University of Reading

A methodological approach to studying restrictive-anorexia nervosa (R-AN) and examining the gut microbiota as a therapeutic strategy

Litai Liu

Declaration

I declare that this thesis is my own work and the contribution of others has been properly and fully acknowledged.

Litai Liu, 2022

……

Acknowledgement

I would like to express my deepest gratitude to my supervisors Dr. Gemma Walton, Dr. Carlos Poveda and Dr. Paul Jenkins for their guidance and constant support during my PhD study.

TABLE OF CONTENTS

An *in vitro* approach to studying the microbial community and impact of pre and probiotics under the anorexia nervosa related dietary restrictions

Liu, L.; Poveda, C.; Jenkins, P.E.; Walton, G.E. *Nutrients* **2021**,*13*,4447.

General abstract

Restricting anorexia nervosa (AN) is characterised by restriction of dietary intake typically leading to low body weight and a distorted perception of body shape. AN individuals frequently suffer from depression, anxiety and gastrointestinal problems. Significant differences in gut microbiota composition have been observed in individuals with AN as compared to healthy controls, this is to be expected as the greatly impacts on this microbial community. There is growing evidence that the gut microbiome affects psychopathologies, such as anxiety and depression. Pre and probiotic intervention, targeting the gut brain axis (GBA) have been postulated to modify microbiota and improve mental well-being and gut symptoms, but there is currently a lack of evidence for such approaches in AN. The aim of this project was to use model systems to explore neurotransmitter (NT) precursor fermentation by the faecal microbiota. Secondly the impact of an *in vitro* model of AN restrictive diet was explored along with the potential role for pre and probiotics for recovering microbial communities. NT precursors fermented with faecal slurry enhanced microbial growth, whist

modulating NT and short chain fatty acid (SCFA) concentrations. An anorexic in vitro model was created, based on restrictive dietary patterns investigating how drastic nutrient changes affect the microbial community using a continuous three-stage colonic fermentation model system. Compared to a healthy model, AN conditions drove significant changes in microbiome, SCFAs and NTs, effects that were largely restored by pre and probiotics. Results suggest that supplementation with pre and probiotics could be used to restore an unbalanced microbial whilst potentially supporting GBA.

Abbreviations

AAA – aromatic amino acid

AN – Anorexia nervosa

AD – Alzheimer's disease

ADHD - attention deficit hyperactivity disorder

α-MSH - alpha-melanocyte-stimulating hormone

ANOVA – Analysis of variance

AS – Asperger syndrome

ASDs – autism spectrum disorders

autoAbs - autoantibodies

BBB – blood-brain barrier

BB-12 - *Bifidobacterium animalis* subsp. *lactis* BB-12

BCAAs - branched chain amino acids

BCFA – branched chain fatty acid

BDNF - brain-derived neurotrophic factor

BLAST – Basic Local Alignment Search Tool

BMI – Body Mass Index

BN – Bulimia nervosa

BNF – British Nutrition Foundation

bp – base pair

CA – catecholamines

CES-D - Center for Epidemiological Studies Depression Scale

CFU – colony forming units

CGI-I - Clinical Global Impression- Improvement rating Scale

CGI-S - Clinical Global Impression Severity rating Scale

CIpB – Caseinolytic protease B

CNS – central nervous system

CO_2 – carbon dioxide

CSF - cerebrospinal fluid

DA – dopamine

ddH_2O – Double-distilled water

ECs - enterochromaffin cells

EDI-2 - Eating Disorder Inventory

EDs – Eating disorders

EDTA – Ethylenediaminetetraacetic acid

ENS – enteric nervous system

EPI – epinephrine

ESI – Electrospray ionisation

Ex-GF – Ex germ free

FC – fold change

FCM – flow cytometry

FISH - florescence *in situ* hybridisation

FISH-FCM - florescence *in situ* hybridisation flow cytometry

FOS - fructo-oligosaccharides

g - grams

GABA – gamma aminobutyric acid

GAD – glutamate decarboxylase

GBA – gut brain axis

GC – Gas chromatography

GF – germ free

GI – gastrointestinal

DI-DQ - Gastrointestinal Discomfort Questionnaire

GIT – gastrointestinal tract

GLP-1 - Glucagon-like peptide

GOS - galactoologosaccharide

h – hours

HAM-D - Hamilton Rating Scale for Depression

H_2O – water

HC – healthy

HCI – chloride acid

HMGB1- high mobility group B1

HMOs – human milk oligosaccharides

HPA – hypothalamic-pituitary-adrenal

hs-CRP - high-sensitivity C-reactive protein

IBS – irritable bowel syndrome

IBS-QOL - Irritable bowel syndrome quality of life questionnaire

IDO - indoleamine-2,3-dioxygenase

IGN - gluconeogenesis

KYNA - kynurenic acid

L – litre

LAB – lactic acid bacteria

L-AADC - L-aromatic amino acid decarboxylase

LC – Liquid chromatography

LCMS – Liquid chromatography mass spectrometry

LNAA - large neutral amino acids

LHA - lateral hypothalamic area

L. plantarum - *Lactobacillus plantarum* C4

L. plantarum GG - *Lactobacillus rhamnosus* GG

M – molar

MADRS - Montgomery-Asberg Depression Rating Scale

MCP-1 - monocyte chemotactic protein-1

m-ESS - modified Epworth Sleepiness Scale

MIP-1 - macrophage inflammatory protein-1 beta

MGBA – microbiota-gut-brain axis

mg – milli grams

Min – minutes

mL – milli litre

mm – milli metre

mM – milli molar

MRM – multiple reaction monitoring

mRNA – messenger ribonucleic acid

MRS - Man-Rogosa-Sharpe

MS – Mass spectrometry

MSG – monosodium glutamate

MDD – major depressive disorder

m/z – mass/charge number of ions

NaCI – sodium chloride

N_2 - nitrogen

NCBI – National Center for biotechnology Information

NCFM - *Lactobacillus acidophilus* NCFM

NE – norepinephrine

ng – nano grams

NGS – next generation sequencing

NT – neurotransmitter

OD – optical density

OTUs – operational taxonomic Units

O_2 – oxygen

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PD – Parkinson disease

PFA – paraformaldehyde

pH – potential of hydrogen

PNMT - phenylethanolamine N-methyltransferase

PYY – peptide YY

QQQ – Triple Quadrupole

QUIN - quinolinic acid

R-AN – restricting-type anorexics

rRNA – ribosomal ribonucleic acid

S. boulardii – Saccharomyces boulardii

SCFA – short chain fatty acid

SS – steady state

SPF – specific pathogen free

TDO - tryptophan-2,3-dioxygenase

TH – tyrosine hydroxylase

TPH – tryptophan hydroxylase

Tris/ HCI – Tris hydrochloride

TRP – tryptophan

V – vessels

VN – vagus nerve

VMN - ventromedial nucleus

vWF - von Willebrand factor

WHO – World Health Organization

µg – micro gram

µL – micro litre

µm – micro metre

xg - relative centrifugal force

°C - Degree Celsius

5-HT - serotonin

5-HTP - 5-hydroxytryptophan

List of publications

Liu, L.; Poveda, C.; Jenkins, P.E.; Walton, G.E. An *in vitro* approach to studying the microbial community and impact of pre and probiotics under the anorexia nervosa related dietary restrictions. *Nutrients* 2021,*13*,4447.

Chapter 1

Introduction

1. Anorexia nervosa

Anorexia nervosa (AN) is a serious eating and psychiatric disorder. AN is characterised by a distorted perception of body shape, leading to self-forced starvation and refusal to eat. Patients usually exhibit very low weight with self-induced hunger due to food restriction, driven by a strong fear of weight gain (Association, 2013). AN has the highest mortality rate out of all mental disorders, furthermore, relapse is common, with only about half of patients fully recovering (Arcelus *et al*., 2011; Zipfel *et al*., 2014; Mehler and Brown, 2015). The treatment of AN is prolonged and based on psychotherapy and refeeding, often applied in hospitalised and outpatient settings (Dejong *et al*., 2012). In addition, gastrointestinal problems are frequently found in AN patients (Mack *et al*., 2016). In the UK, there are between 1.25 to 3.4 million people affected by an eating disorder with the average age of onset of AN being 16-17 years of age (Priorygroup, 2022). The lifetime prevalence rates of AN might be up to 4% among females and 0.3% among males. It is reported that AN patients suffer from gastrointestinal problems and the most frequently reported symptoms being postprandial fullness (96%) and abdominal distention (90%) (Salvioli *et al*., 2013).

In general diets are made up of macronutrients (protein, carbohydrate, fat and dietary fibre) and micronutrients (minerals and vitamins), these enable growth and appropriate functioning of the human body. Diet has repeatedly been proven to be one of the main factors influencing the establishment and composition of the gut microbiota (consortium of bacteria, viruses,

fungi and parasites living within us) throughout life (Wu *et al*., 2011; Moles and Otaegui, 2020; Redondo-Useros *et al*., 2020). Dietary composition determines the type of nutrients that reach the gastrointestinal tract, specific nutrients can be utilised by gut microbes, promoting bacterial metabolism and influencing the microbial community. For example the human gut microbiota composition is known to be modulated in early life by human breast milk oligosaccharides (HMOs) (Praticò *et al*., 2014). HMOs secreted from milk and have a prebiotic protective function and act by increasing levels of bifidobacteria in the infant gut, subsequently this has the effect of reducing pathogens within (Underwood *et al*., 2015), thus HMOs may play a key role in preventing gut dysfunction and enhancing the gut microbial community (Bering, 2018). In addition, the metabolic activity and proliferation of *Bacteroides* is supported by the long-term intake of animal fat and protein as well as high intake of a high-glycaemic and high-carbohydrate foods (Ferguson *et al*., 1999; Fava *et al*., 2013). As such, diet has a significant effect on the human gut microbiota dynamic and thus dietary interventions can effectively regulate function and diversity of gut microbiota, thereby altering the production of metabolites.

The dietary pattern of AN relates it to severe malnutrition, as such the numbers of intestinal bacteria in AN patients are reduced because of limited food intake, maintaining this gut microbial community in a starvation state (Jalanka *et al*., 2015; Morita *et al*., 2015). Compared to healthy individuals and obese or overweight individuals, significant differences in gut microbiota composition has been demonstrated in individuals with AN, with an imbalance in relative abundance of Gram positive/Gram negative bacteria (Borgo *et al*., 2017; Kleiman *et al*., 2015; Morita *et al*., 2015; Mack *et al*., 2016). There are several reasons for a dysregulated gut microbiota; one main reason could be insufficient food intake not supporting a flourishing gut microbiota. Another reason could relate to laxative treatments

used for constipation, common in AN patients resulting in alternations to the gut microbiota (Jalanka *et al*., 2015). Individuals with AN have been reported to have significantly decreased numbers of total gut bacteria and this could impact on clinical symptoms of patients. Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia have been found to be the primary phyla in anorexic patients (Borgo *et al*., 2017; Mack *et al*., 2016). AN patients have generally been observed to have higher relative abundance of mucin and protein degrading taxa and lower levels of carbohydrate utilising taxa (Borgo *et al*., 2017). These microbial changes may be of great importance as there is growing evidence that the gut bacteria can affect neurodevelopment and emotional response (Clarke *et al*., 2013). Alterations in the microbiota that have been observed in those with AN are listed in Table 1.

Table 1 The main gut microbial perturbations associated with AN

Phyla	**Genera**	**Physiological differences on patients with AN**	**References**
Bacteroidetes	*Bacteroides*	● *Bacteroides uniformis* was inversely associated with BMI, and *Bacteroidetes* phylum can be considered as a good predictor of BMI.	(Armougom *et al*., 2009) (Borgo *et al*., 2017) (Morita *et al*., 2015)
Verrucomicrobia	*Akkermansia*	● *Verrucomicrobia* are typical mucin-degrading bacteria that are higher in a nutrient-deficient ecosystems. The mucin-degraders feed on mucus covering the intestinal wall and potentially cause the leaky gut thus resulting in chronic low-grade inflammation. ● *Verrucomicrobia* was inversely correlated with body weight in humans.	(Karlsson *et al*., 2012) (Mack *et al*., 2016) (Marcobal *et al*., 2013) (SantaCruz-Calvo *et al*., 2010)
Proteobacteria	*Enterobacteriaceae*	● *Escherichia coli* as appetite regulator in AN ● *E. coli* produces more CIpB which induces a more potent effect of anorexigenic when the nutrients of *E. coli* are stable. Administration of *E. coli* producing CIpB resulted in a short-term reduction in body weight and food intake compared to administration of *E. coli* lacking CIpB. ● *E. coli* was negatively correlated with BMI. ● *Evterbacteriaceae* have been associated with gut inflammation that facilitates bacterial translocation and boosts systemic inflammation	(Borgo *et al*., 2017) (Million *et al*., 2013) (Szalay *et al*., 2010) (Tennoune *et al*., 2014) (Winter and Baumler, 2014)
	Roseburia spp. *R. faecis* *R. inulinivorans* *R. intestinalis* *R. hominis*	● Carbohydrate-fermenter and producer of SCFA (↓*Firmicutes* → ↓SCFA) ● *Roseburia* spp. has an impact on inulin concentration and further affects glycaemic changes. ● Decreased *Roseberia* may be involved in altered gut permeability and low-grade inflammation. ● They are important species of butyrate producer. ● A depletion of *R. inulinivorans* can result in reduced production of propionate. ● *Roseburia intestinalis* is a mucosal-associated species that increases the bioavailability of butyrate as a source of energy for colon cells. It has been suggested that *R. intestinalis* is also	(Armougom *et al*., 2009) (Belmonte *et al*., 2016) (Borgo *et al*., 2017) (Jesus *et al*., 2014) (Louis *et al*., 2010) (Misra and Klibanski, 2014)

Firmicutes		a polyamine producer and therefore essential for the maintenance and function of the gastrointestinal epithelium. Decreased polyamine production can alter the structure of the epithelial layer.	
	Clostridium spp.	• Carbohydrate-fermenter and producer of SCFA	(Borgo et al., 2017)
		• *Clostridium* spp. was inversely correlated with depression and anxiety scores.	(Morita et al., 2015)
	Lactobacillus	• *Lactobacillus* are part of the normal human gut microbiota that colonizes the mouth, gastrointestinal (GI) tract, and female genitourinary tract.	(Armougom *et al*., 2009) (Bravo *et al*., 2011) (Dinan and Cryan, 2013)
		• Neurotransmitter producer	
		• SCFAs producer	(Million *et al*., 2013)
		• *L. rhamnosus* is involved in modulating the central expression of GABA receptors in key CNS brain regions in mice and therefore may have beneficial impacts on the symptoms of depression and anxiety.	(Morita *et al*., 2015) (Nicholson et al., 2012)
		• *L. rhamnosus GG* inhibits biofilm formation by various pathogens, including *Salmonella* spp. and pathogenic *E. coli*.	
	Ruminococcus	• Carbohydrate-fermenter and producer of SCFA • *Ruminococcus* spp. are found as abundant members of a "core gut microbiome" in most humans	(Borgo *et al*., 2017) (Leschine, 1995) (García-Mantrana *et al*., 2020)
		• Main roles in the degradation and fermentation of dietary polysaccharides.	(Qin et al., 2010)
		• High intake of dietary fibre, omega-3 fatty acids and polyphenols resulted in a large number of *Ruminococcus*.	
		• Mucin degrading bacteria	
	Methanobrevibacter smithii	• Mucin-degrading bacteria	(Armougom *et al*., 2009)
		• *M. smithii* is capable of converting hydrogen on methane and allowing extra calories to be extracted from nutrients to exploit energy.	(Borgo *et al*., 2017) (Morita *et al*., 2015) (Mack *et al*., 2016)
Archaea		• *M. smithii* has been associated with chronic constipation which is dominant in harder stools.	
		• *M. smithii* was negatively correlated with BMI.	

2. Dominant gut microbiota in patients with AN

2.1. The gastrointestinal tract and the gut microbiota

The human colonic microbiota is a diverse and large microbial community. Over 1000 bacterial species have been identified of which many remain uncultured, with about 160 species being found in the gut of any individual (Rajilić-Stojanović and de Vos, 2014). The gene set of the gut microbiota is estimated to be about 3 million genes –150 times larger than that of the human genome and this is known as the microbiome (Zhang *et al*., 2010). The role of gut microbiota in regulating health and disease physiology is attracting increased

attention (Goulet, 2015; Marchesi *et al*., 2016). Diverse and dense gut microbial inhabitants form a dynamic relationship with the host (Ley *et al*., 2008). The gut microbiota can breakdown and transform dietary substrates, often resulting in the formation of short chain fatty acids (SCFAs) (Gill *et al*., 2006). SCFA have effects all around the body, including provision of energy to cells, these will be discussed in more details in the coming sections.

It is becoming apparent that the gut microbiome plays a major role in metabolism, calorie extraction and harvest, mood regulation, appetite, behaviour and gastrointestinal symptoms (Backhed *et al*., 2004; Dinan *et al*., 2015; Guinane and Cotter, 2013; Mithieux, 2018; Slyepchenko *et al*., 2017; van de Wouw *et al*., 2017). Indeed, it has been determined that the gut microbiome can impact on the risk and pathogenesis of malnutrition through immune function and nutrient metabolism (Krajmalnik-Brown *et al*., 2012). In addition to this more information is accumulating on the role of the microbiota in cognitive function; the microbiota have been implicated in the production of neurohormones such as NTs, vitamins B and K and polyamines (Chow *et al*., 2010; Kastin and Pan, 2010; Ouwehand *et al*., 2002). Therefore, this community, rather than being passive inhabitants, seem to be involved in many different aspects of host health.

2.2. Key mucin-degrading bacteria (Verrucomicrobia, Bifidobacterium and Firmucutes)

Mucins are important as a barrier to protect enterocytes against pathogens, as well as against physical and biochemical damage (Marcobal *et al*., 2013). Several mucin-degrading bacteria including Verrucomicrobia, *Bifidobacterium* and Firmucutes were found to be higher in patients with nutritional deficiencies (Marcobal *et al*., 2013; Crost *et al*., 2013). These bacteria live in the intestinal mucus layer, taking nutrients from the layers leading to mucin

degradation (Mithieux, 2012). As such it is likely that in AN patients mucin becomes a more predominant source of sustenance to the gut bacteria compared to other nutrients. Mack and colleagues reported that *Bifidobacterium* were a core gut microbe in patients with AN but not in normal weight group. This suggests that the majority of patients with AN have specific bifidobacterial taxa in their gut microbiome compared to healthy normal weight group, and already established *Bifidobacterium* may benefit from diet-derived carbohydrates in the form of fibre that often form a large component of the anorexic diet (Mack *et al*., 2016).

2.3. Altered methane-producing archaea species in patients with AN

A significant change of higher amounts of methanogenic archaea species in patients with AN has been observed (Armougom *et al*., 2009; Borgo *et al*., 2017; Million *et al*., 2013; Mack *et al*., 2016). Also, a study indicated decreases in Firmicutes and SCFAs with increases in *Methanobrevibacter smithii* in AN (Aurigemma *et al*., 2018). *Methanobrevibacter smithii* is able to use hydrogen to reduce carbon dioxide to methane which produces cellular energy and extracts additional calories from ingested carbohydrates (Flourie *et al*., 1990; Sahakian *et al*., 2010). Decreased *M. smithii* has been implicated in an obese group, being opposite to increased *M. smithii* in anorexic patients, which maybe due to the opposite energetic spectrum in the two groups (Million *et al*., 2013). An increase in *M. smithii* indicates that patients may have an adaptive response to long-term nutrient and energy deficits (Aurigemma *et al*., 2018). Furthermore, this bacterial species is associated with mucin, which further supports the increased value of mucin as a microbial energy source during AN.

2.4. Enterobacteriaceae family and Alpha-melanocyte-stimulating hormone (α-MSH)

Gram negative bacteria, especially Enterobacteriaceae, are connected with elevated levels of alpha-melanocyte-stimulating hormone (α-MSH), a strong inhibitor of food intake. α-MSH is

anorexigenic and anxiogenic with a 13 amino acid neuropeptide that modulates appetite and metabolism through the MC4 receptor (Cone, 2005), suggesting that it may play a role in pathophysiology of eating disorders (ED) (Fetissov *et al*., 2002; Tennoune *et al*., 2015). α-MSH-reactive autoantibodies (autoAbs) were initially found in patients with eating disorders, with altered α-MSH signalling leading to abnormal feeding behaviour, and appetite suppression by activation of anorexigenic neurons (Adan and Vink, 2001; Cone, 2005). Yehuda and colleagues stated that the effects of α -MSH on feeding behaviour are regulated by the dopaminergic system (Yehuda and Sheleff, 1985). However, as levels of this stimulating hormone are related to Enterobactriaceae this further suggests that the microbiota could have a key role to play in pathways of appetite.

2.5. Caseinolytic protease B (CIpB)

Enterobacteria such as *Escherichia coli* are prominent residents of the gut microbiota and are capable of producing small protein sequences, caseinolytic protease B (CIpB) (Breton *et al*., 2016; Tennoune *et al*., 2014; Tennoune *et al*., 2015). CIpB is a conformational antigen mimic of α-MSH that seems to induce α-MSH and activate anorexigenic brain neurones involved in anxiety and satiety signals (Adan and Vink, 2001; Kishi and Elmquist, 2005). For example, a mouse study showed that mice immunised with CIpB bacterial protein had decreased food consumption, bodyweight and increased anxiety (Breton *et al*., 2016). CIpB has been observed to be increased in the plasma of patients with eating disorders associated with insufficient food intake such as AN. Furthermore, the concentration of CIpB is associated with a variety of psychopathological features (Breton *et al*., 2016). For instance, compared with healthy participants, ClpB in plasma correlates with Eating Disorder Inventory (EDI-2) subscales, Montgomery-Asberg Depression Rating Scale (MADRS) total score in patients

with AN (Breton *et al*., 2016). Hence, CIpB may be involved in the psychopathological symptoms of patients with AN and it should be investigated as to whether ClpB in plasma may provide a link on the communication of GBA.

2.6. Gut microbial changes in Bacteroidetes and Firmicutes Phyla

Individuals with AN exhibit an overall change in balance of the gut microbiome. These microbial changes also implicate gut bacterial-mediated metabolic processes in AN (Aurigemma *et al*., 2018). For example, the imbalanced ratio of Firmicutes/Bacteroidetes (F/B) could be associated with psychological symptoms. In IBS patients were found to have a higher F/B ratio which correlated with clinically significant anxiety and depression (Jeffery *et al*., 2012).

