Task; MANT) were assessed at baseline, after 4-weeks daily consumption and then after a 4-week wash-out period. The cognitive tests were performed through E-Prime V3 (Psychology Software Tools, Inc.) to display the stimuli and record participant responses. To present the audio stimuli during the RAVLT task, participants wore enclosed headphones throughout all tasks.

2.4.1. Rey's Auditory Verbal Learning Task (RAVLT):

This task assessed learning, memory recall and word recognition. Participants were presented an auditory recording of 15 nouns (list A). Each presentation was followed by a free recall of this list (recalls A1–A5). A further list of fifteen nouns (list B) was then presented as an interference list and recalled once only (Recall B). There was then a further free recall of list A (Recall 6) followed by a 15-min delay and then a final free recall of list A (Recall 7). After recall A7, participants were visually presented with 50 nouns containing: words from lists A and B, and 20 additional nouns and asked to press the button B for the words from list A and button N for the words that have not been heard in either List A or in List B. For each test session, the following outcomes were calculated as specified in Lezak et al. (2012): immediate word span (Recall 1)— showing immediate free recall ability; number of words learned (Recall 5 minus Recall 1)—showing learning over the session; final acquisition (Recall 5)—showing the total number of words learned, delayed word recall (Recall 7)- showing the ability to recall specific information acquired earlier (Whyte et al., 2016). Outcomes including, wordspan, final acquisition, total acquisition, amount learned, and delayed recall were recorded.

2.4.2. Serial Three Task:

The Serial 3s measures attention/working memory function. Participants were required to count backwards in threes from a given number as quickly and as accurately as possible using the computer keyboard linear number keys to enter each response. A random starting number between 800 and 999 was presented on the computer screen, which was cleared by the entry of the first response. The task was scored for number of correct responses and number of errors. In the case of incorrect responses, subsequent responses were scored as positive if they were scored as correct in relation to the new number. The duration of this task was 2 min (Scholey et al., 2010).

2.4.3. Modified Attention Network Task:

The task is a modification of the flanker task and measures executive function. A central arrow is responded to by participants which can either be congruent, incongruent or none (neutral) depending on the direction of the arrows surrounding it. Additional cues are presented before the stimulus in each trial. The stimuli were displayed either above or below the fixation point. In this task, participants were instructed to press the left or right arrow key on the keyboard according to the direction of the target stimulus arrow for each trial. A schematic for this task can be seen in **Figure 2.** Neutral (none) trials were considered to be low visual load and congruent and incongruent trials either being medium

visual load (one row of 5 arrows) or high visual load (two rows of 5 arrows). The stimuli position, congruence, and load were randomised so that each was displayed with equiprobability. Participants were instructed to press the arrow button on the keyboard corresponding to the direction the central arrow was facing and had a response window extending into the following fixation slide. Accuracy and response times for each treatment were measured for congruency and load. The combination of speed and accuracy measures gave an indication of the participant's attention and inhibitory control. From these manipulations, measures of executive attention were recorded by comparing congruency; measures of alerting were recorded by comparing trials where cues or no cues are presented; and measures of orienting were recorded by comparing trials where location or double cues are given (Whyte et al., 2017).

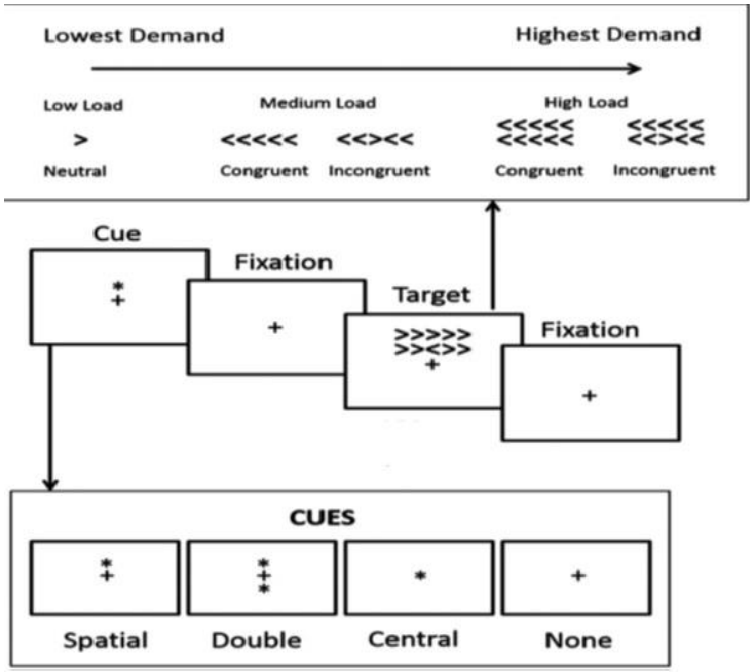

Figure 2. Schematic of the modified attention network task (Whyte et al., 2017).

2.5. Microbial Analysis from the Stool Samples:

Microbiota was the secondary outcome of this study. Stool samples were collected from participants and the fecal microbiota were investigated by 16S ribosomal RNA (rRNA) amplicon sequencing to characterise and quantify the proportion of bacteria present within genera.

A 250 mg quantity of faecal pellet was weighed as instructed in the DNeasy PowerSoil Kit (Qiagen, Manchester, UK) extraction kit protocol. The samples were homogenized with vortex in the PowerBead Tube provided. The subsequent steps of DNA extraction were performed as in the

protocol. The DNA yield was measured using a NanoDropH ND-1000 UV spectrophotometer (Nano-Drop Technologies). A 1 µL volume of sample was placed on the NanoDroph pedestal and the DNA purity by reading 260/280 nm making sure each sample concentration is 10-50 ng/µL. The primers were used to amplify the V3/V4 variable region of the 16S rRNA gene for the profiling of the bacterial faecal microbiota performed in Eurofins Genomics Europe Sequencing GmbH. For taxonomical classification non-chimeric, unique clusters were subjected to BLASTn analysis using non-redundant 16S rRNA reference sequences with an E-value cutoff of 1e-06. 16S rRNA sequences were obtained from Ribosomal Database Project. Only good quality and unique 16S rRNA sequences with a taxonomic assignment were considered and used as a reference database to assign operational taxonomic unit (OTU) status to the clusters.

2.6. Statistical analysis:

2.6.1. Cognitive data:

RAVLT, MANT, and Serial 3s data were analysed by Linear Mixed Models using an unstructured covariance matrix to model repeat measures performing with SPSS (Version 22.0). Thirteen participants (5 inulin, 4 WBB, 4 placebo) were recruited and separate Linear Mixed Models (LMMs) were performed for each dependent variable. For RAVLT, outcomes including, wordspan, final acquisition, total acquisition, amount learned and delayed recall; for MANT, accuracy and RTs; and for Serial 3s the number of correct and incorrect answers were included as dependant variables. Baseline performances were included as a covariate in Cognitive tests. Drink (placebo, inulin, WBB), Time (chronic, wash-out) and Drink × Time were included as Fixed Factors to compare the effects of treatment across the intervention period. In addition, for the MANT task, Congruency (congruent, incongruent) and Load (high load, medium load)) were also included as Fixed Factors in the model to detect changes in relation to cognitive demand as in Whyte et al. (2017) and Khalid et al. (2017). All post-hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each LMMs.