3. The gut-brain axis

The gut-brain axis (GBA) is a bidirectional communication system and an integrative physiological network between the gastrointestinal tract (GIT) and the brain. GBA involves different communication pathways: (1) immune; (2) endocrine (hypothalamic-pituitary-adrenal axis); (3) autonomic nervous system (vagus nerve) (Borre *et al*., 2014; Mayer, 2011). The vagus nerve (VN) is the main nerve of the autonomic nervous system and seems essential for mediating the influence of gut microbiota on neurophysiological functions. For example, probiotic *Lactobacillus rhamnosus* intervention in mice induced alterations in GABA mRNA in the brain, also impacting on hippocampus expression whilst reducing anxiety and depression related behaviour; however, an experiment in mice undergoing a vagotomy failed to result in relief to depressive or anxiety-like behaviours (Bravo *et al*., 2011). As such, it can be seen that the VN is one of the communication routes responsible for

conveying information such as gut microbiota-derived NTs from the intestine to the brain (Bonaz *et al*., 2018; Latorre *et al*., 2016).

4. Bacterial fermentation products

4.1. Branched-chain fatty acids (BCFA)

BCFAs are products of protein fermentation of the colonic microbiota and are mainly formed by branched chain amino acids (BCAAs) including valine, isoleucine and leucine (Macfarlane *et al*., 1992). Increased concentrations of total BCFAs particularly iso-butyrate and iso-valerate have been observed in stool samples of patients with AN (Mack *et al*., 2016), indicating an increased in intestinal protein fermentation. Increased BCFA has been demonstrated to have a detrimental effect on gut motility, physiology (Macfarlane and Gibson, 1994) and also psychopathology, such as impacting on gut hormone release and accelerating symptoms of depression (Holzer and Farzi, 2014; Mack *et al*., 2016).

4.2. Short chain fatty acids (SCFA)

As important indigestible carbohydrate and protein fermentation metabolites, SCFAs are probably the most widely studied. The most abundant are acetate, butyrate and propionate which account for about 90-95% of total SCFAs and act as signalling molecules to affect physiology of the host, such as colonic pH, energy homeostasis and appetite regulation (Stilling *et al*., 2014; Sherwin *et al*., 2016; Wong *et al*., 2003). SCFAs are mainly produced by the gut microbial fermentation of non-host digestive dietary fibres and resistant starch in the colon and can provide less than or equal to 10% of daily total caloric intake (Mcneil, 1984; Turnbaugh *et al*., 2006). Individuals with AN have been seen to have reduced concentrations of SCFAs in faecal samples (Borgo *et al*., 2017; Morita *et al*., 2015; Speranza

et al., 2018). Additionally, cumulative studies have indicated an increase in SCFA concentrations in individuals who are overweight and obese (Fernandes *et al*., 2014; Rahat-Rozenbloom *et al*., 2014; Schwiertz *et al*., 2010; Turnbaugh and Gordon, 2009). Therefore, the gut microbiota of obese people may harvest increased energy within the gut resulting in increased SCFA levels (Turnbaugh and Gordon, 2009). Faecal SCFA concentration can be modulated by several factors including colonic SCFA absorption, colonic microbiota, colonic transit time and composition of dietary intake. A lower concentration of SCFA in faeces is also associated with a lower BMI (Shortt *et al*., 2018).

5. Psychotropic medications treatment of AN

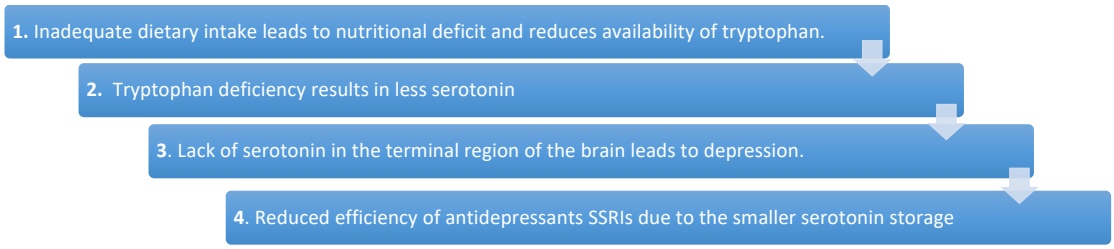
Currently, an effective pharmacotherapy in patients with AN has not been confirmed (Miniati *et al*., 2016). Antidepressants are commonly used to treat psychiatric symptoms and comorbidities such as anxiety and depression in AN patients (Becker, 2004; Kaye *et al*., 2004). However, in most patients with AN response to these treatment options is unsatisfactory (Miniati *et al*., 2016). From the perspective of pathophysiology, antidepressants are worthy of clinical research in AN as they have pharmacological effects on the serotonin system and a dysregulated serotonergic nervous system is assumed in AN patients (Phillipou *et al*., 2014). For instance, AN individuals have significantly reduced cerebral spinal fluid (CSF) basal levels of 5-HT and its metabolites (Kaye *et al*., 1984).

6. Precursors and neurotransmitters (NTs)

The effects of selective serotonin reuptake inhibitor (SSRI) are related to a significant reduction in core mental health symptoms such as anxiety, depression, behaviours of compulsions and obsession usually present in AN patients (Becker, 2004; Kaye *et al*., 2004;

Milos *et al*., 2004). However, antidepressants seem to be ineffective in treating malnourished AN patients (Attia *et al*., 1998; Ferguson *et al*., 1999). However, they may treat depressive symptoms in patients with AN and prevent relapse after weight recovery but currently available data also shows limited effectiveness of SSRIs (Barbarich *et al*., 2004; Marzola *et al*., 2015; Walsh, 2006). It can be pointed out that deficits in serotonin neurotransmission affects the mechanism of action of antidepressants (Haleem, 2012; Haleem *et al*., 2015). Hence, it is possible that inadequate levels of serotonin storage in patients with AN limits therapeutic effects (Haleem, 2016) as seen in Figure 1. Thus it is also necessary to investigate the tryptophan and serotonin administration in individuals with AN.

Figure 1. Pathway shows the effect of brain serotonin storage level on the efficacy of SSRIs



6. 1. Essential amino acid-Tryptophan

Tryptophan is a non-polar hydrophobic essential amino acid that must be provided in the diet. It has a beta carbon attached to the 3 position of an indole group. Among the 20 common amino acids, tryptophan has the largest molecular weight. It is a biosynthetic precursor of many gut microbial and host metabolites, although tryptophan is the least abundant amino acid in cells. It cannot be synthesised endogenously, therefore, it is sourced from the diet. Since the body cannot synthesise tryptophan itself, reducing dietary intake of tryptophan reduces plasma tryptophan levels. The fundamental importance of tryptophan is mainly reflected in its relationship with serotonin. Past studies have demonstrated that a direct link between circulating total tryptophan levels and serotonin production, therefore potentially

providing a marker of serotonin-related disorders in mental illness (Comai *et al*., 2016). Some researchers suggest that supplementation with tryptophan in daily diet may improve drug therapy for certain diseases. Because tryptophan has relatively low tissue storage and low concentration in the body, only a small amount is needed compared to other amino acids (Richard *et al*., 2009). The recommended tryptophan daily intake is 2.50-4.25mg/kg per day (Young, 1996), and the WHO set the recommended tryptophan intake as 4mg/kg/day, to date, no side effects of excessive intake of tryptophan have been reported.

The plasma concentration of tryptophan is directly related to dietary intake (Figure 2). After ingestion of food all amino acids, including tryptophan, are absorbed from the gut then absorbed into the blood circulation. Digested tryptophan is metabolised along three major pathways in the gut: (1) about 90% of digested tryptophan is metabolised along the kynurenine biosynthesis pathway for production of serotonin (Figure 3) (Badawy, 2017); (2) about 3% of digested tryptophan is metabolised into serotonin by the microbiota (Figure 4 and 5); (3) and the rest is degraded by the intestinal microbiota to generate indole and its derivatives (Richard *et al*., 2009) (Richard et al., 2009). Serotonin and kynurenine are important signaling molecules in gut-brain axis communication and immune response (Le Floc'h *et al*., 2011; Nguyen *et al*., 2014; O'Mahony *et al*., 2015). The GBA is a bidirectional communication network serotonin derived in the gut acts as a major signaling molecule in both the enteric nervous system (ENS) and central nervous system (CNS) as seen Table 2.

Figure 2. The role of ingested tryptophan in main three tryptophan metabolic pathways in the gut

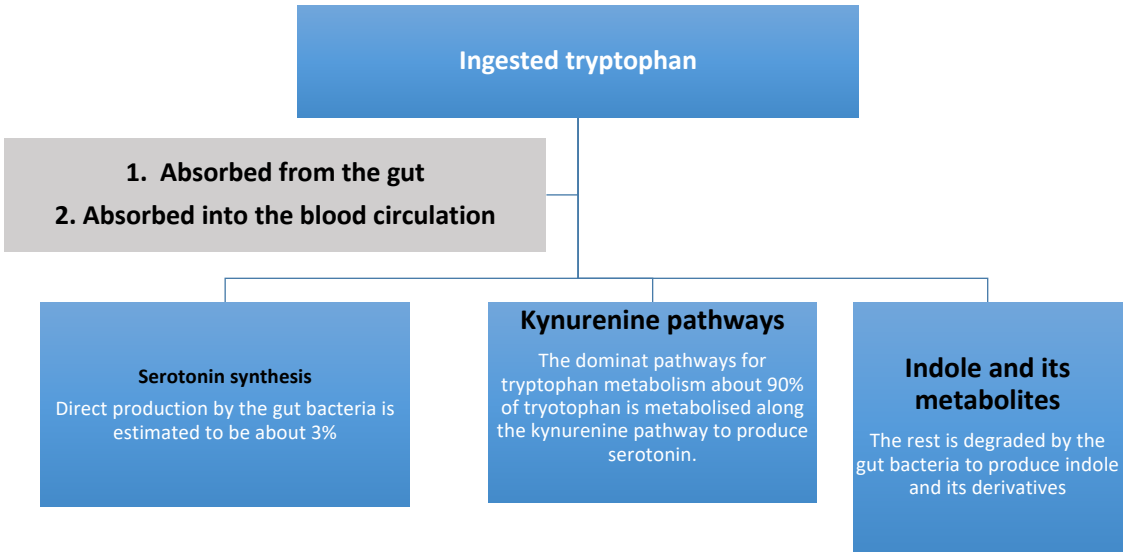
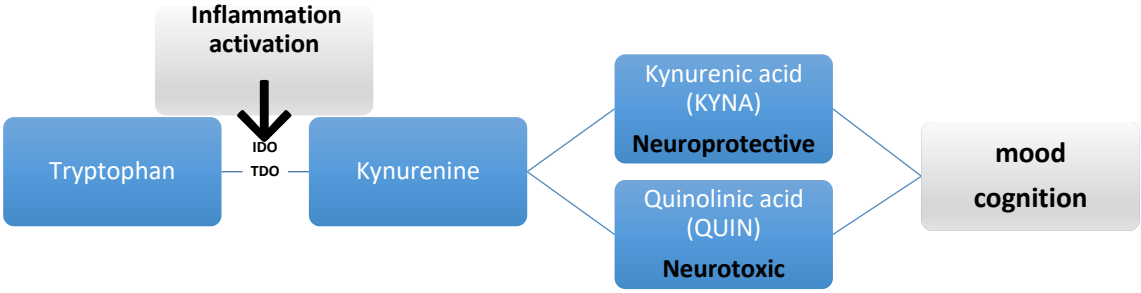


Figure 3. The Kynurenine pathway of tryptophan metabolism (Kynurenine pathway: The rate of tryptophan (TRP) metabolism along the kynurenine (TRP) pathway is mediated by the rate-limiting enzyme expression of tryptophan-2,3-dioxygenase (TDO) which is localised to the liver and indoleamine-2,3-dioxygenase (IDO) found in all tissues (Clarke *et al*., 2012). Inflammatory cytokines and glucocorticoids can be triggered by the stress response which activates the expression of IDO and TDO, activation of TDO and IDO can have an impact on the reduction of tryptophan availability, further limiting serotonin synthesis leading to interference with gastrointestinal function. Tryptophan is metabolised in the kynurenine pathways then Kynurenine is carried out along two different branches of the pathway, one leading to neuroprotective effects in Kynurenic acid (KYNA) production and one causing neurotoxic effects in quinolinic acid (QUIN) production (Kennedy *et al*., 2017). Accumulative evidence suggests that the intestinal microbiota regulates the metabolism of the kynurenine pathway, thus this is a humoral pathway through which the gut microbiota can affect cognition and mood at the central nervous system (CNS) and gastrointestinal function (O'Mahony *et al*., 2015). KYNA is also an antioxidant and its potential use in neuroprotection has been indicated for complex neurodegenerative processes such as

Parkinson's disease and Alzheimer's disease (Tajti *et al*., 2015). This beneficial action is attributed to irreversible changes in the brain caused by neuroinflammatory processes, excitotoxicity and apoptosis (Klein *et al*., 2013). Conversely, QUIN is a neurotoxic metabolite that is normally present in the brain and cerebrospinal fluid in small concentrations (nmol) but can be increased under pathological conditions (Heyes *et al*., 1996; Pérez-De La Cruz *et al*., 2012).



It has been seen that the gut microbiota can impact on quinolinic acid (QUIN) production, via production of 3-hydroxyanthranilic acid dehydrogenase; an enzyme used in the kynurenine pathway that can be of microbial origin (Kennedy *et al*., 2017). For example, supplementation of *B. infantis* to rodents resulted in anti-inflammatory effects and increased concentrations of tryptophan and kynurenic acid (neuroprotective direction) (Desbonnet *et al*., 2008). Therefore, targeting the gut bacteria could provide a strategy for increasing the availability of circulating tryptophan that affects kynurenine metabolism (Kennedy *et al*., 2017). As such, promoting different gut microbial species might improve serotonin related disease symptoms including mood, cognition, gut permeability and stress response (Claesson *et al*., 2012; Dinan and Cryan, 2013; Kelly *et al*., 2015; Yano *et al*., 2015). Serotonin does not cross the BBB, instead the gut derived serotonin exerts its actions via nerves and locally.

Figure 4. Indirect and direct gut microbial affect tryptophan and serotonin synthesis.

The gut microbiome can either indirectly or directly affect tryptophan metabolism and serotonergic signaling system within the network of the gut-brain axis to regulate host behaviour.

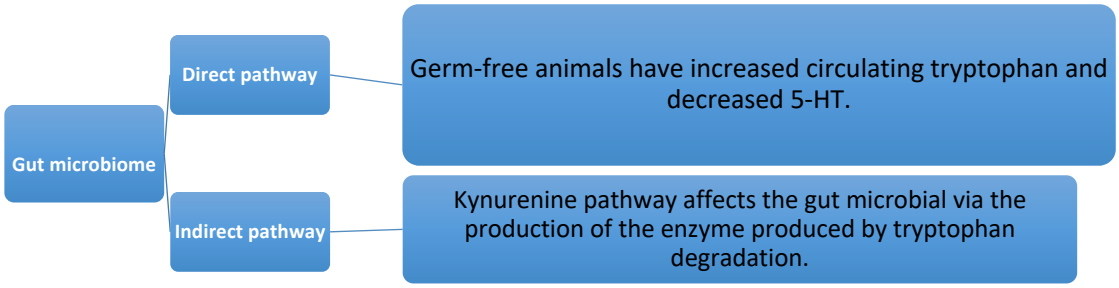


Figure 5. Serotonin synthesis pathway. Tryptophan in blood is transported into brain via a carrier, in brain neurons, hydroxylation of tryptophan converted to 5-hydroxytryptophan (5-HTP) occurs and this is the rate limiting step. Serotonin synthesis in the brain depends on the availability of its precursor tryptophan (the concentrations are at much lower levels than those found in the gut). Tryptophan can only be obtained from peripheral supply of the diet. The rate-limiting enzyme tryptophan hydroxylase (TPH) that is unsaturated at normal concentrations of tryptophan, therefore increased availability of tryptophan concentrations could be enhanced leading to increased in 5-HT synthesis. This is a catalytic biosynthesis step and the conversion of 5-HTP to 5-HT in the presence of an L-aromatic amino acid decarboxylase (L-AADC). These reactions occur both in the ENS and CNS system where serotonin modulates a number of functions including gastrointestinal, behavioural and CNS effects as seen in the table.

Table 2. Physiological effects of serotonin (5-HT) both in gut and the brain

Serotonin	**5-HT in the gut**	**5-HT in the brain**	**Disturbances 5-HT**	**References**
(5-HT)	(ENS and GI effects)	(Behavioural and CNS effects)		
Physiological effects both in the CNS and the gut	Gastrointestinal secretion; Gut motility; Intestinal transit; Pain perception; Absorption of nutrients; Vasodilatation	Mood; Anxiety; Appetite; Cognition	Anxiety; Impulse control; Appetite dysregulation; Obsessional behaviours	(Costedio *et al*., 2008) (Folks, 2004) (McLean *et al*., 2007)
Type of TPH enzyme	TpH1	TpH2		

Changes in the supply and availability of tryptophan has a number of implications for CNS and ENS functioning and thus called gut-brain axis signaling. Serotonin is produced both in the gut and the brain, and 90% of serotonin is produced in the gut particularly in enterochromaffin cells (ECs). Alteration in serotonin transmission may be due to the pathological symptom changes including some psychiatric and gastrointestinal disorders, and may indicate their high co-morbidity. Peripheral 5-HT does not cross the BBB.

6.2. Tryptophan in the modulation of serotonin synthesis pathway

Tryptophan is a sole biochemical precursor in production of neurochemically active compounds such as serotonin (Kałużna-Czaplińska *et al*., 2019). Tryptophan is an important target that plays a major role in the interaction between the gut and the brain, and alterations in brain levels of tryptophan can have a profound impact on serotonin synthesis (Haleem, 2017). Tryptophan and its derivative metabolites have a wide range of physiological properties, and there are many links between alterations in this serotonergic system and disease. For example, individuals with eating disorders and children with IBS or autism spectrum disorder have been reported to have a dysregulated serotonergic system. Accumulated studies indicate the gut microbiome is a critical component in the modulation of the brain and behaviour and this can occur via tryptophan metabolism and the serotonergic system (Haleem, 2017). It can be seen that nutrient intake is an important source which supports the tryptophan and serotonergic system.

6.3. Feeding behaviour and tryptophan in AN

Haleem and colleagues suggested that females are more susceptible to food restriction, which may begin with a long-term deficiency in levels of tryptophan and its bioavailability (Haleem, 2012). Monoaminergic NT such as 5-HT, dopamine and noradrenaline are related to feeding behaviour, while the availability of NT precursor in the blood strongly affects monoamine synthesis in the brain (Ehrlich *et al*., 2009).

For patients with AN common features include dysfunctional cognition associated with distorted self-image of shape and weight; leading to restrictive eating behaviours (Haleem, 2012; Strober *et al*., 2001). Behavioural changes observed in patients with AN present alongside a dysregulated serotonergic pathway. In patients with AN, individual serotonin levels relate to almost all behavioural changes (Haleem, 2012). Patients with AN exhibit a higher rate of compulsive movement compared to patients with bulimia nervosa (BN) (Haleem, 2012). Dysregulation of appetite and mood in individuals with AN is associated with alterations in serotonin function. Furthermore, the occurrence of neuronal serotonin dysregulation in AN promotes pre-symptoms of anxiety (Kaye *et al*., 2005; Kaye, 2008).

6.4. The proportion of TRP (Tryptophan) / LNAA (Large Neutral Amino Acids) impacts on tryptophan concentration

Tryptophan concentration is affected by the proportion of TRP (Tryptophan) / LNAA (Large Neutral Amino Acids). Briefly, carbohydrate intake leads to an insulin-mediated fall in plasma levels of the LNAA (tyrosine; phenylalanine; valine; leucine; isoleucine) which compete with TRP for uptake into the brain. The increases the plasma TRP/LNAA, and thus brain TRP, which rapidly boosts 5-HT synthesis and release in brain. Studies indicated that

malnourished AN females have a decrease of plasma TRP availability, TRP deficiency could significantly alter serotonergic neurotransmission (Figure 6). The increased proportion of Trp/LNAA is associated with relief of depressive symptoms (Gauthier et al., 2014).

Figure 6. Pathway shows the effect of dietary intake on the efficacy of serotonin neurotransmission (A lot of evidence of anxiety, dysregulated appetite, extremes of impulse control and compulsive behaviours are caused by 5-HT dysregulation within eating disorders. Enhancement of serotonin release in the brain, which can influence appetite regulation and impact on food consumption, relying on the amount of carbohydrate and protein in the meal. Carbohydrate consumption results in a reduction of the large neutral amino acids (LNAA). For example, a reduction in plasma ratio of Trp/LNAA and tryptophan concentrations have been observed in patients that are acutely underweight (Comai *et al*., 2010; Ehrlich *et al*., 2009; Gauthier *et al*., 2014). In contrast, an enhanced protein diet can block those effects, leading to the large amounts of LNAA (Fernstrom and Faller, 1978; Kaye *et al*., 2005; Kaye, 2008).

7. Microorganism-derived NTs

Gut microbiota has recently been regarded as a considerable exogenous source of NTs, symbionts living in the GI tract have been observed to actively promote the production of NTs, which may have an effect on the nervous system (Lyte, 2011). It could be that secreted NTs from gut bacteria in the gut lumen may induce epithelial cells to release molecules, thereby regulating neural signalling in the nervous system (Forsythe *et al*., 2014). *Bifidobacterium* and *Lactobacillus* species were found in the human intestine that are able to produce Gamma-Aminobutric acid (GABA). In particularly, the specific strain of

Lactobacillus brevis DPC6108 has a 100% conversion MSG to GABA showing considerable GABA production capacity (Barrett *et al*., 2012; Xu *et al*., 2017).

7.1. Gamma-Aminobutric Acid (GABA)

GABA is an important inhibitory NT in the CNS involved in many physiological and psychological processes. Dysregulated GABA signalling has been associated with mental illness including depression and anxiety (Schousboe and Waagepetersen, 2007). GABA also plays a role in gastrointestinal motility from the stomach to the ileum and the peristaltic reflex in the colon (Auteri *et al*., 2015). GABA can be synthesised from the amino acid glutamate in the catalytic reaction by the action of L-glutamic acid decarboxylase (GAD) (Figure 4). Some studies found that several bacteria including a number of *Lactobacillus* and *Escherichia coli* can provide enzyme GAD to convert glutamate to GABA (Nomura *et al*., 1998; Yokoyama *et al*., 2002). Studies have indicated that human intestinal-derived strains of *Bifidobacterium* and *Lactobacillus* produce GABA from monosodium glutamate (MSG) in culture (Barrett *et al*., 2012), and it has been shown that GABA produced by commensal bacteria may have an impact on the GBA (Bienenstock *et al*., 2010). Additionally, compared to Ex-GF (Ex germ free) and GF-mice (germ free), in the former GABA levels in the gut lumen were significantly higher than the latter, indicating that GABA produced by bacteria seems to occur naturally under physiological conditions (Matsumoto *et al*., 2013). Hence, GABA locally produced by resident gut microbiota may make an important contribution to interactive signals in the gastrointestinal tract (Chevrot *et al*., 2006). The receptors gene expression can be altered by *Lactobacillus*, for example, intake of *Lactobacillus rhamnosus* (JB-1) altered the mRNA expression of GABAA and GABAB receptors. These receptors are associated with depression and anxiety and are widely expressed in brain regions that maintain fear and emotional response (Bravo *et al*., 2011).