2.6.2. Microbiota Data:

16 S rRNA gene sequencing data were analysed by Linear Mixed Models using an unstructured covariance matrix to model repeat measures performing with SPSS (Version 22.0). Thirteen participants (5 inulin, 4 WBB, 4 placebo) were recruited **(as reported in Table 1)** and separate Linear Mixed Models (LMMs) were performed for each dependent variable. The % abundancy of each genus and phylum were included as dependant variables. Baseline performance was

included as a fixed factor. Drink (placebo, inulin, WBB), Time (chronic, wash-out) and Drink × Time were included as Fixed Factors to compare the effects of treatment across the intervention period. All post-hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each LMMs.

3. RESULTS

Baseline characteristics for the volunteers are reported in Table 1.

3.1. Cognitive Changes:

Changes in the cognitive behaviours are shown in **Table 3 and Figure 3.** Both inulin and the WBB group displayed significantly improved delayed recall memory performance on the RAVLT with participants remembering 9.5 and 9.6 words respectively compared to only 5 words following placebo treatment. These groups also learnt more words during the immediate recalls compared to those participants administered placebo showing better final acquisition after the 4-week chronic intervention period and the wash-out period ($p \leq 0.05$). The inulin group also showed better total acquisition performance compared to placebo. There were no significant differences between the treatment groups and the placebo group on any RAVLT measures at baseline.

Chronic and wash-out WBB and inulin-related benefits were seen on cognitively demanding trials on the modified attention network task (MANT), a task measuring executive function. Inulin group maintained significantly higher accuracy on congruent medium load trials compared to placebo-treated participants ($p \leq 0.05$) after the 4-week intervention (chronic period) whilst during the wash out period (4 weeks after the intervention) significant increases in accuracy on congruent high load trials for WBB group compared with placebo group were maintained ($p \leq 0.05$). Quicker reaction times (RTs) were observed for inulin and WBB participants compared with placebo groups without cost to accuracy, no significant decrease was observed for RTs neither in the WBB nor in the inulin groups. Significant benefits might be observed with a greater number of participants both with WBB and inulin groups. No significant WBB and/or Inulin-related effects were observed on the Serial 3s and on Word Recognition tasks. Additionally, no significant differences were observed between any treatment group and the placebo group on any MANT measures at baseline level.

Table 3. Mean (SD) data for Inulin, WBB and Placebo participants' performance for cognitive outcome variables (RAVLT, Serial 3s, Word Recognition and Modified Attention Network Task (MANT) outcome measures at baseline, chronic (4 weeks) and wash-out (8 weeks). *Significantly different from Placebo participants P<0·05 (LMMs).

	Baseline			Chronic			Wash-out		
	Inulin (n=5)	WBB (n=4)	Placebo (n=4)	Inulin (n=5)	WBB (n=4)	Placebo (n=4)	Inulin (n=5)	WBB (n=4)	Placebo (n=4)
RAVLT									
Wordspan	5.2 (1.10)	4.5 (1.00)	3.75 (0.96)	6.4 (1.69)	5.25 (0.96)	4.75 (1.50)	7.2 (2.49)	5.25 (0.50)	4.75 (0.96)
Total Acquisition	42.6 (8.44)	36.25 (7.23)	35.5 (5.51)	48.8 (10.89) *	40.25 (7.23)	31.25 (5.56)	52 (8.15) *	38.5 (3.70)	29.25 (3.40)
Final Acquisition	9.4 (2.88)	9.25 (1.50)	6.75 (1.26)	10.2 (2.95) *	10 (1.41) *	5.75 (0.96)	12 (1.41) *	9.25 (0.50) *	6.25 (0.96)
Total Amount Learned	4.2 (1.92)	4.75 (2.06)	3 (1.83)	3.8 (2.17)	4.75 (0.96)	2 (0.82)	4.8 (2.17)	4 (0.01)	1.5 (1.00)
Delayed Recall	6.4 (2.61)	5 (3.27)	5 (0.82)	9.6 (1.82) *	9.5 (1.29) *	4 (0.82)	9 (1.58)	7 (0.82)	5 (0.82)
Serial 3s									
Correct	7.2 (3.96)	10.25 (1.71)	9.75 (1.71)	48.6 (2.97)	60 (12.99)	39.75 (6.40)	47.6 (16.32)	51 (14.35)	30.25 (6.13)
Error	6 (3.16)	2.25 (0.50)	2.75 (1.50)	4 (3.74)	3 (1.83)	4 (2.16)	7 (2.55)	7.25 (1.26)	6.75 (1.71)
Word Recognition									
Yes Trials	10 (2.92)	14.5 (2.08)	9.25 (3.30)	17.6 (6.66)	20 (6.06)	19.25 (4.99)	17.2 (5.76)	11 (4.08)	20.25 (6.18)
No Trials	39 (13.73)	36.5 (20.95)	29.5 (5.80)	86.4 (5.98)	98.25 (2.22)	94.25 (5.74)	77.2 (22.35)	86.5 (17.82)	80.75 (16.52)
MANT									
Accuracy									
Congruent High Load	0.55 (0.28)	0.44 (0.42)	0.25 (0.06)	0.73 (0.10)	0.81 (0.13)	0.59 (0.14)	0.67 (0.12)	0.81 (0.06) *	0.59 (0.11)
Congruent medium Load	0.59 (0.29)	0.45 (0.39)	0.22 (0.14)	0.68 (0.19) *	0.56 (0.12)	0.41 (0.11)	0.75 (0.07)	0.77 (0.09)	0.73 (0.04)
Incongruent High Load	0.48 (0.31)	0.41 (0.05)	0.34 (0.19)	0.32 (0.10)	0.47 (0.14)	0.43 (0.10)	0.36 (0.19)	0.40 (0.13)	0.51 (0.19)
Incongruent Medium Load	0.48 (0.29)	0.35 (0.18)	0.37 (0.10)	0.26 (0.22)	0.24 (0.16)	0.26 (0.11)	0.27 (0.18)	0.34 (0.11)	0.41 (0.13)
None Low	0.58 (0.27)	0.24 (0.22)	0.19 (0.26)	0.31 (0.05)	0.41 (0.10)	0.66 (0.33)	0.44 (0.14)	0.40 (0.04)	0.31 (0.03)
Reaction Time (RT)									
Congruent High Load	701.65 (102.81)	577.09 (162.95)	708.42 (117.42)	743.09 (61.84)	644.17 (186.71)	755.97 (63.19)	768.22 (104.02)	686.97 (87.05)	791.82 (103.49)
Congruent medium Load	702.88 (105.53)	560.86 (171.07)	711.75 (119.68)	752.84 (68.62)	677.86 (126.32)	769.95 (65.78)	830.37 (91.58)	654.56 (116.85)	863.37 (62.67)
Incongruent High Load	674.19 (57.82)	666.57 (130.91)	687.55 (57.17)	772.12 (124.59)	708.57 (145.81)	805.72 (114.77)	759.51 (64.04)	709.80 (143.64)	782.98 (42.37)
Incongruent Medium Load	726.26 (85.54)	694.69 (172.72)	716.21 (95.31)	830 (117.55)	736.69 (153.39)	841.64 (132.37)	790.62 (110.35)	704.56 (128.19)	828.16 (82.72)
None Low	686.77 (125.49)	584.74 (162.76)	693.47 (143.87)	767.56 (91.84)	676.74 (82.76)	790.21 (88.47)	782.06 (141.02)	665.18 (104.35)	843.75 (33.91)

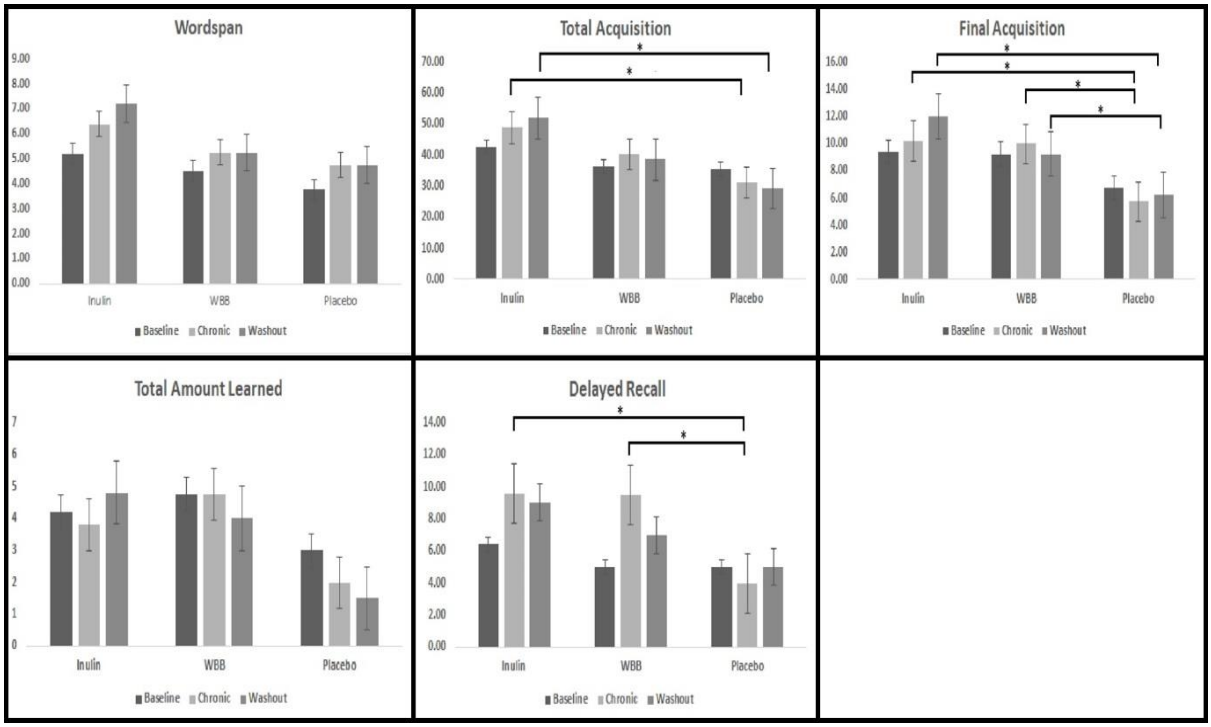

Figure 3. Number of Recall words from Total Acquisition, Final Acquisition and Delayed Recall from RAVLT among Inulin, WBB and Placebo participants at baseline, post consumption and wash-out periods. *Significantly different from the placebo group P<0·05 (LMMs).

3.2. Microbiota Changes:

Changes in the % abundance of the bacterial genus and phylum levels are displayed in **Figure 4.** No significant differences were observed in *Alistipes* spp. and in *Bifidobacterium* spp. On the other hand, the % abundance of *Bifidobacterium* were significantly higher at baseline in the WBB group compared to the placebo group. This might be due to low number of participants and higher variance in baseline levels between participants. A significant increase in the % abundance of *Faecalibacterium prausnitzii* was seen in the inulin group post treatment (after the chronic period) ($p \leq 0.05$) compared the placebo whilst an increasing trend was observed in the WBB group post treatment ($p \leq 0.1$). Additionally, the % abundance of *F. prausnitzii.* was significantly lower in the inulin and the WBB group compared to the placebo group at the beginning of the study. There was a significant decrease in the % abundance of *Blautia* spp. post consumption of inulin compared to placebo ($P \leq 0.05$). Despite having neither significant nor trend of alteration in the WBB group, the % abundance of *Blautia* spp. decreased in inulin group compared to the placebo. Interestingly, % abundance of *Clostridium* spp. in the WBB group increased significantly after the chronic period and the % abundance of *Clostridium* spp. was significantly higher in the WBB group at wash-out period ($P \leq 0.05$) whilst no significant changes were

observed neither in the placebo or in the inulin group. The % abundance of the *Coprococcus* spp. decreased significantly post treatment (chronic period) and wash out periods in WBB group compared to placebo group (P<0.05). In addition, the % abundances of *Coprococcus* spp. were significantly higher initially both in the inulin and the WBB group compared to the placebo group. Alterations of the % abundance of the bacterial phylum was shown in **Figure 5.** There were significant changes in WBB group in the % abundance of phylum post consumption. As such, a significant decrease was observed in Actinobacteria (P≤0.05), and a significant increase was observed in the Bacteroidetes (P<0.05), whilst no significant changes were seen either in the placebo or in the inulin groups.