In brief, there may be two potential pathways for increasing a person's GABA yields. One approach is via lactic acid bacteria (LAB) that can produce GABA within food (Siragusa *et al*., 2007). GABA-producing LAB have been isolated from food such as fermented dairy products (Inoue *et al*., 2003; Nomura *et al*., 1998). For example, GABA can be generated by cheese starters during cheese ripening (Siragusa *et al*., 2007). Thus, elevated GABA levels in humans is can be achieved by consuming GABA-rich foods. Another way to increase GABA levels in the gut may be utilise the commensal gut bacteria or ingesting probiotics that can act on dietary monosodium glutamate (MSG) to produce GABA. These approaches are highlighted in Figure 7 (Lyte, 2011). Strains of *Bifidobacterium* and *Lactobacillus* can be regarded as natural residents of the intestine, which can enhance GABA concentration (Barrett *et al*., 2012).

Figure 7. GABA-producing pathway (Decarboxylation of glutamate to GABA)

7.3. Catecholamines

Catecholamines including dopamine (DA), norepinephrine (NE) and epinephrine (EPI) are derived from amino acid tyrosine. DA is degraded into EPI and NE. All three NTs have been identified as playing a crucial role in maintaining the GI tract, including nutrient absorption, innate immune function and gut motility (Mittal *et al*., 2017). Several bacteria have been shown to produce CAs (Özoğul, 2004; Shishov *et al*., 2009; Tsavkelova *et al*., 2000). A previous *in vitro* study demonstrated that relatively high concentrations of NE were harboured in the biomass of *E. coli*, *Serratia marcescens*, *Bacillus subtilis* (Tsavkelova *et al*., 2000). An animal study indicated that microorganisms are involved in NE production in the

lumen, compared to wild-type mice, germ free mice harboured decreased NE levels in the lumen and tissue, whilst NE could be recovered via a mixture of 46 *Clostridium* species or colonisation with a microbiota (Asano *et al*., 2012).

NTs levels related to human sites of the body are shown in Table 3.

Table 3 NT levels reported in healthy individuals (Dugdale, 2021; Haldeman *et al*., 2021).

NTs	Blood (ng/ml)	Urine (ng/24hrs)	Faecal (ng/g)
5-HT (95% produced in gut)	101 to 283	2000000 to 9000000	-
GABA	400543	Morning: 0.345 Evening: 0.271	0 to 330000
DA (50% produced in gut)	0.03	65000 to 400000	-
NE	0.07 to 1.7	15000 to 80000	-
EPI	0 to 0.14	500 to 20000	-

8. Appetite hormones

Many studies indicate a key role for gut microbiota in regulating host central appetite and food intake (Alcock et al., 2014; Fetissov et al., 2017; Torres-Fuentes et al., 2017). Due to the effect of the altered composition of gut microbiota and its metabolites on host energy metabolism and eating-related behaviour, thus metabolic dysregulation and diet-related disorders may be established/exacerbated such as AN (Le Chatelier *et al*., 2013; Turnbaugh and Gordon, 2009; Morita *et al*., 2015; Mack *et al*., 2016). A low energy state caused by insufficient calorie and fat intake resulting in low available energy, impacting on the microbiota and is also damaging to the endocrine system. These disturbances can have deleterious effect on neuropsychiatric comorbidities and bone health due to the dysregulation of HPA axis hormones and appetite-regulated hormones (Löwe *et al*., 2001). Dysregulated hormones including oestrogen, androgen, cortisol, leptin, PYY and oxytocin levels are associated with eating disorder psychopathology, neurocognition, anxiety and depression (Lawson and Klibanski, 2008; Lawson *et al*., 2011; Lawson *et al*., 2013; Miller *et al*., 2007).

Depression and anxiety measures were also positively correlated with levels of serum cortisol and negatively correlated with serum leptin levels (Lawson and Klibanski, 2008; Lawson *et al*., 2012). Psychopathology of eating disorders measured in patients with AN have been positively correlated with PYY levels and serum cortisol, independent of BMI, and negatively correlated with elevated serum leptin levels (Lawson *et al*., 2011). Appetite hormone changes in AN are shown in Table 4. The following section will emphasise the role of gut microbiota on appetite hormones. It can be observed that some of the changes, e.g. increased ghrelin, would be associated with a greater appetite, but this in parallel with elevated PYY (increased satiation) demonstrates the dis-balance that occurs with these hormones in AN.

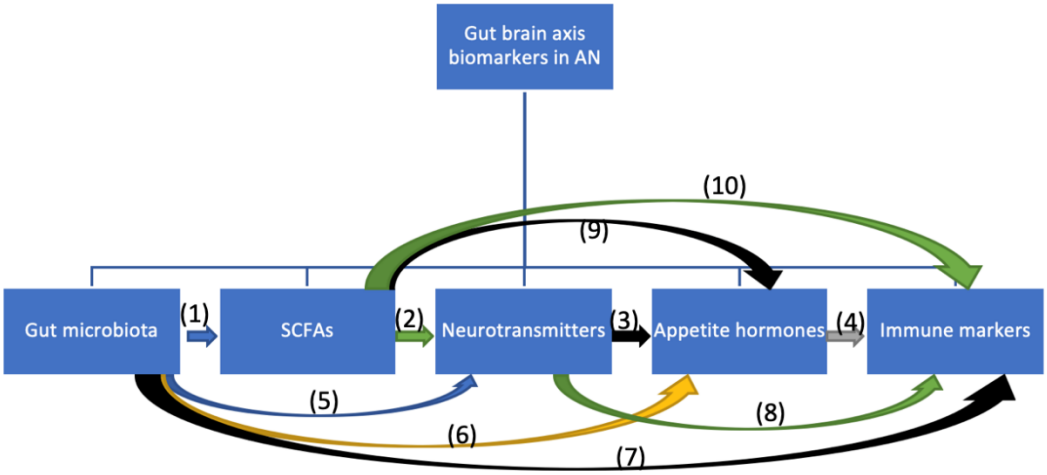
Table 4. Representative gastrointestinal hormones linked to the psychopathology of AN individuals

	Difference in patients with AN compared with healthy controls	**Type of hormone and secretion site**	**Major regulatory activity**	**Physiological relevance**	**Reference**
Cortisol	Decreased	Gluconeogenic	Stress response Menstrual recovery Host metabolism Anti-inflammatory	Elevated cortisol secretion is inversely correlated with fat mass and BMI. Cortisol and growth hormone may together maintain euglycemia in AN	(Misra *et al*., 2006)
Leptin	Decreased	Anorexigenic hormones	Appetite regulation	• Reduction in leptin is associated with a reduction in subcutaneous fat, which is an appropriate adaptive response to starvation and reduced appetite suppression. • Satiety hormone leptin was related to an increased number of *Bifidobacterium* and *Lactobacillus* and a decreased number of *Prevotella* and *Bacteroidetes*. • Associated with NT levels	(Queipo-Ortuño *et al*., 2013)
Ghrelin	Increased	Orexigenic hormone Appetite-stimulating peptide secreted in stomach	Increase appetite and growth hormone secretion	• Higher ghrelin associated with lower BMI and fat mass in patients with AN • Induces hunger • Ghrelin is involved in regulation of the dopaminergic reward system. • Decreased intestinal permeability	(Misra *et al*., 2006) Méquinion (Méquinion *et al*., 2013)
PYY	Increased	Anorexigenic hormone secreted by L or endocrine cells in distal gut	Reduces gastric empting, pancreatic exocrine, and delays intestinal transit	• PYY levels are inversely correlated with fat mass, body weight and BMI. • Inhibit gastric motility and improve glucose homeostasis • Induce satiety	(Misra *et al*., 2006) Painsipp (Painsipp *et al*., 2011)

				• Associated with increased anxiety and depression	
GLP-1	Increased	Incretin hormone Upper intestine	• Inhibits glucagon and stimulates insulin and gastric emptying • By activating GLP-1 receptors involved in body weight, food intake, regulation of the HPA axis and neuromodulation of the overall response to stress	• Increase anxiety-related behaviour • Regulate small intestine transit time • Inhibit gastric motility and improve glucose homeostasis • Induce satiety and reduce energy intake	(Kinzig *et al*., 2003) (Ghosal *et al*., 2013)
Oxytocin			Anxiolytic and antidepressant effects	Eating disorder psychopathology, depression and anxiety	(Lawson and Klibanski, 2008) (Lawson *et al*., 2013)

9. Biomarkers linked with AN and associated GBA communication

Figure 8. There are five potential GBA biomarkers linked with AN individuals including gut microbiota, SCFAs, NTs, appetite hormones and immune markers. Ten routes of communication are (1) Gut microbiota effects on SCFAs regulation; (2) SCFAs effects on NTs regulation; (3) NTs effects on appetite hormones regulation; (4) Appetite effects on immune markers regulation; (5) Gut microbiota effects on NTs regulation; (6) Gut microbiota effects on appetite hormone regulation; (7) Gut microbiota effects on immune markers regulation; (8) NTs effects on immune markers regulation; (9) SCFAs effects on appetite hormone regulation; (10) SCFAs effects on immune markers regulation.



9.1. Gut microbiota effects on SCFAs regulation

The three most abundant SCFAs detected in faeces are acetate, butyrate, and propionate (Macfarlane *et al*., 1992). Within the gastrointestinal tract, butyrate is produced by Firmicutes including *Faecalibacterium prausnitzii* and Lachnospiraceae, and predominant propionate producers are *Bacteroides* species, *Clostridium* species and Negativicutes (Vital *et al*., 2014).

When considering the brain function neuroinflammation is a crucial factor. Some studies have indicated that gut microbiota perturbations by antibiotics systemically produce altered

immune responses, these are pro-inflammatory profiles (Erny *et al*., 2015). In the CNS, inflammatory responses are activated when the microbiota is depleted by antibiotic (Stanisavljević *et al*., 2019). It was shown that neuroinflammation triggered alteration of microglial morphology affected by antibiotic-induced disturbances in gut microbial community (Jang *et al*., 2018; Minter *et al*., 2016; Minter *et al*., 2017). In addition, studies have shown that sodium butyrate is capable of inhibiting microglial activation and secretion of pro-inflammatory cytokines (Patnala *et al*., 2017; Yamawaki *et al*., 2018). For instance, sodium butyrate inhibits lipopolysaccharide-induced depression-like symptoms via microglial activation in mice (Yamawaki *et al*., 2018). Therefore, considering the role of microglia in shaping neuronal networks and the impact of the microbiota on this process, SCFAs might provide new clues to regulate the immune-system dis-regulation associated neurodegenerative and neurodevelopmental disorders (Silva *et al*., 2020).

9.2. SCFAs effects on NTs regulation

Gut microbiota acting through SCFAs can upregulate enteric 5-HT production and homeostasis by the enterochromaffin cells (ECs) (Reigstad *et al*., 2015). 5-HT synthesis is regulated by tryptophan hydroxylase (TPH) that is a rate-limiting enzyme that participates 5-HT synthesis (O'Mahony *et al*., 2015). A study showed both mouse and human derived gut microbiota promote colonic TPH expression and 5-HT amounts through SCFA activities on EC cells (Reigstad *et al*., 2015). Also, SCFAs have been observed to increase DA synthesis through tyrosine hydroxylase, a major enzyme in catecholamine synthesis (Shah *et al*., 2006).

9.3. NTs effects on appetite hormone regulation

Accumulated studies have indicated that NTs participate in hypothalamic appetite regulation (Meister, 2007; Schwartz, 2000; Saper *et al*., 2002) (Saper et al., 2002; Van et al., 2003; Schwartz et al., 2000; Meister et al., 2007). DA and 5-HT are essential NTs in the regulation of food intake; alteration in amounts of DA and 5-HT in the ventromedial nucleus (VMN) and lateral hypothalamic area (LHA) relate to the impact on food choice including meal size and meal number (Meguid *et al*., 2000). Levels of NE within the brain may directly alter the leptin activity. There appears to be an inverse relationship between NE and leptin activity. Enhanced leptin may decrease NE activity to induce satiety, whereas the absence of leptin may stimulate increased NE secretion and subsequently to release hunger signals (Wellman, 2000).

9.4. Appetite hormones effects on immune markers regulation

There is still limited research focusing on appetite hormone effects on immune markers. Sepsis has been observed to reduce levels of ghrelin, as such a rat study performed caecal ligation on males to administer sepsis. Subsequently ghrelin was infused. The ghrelin infusion restored brain levels and attenuated gut barrier dysfunction through high mobility group B1 (HMGB1) serum levels and the vagus nerve (Wu *et al*., 2009). Therefore, satiety hormones may also impact on gut barrier function and subsequently immune function. This closely linked network is likely to impact on many illnesses, and AN is a good target, as this condition is highly linked to diet.

9.5. Gut microbiota effects on NTs regulation

The gut microbiota has been related to the production of not only SCFAs but also gut microbial-derived NTs including 5-HT, GABA, DA, NE and EPI (Clarke *et al*., 2013; Lyte, 2011; Walls *et al*., 2009). There have been studies on the potential role of gut microbiome on host NTs and their related pathways with outcomes for behaviour and host physiology. For instance, compared to specific pathogen free (SPF) mice, germ-free mice have decreased 5-HT receptors and circulating 5-HT in the hippocampus and this is accompanied by altered anxiety-like behaviour (Neufeld *et al*., 2011). Some studies have indicated altered concentrations of NTs in Germ free mice following supplementation with defined gut bacteria (Sampson *et al*., 2016; Yano *et al*., 2015). DA and 5-HT are reported to be produced by several gut bacteria and this is likely to have an impact on the brain, as the total concentration of tryptophan, glutamine and tyrosine in the brain of germ-free mice is lower than the mice with gut microbiota re-colonisation (Matsumoto *et al*., 2013), whilst strains of *Bifidobacterium* and *Lactobacillus* can be regarded as natural residents of the intestine with beneficial impacts on GABA production (Barrett *et al*., 2012; Pokusaeva *et al*., 2017; Siragusa *et al*., 2007; Strandwitz *et al*., 2019). The gut microbiota is considered to be a modulator of NT levels, which then operate through the gut-brain axis (VN pathway). The variation in gut microbiota associated communication in gut-microbiota-brain axis has been implicated in aspects of physiological and psychological including neurologic, immunologic and psychiatric conditions (Collins *et al*., 2012; Foster and Neufeld, 2013; Mayer, 2011). For example, variation in the gut microbial community has been reported in CNS disorders, including autistic spectrum disorders (ASD), anxiety and depression (Borre *et al*., 2014). Neurological diseases such as depression (decreased serotonin and catecholamines), Parkinson's (decreased dopamine), insomnia and anxiety (decreased GABA) are linked to deficits in certain NTs (Collins *et al*., 2012; Desbonnet *et al*., 2015; Logan and Jacka, 2014;

O'Mahony *et al*., 2015; Sugama and Kakinuma, 2016). The changes observed in mental well-being may therefore be caused by signal transduction from the intestine to the brain (Kennedy *et al*., 2014).

9.6. Gut microbiota effects on appetite hormones regulation

Gut microbiota plays a key role in host appetite control via gut hormone regulation (Breton et al., 2016a). Gut commensal *Escherichia coli* are capable of producing ClpB as has been stated in previous section. Additionally, ghrelin has been implicated in the regulation of food intake and energy homeostasis in mammals (Nakazato *et al*., 2001; Wren *et al*., 2001). Galactooligosaccharides (GOS) are enzymatically produced from lactose by the food industry. They are widely used in infant nutrition formulations to mimic the biological functions of human milk oligosaccharides, such as effects on gut microbiota and the immune system (Underwood *et al*., 2015). GOS escapes digestion and absorption due to lack of the appropriate digestive enzymes in the small intestine. After arriving in the colon, GOS are metabolised by resident microbial species (Williams and Jackson, 2002). A study in 2013 confirmed that GOS-fed mice displayed increased gene expression of satiety-related peptides, so it was observed gut microbiota regulated by prebiotics had enhanced genes of GLP-1 precursor proglucagon expression by 3.5-fold and PYY (1.5 fold) in the colonic mucosal (Overduin *et al*., 2013). Accumulating evidence suggests that gut microbiota composition can help in regulation of appetite hormone.

9.7. Immune markers effects on gut microbiota and NTs regulation

Accumulated animals studies emphasised that a high fat diet leads to alternations in gut microbiota composition and enhanced intestinal permeability (Moreira *et al*., 2012; Serino *et*

al., 2012; Seki and Schnabl, 2012). In a murine study a high fat diet led to higher Firmicutes phylum and lower Bacteroides in faeces, along with higher pro-inflammatory cytokines such as TNF-a, IL-1b and IL-6 in plasma (Cani *et al*., 2007a; Cani *et al*., 2007b; Chen *et al*., 2011; Kim *et al*., 2012; Neyrinck *et al*., 2011; Park *et al*., 2019; Pyndt Jørgensen *et al*., 2014). Currently, there is no research involving immune-neurotransmitter interaction in AN individuals. However as diet can impact on NTs as high fat induced inflammation leads to negative effects on cognition and behaviour via dysregulated neurotransmission (Sen *et al*., 2017). As such, changes in dietary regimens are likely, through the microbiota, to impact on the brain.

9.8. *SCFA effects on appetite hormones regulation*

SCFA are involved in regulating the expression of appetite hormones and energy homeostasis (Byrne *et al*., 2015). It has been reported that propionate stimulates both PYY and GLP-1 secretions from wild type primary murine colonic crypt cultures (Psichas et al., 2015). As such there are communication pathways among the gut microbiome, SCFA and anorexigenic/orexigenic hormones, which could have potential for normalising the satiety hormone levels in AN and therefore having a potential role in therapeutic feeding regimes.

Propionate is an energy source for the epithelial cells, also transferred to the liver where it plays a role in gluconeogenesis (IGN) and thus is considered beneficial for glucose and energy homeostasis. A murine study indicated that the higher physiological levels of propionate significantly stimulated anorexigenic hormones including GLP-1 and PYY

(Psichas *et al*., 2015), suggesting that increased levels of SCFA would stimulate gut hormone profiles thus impacting on appetite.

9.9. *SCFAs effects on immune markers regulation*

A study has reported that patients with AN have increased pro-inflammatory cytokines levels in plasma, these are IL-6, IL-17 and TNF-α when compared to healthy controls (Roubalova *et al*., 2021). SCFA especially butyrate, as the main energy source of colonocytes, supports gut barrier function and exerts anti-inflammatory effects (Blaak *et al*., 2020; Machate *et al*., 2020). For example, TNF-α production induced by stimuli in vitro could be suppressed by acetate and butyrate (Liu *et al*., 2012; Segain *et al*., 2000; Usami *et al*., 2008; Vinolo *et al*., 2011). An in vitro study indicated that butyrate could inhibit proinflammatory cytokines by restricting lipopolysaccharide-induced nuclear factor-jB activity (Liu *et al*., 2012; Segain *et al*., 2000; Usami *et al*., 2008; Vinolo *et al*., 2011).

10. BBB and AN

BBB is a selective protective barrier composed of cell appendages that surround each individual blood vessel and capillary in the brain and is located between the peripheral blood system and cerebral blood system within a tight junction. The main role of barrier is designed to keep pathogens out while simultaneously allowing dietary nutrients to move by facilitated diffusion (Logsdon *et al*., 2018). Therefore, BBB manages molecules and substances from peripheral blood into the brain, thus BBB is an important factor in the bioavailability of essential molecules to the brain, especially molecules that the brain cannot synthesise. Some studies have indicated stress and obesity seem to be related to increased permeability of BBB

(Banks *et al*., 2004; Banks, 2008; Esposito *et al*., 2002). This relates well to the fact that gut microbiota have been reported to regulate tight junctions, enterically and also in the brain. As such alterations in the microbiota can support the BBB. Proinflammatory cytokines have been associated with AN and their involvement may depend on their ability to cross the BBB and modify its function (Banks *et al*., 2002). For example, inflammation status induced by iron deficiency in AN patients severely alters BBB transport of amino acids, insulin, glucose, and NE (Ben-Shachar *et al*., 1988). Some studies also indicate that the BBB blocks amino passage into the CNS under physiological conditions, indeed glutamate has been reported not to cross this barrier (Janik *et al*., 2016; Mazzoli and Pessione, 2016; Moloney *et al*., 2016). 5-HT is also reported to be unable to cross the BBB, however, tryptophan once in the bloodstream, can cross the BBB and participate in 5-HT synthesis in the CNS (O'Mahony 2015). There are some metabolites and amino acids that can pass through the BBB are shown in Table 5.

Table 5 There are some metabolites and NTs that can pass through the BBB and can improve the cognitive function & anxiety & depression

	Can cross the BBB	**Physiological and psychological effects**	**References**
Microbial metabolites	SCFAs	SCFA are involved in regulating the expression of neuropeptides such as PYY and ghrelin and are associated with antidepressant effects.	(Banks, 2008) (Erny *et al*., 2015) (Karuri *et al*., 1993) (Rudzki *et al*., 2019)
Gut hormones	Ghrelin	Decreases intestinal permeability	(Banks *et al*., 2004) (Bansal *et al*., 2010)
Amino acids	Aromatic amino acid (AAA) such as tryptophan and tyrosine; Glutamate	AAA are transported across the BBB by a competitive transport mechanism. For example, there is a competition between tryptophan and other amino acids to transport across the BBB.	

11. Treatment for AN patients

11.1. Nutritional and weight restoration treatment

Weight restoration and nutritional rehabilitation is the first line of treatment for AN patients with malnourished and underweight, with the aim of restoring psychological and physiological functions by reversing malnutrition (Aigner *et al*., 2011; American Psychiatric Association, 2013; Hay *et al*., 2014). Weight restoration can be beneficial for the cognitive function improvement and the effectiveness of psychological interventions (Marzola *et al*., 2015). Additionally, malnutrition in AN patients may exacerbate the main symptoms of depression and anxiety, these symptoms can be improved or reversed after weight restoration and nutritional rehabilitation (Brown and Mehler, 2015). The dietary composition has an impact on composition of gut microbiota and microbial metabolites. Feeding regimens of low fibre or fibre-free and resistant starch limit the availability of energy substrates for gut microbes; abnormal fluid secretion caused by enteric formulation infusion into the colon can also have deleterious effects on gut microbes (O'Keefe, 2010; Whelan *et al*., 2004). For example, an increase in *Ruminococcus* levels after weight gain is correlated with high intake of resistant starch and fibre (Kleiman *et al*., 2015; Mack *et al*., 2016). A diet with high fibre and high fat may potentially improve gastrointestinal symptoms as the increase of dietary substrates could create good conditions for higher abundance of carbohydrate utilising taxa (Mack *et al*., 2016). A diet plan of two weeks fibre-free provided to healthy volunteers has shown a reduction in total faecal bacteria and SCFAs (Whelan *et al*., 2005). The institutionalised programs of nutritional rehabilitation adaptation of AN patients indicate that gut microbial composition and diversity are altered by refeeding but still significantly different from healthy control groups (Kleiman *et al*., 2015; Mack *et al*., 2016). One study showed an increase the numbers of *Bacteroides* after fibre supplements. Fibre intake influences the composition of gut microbiota and its metabolites are related to positive effects

on gut barrier function in humans (Shortt *et al*., 2018). Dietary macronutrients and micronutrients have been widely found to affect the gut microbiota composition and diversity in the host are shown in Table 6.

Table 6 The impact of macronutrient and micronutrient intake on the gut microbiota (Ramos and Martín, 2021).

Carbohydrates		**Proteins**		**Fats**		**Others**
Microbiota accessible carbohydrates	**Oligosaccharides**	**Animal proteins**	**Plant proteins**	**Saturated fats**	**Unsaturated fat**	**Vitamins**
Increased	**Increased**	**Increased**	**Increased**	**Increased**	**Increased**	**Increased**
Bifidobacterium	*Firmcutes*	*Enterococcus*	*Bifidobacteriaceae*	*Bilophyla*	*Bifidobacterium*	*Bifidobacterium*
Lactobacillus	*Proteobacteria*	*Streptococcus*	*Desulfovibrionaceae*	**Decreased**	*Roseburia*	*Lactobacillus*
Akkermansia	*Bacteroides*	*Peptostreptococcus*	*Latobacilaceae*	*Bifidobacterium*	*Fecalibacterium*	*Bacteroidetes*
Fecalibacterium	**Decreased**	**Decreased**	*Erysipelotrichaceae*	*Bacteroidetes*		*Parabacteroides*
Bacteroides	*Bacteroides*	*Bifidobacterium*		*Prevotella*		
Prevotella	*Verrucomicrobia*			*Lactobacillus*		
Roseburia						
Rumincoccus						
Clostridium						

11.2. Prebiotics, probiotics and psychobiotics

Probiotics refer to strains of live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Pre and probiotics that work by targeting the gut to modulate neurological and psychiatric disorders have been termed psychobiotics (Dinan and Cryan, 2013), examples of studies involving these are summarised in Table 7 have introduced the psychobiotic concept for the treatment of various neurological and psychiatric disorders through targeting of the gut microbiota. Psychobiotics are defined

as microbiota-targeted interventions such as 'beneficial bacteria (probiotics) or support for such bacteria (prebiotics) that impact on bacteria–brain relationships' (Sarkar *et al*., 2016). Psychobiotics could function through SCFA production, production of neuroactive substances such as GABA and 5-HT, the management of the immune system and regulation of cytokine, the reinforcement of intestinal barrier function (Dinan and Cryan, 2013; Kho and Lal, 2018).

As such, the studies mentioned indicate the microbiota holds great potential for influences the GBA, impacting on neuropathologies and also appetite. Therefore, there is a chance that gut microbial modulation could positively impact on eating disorders such as AN.

Table 7 Psychobiotic interventions on Gut brain axis related mental disorders

Study title and author	Population	Study design	Psychobiotics	Methods	Results
(Tomasik *et al*., 2015)	Schizophrenia and schizoaffective (total n=58; probiotic: n=31; placebo: n=27)	Randomized, placebo- controlled trial	Probiotic: *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis subsp. lactis* BB-12	Schizophrenia and schizoaffective patients randomly received the probiotic supplements or placebo for 14 weeks.	(1). Compared to the placebo, probiotic treated had significantly reduced levels of pro-inflammatory biomarkers related von Willebrand factor (vWF), and significantly increased levels of anti-inflammatory biomarkers, these are monocyte chemotactic protein-1 (MCP-1), brain-derived neurotrophic factor (BDNF), RANTES, and macrophage inflammatory protein-1 beta (MIP-1). (2). Probiotic treated alterations are related to immune and intestinal epithelial cells regulation via the IL-17 family of cytokines.
(Akkasheh *et al*., 2016)	Major depressive disorder (MDD) (total n=40; probiotic: n=20; placebo: n=20)	Randomized, double-blind, placebo-controlled clinical trial	Probiotic: *Lactobacillus acidophilus, Lactobacillus casei,* and *Bifidobacterium bifidum* intervention for 8 weeks	Patients were randomly allocated into two groups to receive either probiotic supplements or placebo for 8 weeks.	(1). Patients who took probiotic supplements had significantly decreased Beck depression inventory (BDI) total scores when compared to the placebo; (2). Compared to placebo, significant decreases in serum insulin levels and serum hs-CRP concentrations.
(Kazemi *et al*., 2019)	Major depressive disorder (MDD) (total n=110; probiotic: n=38; placebo: n=36; prebiotic: n=38)	Randomized, double-blind, placebo-controlled clinical trial	Probiotic: *L. helveticus R0052* and *B. longum R0175* Prebiotic: Galactooligosaccharides (GOS)	Patients randomly received the probiotic and prebiotic or placebo for 8 weeks.	(1). Compared to placebo and prebiotic, probiotic supplementation resulted in a significant decrease in BDI score. (2). Inter-group comparison indicated no significant differences among the groups in terms of serum kynurenine/tryptophan ratio and tryptophan/BCAAs ratio. (3). Kynurenine/tryptophan ratio decreased significantly in the probiotic group compared to the placebo group

					(4). Tryptophan/isoleucine ratio increased significantly in the probiotic group when compared to the placebo group
(Majeed *et al*., 2018)	MDD in irritable bowel syndrome (IBS) patients (total n=40; probiotic: n=20; placebo: n=20)	Randomised, double- blind, placebo controlled, multi-centre, pilot clinical study	Probiotic: *Bacillus coagulans* MTCC 5856	MDD in IBS patients randomly received probiotics and placebo for 90 days.	Probiotic treated MDD in IBS patients showed a significant improvement in the scores of Hamilton Rating Scale for Depression (HAM-D), Montgomery-Asberg Depression Rating Scale (MADRS), Center for Epidemiological Studies Depression Scale (CES-D) and Irritable bowel syndrome quality of life questionnaire (IBS-QOL) when compared to the placebo. (2). Compared to placebo group, probiotic treated MDD in IBS patients showed a significant improvement in the scores of Clinical Global Impression- Improvement rating Scale (CGI-I), Clinical Global Impression Severity rating Scale (CGI-S), Gastrointestinal Discomfort Questionnaire (GI-DQ) and Modified Epworth Sleepiness Scale (mESS). (3). Compared to the placebo group, probiotic significant reduced inflammatory biomarker.
(Partty *et al*., 2015)	Attention deficit hyperactivity disorder (ADHD)and Asperger syndrome (AS) (total n=75; probiotic: n=40; placebo: n=35)	Randomized, double-blind, placebo-controlled clinical trial	Probiotic: *Lactobacillus rhamnosus* GG	75 infants randomly received probiotics or placebo during the first 6 months of life were followed-up for 13 years	(1). At the age of 13 y, ADHD or AS was diagnosed in 6/35 (17.1%) children in the placebo and none in the probiotic group. (2). The numbers of *Bifidobacterium* in faecal during the first 6 months of life was lower in affected children than in healthy children.
(Akbari *et al*., 2016)	Alzheimer's disease (AD) (total n=60; probiotic: n=30; placebo: n=30)	Randomized, double-blind, and controlled clinical trial	Probiotic: *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum,* and *Lactobacillus fermentum*	60 AD patients randomly received probiotics and control (milk) for 12 weeks.	(1). Probiotic treated AD patients showed a significant improvement in the Mini-mental state examination (MMSE) score when compared to the control. (2). Levels of plasma, homeostasis model of assessment-estimated insulin resistance, serum high-sensitivity C-reactive protein, Beta cell function, serum triglycerides, quantitative insulin sensitivity check index were significantly varied in the probiotic treated group when compared to the control. (3). Probiotic consumption positively affect metabolic statuses and cognitive function.

(Rudzki *et al*., 2019)	MDD patients taking SSRIs (total n=79; probiotic: n=40; placebo: n=39)	Randomized, double-blind, and controlled clinical trial	Probiotic*: L. plantarum* 299v (10×10^9 CFU/capsule)	79 patients randomly received probiotics and placebo for 8 weeks.	Significant improvements in cognitive functions were observed: Work Speed in Attention and Perceptivity Test, California Verbal Learning Test (CVLT) total recall of trials 1–5.
(Wallace *et al*., 2020)	MDD patients (total n=12)	Pilot, open-label study	Combination of *L. helveticus Rosell*®-52 and *B. longum Rosell*®-175 (3×10^9 CFU/day)	Patients received probiotics for 8 weeks.	Significant improvements in mood: MADRS, Quick Inventory of Depressive Symptomatology 16-item self-report (QIDS-SR16), anhedonia: Snaith Hamilton Pleasure Scale (SHAPS), anxiety: Generalized Anxiety Disorder 7-item scale (GAD-7), State-Trait Anxiety Inventory (STAI), and subjective sleep quality: Pittsburgh Sleep Quality Index (PSQI).
(Tian *et al*., 2022)	Mild to moderate IBS and MDD (total n=45)	Placebo-controlled, double-blind randomized controlled trial	*Bifidobacterium breve* CCFM1025 (total CFU 10^{10}) (1 sachet per day)	Patients received probiotics for 4 weeks.	(1). Better antidepressant-like effect. (2). Reduced gastrointestinal symptoms
(Okubo *et al*., 2019)	Outpatients with schizophrenia with anxiety and depressive symptoms (total n=29)	Open-label single-arm study	*Bifidobacterium breve* A-1 (5.0×10^{10} CFU) (2 sachets per day)	Patients received probiotics for 8 weeks.	Potential effect in improving anxiety and depressive symptoms.
(Dao *et al*., 2021)	Participants with symptoms suggesting anxiety/depression (total n=83)	Single-center uncontrolled trial	*Bifidobacterium bifidum W23*, *Bifidobacterium lactis W52*, *Lactobacillus acidophilus W37*, *Lactobacillus brevis W63*, *Lactobacillus casei W56*, *Lactobacillus salivarius W24*, *Lactococcus lactis W19*, and *Lactococcus lactis W58* (over 2.5×10^9 CFU/g) (1 sachet per day)	Participants received probiotics for 8 weeks.	Anxiety and depression symptoms significantly improved.
(Heidarzadeh-Rad *et al*., 2020)	Participants with low to moderate depression (total n=78)	Double-blind, randomized controlled trial	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 ($\geq 10 \times 10^9$CFU) (1 sachet per day)	Participants received probiotics for 8 weeks.	Improved depression symptoms
(Alli *et al*., 2022)	Participants with a mild or moderate depressive (total n=119)	Randomized placebo-controlled study	*Lactobacillus casei* PXN 37, *Lactobacillus plantarum* PXN 47, *Lactobacillus rhamnosus* PXN 54, *Lactobacillus acidophilus* PXN 35, *Lactobacillus bulgaricus* PXN 39, *Lactobacillus helveticus* PXN 45,	Participants received probiotics for 6 weeks.	(1). Reduction in depression symptoms. (2). Decrease in the levels of cortisol, dopamine, Il-6 and TNF-α

			Lactobacillus salivarius PXN 57, *Lactobacillus fermentum* PXN 44, *Lactococcus lactis* ssp. *Lactis* PXN 63, *Streptococcus thermophilus* PXN 66, *Bifidobacterium bifidum* PXN 23, *Bifidobacterium breve* PXN 25, *Bifidobacterium longum* PXN 30, and *Bifidobacterium infantis* PXN 27 (2×10^9 CFU) (3 capsules per day)		
(Mensi *et al*., 2021)	Autistic children and adolescents (n=131)	Read-world experience	*Lactobacillus plantarum* PS128 (105 patients) (6×10^{10} CFU or 3×10^{10} CFU	Participants received probiotics for 6 months.	(1). Significant improvements in terms of global functioning of the patient. (2) Greater improvement in neurodevelopmental impairment scores in patients taking *Lactobacillus plantarum* PS128 than in those taking other probiotics
(Santocchi *et al*., 2020)	Preschoolers with ASD	Randomized, double-blind, controlled placebo trial	"*Vivomixx*" (*Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* DSM 24732, *Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, *Lactobacillus acidophilus* DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM 24733, and *Lactobacillus delbrueckii subsp. bulgaricus* DSM 24734) (4.5×10^{11} CFU) (2 packets or 1 packet/day	Participants received probiotics for 6 months.	(1). No statistically significant changes in autism symptoms between probiotics and placebo group. (2). Significant modification of core ASD symptoms in group without gastrointestinal symptoms. (3). Alleviation of gastrointestinal symptoms, greater improvements in adaptive functioning, and sensory profiles than in the GI group.

12. The usefulness of animal models

Animal models have been used to investigate the dynamic and diverse community of microorganisms that inhibit in the GIT and provide an understanding of the biological complexities of the processes that govern host-microbiota symbiosis. Animal models provide a useful tool to study microorganism to establish unique roles for the gut microbiota in the context of different health and disease states (Sekirov *et al*., 2010). The mouse model is most commonly used, and hold many advantages compared to working with human volunteers, including (1) fewer ethical restrictions; (2) complete environmental control (diet; stress, etc.); (3) genetic control of subject population; and (4) accessibility of intestinal contents, tissues, and organs at autopsy (Boureau, 2000). However, the additional layers of complexity introduced in animal models can hinder the interpretation of the data, especially when modeling gastrointestinal diseases. Even the most widely used mice model is physiologically very different from the human model (Gibbons and Spencer, 2011). For example, there are differences in the microbial community, secondly, paneth cells in the mouse small intestine produce more than 10 times more defensins (cryptdins) than human cells, which may affect microbial colonisation and survival (Mestas and Hughes, 2004).

13. Aims and Objectives

Limited nutrient intake in AN individuals affects the gut microbial community and further affects the brain, so psychological changes undergone by AN individuals may have a microbial origin. As such, the gut microbiota could be a functional target for improving AN symptoms through regulating SCFAs, NTs and appetite hormones. New therapeutic options in the management of AN are currently being investigated as direct and/or adjunctive therapies (Long-Smith *et al*., 2020).

The aims and objectives of this research were:

1. To investigate the impact of NT precursors (dietary intervention) on faecal bacterial populations and neuroactive metabolites using *in vitro* batch culture fermentation systems under physiological relevant conditions. To develop a sensitive LC-MS method for selective analysis of dietary NTs, covering the level range found in human faecal fluid.

2. To investigate the impact of pre and probiotics combined with NTs precursors within a mixed faecal community to impact on microbial growth, SCFA and NTs under pH related to proximal and distal colon.

3. (1) to establish an anorexic three-stage continuous culture system based on nutrient intake and gastrointestinal transit times in AN patients,

(2) to determine the impact of anorexic nutrient intake on the gastrointestinal microbial community,

(3) compare changes observed within our system to current data of AN individuals, (4) assess the levels of gut microbial community and metabolites in the models (comparing healthy, anorexic and psychobiotic intervention arms of the experiment) within different regions of colon model (proximal, transverse and distal).

(4) to evaluate these in vitro results to assess impact of pre and probiotics on microbial communities and metabolites, associated with AN.

References

Adan, R. A. H. & Vink, T. (2001). Drug target discovery by pharmacogenetics: mutations in the melanocortin system and eating disorders. *European Neuropsychopharmacology,* **11**(6), 483-490.

Aigner, M., Treasure, J., Kaye, W., Kasper, S. & Disorders, W. T. F. E. (2011). World Federation of Societies of Biological Psychiatry (WFSBP) Guidelines for the Pharmacological Treatment of Eating Disorders. *World Journal of Biological Psychiatry,* **12**(6), 400-443.

Akbari, E., Asemi, Z., Kakhaki, R. D., Bahmani, F., Kouchaki, E., Tamtaji, O. R., Hamidi, G. A. & Salami, M. (2016). Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. *Frontiers in Aging Neuroscience,* **8**.

Akkasheh, G., Kashani-Poor, Z., Tajabadi-Ebrahimi, M., Jafari, P., Akbari, H., Taghizadeh, M., Memarzadeh, M. R., Asemi, Z. & Esmaillzadeh, A. (2016). Clinical and metabolic response to probiotic administration in patients with major depressive disorder: A randomized, double-blind, placebo-controlled trial. *Nutrition,* **32**(3), 315-320.

Alkhalaf, L. M. & Ryan, K. S. (2015). Biosynthetic Manipulation of Tryptophan in Bacteria: Pathways and Mechanisms. *Chemistry & Biology,* **22**(3), 317-328.

American Psychiatric Association (2013). *Diagnostic and statistical manual of mental disorders : DSM-5*. Arlington: American Psychiatric Association.

Arcelus, J., Mitchell, A. J., Wales, J. & Nielsen, S. (2011). Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies. *Arch Gen Psychiatry,* **68**(7), 724-31.

Armougom, F., Henry, M., Vialettes, B., Raccah, D. & Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. *PLoS One,* **4**(9), e7125.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y. & Sudo, N. (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *Am J Physiol Gastrointest Liver Physiol,* **303**(11), G1288-95.

Association, A. P. (2013). *Desk reference to the diagnostic criteria from DSM-5 (R)*. American Psychiatric Publishing.

Attia, E., Haiman, C., Walsh, T. & Flater, S. R. (1998). Does fluoxetine augment the inpatient treatment of anorexia nervosa? *American Journal of Psychiatry,* **155**(4), 548-551.

Aurigemma, N. C., Koltun, K. J., VanEvery, H., Rogers, C. J. & De Souza, M. J. (2018). Linking the Gut Microbiota to Bone Health in Anorexia Nervosa. *Current Osteoporosis Reports,* **16**(1), 65-75.

Auteri, M., Zizzo, M. G. & Serio, R. (2015). GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. *Pharmacological Research,* **93**, 11-21.

Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F. & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America,* **101**(44), 15718-15723.

Badawy, A. A. B. (2017). Kynurenine Pathway of Tryptophan Metabolism: Regulatory and Functional Aspects. *International Journal of Tryptophan Research,* **10**.

Banks, W. A. (2008). The blood-brain barrier as a cause of obesity. *Current Pharmaceutical Design,* **14**(16), 1606-1614.
Banks, W. A., Goulet, M., Rusche, J. R., Niehoff, M. L. & Boismenu, R. (2002). Differential transport of a secretin analog across the blood-brain and blood-cerebrospinal fluid barriers of the mouse. *Journal of Pharmacology and Experimental Therapeutics,* **302**(3), 1062-1069.
Banks, W. A., Niehoff, M. L. & Zalcman, S. S. (2004). Permeability of the mouse blood-brain barrier to murine interleukin-2: predominance of a saturable efflux system. *Brain Behavior and Immunity,* **18**(5), 434-442.
Bansal, T., Alaniz, R. C., Wood, T. K. & Jayaraman, A. (2010). The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U S A,* **107**(1), 228-33.
Barbarich, N. C., McConaha, C. W., Gaskill, J., La Via, M., Frank, G. K., Achenbach, S., Plotnicov, K. H. & Kaye, W. H. (2004). An open trial of olanzapine in anorexia nervosa. *Journal of Clinical Psychiatry,* **65**(11), 1480-1482.
Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F. & Stanton, C. (2012). gamma-Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology,* **113**(2), 411-417.
Becker, A. E. (2004). Television, disordered eating, and young women in Fiji: negotiating body image and identity during rapid social change. *Cult Med Psychiatry,* **28**(4), 533-59.
Belmonte, L., Achamrah, N., Nobis, S., Guerin, C., Riou, G., Bole-Feysot, C., Boyer, O., Richard, V., Do Rego, J. C., Dechelotte, P., Goichon, A. & Coeffier, M. (2016). A role for intestinal TLR4-driven inflammatory response during activity-based anorexia. *Scientific Reports,* **6**.
Ben-Shachar, D., Yehuda, S., Finberg, J. P., Spanier, I. & Youdim, M. B. (1988). Selective alteration in blood-brain barrier and insulin transport in iron-deficient rats. *J Neurochem,* **50**(5), 1434-7.
Bering, S. B. (2018). Human Milk Oligosaccharides to Prevent Gut Dysfunction and Necrotizing Enterocolitis in Preterm Neonates. *Nutrients,* **10**(10).
Bienenstock, J., Forsythe, P., Karimi, K. & Kunze, W. (2010). Neuroimmune aspects of food intake. *International Dairy Journal,* **20**(4), 253-258.
Blaak, E. E., Canfora, E. E., Theis, S., Frost, G., Groen, A. K., Mithieux, G., Nauta, A., Scott, K., Stahl, B., van Harsselaar, J., van Tol, R., Vaughan, E. E. & Verbeke, K. (2020). Short chain fatty acids in human gut and metabolic health. *Benef Microbes,* **11**(5), 411-455.
Bonaz, B., Bazin, T. & Pellissier, S. (2018). The Vagus Nerve at the Interface of the Microbiota-Gut-Brain Axis. *Frontiers in Neuroscience,* **12**.
Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., Anselmetti, S., Scarone, S., Pontiroli, A. E., Morace, G. & Borghi, E. (2017). Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. *Plos One,* **12**(6).
Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G. & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in Molecular Medicine,* **20**(9), 509-518.
Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J. & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behaviour and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America,* **108**(38), 16050-16055.

Breton, J., Legrand, R., Akkermann, K., Jarv, A., Harro, J., Dechelotte, P. & Fetissov, S. O. (2016). Elevated Plasma Concentrations of Bacterial ClpB Protein in Patients with Eating Disorders. *International Journal of Eating Disorders,* **49**(8), 805-808.

Brown, C. & Mehler, P. S. (2015). Medical complications of anorexia nervosa and their treatments: an update on some critical aspects. *Eating and Weight Disorders-Studies on Anorexia Bulimia and Obesity,* **20**(4), 419-425.

Butel, M. J. (2014). Probiotics, gut microbiota and health. *Medecine Et Maladies Infectieuses,* **44**(1), 1-8.

Byrne, C. S., Chambers, E. S., Morrison, D. J. & Frost, G. (2015). The role of short chain fatty acids in appetite regulation and energy homeostasis. *International Journal of Obesity,* **39**(9), 1331-1338.

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A. l., Delmée, E., Cousin, B. a., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J.-F. o., Gibson, G. R., Casteilla, L., Delzenne, N. M., Alessi, M. C. & Burcelin, R. m. (2007a). Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes,* **56**(7), 1761-1772.

Cani, P. D., Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R. G., Tuohy, K. M., Gibson, G. R. & Delzenne, N. M. (2007b). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia,* **50**(11), 2374-2383.

Chen, J. J., Wang, R., Li, X. F. & Wang, R. L. (2011). Bifidobacterium longum supplementation improved high-fat-fed-induced metabolic syndrome and promoted intestinal Reg I gene expression. *Exp Biol Med (Maywood),* **236**(7), 823-31.

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E. & Faure, D. (2006). GABA controls the level of quorum-sensing signal in Agrobacterium tumefaciens. *Proceedings of the National Academy of Sciences,* **103**(19), 7460-7464.