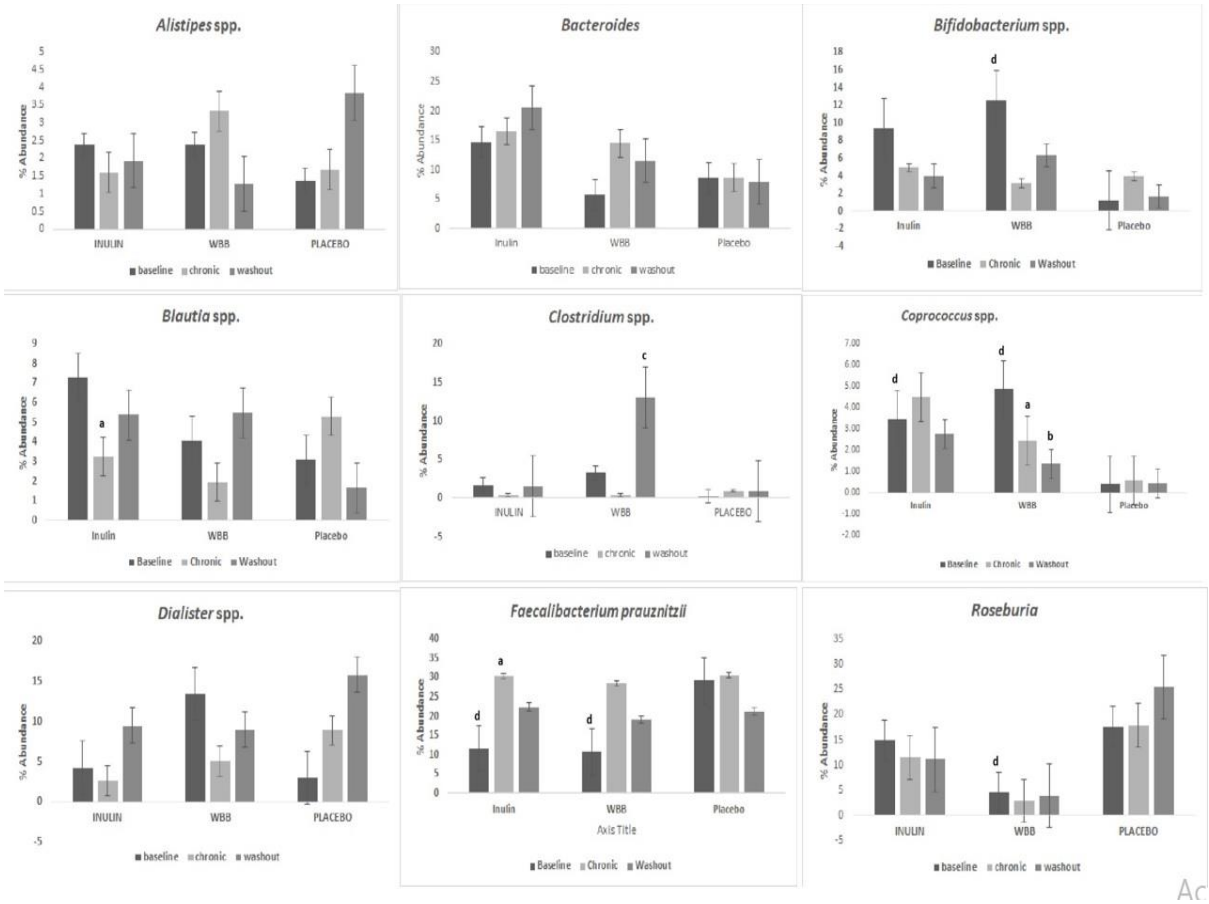

Figure 4. Microbiome (genus) composition (% Abundance) at genus level among Inulin, WBB (Freezed Dried Wild Blueberries) and Placebo (Maltodextrin) participants at baseline, post-consumption, and wash-out periods. Results are reported as means (% abundance) of the data (n=3). [a] Significant differences between baseline and chronic with P≤0.05; [b] Significant differences between baseline and wash-out with P≤0.05; [c] Significant differences between chronic and wash-out with P≤0.05; [d] Significant differences between placebo group and the treatment group (Inulin or WBB) at baseline with P≤0.05; In italics type: Significant differences with P≤0.01.

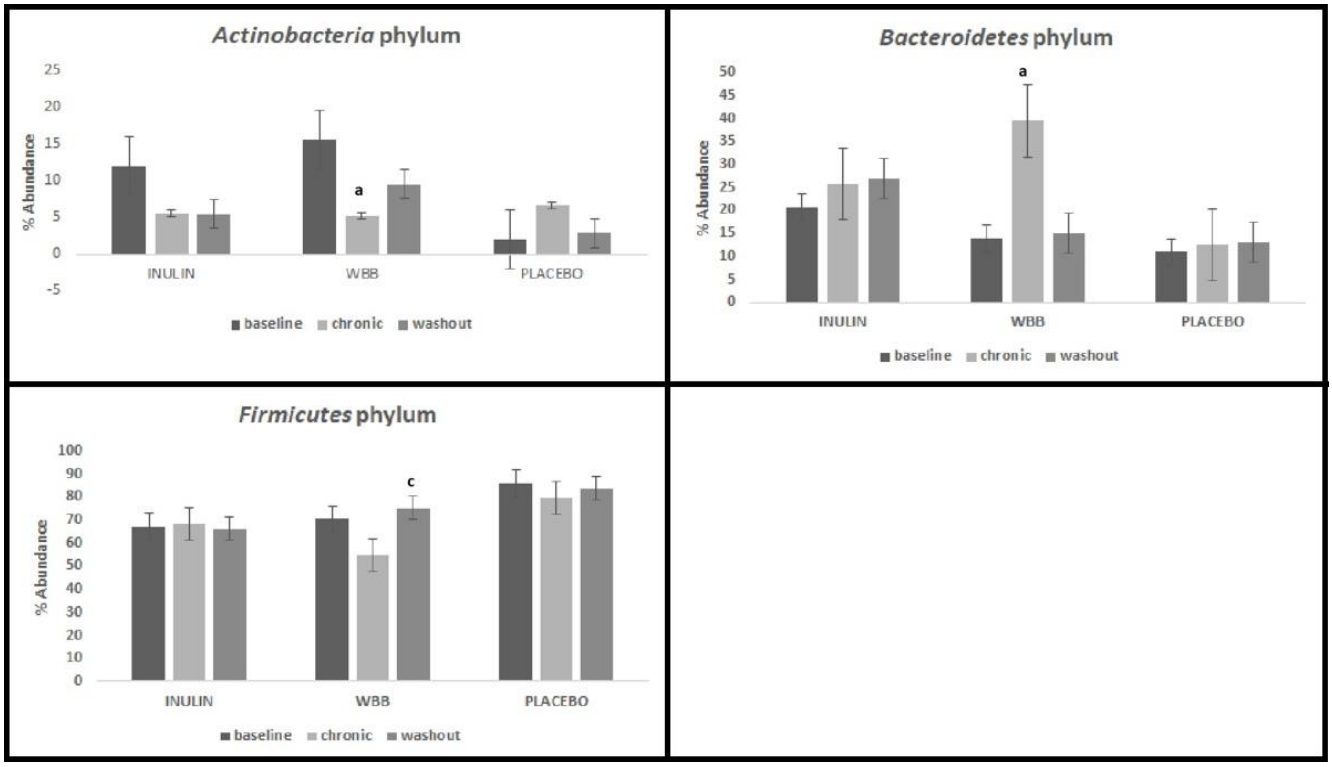

Figure 5. Microbiome (Phlyum) composition (% Abundance) at phylum level among Inulin, WBB (Freeze-dried Wild Blueberries) and Placebo (Maltodextrin) participants at baseline, post-consumption, and wash-out periods. Results are reported as means (mM) of the data (n=3). [a]Significant differences between baseline and chronic with P≤0.05; [b] Significant differences between baseline and wash-out with P≤0.05; [c] Significant differences between chronic and wash-out with P≤0.05; [d] Significant differences between placebo group and the treatment group (Inulin or WBB) at baseline with P≤0.05; In italics type: Significant differences with P≤0.01.

4. DISCUSSION

The aim of this study was to investigate the cognitive benefits of a chronic flavonoid (WBB) and prebiotic (inulin) intervention in a sample of children aged 7 to 10 years old. Both the inulin and WBB group experienced significantly improved memory performance on the RAVLT compared to placebo indicating the potential for these ingredients to positively impact on cognitive function, and more specifically, the episodic memory domain of cognitive function. Moreover, potential improvements were seen in accuracy in MANT showing better performance both in the inulin and the WBB group in the executive domain of cognitive function. Changes in the microbiota were also observed, as such the treatments had an impact on both the microbiota and cognitive factors.

In contrast, to the cognitive observations of the current study, Barfoot et al. (2021) studied the chronic effects of WBB as well and did not observe any significant alterations in delayed recall memory. Barfoot et al. (2021) however did observe significant improvements on MANT in accuracy, specifically in more demanding incongruent trials. However, these improvement effects were considered with caution as the placebo group might have, by chance, a lower potential ability. As such, it was observed that placebo participants failed to respond to non-target stimuli at significantly higher rate than WBB participants on the demographic measure of sustained attention (Barfoot et al., 2021). The ability of WBB to improve episodic memory performance was also observed in a study of Whyte and Williams (2015) who observed that flavonoid intervention resulted in significant improvements on delayed recall memory.

In a study performed by Whyte et al. (2017), it was revealed that executive functioning performance in 7-10 year old children was found to be significantly faster with WBB group compared to placebo particularly on more cognitively demanding incongruent and high load trials in MANT. Furthermore, another study conducted with 7–10-year-old children by Whyte et al. (2016) also found a significant increase in accuracy in the more demanding incongruent trials whilst consuming WBB, however, in our current study no significant alterations in incongruent trials were observed. This might be due to the small number of participants of the current study. Additionally, in the study of Whyte et al. (2016) investigated the acute effects of flavonoids whilst in this current study the chronic impact of the flavonoids was investigated. So, it might be the case that the acute and the chronic effects of flavonoids differ. As such, trials in which a visual cue alerted participants to the imminent appearance of the target also led to better performance following WBB administration compared with placebo administration (Whyte et al., 2017). Thus, the complexity and demanding nature of the task might also have an importance on investigating dietary intervention impact on executive function. It can be hypothesised that while the cognitive difficulty or demanding nature of a task increases, participants will initially display a similar reduction in performance regardless of dietary intervention. However, according to the several studies conducted by Whyte et al. (2017), flavonoid interventions are considered to produce more advantages in performance when the task is sufficiently demanding, but this point should not be as demanding as being impossible to perform (Whyte et al., 2017). However, for prebiotic intervention studies the most relevant cognitive outcomes still need to be determined. Prebiotics are also known to impact on cognitive functions which was also the case for our findings. As such, significant improvements in memory and executive functions were seen in our study following prebiotic intervention, which is a very promising outcome. Grimaldi et al. (2018) found a significant improvement in social behaviour of children (<12 years of age) suffering from autism

spectrum disorder (ASD) following a 6-week supplementation with the prebiotic galactooligosaccharides (1.8 g/day), especially in those following a restricted diet (casein and/or gluten-free), along with a non-significant reduction in GI symptoms, (Grimaldi et al., 2018). A human dietary intervention study conducted with B-GOS showed that there was a significant pre- versus post-effect of B-GOS on executive domains. 39 participants (18-60 years old) were recruited in this study, and they were hospitalised with psychosis, with a global cognitive score below the healthy average. Participants performed the Brief Assessment of Cognition in Schizophrenia (BACS) (Keefe et al., 2008), at baseline, 12 and 24 weeks, and results were expressed as a standardized T-score to account for age and gender. Placebo supplement had no influence on the executive domains. The overall effect of B-GOS on the composite T-scores was driven by subtests of executive function (Gronier et al., 2018). Moreover, an acute effect of (4 hours after consumption) oligofructose-enriched inulin (5 g) on subjective wellbeing, mood and cognitive performance of healthy younger adults (aged between 19-30) was investigated. Results showed that participants felt happier, had less indigestion and were less hungry than when they consumed the placebo. The consumption of inulin was associated with greater accuracy on a recognition memory task, and improved recall performance (immediate and delayed) which is also consistent with our current study (Smith et al., 2015).

It is also worth considering the limitation as the results could be affected by several factors including the number of participants, the randomization type of the groups, the period of the study (chronic or acute), dose consumed and the sensitivity levels of each cognitive tests. As such, the effects of interventions on cognitive function could be the result of many different factors, WBB has been observed to result in significant positive changes on cognitive domains including memory and executive functioning. This promising change might be explained by several potential mechanisms of action, for example: stimulating an increase in cerebral blood flow (CBF) following acute intervention or by facilitating an up-regulation of brain-derived neurotropic factor (BDNF) (Rendeiro et al., 2015). As such, an increased CBF could facilitate an increase in oxygen, which could be related to improved attention ability and memory functions (Rendeiro et al., 2012). On the other hand, flavonoids might promote the modulation of the gut microbiota, stimulating neurotransmitter and SCFAs production, which could have positive cognitive and memory impacts (Rodriguez-Daza et al., 2020). In the current study the microbiota was monitored to ascertain whether these may be involved in the changes observed along with the cognitive tests.

Flavonoids are known as to accumulate in the large intestinal lumen up to the mM range and might impact on the gut microbial community, modulating the composition of microbiota and metabolic

activities to impact on health status (Cueva et al., 2017). Red wine, which is a source of flavonoid, was reported to increase the faecal microbial diversity in healthy volunteers and has been reported to increase the number of faecal protective species such as *Bifidobacterium* and *Lactobacillus* and butyrate-producing bacteria such as *Faecalibacterium prausnitzii* and *Roseburia* (Chiva-Blanch & Badimon, 2017). Another dietary intervention human study with wild blueberries showed significant increases in *Bifidobacterium* spp. compared to placebo drink whilst significant increases in *Lactobacillus* spp. were observed for both blueberry and placebo groups (Vendrame et al., 2011). Bifidobacteria, which have been largely proposed to be of benefit for the host, appeared to be selectively favoured suggesting an important role for the polyphenols and fibre present in wild blueberries. Results obtained suggest that regular consumption of a wild blueberry drink can positively modulate the composition of the intestinal microbiota (Berding et al., 2021; Guglielmetti et al., 2013).