Chow, J., Lee, S. M., Shen, Y., Khosravi, A. & Mazmanian, S. K. (2010). Host-Bacterial Symbiosis in Health and Disease. *Mucosal Immunity,* **107**, 243-274.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., Harris, H. M., Coakley, M., Lakshminarayanan, B., O'Sullivan, O., Fitzgerald, G. F., Deane, J., O'Connor, M., Harnedy, N., O'Connor, K., O'Mahony, D., van Sinderen, D., Wallace, M., Brennan, L., Stanton, C., Marchesi, J. R., Fitzgerald, A. P., Shanahan, F., Hill, C., Ross, R. P. & O'Toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature,* **488**(7410), 178-84.

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

Clarke, G., McKernan, D. P., Gaszner, G., Quigley, E. M., Cryan, J. F. & Dinan, T. G. (2012). A distinct profile of tryptophan metabolism along the kynurenine pathway downstream of toll-like receptor activation in irritable bowel syndrome. *Frontiers in Pharmacology,* **3**.

Collins, S. M., Surette, M. & Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nature Reviews Microbiology,* **10**(11), 735-742.

Comai, S., Bertazzo, A., Carretti, N., Podfigurna-Stopa, A., Luisi, S. & Costa, C. V. L. (2010). Serum levels of tryptophan, 5-hydroxytryptophan and serotonin in patients affected with different forms of amenorrhea. *International journal of tryptophan research : IJTR,* **3**, 69-75.

Comai, S., Bertazzo, A., Vachon, J., Daigle, M., Toupin, J., Cote, G., Turecki, G. & Gobbi, G. (2016). Tryptophan via serotonin/kynurenine pathways abnormalities in a large cohort of aggressive inmates: markers for aggression. *Progress in Neuro-Psychopharmacology & Biological Psychiatry,* **70**, 8-16.

Cone, R. D. (2005). Anatomy and regulation of the central melanocortin system. *Nature Neuroscience,* **8**(5), 571-578.

Costedio, M. M., Coates, M. D., Danielson, A. B., Buttolph, T. R., 3rd, Blaszyk, H. J., Mawe, G. M. & Hyman, N. H. (2008). Serotonin signaling in diverticular disease. *J Gastrointest Surg,* **12**(8), 1439-45.

Crost, E. H., Tailford, L. E., Le Gall, G., Fons, M., Henrissat, B. & Juge, N. (2013). Utilisation of Mucin Glycans by the Human Gut Symbiont Ruminococcus gnavus Is Strain-Dependent. *Plos One,* **8**(10).

Dejong, H., Broadbent, H. & Schmidt, U. (2012). A systematic review of dropout from treatment in outpatients with anorexia nervosa. *Int J Eat Disord,* **45**(5), 635-47.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D., Cotter, P. D., Dinan, T. G. & Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun,* **48**, 165-73.

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

Dinan, T. G. & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil,* **25**(9), 713-9.

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

Dugdale, D. C. (2021). *catecholamine blood test*.

Ehrlich, S., Franke, L., Schneider, N., Salbach-Andrae, H., Schott, R., Craciun, E. M., Pfeiffer, E., Uebelhack, R. & Lehmkuhl, U. (2009). Aromatic amino acids in weight-recovered females with anorexia nervosa. *Int J Eat Disord,* **42**(2), 166-72.

Erny, D., Hrabě de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., Schwierzeck, V., Utermöhlen, O., Chun, E., Garrett, W. S., McCoy, K. D., Diefenbach, A., Staeheli, P., Stecher, B., Amit, I. & Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci,* **18**(7), 965-77.

Esposito, P., Chandler, N., Kandere, K., Basu, S., Jacobson, S., Connolly, R., Tutor, D. & Theoharides, T. C. (2002). Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J Pharmacol Exp Ther,* **303**(3), 1061-6.

Fava, F., Gitau, R., Griffin, B. A., Gibson, G. R., Tuohy, K. M. & Lovegrove, J. A. (2013). The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk' population. *International Journal of Obesity,* **37**(2), 216-223.

Ferguson, C. P., La Via, M. C., Crossan, P. J. & Kaye, W. H. (1999). Are serotonin selective reuptake inhibitors effective in underweight anorexia nervosa? *International Journal of Eating Disorders,* **25**(1), 11-17.

Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T. M. & Comelli, E. M. (2014). Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutr Diabetes,* **4**, e121.

Fernstrom, J. D. & Faller, D. V. (1978). Neutral amino acids in the brain: changes in response to food ingestion. *J Neurochem,* **30**(6), 1531-8.

Fetissov, S. O., Hallman, J., Oreland, L., af Klinteberg, B., Grenback, E., Hulting, A. L. & Hokfelt, T. (2002). Autoantibodies against alpha-MSH, ACTH, and LHRH in anorexia and bulimia nervosa patients. *Proceedings of the National Academy of Sciences of the United States of America,* **99**(26), 17155-17160.

Flourie, B., Etanchaud, F., Florent, C., Pellier, P., Bouhnik, Y. & Rambaud, J. C. (1990). Comparative-Study of Hydrogen and Methane Production in the Human Colon Using Cecal and Fecal Homogenates. *Gut,* **31**(6), 684-685.

Folks, D. G. (2004). The interface of psychiatry and irritable bowel syndrome. *Current Psychiatry Reports,* **6**(3), 210-215.

Forsythe, P., Bienenstock, J. & Kunze, W. A. (2014). Vagal Pathways for Microbiome-Brain-Gut Axis Communication. *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease,* **817**, 115-133.

Foster, J. A. & Neufeld, K. A. M. (2013). Gut-brain: how the microbiome influences anxiety and depression. *Trends in Neurosciences,* **36**(5), 305-312.

García-Mantrana, I., Selma-Royo, M., González, S., Parra-Llorca, A., Martínez-Costa, C. & Collado, M. C. (2020). Distinct maternal microbiota clusters are associated with diet during pregnancy: impact on neonatal microbiota and infant growth during the first 18 months of life. *Gut Microbes,* **11**(4), 962-978.

Gauthier, C., Hassler, C., Mattar, L., Launay, J.-M., Callebert, J., Steiger, H., Melchior, J.-C., Falissard, B., Berthoz, S., Mourier-Soleillant, V., Lang, F., Delorme, M., Pommereau, X., Gerardin, P., Bioulac, S., Bouvard, M. & Godart, N. (2014). Symptoms of depression and anxiety in anorexia nervosa: Links with plasma tryptophan and serotonin metabolism. *Psychoneuroendocrinology,* **39**, 170-178.

Ghosal, S., Myers, B. & Herman, J. P. (2013). Role of central glucagon-like peptide-1 in stress regulation. *Physiol Behav,* **122**, 201-7.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K. & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology,* **14**(8), 491-502.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science,* **312**(5778), 1355-1359.

Goulet, O. (2015). Potential role of the intestinal microbiota in programming health and disease. *Nutrition Reviews,* **73**, 32-40.

Guinane, C. M. & Cotter, P. D. (2013). Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology,* **6**(4), 295-308.

Haldeman, C., Turley, K. & Foley, M. (2021). *Catecholamines (Urine)*. URL: https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=167&contentid=catecholamines_urine [26 October 2021].

Haleem, D. J. (2012). Serotonin neurotransmission in anorexia nervosa. *Behav Pharmacol,* **23**(5-6), 478-95.

Haleem, D. J. (2016). Drug Targets for Obesity and Depression: From Serotonin to Leptin. *Current Drug Targets,* **17**(11), 1282-1291.

Haleem, D. J. (2017). Improving therapeutics in anorexia nervosa with tryptophan. *Life Sci,* **178**, 87-93.

Haleem, D. J., Haque, Z., Inam, Q. U. A., Ikram, H. & Haleem, M. A. (2015). Behavioral, hormonal and central serotonin modulating effects of injected leptin. *Peptides,* **74**, 1-8.

Hay, P., Chinn, D., Forbes, D., Madden, S., Newton, R., Sugenor, L., Touyz, S. & Ward, W. (2014). Royal Australian and New Zealand College of Psychiatrists Clinical practice guidelines for the treatment of eating disorders. *Australian and New Zealand Journal of Psychiatry,* **48**(11), 977-1008.

Heyes, M. P., Achim, C. L., Wiley, C. A., Major, E. O., Saito, K. & Markey, S. P. (1996). Human microglia convert L-tryptophan into the neurotoxin quinolinic acid. *Biochemical Journal,* **320**, 595-597.

Holzer, P. & Farzi, A. (2014). Neuropeptides and the Microbiota-Gut-Brain Axis. *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease,* **817**, 195-219.

Inoue, K., Shirai, T., Ochiai, H., Kasao, M., Hayakawa, K., Kimura, M. & Sansawa, H. (2003). Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (GABA) in mild hypertensives. *Eur J Clin Nutr,* **57**(3), 490-5.

Jalanka, J., Salonen, A., Salojarvi, J., Ritari, J., Immonen, O., Marciani, L., Gowland, P., Hoad, C., Garsed, K., Lam, C., Palva, A., Spiller, R. C. & de Vos, W. M. (2015). Effects of bowel cleansing on the intestinal microbiota. *Gut,* **64**(10), 1562-8.

Jang, H. M., Lee, H. J., Jang, S. E., Han, M. J. & Kim, D. H. (2018). Evidence for interplay among antibacterial-induced gut microbiota disturbance, neuro-inflammation, and anxiety in mice. *Mucosal Immunol,* **11**(5), 1386-1397.

Janik, R., Thomason, L. A. M., Stanisz, A. M., Forsythe, P., Bienenstock, J. & Stanisz, G. J. (2016). Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *Neuroimage,* **125**, 988-995.

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

Jesus, P., Ouelaa, W., Francois, M., Riachy, L., Guerin, C., Aziz, M., Do Rego, J. C., Dechelotte, P., Fetissov, S. O. & Coeffier, M. (2014). Alteration of intestinal barrier function during activity-based anorexia in mice. *Clinical Nutrition,* **33**(6), 1046-1053.

Kałużna-Czaplińska, J., Gątarek, P., Chirumbolo, S., Chartrand, M. S. & Bjørklund, G. (2019). How important is tryptophan in human health? *Crit Rev Food Sci Nutr,* **59**(1), 72-88.

Karlsson, F. H., Fak, F., Nookaew, I., Tremaroli, V., Fagerberg, B., Petranovic, D., Backhed, F. & Nielsen, J. (2012). Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nature Communications,* **3**.

Karuri, A. R., Dobrowsky, E. & Tannock, I. F. (1993). Selective cellular acidification and toxicity of weak organic acids in an acidic microenvironment. *Br J Cancer,* **68**(6), 1080-7.

Kastin, A. J. & Pan, W. H. (2010). Concepts for Biologically Active Peptides. *Current Pharmaceutical Design,* **16**(30), 3390-3400.

Kaye, W. (2008). Neurobiology of anorexia and bulimia nervosa. *Physiol Behav,* **94**(1), 121-35.

Kaye, W. H., Bulik, C. M., Thornton, L., Barbarich, N., Masters, K. & Grp, P. F. C. (2004). Comorbidity of anxiety disorders with anorexia and bulimia nervosa. *American Journal of Psychiatry,* **161**(12), 2215-2221.

Kaye, W. H., Ebert, M. H., Gwirtsman, H. E. & Weiss, S. R. (1984). Differences in Brain Serotonergic Metabolism between Nonbulimic and Bulimic Patients with Anorexia-Nervosa. *American Journal of Psychiatry,* **141**(12), 1598-1601.

Kaye, W. H., Frank, G. K., Bailer, U. F., Henry, S. E., Meltzer, C. C., Price, J. C., Mathis, C. A. & Wagner, A. (2005). Serotonin alterations in anorexia and bulimia nervosa: new insights from imaging studies. *Physiol Behav,* **85**(1), 73-81.

Kazemi, A., Noorbala, A. A., Azam, K., Eskandari, M. H. & Djafarian, K. (2019). Effect of probiotic and prebiotic vs placebo on psychological outcomes in patients with major depressive disorder: A randomized clinical trial. *Clin Nutr,* **38**(2), 522-528.

Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G. & Hyland, N. P. (2015). Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Frontiers in cellular neuroscience,* **9**, 392-392.

Kennedy, P. J., Cryan, J. F., Dinan, T. G. & Clarke, G. (2014). Irritable bowel syndrome: a microbiome-gut-brain axis disorder? *World J Gastroenterol,* **20**(39), 14105-25.

Kennedy, P. J., Cryan, J. F., Dinan, T. G. & Clarke, G. (2017). Kynurenine pathway metabolism and the microbiota-gut-brain axis. *Neuropharmacology,* **112**(Pt B), 399-412.

Kho, Z. Y. & Lal, S. K. (2018). The Human Gut Microbiome – A Potential Controller of Wellness and Disease. *Frontiers in Microbiology,* **9**.

Kim, K. A., Gu, W., Lee, I. A., Joh, E. H. & Kim, D. H. (2012). High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One,* **7**(10), e47713.

Kinzig, K. P., D'Alessio, D. A., Herman, J. P., Sakai, R. R., Vahl, T. P., Figueiredo, H. F., Murphy, E. K. & Seeley, R. J. (2003). CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors. *J Neurosci,* **23**(15), 6163-70.

Kishi, T. & Elmquist, J. K. (2005). Body weight is regulated by the brain: a link between feeding and emotion. *Molecular Psychiatry,* **10**(2), 132-146.

Kleiman, S. C., Watson, H. J., Bulik-Sullivan, E. C., Huh, E. Y., Tarantino, L. M., Bulik, C. M. & Carroll, I. M. (2015). The Intestinal Microbiota in Acute Anorexia Nervosa and During Renourishment: Relationship to Depression, Anxiety, and Eating Disorder Psychopathology. *Psychosomatic Medicine,* **77**(9), 969-981.

Klein, C., Patte-Mensah, C., Taleb, O., Bourguignon, J.-J., Schmitt, M., Bihel, F., Maitre, M. & Mensah-Nyagan, A. G. (2013). The neuroprotector kynurenic acid increases neuronal cell survival through neprilysin induction. *Neuropharmacology,* **70**, 254-260.

Krajmalnik-Brown, R., Ilhan, Z. E., Kang, D. W. & DiBaise, J. K. (2012). Effects of Gut Microbes on Nutrient Absorption and Energy Regulation. *Nutrition in Clinical Practice,* **27**(2), 201-214.

Latorre, R., Sternini, C., De Giorgio, R. & Greenwood-Van Meerveld, B. (2016). Enteroendocrine cells: a review of their role in brain-gut communication. *Neurogastroenterology and Motility,* **28**(5), 620-630.

Lawson, E. A., Eddy, K. T., Donoho, D., Misra, M., Miller, K. K., Meenaghan, E., Lydecker, J., Herzog, D. & Klibanski, A. (2011). Appetite-regulating hormones cortisol and peptide YY are associated with disordered eating psychopathology, independent of body mass index. *Eur J Endocrinol,* **164**(2), 253-61.

Lawson, E. A., Holsen, L. M., Desanti, R., Santin, M., Meenaghan, E., Herzog, D. B., Goldstein, J. M. & Klibanski, A. (2013). Increased hypothalamic-pituitary-adrenal drive is associated with decreased appetite and hypoactivation of food-motivation neurocircuitry in anorexia nervosa. *Eur J Endocrinol,* **169**(5), 639-47.

Lawson, E. A. & Klibanski, A. (2008). Endocrine abnormalities in anorexia nervosa. *Nat Clin Pract Endocrinol Metab,* **4**(7), 407-14.

Lawson, E. A., Miller, K. K., Blum, J. I., Meenaghan, E., Misra, M., Eddy, K. T., Herzog, D. B. & Klibanski, A. (2012). Leptin levels are associated with decreased depressive symptoms in women across the weight spectrum, independent of body fat. *Clin Endocrinol (Oxf),* **76**(4), 520-5.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam, M., Batto, J. M., Kennedy, S., Leonard, P., Li, J., Burgdorf, K., Grarup, N., Jørgensen, T., Brandslund, I., Nielsen, H. B., Juncker, A. S., Bertalan, M., Levenez, F., Pons, N., Rasmussen, S., Sunagawa, S., Tap, J., Tims, S., Zoetendal, E. G., Brunak, S., Clément, K., Doré, J., Kleerebezem, M., Kristiansen, K., Renault, P., Sicheritz-Ponten, T., de Vos, W. M., Zucker, J. D., Raes, J., Hansen, T., Bork, P., Wang, J., Ehrlich, S. D. & Pedersen, O. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature,* **500**(7464), 541-6.

Le Floc'h, N., Otten, W. & Merlot, E. (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids,* **41**(5), 1195-1205.

Leschine, S. B. (1995). Cellulose degradation in anaerobic environments. *Annu Rev Microbiol,* **49**, 399-426.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. & Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology,* **6**(10), 776-788.

Liu, T., Li, J., Liu, Y., Xiao, N., Suo, H., Xie, K., Yang, C. & Wu, C. (2012). Short-Chain Fatty Acids Suppress Lipopolysaccharide-Induced Production of Nitric Oxide and Proinflammatory Cytokines Through Inhibition of NF-κB Pathway in RAW264.7 Cells. *Inflammation,* **35**(5), 1676-1684.

Logan, A. C. & Jacka, F. N. (2014). Nutritional psychiatry research: an emerging discipline and its intersection with global urbanization, environmental challenges and the evolutionary mismatch. *J Physiol Anthropol,* **33**(1), 22.

Logsdon, A. F., Erickson, M. A., Rhea, E. M., Salameh, T. S. & Banks, W. A. (2018). Gut reactions: How the blood-brain barrier connects the microbiome and the brain. *Exp Biol Med (Maywood),* **243**(2), 159-165.

Long-Smith, C., O'Riordan, K. J., Clarke, G., Stanton, C., Dinan, T. G. & Cryan, J. F. (2020). Microbiota-Gut-Brain Axis: New Therapeutic Opportunities. *Annu Rev Pharmacol Toxicol,* **60**, 477-502.

Louis, P., Young, P., Holtrop, G. & Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environmental Microbiology,* **12**(2), 304-314.

Löwe, B., Zipfel, S., Buchholz, C., Dupont, Y., Reas, D. L. & Herzog, W. (2001). Long-term outcome of anorexia nervosa in a prospective 21-year follow-up study. *Psychol Med,* **31**(5), 881-90.

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

Macfarlane, G. T. & Gibson, G. R. (1994). Metabolic activities of the normal colonic flora. *In:* Gibson, S. A. W. (ed.) *Human Health - The Contribution of Microorganisms*. London: Springer-Verlag.

Macfarlane, G. T., Gibson, G. R. & Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol,* **72**(1), 57-64.

Machate, D. J., Figueiredo, P. S., Marcelino, G., Guimarães, R. C. A., Hiane, P. A., Bogo, D., Pinheiro, V. A. Z., Oliveira, L. C. S. & Pott, A. (2020). Fatty Acid Diets: Regulation of Gut Microbiota Composition and Obesity and Its Related Metabolic Dysbiosis. *Int J Mol Sci,* **21**(11).

Mack, I., Cuntz, U., Grämer, C., Niedermaier, S., Pohl, C., Schwiertz, A., Zimmermann, K., Zipfel, S., Enck, P. & Penders, J. (2016). Weight gain in anorexia nervosa does not ameliorate the faecal microbiota, branched chain fatty acid profiles, and gastrointestinal complaints. *Sci Rep,* **6**, 26752.

Majeed, M., Nagabhushanam, K., Arumugam, S., Majeed, S. & Ali, F. (2018). Bacillus coagulans MTCC 5856 for the management of major depression with irritable bowel syndrome: a randomised, double-blind, placebo controlled, multi-centre, pilot clinical study. *Food & Nutrition Research,* **62**.

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M., Hold, G., Quraishi, M. N., Kinross, J., Smidt, H., Tuohy, K. M., Thomas, L. V., Zoetendal, E. G. & Hart, A. (2016). The gut microbiota and host health: a new clinical frontier. *Gut,* **65**(2), 330-339.

Marcobal, A., Southwick, A. M., Earle, K. A. & Sonnenburg, J. L. (2013). A refined palate: Bacterial consumption of host glycans in the gut. *Glycobiology,* **23**(9), 1038-+.

Marzola, E., Desedime, N., Giovannone, C., Amianto, F., Fassino, S. & Abbate-Daga, G. (2015). Atypical Antipsychotics as Augmentation Therapy in Anorexia Nervosa. *Plos One,* **10**(4).

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Sawaki, E., Koga, Y. & Benno, Y. (2013). Cerebral Low-Molecular Metabolites Influenced by Intestinal Microbiota: A Pilot Study. *Frontiers in Systems Neuroscience,* **7**.

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut-brain communication. *Nature Reviews Neuroscience,* **12**(8), 453-466.

Mazzoli, R. & Pessione, E. (2016). The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling. *Frontiers in Microbiology,* **7**.

McLean, P. G., Borman, R. A. & Lee, K. (2007). 5-HT in the enteric nervous system: gut function and neuropharmacology. *Trends in Neurosciences,* **30**(1), 9-13.

Mcneil, N. I. (1984). The Contribution of the Large-Intestine to Energy Supplies in Man. *American Journal of Clinical Nutrition,* **39**(2), 338-342.

Meguid, M. M., Fetissov, S. O., Varma, M., Sato, T., Zhang, L., Laviano, A. & Rossi-Fanelli, F. (2000). Hypothalamic dopamine and serotonin in the regulation of food intake. *Nutrition,* **16**(10), 843-57.

Mehler, P. S. & Brown, C. (2015). Anorexia nervosa - medical complications. *Journal of Eating Disorders,* **3**.

Meister, B. (2007). Neurotransmitters in key neurons of the hypothalamus that regulate feeding behaviour and body weight. *Physiology & Behavior,* **92**(1-2), 263-271.

Méquinion, M., Langlet, F., Zgheib, S., Dickson, S., Dehouck, B., Chauveau, C. & Viltart, O. (2013). Ghrelin: Central and Peripheral Implications in Anorexia Nervosa. *Frontiers in Endocrinology,* **4**.

Miller, K. K., Wexler, T. L., Zha, A. M., Lawson, E. A., Meenaghan, E. M., Misra, M., Binstock, A. B., Herzog, D. B. & Klibanski, A. (2007). Androgen deficiency: Association with increased anxiety and depression symptom severity in anorexia nervosa. *Journal of Clinical Psychiatry,* **68**(6), 959-965.

Million, M., Angelakis, E., Maraninchi, M., Henry, M., Giorgi, R., Valero, R., Vialettes, B. & Raoult, D. (2013). Correlation between body mass index and gut concentrations of Lactobacillus reuteri, Bifidobacterium animalis, Methanobrevibacter smithii and Escherichia coli. *International Journal of Obesity,* **37**(11), 1460-1466.

Milos, G., Spindler, A., Schnyder, U., Martz, J., Hoek, H. W. & Willi, J. (2004). Incidence of severe anorexia nervosa in Switzerland: 40 years of development (vol 35, pg 250, 2004). *International Journal of Eating Disorders,* **36**(1), 118-119.

Miniati, M., Mauri, M., Ciberti, A., Mariani, M. G., Marazziti, D. & Dell'Osso, L. (2016). Psychopharmacological options for adult patients with anorexia nervosa. *Cns Spectrums,* **21**(2), 134-142.

Minter, M. R., Hinterleitner, R., Meisel, M., Zhang, C., Leone, V., Zhang, X., Oyler-Castrillo, P., Zhang, X., Musch, M. W., Shen, X., Jabri, B., Chang, E. B., Tanzi, R. E.

& Sisodia, S. S. (2017). Antibiotic-induced perturbations in microbial diversity during post-natal development alters amyloid pathology in an aged APP(SWE)/PS1(ΔE9) murine model of Alzheimer's disease. *Sci Rep,* **7**(1), 10411.

Minter, M. R., Zhang, C., Leone, V., Ringus, D. L., Zhang, X., Oyler-Castrillo, P., Musch, M. W., Liao, F., Ward, J. F., Holtzman, D. M., Chang, E. B., Tanzi, R. E. & Sisodia, S. S. (2016). Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. *Scientific Reports,* **6**(1), 30028.

Misra, M. & Klibanski, A. (2014). Endocrine consequences of anorexia nervosa. *Lancet Diabetes & Endocrinology,* **2**(7), 581-592.

Misra, M., Miller, K. K., Tsai, P., Stewart, V., End, A., Freed, N., Herzog, D. B., Goldstein, M., Riggs, S. & Klibanski, A. (2006). Uncoupling of cardiovascular risk markers in adolescent girls with anorexia nervosa. *Journal of Pediatrics,* **149**(6), 763-769.

Mithieux, G. (2012). Comment about intestinal gluconeogenesis after gastric bypass in human in relation with the paper by Hayes et al., Obes. Surg. 2011. *Obesity Surgery,* **22**(12), 1920-1922.

Mithieux, G. (2018). Gut Microbiota and Host Metabolism: What Relationship? *Neuroendocrinology,* **106**(4), 352-356.

Mittal, R., Debs, L. H., Patel, A. P., Nguyen, D., Patel, K., O'Connor, G., Grati, M., Mittal, J., Yan, D., Eshraghi, A. A., Deo, S. K., Daunert, S. & Liu, X. Z. (2017). Neurotransmitters: The Critical Modulators Regulating Gut-Brain Axis. *J Cell Physiol,* **232**(9), 2359-2372.

Moles, L. & Otaegui, D. (2020). The Impact of Diet on Microbiota Evolution and Human Health. Is Diet an Adequate Tool for Microbiota Modulation? *Nutrients,* **12**(6).

Moloney, R. D., Johnson, A. C., O'Mahony, S. M., Dinan, T. G., Greenwood-Van Meerveld, B. & Cryan, J. F. (2016). Stress and the Microbiota-Gut-Brain Axis in Visceral Pain: Relevance to Irritable Bowel Syndrome. *CNS Neurosci Ther,* **22**(2), 102-17.

Moreira, A. P., Texeira, T. F., Ferreira, A. B., Peluzio Mdo, C. & Alfenas Rde, C. (2012). Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. *Br J Nutr,* **108**(5), 801-9.

Morita, C., Tsuji, H., Hata, T., Gondo, M., Takakura, S., Kawai, K., Yoshihara, K., Ogata, K., Nomoto, K., Miyazaki, K. & Sudo, N. (2015). Gut Dysbiosis in Patients with Anorexia Nervosa. *PLoS One,* **10**(12), e0145274.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K. & Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature,* **409**(6817), 194-198.

Neufeld, K. M., Kang, N., Bienenstock, J. & Foster, J. A. (2011). Reduced anxiety-like behaviour and central neurochemical change in germ-free mice. *Neurogastroenterol Motil,* **23**(3), 255-64, e119.

Neyrinck, A. M., Possemiers, S., Druart, C., Van de Wiele, T., De Backer, F., Cani, P. D., Larondelle, Y. & Delzenne, N. M. (2011). Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, Roseburia and Bacteroides/Prevotella in diet-induced obese mice. *PLoS One,* **6**(6), e20944.

Nguyen, A. T., Mandard, S., Dray, C., Deckert, V., Valet, P., Besnard, P., Drucker, D. J., Lagrost, L. & Grober, J. (2014). Lipopolysaccharides-Mediated Increase in Glucose-Stimulated Insulin Secretion: Involvement of the GLP-1 Pathway. *Diabetes,* **63**(2), 471-482.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W. & Pettersson, S. (2012). Host-Gut Microbiota Metabolic Interactions. *Science,* **336**(6086), 1262-1267.

Nomura, M., Kimoto, H., Someya, Y., Furukawa, S. & Suzuki, I. (1998). Production of gamma-aminobutyric acid by cheese starters during cheese ripening. *J Dairy Sci,* **81**(6), 1486-91.

O'Keefe, S. J. (2010). Tube feeding, the microbiota, and Clostridium difficile infection. *World J Gastroenterol,* **16**(2), 139-42.

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

Ouwehand, A. C., Salminen, S. & Isolauri, E. (2002). Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* **82**(1-4), 279-289.

Overduin, J., Schoterman, M. H., Calame, W., Schonewille, A. J. & Ten Bruggencate, S. J. (2013). Dietary galacto-oligosaccharides and calcium: effects on energy intake, fat-pad weight and satiety-related, gastrointestinal hormones in rats. *Br J Nutr,* **109**(7), 1338-48.

Özoğul, F. (2004). Production of biogenic amines by Morganella morganii, Klebsiella pneumoniae and Hafnia alvei using a rapid HPLC method. *European Food Research and Technology,* **219**(5), 465-469.

Painsipp, E., Herzog, H., Sperk, G. & Holzer, P. (2011). Sex-dependent control of murine emotional-affective behaviour in health and colitis by peptide YY and neuropeptide Y. *British journal of pharmacology,* **163**(6), 1302-1314.

Park, J., Wang, Q., Wu, Q., Mao-Draayer, Y. & Kim, C. H. (2019). Bidirectional regulatory potentials of short-chain fatty acids and their G-protein-coupled receptors in autoimmune neuroinflammation. *Scientific Reports,* **9**(1), 8837.

Partty, A., Kalliomaki, M., Wacklin, P., Salminen, S. & Isolauri, E. (2015). A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatric Research,* **77**(6), 823-828.

Patnala, R., Arumugam, T. V., Gupta, N. & Dheen, S. T. (2017). HDAC Inhibitor Sodium Butyrate-Mediated Epigenetic Regulation Enhances Neuroprotective Function of Microglia During Ischemic Stroke. *Mol Neurobiol,* **54**(8), 6391-6411.

Pérez-De La Cruz, V., Carrillo-Mora, P. & Santamaría, A. (2012). Quinolinic Acid, an endogenous molecule combining excitotoxicity, oxidative stress and other toxic mechanisms. *Int J Tryptophan Res,* **5**, 1-8.

Perry, R. J., Peng, L., Barry, N. A., Cline, G. W., Zhang, D., Cardone, R. L., Petersen, K. F., Kibbey, R. G., Goodman, A. L. & Shulman, G. I. (2016). Acetate mediates a microbiome-brain-beta-cell axis to promote metabolic syndrome. *Nature,* **534**(7606), 213-7.

Phillipou, A., Rossell, S. L. & Castle, D. J. (2014). The neurobiology of anorexia nervosa: A systematic review. *Australian and New Zealand Journal of Psychiatry,* **48**(2), 128-152.

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., Matsunami, R. K., Lugo, M., Major, A., Mori-Akiyama, Y., Hollister, E. B., Dann, S. M., Shi, X. Z., Engler, D. A., Savidge, T. & Versalovic, J. (2017). GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterol Motil,* **29**(1).

Praticò, G., Capuani, G., Tomassini, A., Baldassarre, M. E., Delfini, M. & Miccheli, A. (2014). Exploring human breast milk composition by NMR-based metabolomics. *Nat Prod Res,* **28**(2), 95-101.

Psichas, A., Sleeth, M. L., Murphy, K. G., Brooks, L., Bewick, G. A., Hanyaloglu, A. C., Ghatei, M. A., Bloom, S. R. & Frost, G. (2015). The short chain fatty acid propionate

stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. *Int J Obes (Lond),* **39**(3), 424-9.

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

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D. & Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* **464**(7285), 59-65.

Queipo-Ortuño, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., Casanueva, F. & Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One,* **8**(5), e65465.

Rahat-Rozenbloom, S., Fernandes, J., Gloor, G. B. & Wolever, T. M. S. (2014). Evidence for greater production of colonic short-chain fatty acids in overweight than lean humans. *International Journal of Obesity,* **38**(12), 1525-1531.

Ramos, S. & Martín, M. Á. (2021). Impact of diet on gut microbiota. *Current Opinion in Food Science,* **37**, 83-90.

Redondo-Useros, N., Nova, E., González-Zancada, N., Díaz, L. E., Gómez-Martínez, S. & Marcos, A. (2020). Microbiota and Lifestyle: A Special Focus on Diet. *Nutrients,* **12**(6), 1776.

Reigstad, C. S., Salmonson, C. E., Rainey, J. F., 3rd, Szurszewski, J. H., Linden, D. R., Sonnenburg, J. L., Farrugia, G. & Kashyap, P. C. (2015). Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *Faseb j,* **29**(4), 1395-403.

Riccio, P. & Rossano, R. (2019). Undigested Food and Gut Microbiota May Cooperate in the Pathogenesis of Neuroinflammatory Diseases: A Matter of Barriers and a Proposal on the Origin of Organ Specificity. *Nutrients,* **11**(11).

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N. & Dougherty, D. M. (2009). L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *Int J Tryptophan Res,* **2**, 45-60.

Roubalova, R., Prochazkova, P., Dvorak, J., Hill, M., Papezova, H., Kreisinger, J., Bulant, J., Lambertova, A., Holanova, P., Bilej, M. & Tlaskalova-Hogenova, H. (2021). Altered Serum Immunological and Biochemical Parameters and Microbiota Composition in Patients With AN During Realimentation. *Frontiers in Nutrition,* **8**.

Rudzki, L., Ostrowska, L., Pawlak, D., Malus, A., Pawlak, K., Waszkiewicz, N. & Szulc, A. (2019). Probiotic Lactobacillus Plantarum 299v decreases kynurenine concentration and improves cognitive functions in patients with major depression: A double-blind, randomized, placebo controlled study. *Psychoneuroendocrinology,* **100**, 213-222.

Sahakian, A. B., Jee, S. R. & Pimentel, M. (2010). Methane and the Gastrointestinal Tract. *Digestive Diseases and Sciences,* **55**(8), 2135-2143.

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., Challis, C., Schretter, C. E., Rocha, S., Gradinaru, V., Chesselet, M. F., Keshavarzian, A., Shannon, K. M., Krajmalnik-Brown, R., Wittung-Stafshede, P., Knight, R. &

Mazmanian, S. K. (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell,* **167**(6), 1469-1480.e12.
SantaCruz-Calvo, L., Vilchez, J. I., Narvaez-Reinaldo, J. J., Picazo-Espinosa, R., Gonzalez-Lopez, J. & Manzanera, M. (2010). Biotechnological uses of desiccation tolerant microorganisms for the rhyzoremediation of soils subjected to seasonal drought. *Journal of Biotechnology,* **150**, S220-S220.
Saper, C. B., Chou, T. C. & Elmquist, J. K. (2002). The need to feed: homeostatic and hedonic control of eating. *Neuron,* **36**(2), 199-211.
Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F. & Burnet, P. W. J. (2016). Psychobiotics and the Manipulation of Bacteria-Gut-Brain Signals. *Trends in Neurosciences,* **39**(11), 763-781.
Scheppach, W. (1996). Treatment of distal ulcerative colitis with short-chain fatty acid enemas. A placebo-controlled trial. German-Austrian SCFA Study Group. *Dig Dis Sci,* **41**(11), 2254-9.
Schousboe, A. & Waagepetersen, H. S. (2007). GABA: Homeostatic and pharmacological aspects. *Gaba and the Basal Ganglia: From Molecules to Systems,* **160**, 9-19.
Schwartz, G. J. (2000). The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition,* **16**(10), 866-73.
Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C. & Hardt, P. D. (2010). Microbiota and SCFA in Lean and Overweight Healthy Subjects. *Obesity,* **18**(1), 190-195.
Segain, J.-P., de la Blétière, D. R., Bourreille, A., Leray, V., Gervois, N., Rosales, C., Ferrier, L., Bonnet, C., Blottière, H. M. & Galmiche, J.-P. (2000). Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. *Gut,* **47**(3), 397-403.
Seki, E. & Schnabl, B. (2012). Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. *J Physiol,* **590**(3), 447-58.
Sen, T., Cawthon, C. R., Ihde, B. T., Hajnal, A., DiLorenzo, P. M., de La Serre, C. B. & Czaja, K. (2017). Diet-driven microbiota dysbiosis is associated with vagal remodeling and obesity. *Physiol Behav,* **173**, 305-317.
Serino, M., Luche, E., Gres, S., Baylac, A., Bergé, M., Cenac, C., Waget, A., Klopp, P., Iacovoni, J., Klopp, C., Mariette, J., Bouchez, O., Lluch, J., Ouarné, F., Monsan, P., Valet, P., Roques, C., Amar, J., Bouloumié, A., Théodorou, V. & Burcelin, R. (2012). Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut,* **61**(4), 543-53.
Shah, P., Nankova, B. B., Parab, S. & La Gamma, E. F. (2006). Short chain fatty acids induce TH gene expression via ERK-dependent phosphorylation of CREB protein. *Brain Res,* **1107**(1), 13-23.
Sherwin, E., Rea, K., Dinan, T. G. & Cryan, J. F. (2016). A gut (microbiome) feeling about the brain. *Current Opinion in Gastroenterology,* **32**(2), 96-102.
Shishov, V. A., Kirovskaia, T. A., Kudrin, V. S. & Oleskin, A. V. (2009). [Amine neuromediators, their precursors, and oxidation products in the culture of Escherichia coli K-12]. *Prikl Biokhim Mikrobiol,* **45**(5), 550-4.
Shortt, C., Hasselwander, O., Meynier, A., Nauta, A., Fernandez, E. N., Putz, P., Rowland, I., Swann, J., Turk, J., Vermeiren, J. & Antoine, J. M. (2018). Systematic review of the effects of the intestinal microbiota on selected nutrients and non-nutrients. *European Journal of Nutrition,* **57**(1), 25-49.
Siavoshian, S., Blottière, H. M., Le Foll, E., Kaeffer, B., Cherbut, C. & Galmiche, J. P. (1997). Comparison of the effect of different short chain fatty acids on the growth and

differentiation of human colonic carcinoma cell lines in vitro. *Cell Biol Int,* **21**(5), 281-7.

Silva, Y. P., Bernardi, A. & Frozza, R. L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Frontiers in endocrinology,* **11**, 25-25.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R. & Gobbetti, M. (2007). Synthesis of gamma-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Applied and environmental microbiology,* **73**(22), 7283-7290.

Slyepchenko, A., Maes, M., Jacka, F. N., Kohler, C. A., Barichello, T., McIntyre, R. S., Berk, M., Grande, I., Foster, J. A., Vieta, E. & Carvalho, A. F. (2017). Gut Microbiota, Bacterial Translocation, and Interactions with Diet: Pathophysiological Links between Major Depressive Disorder and Non Communicable Medical Comorbidities. *Psychotherapy and Psychosomatics,* **86**(1), 31-46.

Speranza, E., Cioffi, I., Santarpia, L., Del Piano, C., De Caprio, C., Naccarato, M., Marra, M., De Filippo, E., Contaldo, F. & Pasanisi, F. (2018). Fecal Short Chain Fatty Acids and Dietary Intake in Italian Women With Restrictive Anorexia Nervosa: A Pilot Study. *Front Nutr,* **5**, 119.

Stanisavljević, S., Čepić, A., Bojić, S., Veljović, K., Mihajlović, S., Đedović, N., Jevtić, B., Momčilović, M., Lazarević, M., Mostarica Stojković, M., Miljković, Đ. & Golić, N. (2019). Oral neonatal antibiotic treatment perturbs gut microbiota and aggravates central nervous system autoimmunity in Dark Agouti rats. *Sci Rep,* **9**(1), 918.

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

Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J. & Lewis, K. (2019). GABA-modulating bacteria of the human gut microbiota. *Nature Microbiology,* **4**(3), 396-403.

Strober, M., Freeman, R., Lampert, C., Diamond, J. & Kaye, W. (2001). Males with anorexia nervosa: a controlled study of eating disorders in first-degree relatives. *Int J Eat Disord,* **29**(3), 263-9.

Sugama, S. & Kakinuma, Y. (2016). Loss of dopaminergic neurons occurs in the ventral tegmental area and hypothalamus of rats following chronic stress: Possible pathogenetic loci for depression involved in Parkinson's disease. *Neurosci Res,* **111**, 48-55.

Szalay, C., Aradi, M., Schwarcz, A., Orsi, G., Perlaki, G., Hanna, S., Nemeth, L., Takacs, G., Bajnok, L., Vereczkei, A., Lenard, L. & Karadi, Z. (2010). Taste perception alterations among obese: an fMRI study. *Acta Physiologica Hungarica,* **97**(4), 476-476.

Tajti, J., Majlath, Z., Szok, D., Csati, A., Toldi, J., Fulop, F. & Vecsei, L. (2015). Novel Kynurenic Acid Analogues in the Treatment of Migraine and Neurodegenerative Disorders: Preclinical Studies and Pharmaceutical Design. *Current Pharmaceutical Design,* **21**(17), 2250-2258.

Tennoune, N., Chan, P., Breton, J., Legrand, R., Chabane, Y. N., Akkermann, K., Jarv, A., Ouelaa, W., Takagi, K., Ghouzali, I., Francois, M., Lucas, N., Bole-Feysot, C., Pestel-Caron, M., do Rego, J. C., Vaudry, D., Harro, J., De, E., Dechelotte, P. & Fetissov, S. O. (2014). Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. *Translational Psychiatry,* **4**.

Tennoune, N., Legrand, R., Ouelaa, W., Breton, J., Lucas, N., Bole-Feysot, C., do Rego, J. C., Dechelotte, P. & Fetissov, S. O. (2015). Sex-related effects of nutritional

supplementation of Escherichia coli: Relevance to eating disorders. *Nutrition,* **31**(3), 498-507.
Tomasik, J., Yolken, R. H., Bahn, S. & Dickerson, F. B. (2015). Immunomodulatory Effects of Probiotic Supplementation in Schizophrenia Patients: A Randomized, Placebo-Controlled Trial. *Biomarker Insights,* **10**, 47-54.
Tsavkelova, E. A., Botvinko, I. V., Kudrin, V. S. & Oleskin, A. V. (2000). Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. *Dokl Biochem,* **372**(1-6), 115-7.
Turnbaugh, P. J. & Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *J Physiol,* **587**(Pt 17), 4153-8.
Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature,* **444**(7122), 1027-31.
Underwood, M. A., Gaerlan, S., De Leoz, M. L., Dimapasoc, L., Kalanetra, K. M., Lemay, D. G., German, J. B., Mills, D. A. & Lebrilla, C. B. (2015). Human milk oligosaccharides in premature infants: absorption, excretion, and influence on the intestinal microbiota. *Pediatr Res,* **78**(6), 670-7.
Usami, M., Kishimoto, K., Ohata, A., Miyoshi, M., Aoyama, M., Fueda, Y. & Kotani, J. (2008). Butyrate and trichostatin A attenuate nuclear factor κB activation and tumor necrosis factor α secretion and increase prostaglandin E2 secretion in human peripheral blood mononuclear cells. *Nutrition Research,* **28**(5), 321-328.
van de Wouw, M., Schellekens, H., Dinan, T. G. & Cryan, J. F. (2017). Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite. *J Nutr,* **147**(5), 727-745.
Vinolo, M. A. R., Rodrigues, H. G., Hatanaka, E., Sato, F. T., Sampaio, S. C. & Curi, R. (2011). Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *The Journal of Nutritional Biochemistry,* **22**(9), 849-855.
Vital, M., Howe, A. C., Tiedje, J. M. & Moran, M. A. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. *mBio,* **5**(2), e00889-14.
Walls, A. B., Heimbürger, C. M., Bouman, S. D., Schousboe, A. & Waagepetersen, H. S. (2009). Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. *Neuroscience,* **158**(1), 284-292.
Walsh, B. T. (2006). Fluoxetine after weight restoration in anorexia nervosa (vol 295, pg 2605, 2006). *Jama-Journal of the American Medical Association,* **296**(8), 934-934.
Wellman, P. J. (2000). Norepinephrine and the control of food intake. *Nutrition,* **16**(10), 837-842.
Whelan, K., Judd, P. A., Preedy, V. R., Simmering, R., Jann, A. & Taylor, M. A. (2005). Fructooligosaccharides and fiber partially prevent the alterations in fecal microbiota and short-chain fatty acid concentrations caused by standard enteral formula in healthy humans. *Journal of Nutrition,* **135**(8), 1896-1902.
Whelan, K., Judd, P. A., Preedy, V. R. & Taylor, M. A. (2004). Enteral feeding: the effect on faecal output, the faecal microflora and SCFA concentrations. *Proceedings of the Nutrition Society,* **63**(1), 105-113.
Williams, C. M. & Jackson, K. G. (2002). Inulin and oligofructose: effects on lipid metabolism from human studies. *Br J Nutr,* **87 Suppl 2**, S261-4.
Winter, S. E. & Baumler, A. J. (2014). Dysbiosis in the inflamed intestine Chance favors the prepared microbe. *Gut Microbes,* **5**(1), 71-73.
Wong, C. G., Bottiglieri, T. & Snead, O. C., 3rd (2003). GABA, gamma-hydroxybutyric acid, and neurological disease. *Ann Neurol,* **54 Suppl 6**, S3-12.

Wren, A. M., Small, C. J., Abbott, C. R., Dhillo, W. S., Seal, L. J., Cohen, M. A., Batterham, R. L., Taheri, S., Stanley, S. A., Ghatei, M. A. & Bloom, S. R. (2001). Ghrelin causes hyperphagia and obesity in rats. *Diabetes,* **50**(11), 2540-2547.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H. Z., Bushman, F. D. & Lewis, J. D. (2011). Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science,* **334**(6052), 105-108.

Wu, R., Dong, W., Qiang, X., Wang, H., Blau, S. A., Ravikumar, T. S. & Wang, P. (2009). Orexigenic hormone ghrelin ameliorates gut barrier dysfunction in sepsis in rats. *Crit Care Med,* **37**(8), 2421-6.

Xu, L., Cai, W. X. & Xu, B. J. (2017). A Systematic Assesment on Vitamins (B2, B12) and GABA Profiles in Fermented Soy Products Marketed in China. *Journal of Food Processing and Preservation,* **41**(5).