A prebiotic intervention study conducted with 12 human participants exhibited a significant increase in the growth of *Faecaibacterium prausnitzii* a butyrate-producing bacterial group, during the period of inulin consumption (10 g/d) for a 16-d period (Ramirez-Farias et al., 2009). These assessments are also parallel with our findings where, *Faecalibacterium prausnitzii* were altered significantly in inulin group and had a trend of increase in WBB group post consumption, whilst the baseline levels of this bacteria in the placebo group were higher initially, there is interest in this bacterium when considering cognitive function. Butyrate has been considered to have an impact on cognitive and memory functions which has been also determined in the previous chapters (Stilling et al., 2016). *Faecalibacterium prausnitzii* is one of the most dominant bacterial species detected in the healthy human large intestine (Flint et al., 2015); it is a butyrate producer and is considered health promoting, and also to possess anti-inflammatory attributes (Sokol et al., 2008). Another study conducted with mice, *Faecalibacterium prausnitzii* strains were found to improve cognitive impairment in mice when studying a model for Alzheimer Disease (AD). The study was performed with faecal samples from healthy subjects and those with mild cognitive impairment (MCI) and AD. They found that *Faecalibacterium prausnitzii* correlated with cognitive scores and decreases in the MCI group compared with the healthy group. Two isolated strains of *Faecalibacterium* from the healthy group were observed to improve cognitive impairment in an AD mouse model (Ueda et al., 2021). This correlates well with findings of the current study, where both in the inulin and the WBB group, delayed recall memory was significantly improved, and a significantly quicker Reaction Time (RT) obtained in Modified Attention Network Task (MANT) in the inulin group concurrent with increases in *Faecalibacterium prausnitzii*. As such a possible link between the dietary modifications, the gut microbiota and cognition were observed. In this human study, SCFAs were not measured because SCFAs are rapidly absorbed, making their measurement in faeces unrelated to production. On the

other hand, neurotransmitter (NT) analysis was performed in urine in this study. However, the data was inconsistent, so the results were not included here. Appropriate urinary data could support the investigation of the impacts of these dietary interventions on NT levels and thus potential effects on production.

The potential psychobiotic effects including anxiolytic and anti-depressant-like effects of *Faecalibacterium prausnitzii* were also assessed by several studies. As such, in a study, rats were administered *Faecalibacterium prausnitzii* for 4 weeks prior to testing during each period behaviour, growth status, SCFAs produced, plasma cytokine, endocrinology and bone mineral density (BMD) were assessed. The findings stated that the administration of *F. prausnitzii* had preventive and therapeutic effects on depression-like and anxiety-like behaviour. *F. prausnitzii* administration led to higher levels of SCFAs in the cecum and higher levels of cytokine interleukin-10 (IL-10) in the plasma, prevented the effects on corticosterone, changes that were associated with the effects seen in behaviour. This study supports the idea that *F. prausnitzii* might have significant potential to be psychobiotic (Hao et al., 2019) and also results from studies suggest that compensating the dysbiosis using *F. prausnitzii* as a probiotic is a promising strategy in gastro-intestinal disorder treatment (Sokol et al., 2008).

Flavonoids and prebiotics are shown to increase the *Bifidobacterium* spp. belonging to the Actinobacteria phylum (Parkar et al., 2013) in our study no significant *Bifidobacterium* spp. increase was obtained either in the WBB group or in the inulin group. The possible explanation of not observing any significant increases in *Bifidobacterium* spp. post consumption either in inulin group or in the WBB group might be due to the low numbers of participants, or the participants already having high levels of *Bifidobacterium*. *Bifidobacterium* spp. are considered to have cross-feeding interaction mechanisms including production of acetate, that is used by *F. prausnitzii* to produce butyrate. This cross-feeding interaction has been assessed by several studies (Rios-Covian et al., 2015). Thus, the abundance of *F. prausnitzii* significantly increased upon the administration of inulin, which could therefore be due an indirect pathway. It could be therefore that *F. prausnitzii* found suitable conditions to grow, such as increased availability of acetate that may have been produced by *Bifidobacterium* spp. But not observing any significant increases on *Bifidobacterium* spp. in the current study could be due to variances in as dietary routines for each participant, higher levels of standard deviation resulting in disparities among each participant at baseline, chronic and wash-out levels.

Moreover, in many studies a significant decrease in *Clostridium* spp. was observed with flavonoid administration (Barroso et al., 2017). In our study, a significant increase was observed in *Clostridium* spp. in the washing out period. This might also be the case of the dietary changes of this group or WBB

might have significant impacts on the homeostasis of the gut microbiota through inhibiting the *Clostridium* species and when the WBB administration cut-off these species might have found more suitable conditions to grow. However, with such a low number of volunteers, changes with one volunteer can have a large impact on the overall results, so in a pilot trial as this the results should be viewed with caution.

Several studies observed that phenolic acids and flavonoids might reduce the Firmicutes/Bacteroidetes (F/B) ratio which could be also associated with protection against cognition disorders and obesity (Moorthy et al., 2021; Tomova et al., 2015). This is also the case for our findings. As such, the WBB group showed a significant reduction in the F/B ratio acknowledging a significant increase in % abundance of Bacteroidetes post consumption. As such, a study from the Canadian Healthy Infant Longitudinal Development (CHILD) performed with 405 infants (199 females), determined a link between gut microbiota and neurodevelopment in a general population birth cohort (Tamana et al., 2021). The children participants aged 1-2 years old were assessed their neurodevelopmental outcomes using Bayley Scale of Infant Development (BSID-III) and their microbiota profiling was assessed with 16S rRNA gene sequencing on faecal samples obtained at a mean age of 4 and 12 months. According to the data from this study, the Bacteroidetes-dominant cluster was associated with higher scores for cognitive, language, and motor development at age 2 in models adjusted for covariates (Tamana et al., 2021). A similar link was observed in the current study, where Bacteroidetes at phylum level have been found to increase in WBB group post consumption concomitant with the positive outcomes of memory function and executive function post consumption in the WBB group. To our knowledge, this is the first study investigating the effects of a prebiotic on reading and cognition and the gut microbiota composition in children. Therefore, this study might be expanded as to investigate the synergetic effects of flavonoids and prebiotics through administration of a combination of two of these components.

In conclusion, dietary intake of prebiotics and flavonoids were observed to lead to benefits to cognitive performance, specifically aspects of episodic memory and executive function domains. The changes observed occurred together with changes in the gut microbial community. As such the group of bacteria *Faecalibacterium prausnitzii* could be a key player in gut: brain axis. According to our knowledge, this is the first nutrition intervention study performed with 7–10-year-old children including both inulin as a prebiotic and the flavonoids. However, more clinical studies with greater number of participants are needed to confirm findings and gain a more mechanistic appreciation for how these dietary components elucidate their effect

CHAPTER 6: CONCLUSION CHAPTER

1. GENERAL DISCUSSION:

It has been suggested that the gut microbiota has a significant impact on cognitive function. Indeed, there are several *in-vivo* and *in-vitro* studies supporting the notion that the microbiota operate through several pathways, including metabolic, endocrine, and neural pathways connecting the gut and the brain. This novel connection is known as the gut-brain axis (GBA). As such, the gut microbiota has been stated to have direct and indirect effects on immune activating and other signalling molecules, including neuroactive metabolites and neurotransmitters e.g., γ-aminobutyric acid (GABA), dopamine, epinephrine, norepinephrine, serotonin, and short chain fatty acids (SCFAs). These metabolites may play a significant role in regulating cognitive function. Therefore, dysbiosis of the gut microbial community has been associated with behavioural and cognitive disorders. A number of factors impact on the gut microbiota composition including health status, mode of birth delivery and genetics, but diet is considered the most significant factor. Therefore, this research is focused on dietary approaches to target the gut microbiota to support cognitive health. Within this scope, promising results been obtained within this thesis, where dietary interventions including prebiotic, and flavonoids might have the potential to regulate several cognitive domains including memory and attention functions which could be related with GBA (Dinan & Cryan, 2017). In order to assess the mechanism of how gut microbiota might impact on cognitive functions, a series of *in-vitro* studies and one human trial have been performed.