Yamawaki, Y., Yoshioka, N., Nozaki, K., Ito, H., Oda, K., Harada, K., Shirawachi, S., Asano, S., Aizawa, H., Yamawaki, S., Kanematsu, T. & Akagi, H. (2018). Sodium butyrate abolishes lipopolysaccharide-induced depression-like behaviors and hippocampal microglial activation in mice. *Brain Res,* **1680**, 13-38.

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K. & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell,* **161**(2), 264-276.

Yehuda, S. & Sheleff, P. (1985). The Effects of Mif-I, Beta-Endorphin and Alpha-Msh on D-Amphetamine Induced Paradoxical Behavioral Thermoregulation - Possible Involvement of the Dopaminergic System. *Peptides,* **6**(2), 189-192.

Yokoyama, S., Hiramatsu, J. & Hayakawa, K. (2002). Production of gamma-aminobutyric acid from alcohol distillery lees by Lactobacillus brevis IFO-12005. *J Biosci Bioeng,* **93**(1), 95-7.

Young, S. N. (1996). Behavioral effects of dietary neurotransmitter precursors: Basic and clinical aspects. *Neuroscience and Biobehavioral Reviews,* **20**(2), 313-323.

Zipfel, S., Wild, B., Gross, G., Friederich, H. C., Teufel, M., Schellberg, D., Giel, K. E., de Zwaan, M., Dinkel, A., Herpertz, S., Burgmer, M., Lowe, B., Tagay, S., von Wietersheim, J., Zeeck, A., Schade-Brittinger, C., Schauenburg, H., Herzog, W. & group, A. s. (2014). Focal psychodynamic therapy, cognitive behaviour therapy, and optimised treatment as usual in outpatients with anorexia nervosa (ANTOP study): randomised controlled trial. *Lancet,* **383**(9912), 127-37.

Chapter 2

The impact of neurotransmitter precursors on the faecal microbiota and neurotransmitter production under *in vitro* conditions

Abstract

Neurotransmitters (NTs) are derived from NT precursors which can be obtained from certain foods and may exert crucial effects on the nervous system in humans. For example, glutamate is a precursor for gamma-aminobutyric acid (GABA). The gut microbiota are reported as being considerable sources of NTs in the presence of precursors, however, associated experiments are often unphysiological, In this study, potato starch and tryptone were added to basal media to provide a carbohydrate and protein source, additionally dietary NT precursors (tryptophan, tyrosine and monosodium glutamate) were included with faecal microbiota of healthy female volunteers, using *in vitro* batch culture fermentation systems under physiologically relevant conditions. FISH was used to assess gut microbiota composition and microbial metabolic activities were analysed by LCMS and GC. NT precursors were shown to enhance the microbial community, and altered NT and SCFA concentrations were observed. Therefore, from this study it was determined that *in vitro* models of the human gut could be an appropriate tools for studying NT production and that the microbiota could produce NTs in the presence of precursors.

Keywords

Tryptophan, monosodium glutamate, tyrosine, neurotransmitter precursors, catecholamines, GABA, serotonin, gut-brain axis, gut microbiota.

1. Introduction

It is becoming increasingly clear that macronutrients and micronutrients in the diet can

impact on psychiatric illness such as depression (Gomez-Pinilla and Kostenkova, 2008).Food has been recognised to affect mood depending on the availability of neurotransmitter (NT) precursors, as such dietary modifications for some neurological conditions have been observed to provide relief (Choi *et al*., 2011; Millstine *et al*., 2017). NT precursors are nutritional supplements such as vitamins, minerals and amino acids that can increase the production of NTs (brain body medical, 2018). Levels of NTs in humans, for example, biogenic amines like dopamine (DA), serotonin (5-HT) and gamma-aminobutyric acid (GABA) can be enhanced through consumption of foods containing amino acids including meat, fruits and edible plants(Roshchina *et al*., 2010). 5-HT is a NT derived from its precursor tryptophan via the tryptophan hydroxylase (TPH) enzyme (Bailey *et al*., 2011). 5-HT plays a role in the modulation of host behaviour, GI motility and erythrocyte health (Spohn and Mawe, 2017). There are three main catecholamines (CA), these are DA, norepinephrine (NE) and epinephrine (EPI) which are derived from tyrosine. CA may play a major role in maintaining nutrient absorption rates (Olaleye and Elegbe, 2005) and suppression of inflammation (Farache *et al*., 2013; Abrass *et al*., 1985). GABA is produced primarily from amino acid monosodium glutamate (MSG) by the enzyme glutamate decarboxylase (GAD) (Ueno *et al*., 2000). GABA is a major inhibitory NT in the brain and modulates many physiological and psychological processes. Dysfunctions in the GABA system are implicated in anxiety and depression (Cryan and Kaupmann, 2005; Schousboe and Waagepetersen, 2007). The gastrointestinal tract (GIT) affects the mental status of the host via the bidirectional communication between the gut and brain, which is called microbiota–gut–brain axis (MGBA) (Grenham *et al*., 2011). Gut microbiota inhabits the GIT and has been reported to be involved in production of active NTs that influence the brain via the vagus nerve (Matsumoto *et al*., 2013; Dinan and Cryan, 2013). NTs availability may therefore by influenced by microbial activity (Stickel *et al*., 2009; Naila *et al*., 2010).

The gastrointestinal tract (GIT) has been indicated to actively regulate metabolism and concentration of NTs precursors and production of NTs to then influence the nervous system (Zagajewski *et al*., 2012; Clarke *et al*., 2014; Evrensel and Ceylan, 2015). Members of the gut microbiota have been implicated in the formation of NTs. For example, *Lactobacillus brevis* and *Bifidobacterium dentium* derived from the human intestine are able to utilise MSG to produce GABA (Barrett *et al*., 2012). The genera *Bacillus* and *Serratia* were confirmed play a key role in forming biologically active dopamine in the lumen (Asano *et al*., 2012; Lyte, 2011). Therefore, a considerable exogenous source of NTs could come from gut microbiota (Yunes *et al*., 2016).

Gut microbiota contribute to host metabolism by production of short chain fatty acids (SCFAs) including butyrate, acetate and propionate, which are known to have neuroactive properties (Russell *et al*., 2013). Reduced faecal SCFA concentrations have been confirmed in GBA related disorders where brain physiology and behaviour are altered, including autism spectrum disorders (Liu *et al*., 2019) anorexia nervosa (Morita *et al*., 2015). and Parkinson's disease (Unger *et al*., 2016).

There is some evidence that a subcutaneous injection of MSG (4g/day) led to adult rats being more prone to anxiety and depression-like symptoms (Quines *et al*., 2014). An animal study indicated that intake of tryptophan significantly decreased the frequency of activation of serotonergic neurons (Trulson *et al*., 1976). As such, these studies support that NT precursors can impact on NTs production or activities. Most of the studies discussed did not evaluate the gut microbial community after NT precursors intervention. Therefore, the aim of the current research was to investigate whether, under physiologically relevant conditions, human intestinal microorganisms combined with NTs precursors could generate NTs and end products associated with GBA whilst modulating the microbiota. Those that have tried to

isolate epithelial cells from faeces have experienced difficulties, therefore it is highly unlikely that human epithelial cells can influence the results (Albaugh et al., 1992; Bandaletova et al., 2002). This experiment therefore set out to determine if NT precursors within a faecal community could result in increased levels of NTs in the absence of host cells.

2. Materials and methods

2.1. Faecal inoculation

Faecal samples were provided from three healthy donors (3 female aged between 25-33 years) who were free from known 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 performed 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 collected in a clean white container before being placed in an anaerobic jar (AnaeroJar™ 2.5L; Oxoid Ltd, Basingstoke, Hampshire, UK) with an anaerobic gas generating kit (AnaeroGen™; Oxoid). Within 30 mins of collection samples were diluted 1/10 w/v 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.

2.2. In vitro batch culture fermentation

This method was previously described by Gomez et al. (2010). Sterile vessels were attached to 37 °C water bath with N_2 input and outputs. 135 mL of sterile basal medium was added to each vessel aseptically (Peptone water 2 g, yeast extract 2 g, NaCl 0.1 g, K_2HPO_4 0.04 g, KH_2PO_4 0.04 g, $MgSO_4.7H_2O$ 0.01 g, $CaCl_2.6H_2O$, 0.01 g, $NaHCO_3$ 2 g, Tween 80 2 mL, haemin 0.05 g, Vitamin K 10µL, L-cysteine HCL 0.5 g, bile salt 0.5 g per litre (Sigma Aldrich Ltd, Poole, Dorset, UK). Additionally, to provide a carbon and nitrogen source potato starch 7.5 g, tryptone 4.5 g per litre was added to the media (Sigma Aldrich Ltd, Poole, Dorset, UK) Each fermentation vessel was incubated overnight in anaerobic conditions (oxygen-free nitrogen at a rate of 15 mL/min). The next day, a calibrated probe was placed into the vessel and the pH adjusted at a range between 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 were maintained according to the conditions of the distal part of the human large intestine. 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 (5 mL from each vessel) were collected at 0, 6 and 24 h for analysis of bacterial populations and metabolite production.

The following day the treatments were added to the vessels as outlined below:

Vessel 1: Blank – no treatment added

Vessel 2: Monosodium glutamate (10 mg)

Vessel 3: Tryptophan (10 mg)

Vessel 4: Tyrosine (10 mg)

2.3. Preparation of the samples for NTs, SCFAs and Bacterial community analysis

During fermentation, samples were collected from each vessel over a series of time points (0, 6 and 24 h). A 1 mL sample was collected and centrifuged at 13,000 × *g* for 10 min then stored at −20 °C for NTs and SCFAs analysis. For bacterial community analysis, a 750 µL supernatant of fermentation fluid was centrifuged at 13,000 × *g* for 5 min. The pellet was then resuspended in 375 µL filtered 0.1 M PBS and fixed by 1125 µL filtered paraformaldehyde (PFA 4% w/v) for 4 to 8 h at 4 °C. The sample was washed twice with 1 mL PBS to remove PFA and resuspended in filtered 600 µL ethanol-PBS (1:1, w/v). The samples were kept at −20 °C prior to microbial analysis.

2.4. Enumeration of Bacterial Population by Flow-Fluorescent In Situ Hybridisation (FISH)

The bacterial population was analysed using fluorescent *in situ* hybridisation coupled to flow cytometry (FISH-FCM). (BD Accuri™ C6 Plus, Basingstoke, United Kingdom), detecting at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software. Samples were removed from storage at -20 °C. After defrosting and vortexing for 10 seconds permeabilisation steps were conducted using 500 µL 0.1M PBS added to 75 µL fixed samples and centrifuged at 13,000 x *g* for 3 min. The pellets were resuspended in 100 µL of TE-FISH buffer (Tris-HCl 1 M pH 8, EDTA 0.5 M pH 8, filtered distilled water, 0.22 µm pore size filter with the percentage of 10:10:80) containing lysozyme solution (1 mg/ml of 50,000 U/mg protein) and incubated for 10 min in the dark at room temperature and then centrifuged at 13,000 x *g* for 3 min. Pellets were washed with 0.1 M 500 µL PBS and then washed with 150 µL hybridisation buffer (0.9 M NaCl, 0.2 M Tris/HCl pH 8.0, 30% formamide, ddH_2O,

0.01% sodium dodecyl sulphate) and centrifuged at 13,000 x *g* for 3 min. Pellets were then resuspended in 1 mL of hybridisation buffer, homogenised and 50 µL with 4 µL of different probes aliquoted into Eppendorf tubes (1.5 mL) were incubated at 36°C overnight. Differences in bacterial populations were quantified with oligonucleotide probes aimed to target specific regions of 16S rRNA. The individual probes used (Eurofins, Wolverhampton, UK) in this study are shown in Table 1. Non-EUB and EUB338-I-II-III were linked to fluorescence Alexa 488 at the 5' end, and group specific probes were linked to fluorescence Alexa 647. Non-EUB and EUB338 were linked to Alexa 647 at the 5' end as controls to adjust threshold. 4 µL of EUB338-I-II-III was added together with 4 µL specific probes. 125 µL of hybridisation buffer was added to each Eppendorf tube after incubation samples were vortexed and centrifuged (13,000 x *g*, 3 min). Supernatants were removed and pellets were washed with 175 µL washing buffer solution (0.064 M NaCl, 0.02M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate, 956.2 µL of ddh_2O), vortexed and incubated at 38°C in a heating block for 20 min to remove non-specific binding of the probe. Afterwards samples were centrifuged (13,000 x *g*, 3 min) and supernatants removed. Pellets were resuspended in an appropriate volume of PBS on the basis of FCM load. Number of bacteria were then calculated through determination of FCM reading and PBS dilution.

Table 1. Oligonucleotide probes used in the study for bacterial populations by fluorescent *in situ* hybridisation.

Probe name	**Sequence (5' to 3')**	**Target species**	**Reference**

Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	Wallner et al. (1993)
Eub338I +	GCTGCCTCCCGTAGGAGT	Most Bacteria	Daims et al. (1999)
Eub338II +	GCAGCCACCCGTAGGTGT	*Planctomycetales*	Daims et al. (1999)
Eub338III +	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	Daims et al. (1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk et al. (1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	Harmsen et al. (1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	Manz et al. (1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	Franks et al. (1998)

Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	Walker et al. (2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	Harmsen et al. (2000)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	Walker et al. (2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	Hold et al. (2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Devereux et al. (1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	Franks et al. (1998)

2.5. NTs analysis by Liquid chromatography mass spectrometry (LCMS)

2.5.1. Reagents and chemicals

HPLC Plus grade acetonitrile (≥99.9%) was purchased from Sigma-Aldrich. 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 gut model fluid

samples. Analytical standards powder including LC-MS grade dopamine hydrochloride (99%) and L (-)-Epinephrine (99%) were purchased from Alfa Aesar (Lancashire, UK). L-Noradrenaline (98%), Gamma-Aminobutyric acid (99%) and serotonin were purchased from Sigma-Aldrich Co Ltd.

Separate standard stock solutions (10000 ng/ml) of five analytes including 5-HT, DA, GABA, NE and Epinephrine (EPI) were individually prepared in HPLC water. A 1000 ng/ml mixed standard solution containing the five 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 10 levels: 1, 10, 50, 100, 250, 500, 750, 1000, 2500, 5000 ng/ml. Samples were removed from storage at -20 °C. A 400 µL sample of gut model fluid supernatant was collected in a centrifuge tube filter (Sigma-Aldrich, 0.22 µm, Polypropylene) and then centrifuged at 13,000 X *g* for 10 min at 4 °C (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) and the supernatant remained. 200 µL of HPLC water (blank), calibration standard samples and gut model samples were placed in a 96-well plate.

2.5.2. LCMS system

Samples were measured using online Nexera LC System coupled to LCMS-8050 triple quadrupole (QQQ) mass spectrometry (Shimadzu, Kyoto, Corporation, Japan). Data was processed using LabSolutions LCMS version 5.65 software.

2.5.3. Liquid chromatography (LC) conditions

The chromatographic separation of analytes was obtained from Discovery HS F5-3 column (2.1 mmI.D. x 150 mmL, 3 µm particle size, Sigma-Aldrich Co Ltd, P/N 567503-U). The mobile phase consisted of 0.1% formic acid in water (mobile A) and 0.1% formic acid in acetonitrile (mobile B). For the entire analysis, the flow rates of both mobile phases were 0.25 mL/min, and the autosampler temperature at a maintained constant temperature was set to 4°C. The gradient elution programs were as follows: B conc. 25% (5 min) ➔ 35% (11min) ➔ 95% (15 min) ➔ 95% (20 min) ➔ 0% (20.01- 25 min).

2.5.4 Mass spectrometry (MS) conditions

The LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using 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. The MRM transitions of the native, stable isotopes, retention times and other conditions are shown in Table 2.

Table 2. Optimal conditions of LC-MS/MS used for the quantification of DA, 5-HT, NE, EPI and GABA in faecal supernatant.

Compounds name	**Precursor ion (m/z)**	**Product ion (m/z)**	**Retention time (min)**	**Classification**

5-HT	177.10	160.10	10.527	Amino acid derivative
DA	154.10	91.05	8.078	Amino acid derivative
NE	170.10	152.15	4.988	Catecholamine
EPI	184.10	166.10	7.164	Catecholamine
GABA	104.10	87.05	3.690	Organic acid

Each analyte of ionisation polarity is (+).

2.5.5 Quantification of samples

A linear calibration curve was generated based on the detected signal proportional to the concentration of the analyte. Briefly, results validation was performed following published procedures (Toxicology SwGfF, 2013). Good linearity with R^2 greater than 0.98 was obtained across the set calibration in the range from 1 ng/mL to 5000 ng/mL for each of the analytes, with an accuracy of within 100 % ± 20 %. Quantification of samples was determined by calibration with five analytes including 5-HT, DA, EPI, GABA and NE. Standard calibration concentration and samples of chromatogram peaks were quantified using the software (Requires LabSolutions LCMA version 5.65) following manual inspection.

2.6. Short Chain and Branched Chain Fatty Acid Analysis by Gas Chromatography

The concentration of SCFA was determined by Gas chromatography (GC) as previously described by Richardson et al (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 × 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 chromatograph (Agilent, Cheshire, UK) using an HP-5ms (L × I.D. 30 m × 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 by 5 °C and held at 190 °C for 30 min. Helium was the carrier gas (flow rate, 1.7 mL/min, head pressure, 133 KPa). Peak areas were integrated using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK). SCFA production was quantified by single-point internal standard method as described by *(Liu et al., 2016)*. Peak areas of the standard (acetate, butyrate, propionate, valerate, iso-valerate and iso-butyrate) were used to calculate the response factors for each organic acid with respect to the internal standard.

2.7. Statistical Analyses

All statistical analyses used IBM SPSS version 27 (IBM Corp., USA). The FISH-FCM and NTs production were analysed using one-way mixed ANOVA to compare different test substrates and time points; a *post hoc* Tukey HSD (Honestly Significant Difference) test was performed using to determine significant differences between blank and substrates at respective time point. Repeated measured ANOVA was used to further analyse the data from the same vessel at the multiple time points. difference between vessels at each time point. Statistical analysis was accepted at $p < 0.05$ for all analyses. Graphs were made using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). FISH results are reported as mean ± RSD. Mean of log transformed data is a geometric mean in log, so the SD reported is therefore the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

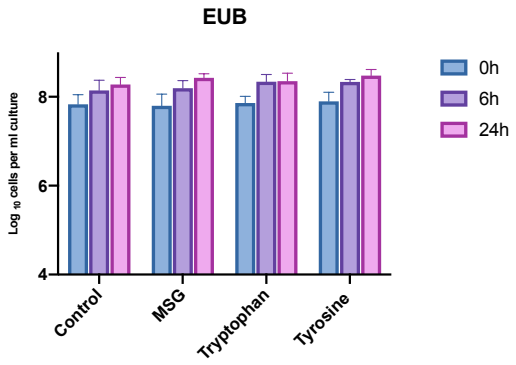
3. Results

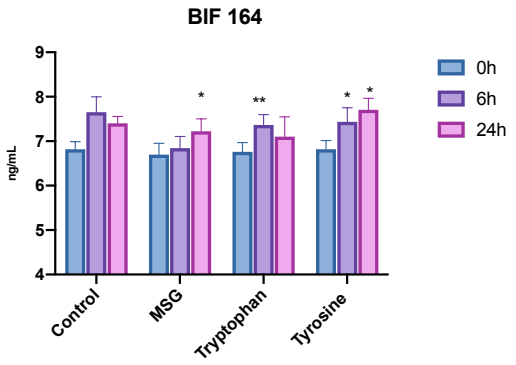
3.1. Effects of different NTs precursors on faecal bacterial growth

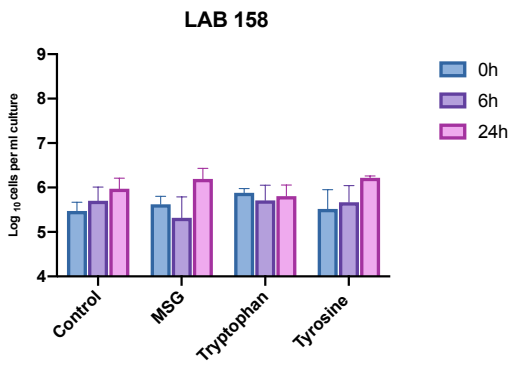
Changes in bacterial compositions in the *in vitro* batch culture fermentation at 0, 6 and 24 h are reported in Figure 1. The numbers of bacterial community were not significantly different to the blank during the fermentation. Compared to the baseline, both fermentation of tryptophan and tyrosine led to significant increased numbers of *Bifidobacterum* at 6 h (6.75 to 7.36 $\log_{10}$ cells/mL, $p < 0.01$ and 6.81 to 7.43 $\log_{10}$ cells/mL, $p < 0.05$). After 24 h, *Bifidobacterium* was significantly increased following both MSG and tyrosine fermentation (6.69 to 6.84 $\log_{10}$ cells/mL, $p < 0.05$ and 6.81 to 7.71 $\log_{10}$ cells/mL, $p < 0.05$). *Clostridium coccoides-Eubacterium rectale* levels were significantly increased over the whole

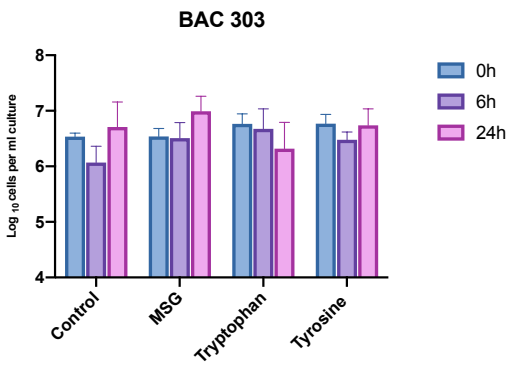
fermentation time after tyrosine administration (7.36 to 7.81 at 6 h, p <0.05 and 7.36 to 7.75 $\log_{10}$ cells/mL at 24 h, $p < 0.05$). Compared to baseline, tyrosine administration led to significant increase both numbers of *Atopobium* group (5.82 to 6.69 $\log_{10}$ cells/mL at 24 h, $p < 0.05$) and *Clostridium histolyticum* group (5.66 to 6.33 $\log_{10}$ cells/mL at 6 h, $p < 0.05$).