In the current work *in-vitro* batch culture studies were performed to ascertain whether the microbiota could produce neurotransmitters under physiologically relevant conditions. Many previous researchers have reported that the microbiota can produce neuro-active molecules, but often the conditions used do not resemble the gut or have artificially high concentrations of precursors. This study is based on assessing whether the production of neurotransmitters by gut bacteria through physiologically relevant concentrations of amino acid precursors such as monosodium glutamate (considered as GABA precursor), tryptophan (considered as serotonin precursor), tyrosine (considered as catecholamine precursor: adrenaline, noradrenaline, dopamine) is possible. Therefore, it was assessed as to whether these conditions could result in the production of neurotransmitters in a mimic of the large intestine environment, but in the absence of human cells. This study established that batch culture fermentation systems are an effective method to investigate growth and the metabolite production of gut bacteria in terms of neuroactive molecules with a sufficient amount of nutrients and an amino acid source. This study revealed that gut microbiota is capable of producing certain

neuroactive metabolites including neurotransmitters (in low amounts) and SCFAs without the need for human cells. This finding is of great importance, as such findings under these relevant conditions are not well studied. Due to these findings, future explorations could be based around whether significant production of neurotransmitters might be possible with additional substrates such as prebiotics and flavonoids.

The second *in-vitro* batch culture study addressed targeting the growth of beneficial gut bacteria to determine if this could result in a significant production of neuroactive metabolites. As such, in the second *in-vitro* batch culture study, prebiotics including inulin and B-GOS and flavonoids (freeze-dried wild blueberry powder (WBB)) and maltodextrin as a control were administered to the pH-controlled, anaerobic batch culture fermentation system. In this study B-GOS led to a significant increase in the growth of *Bifidobacterium* spp. whilst inulin resulted in no significant bifidogenic effects. However, upon inulin fermentation the production of butyrate significantly increased. Current *in-vitro* and *in-vivo* studies also suggest that increasing butyrate might be associated with the cognitive benefits. Several studies have revealed a significant increase in the growth of butyrate-producing bacteria *Faecalibacterium prausnitzii* along with *Bifidobacterium* and *Lactobacillus* after the administration of inulin (Ramirez-Farias et al., 2009). This could be strongly associated with a significant increase in the production of butyrate and accordingly beneficial cognition outcomes. Interestingly, significant increases were also observed in GABA and serotonin following the fermentation of inulin in a more complex gut model fermentation system. Furthermore, a significant increase in SCFAs, specifically in butyrate production, upon fermentation of both inulin and WBB in the gut model fermentation system could also be associated with cognitive benefits and other health benefits such as anti-inflammation (Burokas et al., 2017). Thus, these results highlight those dietary changes impact on the microbiota, and these can support changes in metabolites relevant to cognitive function.

A 3-stage continuous fermentation gut-model system, with more complex nutrients, provides a more realistic representation of a large intestinal system with different pH levels. In the gut model fermentation system fermentation of inulin and WBB led to potentially positive changes in the microbial community. However, inulin fermentation resulted in a significant increase in GABA and serotonin production. Fermentation of WBB did not show any significant increase in neurotransmitters such as GABA, serotonin and catecholamines. Catecholamines in general e.g., dopamine, adrenaline and noradrenaline were not observed to be produced in appreciable amounts and also did not show any significant increase neither in the inulin nor in the WBB vessels. On the other hand, significant increases were observed in SCFAs, specifically in butyrate upon the fermentation of both inulin and WBB. These results are exciting as show a potential for inulin to have

direct effects on GBA through neurotransmitters, whilst the cognitive changes observed of berries may be by a different pathway.

Finally, within a human pilot study, a significant and trend increase in the % abundance of *Faecalibacterium prausnitzii* for the inulin group and WBB group respectively was observed. In accordance with this, both inulin and WBB groups resulted in significantly higher accuracy in the attention network task and significant increases were reported on the memory tasks as well. In WBB group also Bacteroidetes at phylum level were observed to increase significantly, which was associated with positive cognitive functions. This study points out the importance of a dietary intervention studies in order to investigate potential microbially driven cognitive outcomes. Thus, it is very promising that significant increases were observed in SCFAs, specifically in butyrate production upon the fermentation of both inulin and WBB in the gut model fermentation system which could be associated with cognitive benefits and other health benefits such as anti-inflammation (Burokas et al., 2017). It is also worthy to observe significant increases in GABA and serotonin following the fermentation of inulin in batch culture and gut model fermentation system, thus inulin might impact the GBA in a different way to WWB. In this manner, dietary interventions may have the potential to regulate the several cognitive functions, related to GABA and SCFAs.

According to our knowledge, this is the first study to investigate and combine the effects of blueberry and inulin on the gut microbiota composition and the cognitive outcomes of children. Another novel aspect of this project is *in-vitro* studies (batch culture and gut model systems) providing more mechanistic information and enabling the essential comparison and determination between human trials and *in-vitro* studies.

2. RATIONALE & AIM:

The gut microbiota is considered to be shaped in the early stages in life and in childhood the gut microbiota composition is considered to develop as if similar to the adulthood microbiota composition (Cao et al., 2021). As such, it is very important to modulate the gut bacteria composition in early childhood. It has also been reported that there is an interconnection between the early gut microbiota and development of certain cognitive disorders (Tomova et al., 2015). These cognitive disorders including attention deficit and hyperactivity disorder (ADHD), autism spectrum disorder (ASD) is specifically seen in children (Khalid et al., 2017). Therefore, it is essential to address these diseases in the early years of life (Best & Miller, 2010). As such, if cognitive disorders affecting young children, can be treated and/or symptoms attenuated through the modulation of the gut microbiota this could be a powerful tool. Therefore, dietary intervention studies are key to ascertain whether gut bacteria can

have benefits on cognition and might prevent cognitive diseases (Grimaldi et al., 2018; Tomova et al., 2015). This is the major reason why the faecal samples from children are used in our *in-vitro* studies and why children participants are used in our human pilot study.

In conclusion, these *in vitro* studies and the human pilot study showed promising results in that supplementing the microbiota of children with prebiotics and even with a flavonoid the gut bacterial population and metabolic activity was manipulated to target health benefits for the host. According to our knowledge this is the first study which investigates the impact of both inulin and WBB on gut microbiota composition and their neuroactive metabolite production *in-vitro* and investigates the impact of WBB and inulin consumption on gut microbiota composition and possible related outcomes through an *in-vivo* study. This thesis provides an insight into the relationship between gut bacteria composition and the consumption of prebiotics and flavonoids. This also allows us to consider whether there is an association between consuming these substances and specific cognitive domains and the modulation of gut bacteria.

3. LIMITATIONS:

Despite relevant findings, the human study protocol has certain limitations that should be taken into consideration for future studies. One of the limitations of the study was the length of time. As such, to see a stronger effect, longer intervention studies are probably required. Other limitations are the sample size. In this current human pilot study, there were insufficient participants due to COVID-19 pandemic. Therefore, the results, specifically the gut microbiota profiles were quite different at baseline levels for each participant and the standard deviation was relatively high. As such, larger trials really are necessary to counter this large level of variation and to give more power. Furthermore, to get a mechanistic view of the results metabolomic studies might provide a better insight on assessing the relationship between the gut microbiota, metabolite production, and the impacts of these metabolites in cognition.

Further although *in-vitro* studies can be with a great tool for modelling gut microbial communities, there is a lack of human secretions, human cells and also absorption, as such the model system may not be realistic. However, *in vitro* systems allow microbes to grow in a controlled environment, where external factors can be manipulated, therefore answering questions, such as the possibility of microorganisms in a mixed culture to produce neuroactive molecules can be ascertained.

4. FUTURE WORK:

While evidence is growing, this area still needs to be progressed and our knowledge is limited to identify the impact of microbiota produced neuroactive metabolites or microbial roles in production of these neurometabolites and their related effects on cognition. Therefore, it is essential to expand this research with more metabolomic and metagenomic studies including SCFAs and neurotransmitter analysis in the urine and fecal samples along with *in-vitro* and *in-vivo* studies to gain a better insight of the mechanisms of the gut microbiota on impacting on cognitive function. Key questionnaires and food diaries should be used to help a more direct estimation of the impact of the prebiotic and flavonoid intervention on cognition and gut microbiota. In addition, identification of key time points for sample collection is also important to reduce stress in children. Moreover, the human trial should be performed with a greater number of participants as this study ended up with 15 participants in total due to pandemic situation. Additionally, participants should be rewarded for each session completed to engage their attention for the whole period of the study. Cognitive battery testing times could be reduced to maximum of 30 minutes considering the children at the age of 7-10 have maximum 30 minutes of time to give their attention in the task. Furthermore, to investigate the neurotransmitters related to the cognition, and SCFAs in the urine samples would provide a significant insight to assess the effects of nutrition intervention on cognitive performance, their gut microbiota composition and their neuroactive metabolites in their body. Thus, larger trials are essential utilising skills in microbiology, metabolomics and psychology to form a clearer picture of changes a dietary intervention can impact on cognitive function via the GBA. (Everson-Rose, 2003)

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APPENDICES:

School of Psychology & Clinical Language Sciences

Department of Food and Nutritional Sciences

Brief Introduction Letter

The purpose of this study is to investigate the impact of prebiotics and berries on cognitive and memory function in children and to investigate if these alterations are associated with the gut bacteria.

The study will be last for 8 weeks in total. Your child will be asked to consume a special supplemented drink including either prebiotics or berries (flavonoids) or both or placebo (non-supplemented orange juice) every morning with breakfast for 4 weeks. Once the 4 weeks of intervention finish, there will be a wash-out period that your child should not consume any supplemented drinks within this 4 weeks and should continue with their normal routine daily diets. Your child will be asked to perform cognitive tests before the study (baseline), 4 weeks later (chronic study) and another 4 weeks after the 4-weeks of intervention (wash-out study). Along with the cognitive battery tests, you will be asked to assist your child to collect urine and the stool samples before the study, after the study and after the wash-out period.

At the first session, participants and their parents will be invited to School of Psychology & Clinical Language Sciences, University of Reading in the morning after having their breakfast prior to the participant consuming the supplemented drinks. The participant will be asked to complete the cognitive battery tests and Positive and Negative Affect Scale (PANAS). These tests will be for the baseline results. Participants are also required to collect the stool and the urine samples in the sample pots on the morning that they are visiting the department. On the same day, the parents will be provided 4 weeks' worth of the formula drink and sample pots to collect the urine and the stool samples from the participants at the end of the 4th week. The parents will be advised on how to prepare the formula drink to be given to the participant every morning with breakfast during these 4 weeks. 4 weeks later, the participants and their parents/carers will be invited to our department again at the same time in the morning (08.00-10.00 am) and the participants will be asked to complete the tests. The participants should have their breakfast without the supplemented drink, or they will be provided a simple breakfast by our department. This will be the chronic part of the study. The parents will be required not to give the supplemented drinks to their children on that test day in order to avoid the acute effects. For the following 4 weeks of the period, the parents will be advised not to give the supplemented drinks to their children and at the end of this period the parents and the participants will be invited to the department and asked to complete the tests again, representing the non-treatment period. This study aims to collect relevant information about the impact of our children's gut bacteria on their cognitive and memory functions and their moods. Prebiotic and blueberry (flavonoid) supplemented drink are supposed to modulate the gut bacteria in a positive way, and we think this will have beneficial impacts on cognitive and behavioural functions.

University of Reading

School of Psychology & Clinical Language Sciences

Department of Food and Nutritional Sciences

Parental Consent Form

Investigating the impact of prebiotics and flavonoids (berries) on the faecal microbiota and cognitive function

I ……parent/guardian of ……...
agree to my child participating in the study "Investigations of the Impacts of the Prebiotics on Gut Microbiota and the Impacts of Probiotics/Prebiotics and Flavonoids (Berries) on Attention-Deficit-Hyperactivity-Disorder (ADHD) and Cognitive Functions" at the Department of Food and Nutritional Sciences, University of Reading. This study has been reviewed by the University of Reading Research Ethics Committee and has been given ethical approval.

☐ I have seen and read a copy of the "Parents Information Sheet" and have been given the opportunity to ask questions about the study and these have been answered to my satisfaction.

☐ I understand that all personal information will remain confidential to the researcher and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me.

☐ The contents of the drinks have been explained to me and I am happy for my child to consume them.

☐ I understand that participation in this study is voluntary and that I can withdraw my child at any time without having to give an explanation.

☐ I believe that my child understands what is required of them during the study

☐ I am happy for my child to proceed with participation.

Signature …….

Name (in capitals) …….

Date ……

Fruit Juice Administration Log

University of Reading

Day	Consumed	Time Consumed	Not Consumed	Comments
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Food and Drink Record Chart

	DAY 1			DAY 2			DAY 3		
	Food Item	Type of Food (type of bread, type of beans)	Food Size and Amount	Food Item	Type of Food (type of bread, type of beans)	Food Size and Amount	Food Item	Type of Food (type of bread, type of beans)	Food Size and Amount
BREAKFAST									
LUNCH									
DINNER									