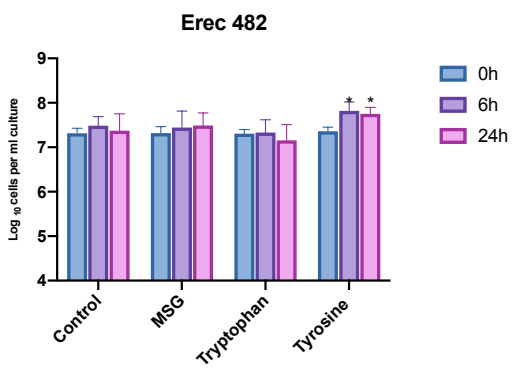
Figure 1. Bacterial groups detected by FISH-FCM (Log_{10} cells /mL) following baseline (0 h), 6 h and 24 h with the administration of MSG, tryptophan and tyrosine of within a pH controlled in vitro faecal (1%) batch culture system. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h and 24 h are indicated. FISH results are reported as mean ± RSD. Mean of log transformed data is a geometric mean in log, so the SD reported is therefore the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

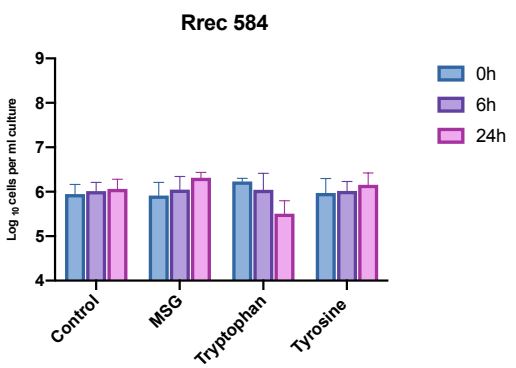

EUB
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine

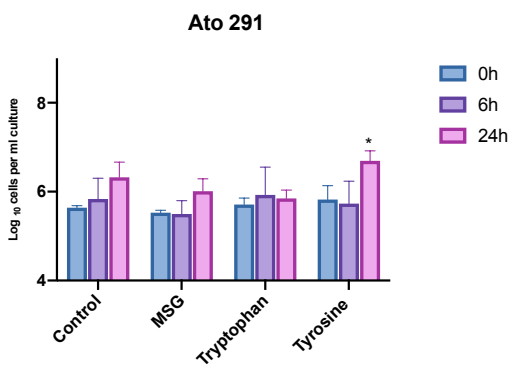

BIF 164
ng/mL
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine

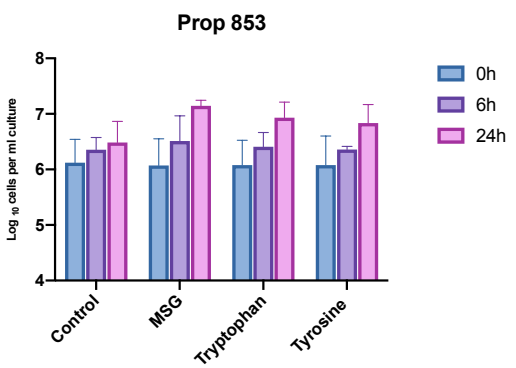

LAB 158
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine

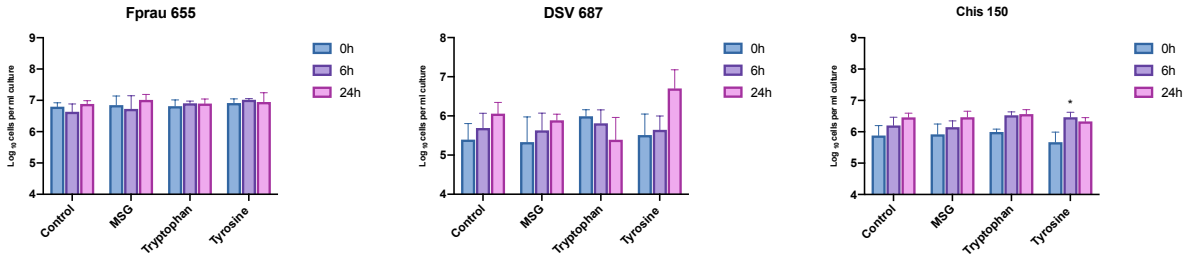

BAC 303
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine


Erec 482
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine


Rrec 584
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine


Ato 291
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine


Prop 853
Log 10 cells per ml culture
0h
6h
24h
Control
MSG
Tryptophan
Tyrosine



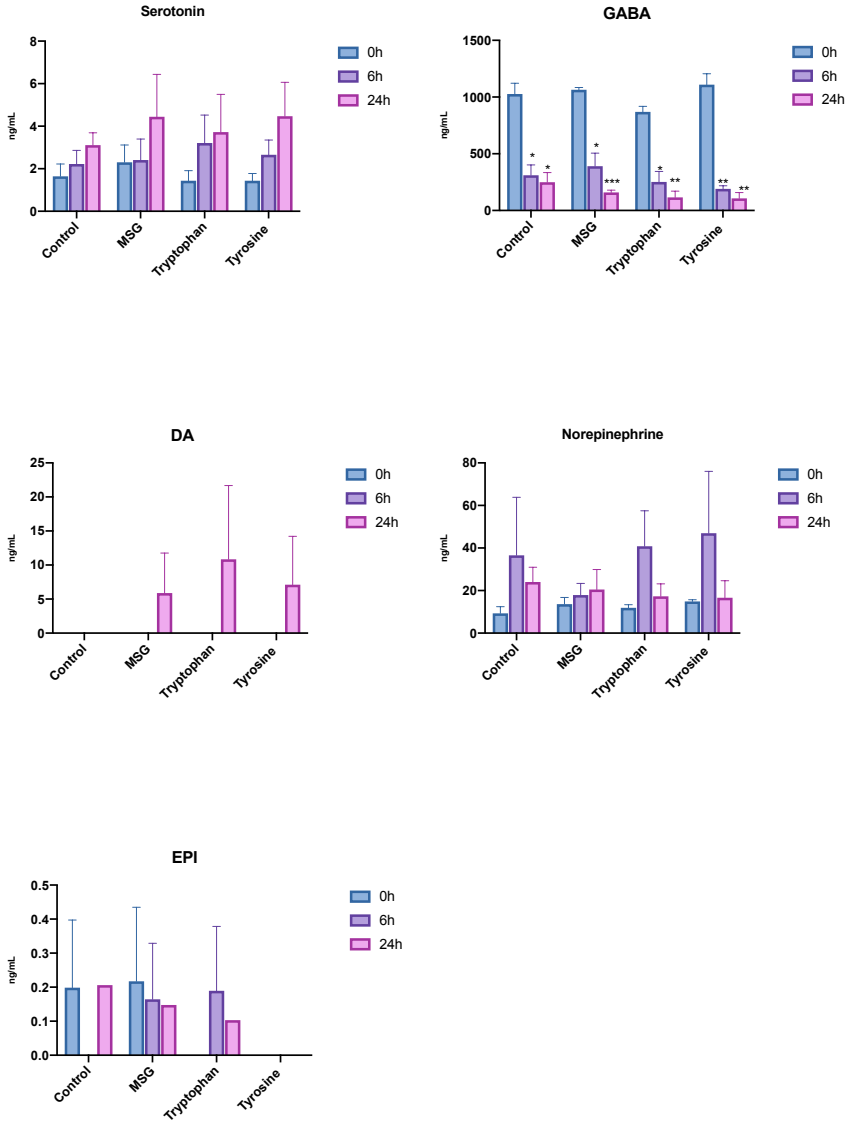
3.2. Effects of different NTs precursors on NTs production

Changes in NTs concentrations in the *in vitro* batch culture fermentation at 0, 6 and 24 h are reported in Figure 2. The amounts of all NTs production were not significantly different to the blank. Compared to time 0, less of a reduction in GABA was observed in the vessel that contained MSG at 6 h and 24 h as compared to the other treatments. Compared to baseline, tyrosine fermentation resulted in a trend of increased 5-HT production at 6h (1.43 to 2.66 ng/mL, $p < 0.1$). DA concentration could not be detected at baseline and 6 h in all treatment vessels and can be only detected in all three NTs precursors vessels at 24 h.

NE production reached peak in all vessels at 6 h when compared to time 0. MSG was the only treatment that elevated increase in NE production at all time points when compared to other treatments (from13.62 to 17.90 ng/mL at 6 h p =0.59; and from 17.90 to 20.52 ng/mL at 24 h p =0.38).

Compared to baseline, GABA amounts significantly decreased in all Vessels at 6 h and 24 h (blank vessel: from 1026.3 to 310.36 ng/mL at 6 h, $p < 0.05$; from 310.36 to 249.86 at 24 h, $p < 0.05$; MSG vessel: from 1064.3 to 391.10 ng/mL at 6 h, $p < 0.05$; from 391.10 to 159.88 at 24 h, $p < 0.001$; tryptophan vessel: from 868.8 to 252.3 ng/mL at 6 h, $p < 0.05$; from 252.3 to 116.07 at 24 h, $p < 0.01$; tyrosine vessel: from 1109.8 to 190.60 ng/mL at 6 h, $p < 0.01$; from 190.60 to 107.41at 24 h, $p < 0.01$).

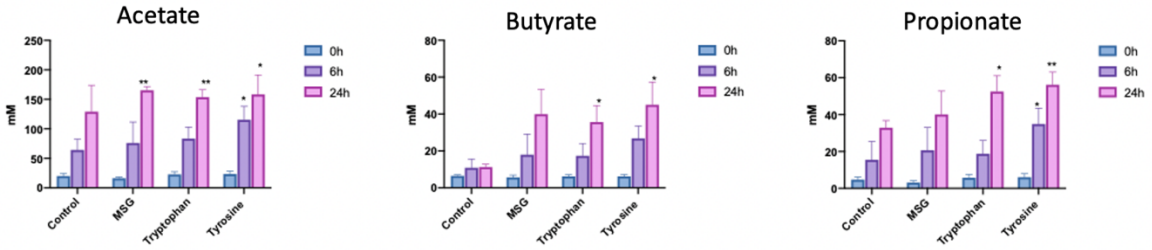
Figure 2. LCMS analysis. 5-HT, GABA, DA, NE and EPI concentration detected by LCMS (ng/mL) following baseline (0 h), 6 h and 24 h with administration of MSG, tryptophan and tyrosine of within a pH controlled in vitro faecal (1%) batch culture system. Results are reported as Means ± SD. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h and 24 h are indicated.



3.3. Effects of different NTs precursors on organic acid production

Changes in organic acid concentrations in the *in vitro* batch culture fermentation at 0, 6 and 24 h are reported in Figure 3. The acetate amounts were not significantly different to the blank. Compared to baseline, acetate significantly increased upon fermentation of all NTs precursors at 24 h (16.40 to 165.33 mM MSG vessel, $p < 0.01$; 22.78 to 153.71 mM tryptophan, $p < 0.01$; 23.53 to 158.33 mM tyrosine, $p < 0.05$). Compared to blank vessel baseline, there was a significant increase in butyrate concentration upon both tryptophan (11.23 versus 35.61 mM, $p < 0.05$) and tyrosine (11.23 versus 45.01 mM, $p < 0.05$) fermentation at 24 h. Compared to time 0, propionate production significantly increased at all time points in the tyrosine vessel (6.11 to 34.87 mM at 6 h, $p < 0.05$; 6.11 to 56.13 mM at 24 h, $p < 0.01$). Tryptophan led to a significant increase in propionate at 24 h (5.77 to 52.47 mM, $p < 0.05$) when compared to time 0.

Figure 3. GC analysis. Acetate, butyrate and propionate amounts detected by GC (mM) following baseline (0 h), 6 h and 24 h with administration of MSG, tryptophan and tyrosine of within a pH controlled in vitro faecal (1%) batch culture system. Results are reported as Means ± SD. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h and 24 h are indicated.



4. Discussion

This study investigated fermentation of NT precursors to determine if they could modulate the microbiota and enhance production of NTs and SCFAs. Whilst authors have previously reported the microbiota can product NTs many experiments have not included physiologically relevant conditions, as such *in vitro* models were used to assess microbial production in the absence of human cells. In initial work by ourselves the gut microbial community did not produce detectable amounts of NTs in the presence of potato starch. However, these experiments lacked NTs precursors. Gut bacteria can breakdown potato starch, which provides them with a growth advantage in the gut at the same time as accumulation of SCFAs (Trachsel *et al*., 2019).For instance, pigs fed a diet containing resistant potato starch exhibited alterations associated with gut health when compared to a control diet (Trachsel *et al*., 2019). As such, within the current study additional potato starch and tryptone (a protein source) was added into defined basil media as to ensure substrates were available for microbial growth as only trace amounts of NT precursors were to be used within the batch cultures.

Butyrate is indicted to stimulate memory and synaptic plasticity by inhibiting histone deacetylases (Vecsey *et al*., 2007; Stefanko *et al*., 2009). Studies have also pointed that propionate produced by gut bacteria with prebiotic intervention protects the blood–brain barrier (BBB) from oxidative stress (Hoyles *et al*., 2018). Additionally, SCFAs can impact on neuroinflammation by regulating the recruitment and production of immune cells such as inflammatory cytokines and neutrophils (Park *et al*., 2019). This current study demonstrated that both tryptophan and tyrosine administration positively affected the production of acetate, butyrate and propionate. This is of good potential in regulating the impact on microbial metabolic activity in the distal colon to improve gut health and impact on the GBA.

MSG is a promising candidate for modulating GBA, for its fermentation resulted in enhanced NE and EPI, as compared to tyrosine (precursor of NE and EPI) and tryptophan treatment. A possible reason could be aromatic amino acid (AAA) including tyrosine and tryptophan can be utilised by the gut bacteria and fermented to phenylacetic acid, phenylpropanoid metabolites and 4-hydroxyphenyl-acetic acid (Russell *et al*., 2013). NTs precursors may be therefore utilised by this pathway prior to the synthesis of NE and EPI. This MSG effect is also corroborated by previous *in vivo* research where Russell observed that MSG infusion led to significantly greater release of NE from rats suffering from spontaneous hypertensive syndrome (Russell *et al*., 2001).

This current study observed a decrease in GABA levels throughout the fermentation time. There are major factors affecting GABA synthesis by microbial fermentation, these are 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. For example, in a study by Kim et al., (2009) black raspberry juice during fermentation by *Lactobacillus brevis* resulted in highest GABA production on the 15th day at 25°C, pH 4.0 where the lowest GABA content was found under the pH 6.0 administration (Kim *et al*., 2009). In addition, nitrogen and carbon sources can affect the amount of GABA production (Blanc *et al*., 1994; Pimentel *et al*., 1996; Wang *et al*., 2003). However, the conversion of GABA in microorganisms is mainly affected by pH which has been observed in many studies (Komatsuzaki *et al*., 2005; Tsai *et al*., 2006; Yang *et al*., 2008). It has been reported that pH regulation at optimum pH for enzyme GAD activity is highest at pH 5.0, and this is when GABA amounts are significantly enhanced (Komatsuzaki *et al*., 2005). The capacity to produce GABA trough glutamate decarboxylation is commonly found in potentially lethal acidic environments. It is generally

held that the hydrogen ion consumed by glutamate during the decarboxylation reaction helps to prevent excessive acidification of the cytoplasm, thereby protecting the cells against acidic environments. In this study the range of pH is 6.7-6.9, which may limit the availability of hydrogen ion to support GABA synthesis (Komatsuzaki et al., 2005). This current study only tested the effects of the MSG on GABA production in a defined batch culture medium at a neutral pH, with temperature and fermentation time associated with that of distal colon physicochemical conditions.

With this current study, it was possible to detect levels of all five of the NTs *in vitro*. However, it must be noted that the levels of these NTs were low and unlikely to be physiologically relevant. For example, a study indicated that the GABA level was presented in a broad range at 0–330 μg/g from healthy human faecal (Altaib *et al*., 2021). These results indicate that the gut microbiota may need a host cell to enhance production of NTs and might involve the production pathways of these NTs by the host. On the other hand, although the NTs production amounts were very low, dietary prebiotics such as FOS and B-GOS might have effects on the growth of the gut microbial community enhancing NTs levels (Flint *et al*., 2012; Verbeke *et al*., 2015; Oriach *et al*., 2016). As such it is worth exploring further within these *in vitro* models whilst including different substrates.

5. Conclusions

Overall, the current study reveals that microbial community and its metabolites differ based on NTs precursors under the physiologically relevant conditions. This suggests the important role of commensal gut microbiota in mediating NTs and SCFAs

production in the presence of NTs precursors. This study highlights the importance of evaluating the neuroactive potential and composition of the gut microbiota, as this information could be essential for positive alteration of the GBA via manipulation of microbes. The finding of this study show that *in vitro* systems can be used as a tool for assessing NT levels, further manipulation with pre and probiotics is warranted to see what impact these other factors can have.

References

(2013). Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol,* **37**(7), 452-74.

Abrass, C. K., O'Connor, S. W., Scarpace, P. J. & Abrass, I. B. (1985). Characterization of the beta-adrenergic receptor of the rat peritoneal macrophage. *J Immunol,* **135**(2), 1338-41.

Altaib, H., Nakamura, K., Abe, M., Badr, Y., Yanase, E., Nomura, I. & Suzuki, T. (2021). Differences in the Concentration of the Fecal Neurotransmitters GABA and Glutamate Are Associated with Microbial Composition among Healthy Human Subjects. *Microorganisms,* **9**(2), 378.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y. & Sudo, N. (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *Am J Physiol Gastrointest Liver Physiol,* **303**(11), G1288-95.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun,* **25**(3), 397-407.

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

Blanc, P., Loret, M., Santerre, A., Pareilleux, A., Prome, D., Prome, J., Laussac, J. & Goma, G. (1994). Pigments of monascus. *Journal of food science,* **59**(4), 862-865.

Choi, S., DiSilvio, B., Fernstrom, M. H. & Fernstrom, J. D. (2011). Effect of chronic protein ingestion on tyrosine and tryptophan levels and catecholamine and serotonin synthesis in rat brain. *Nutr Neurosci,* **14**(6), 260-7.

Clarke, S. F., Murphy, E. F., O'Sullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., Hayes, P., O'Reilly, M., Jeffery, I. B., Wood-Martin, R., Kerins, D. M., Quigley, E., Ross, R. P., O'Toole, P. W., Molloy, M. G., Falvey, E., Shanahan, F. & Cotter, P. D. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut,* **63**(12), 1913-20.

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

Dhakal, R., Bajpai, V. K. & Baek, K. H. (2012). Production of gaba (γ - Aminobutyric acid) by microorganisms: a review. *Braz J Microbiol,* **43**(4), 1230-41.

Dinan, T. G. & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil,* **25**(9), 713-9.

Evrensel, A. & Ceylan, M. E. (2015). The Gut-Brain Axis: The Missing Link in Depression. *Clinical psychopharmacology and neuroscience : the official scientific journal of the Korean College of Neuropsychopharmacology,* **13**(3), 239-244.

Farache, J., Koren, I., Milo, I., Gurevich, I., Kim, K. W., Zigmond, E., Furtado, G. C., Lira, S. A. & Shakhar, G. (2013). Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity,* **38**(3), 581-95.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes,* **3**(4), 289-306.

Gomez-Pinilla, F. & Kostenkova, K. (2008). The influence of diet and physical activity on brain repair and neurosurgical outcome. *Surgical neurology,* **70**(4), 333-336.

Grenham, S., Clarke, G., Cryan, J. F. & Dinan, T. G. (2011). Brain-gut-microbe communication in health and disease. *Frontiers in Physiology,* **2**.
Hoyles, L., Snelling, T., Umlai, U.-K., Nicholson, J. K., Carding, S. R., Glen, R. C. & McArthur, S. (2018). Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier. *Microbiome,* **6**(1), 55.
Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S. & Hwang, K. T. (2009). Production of gamma-aminobutyric acid in black raspberry juice during fermentation by Lactobacillus brevis GABA100. *Int J Food Microbiol,* **130**(1), 12-6.
Komatsuzaki, N., Shima, J., Kawamoto, S., Momose, H. & Kimura, T. (2005). Production of γ-aminobutyric acid (GABA) by Lactobacillus paracasei isolated from traditional fermented foods. *Food Microbiology,* **22**(6), 497-504.
Liu, T.-L., Wang, P.-W., Yang, Y.-H. C., Shyi, G. C.-W. & Yen, C.-F. (2019). Association between Facial Emotion Recognition and Bullying Involvement among Adolescents with High-Functioning Autism Spectrum Disorder. *International journal of environmental research and public health,* **16**(24), 5125.
Liu, Y., Gibson, G. R. & Walton, G. E. (2016). An In Vitro Approach to Study Effects of Prebiotics and Probiotics on the Faecal Microbiota and Selected Immune Parameters Relevant to the Elderly. *PLoS One,* **11**(9), e0162604.
Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays,* **33**(8), 574-581.
Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Sawaki, E., Koga, Y. & Benno, Y. (2013). Cerebral Low-Molecular Metabolites Influenced by Intestinal Microbiota: A Pilot Study. *Frontiers in Systems Neuroscience,* **7**.
Millstine, D., Chen, C. Y. & Bauer, B. (2017). Complementary and integrative medicine in the management of headache. *Bmj,* **357**, j1805.
Morita, C., Tsuji, H., Hata, T., Gondo, M., Takakura, S., Kawai, K., Yoshihara, K., Ogata, K., Nomoto, K., Miyazaki, K. & Sudo, N. (2015). Gut Dysbiosis in Patients with Anorexia Nervosa. *PLoS One,* **10**(12), e0145274.
Naila, A., Flint, S., Fletcher, G., Bremer, P. & Meerdink, G. (2010). Control of biogenic amines in food--existing and emerging approaches. *J Food Sci,* **75**(7), R139-50.
Olaleye, S. B. & Elegbe, R. A. (2005). Catecholamines potentiate the effect of thyroid hormone on intestinal absorption of glucose in the rat. *Afr J Med Med Sci,* **34**(2), 177-83.
Oriach, C. S., Robertson, R. C., Stanton, C., Cryan, J. F. & Dinan, T. G. (2016). Food for thought: The role of nutrition in the microbiota-gut–brain axis. *Clinical Nutrition Experimental,* **6**, 25-38.
Park, J., Wang, Q., Wu, Q., Mao-Draayer, Y. & Kim, C. H. (2019). Bidirectional regulatory potentials of short-chain fatty acids and their G-protein-coupled receptors in autoimmune neuroinflammation. *Scientific Reports,* **9**(1), 8837.
Pimentel, M. C., Melo, E. H., Lima Filho, J. L. & Durán, N. (1996). Production of lipase free of citrinin by Penicillium citrinum. *Mycopathologia,* **133**(2), 119-21.
Quines, C. B., Rosa, S. G., Da Rocha, J. T., Gai, B. M., Bortolatto, C. F., Duarte, M. M. & Nogueira, C. W. (2014). Monosodium glutamate, a food additive, induces depressive-like and anxiogenic-like behaviors in young rats. *Life Sci,* **107**(1-2), 27-31.
Richardson, A. J., Calder, A. G., Stewart, C. S. & Smith, A. (1989). Simultaneous Determination of Volatile and Non-Volatile Acidic Fermentation Products of Anaerobes by Capillary Gas-Chromatography. *Letters in Applied Microbiology,* **9**(1), 5-8.

Roshchina, G., Koroleva, V. I. & Davydov, V. I. (2010). [Changes in the high-frequency electrical brain activity in the animal hypnosis state in rabbits]. *Zh Vyssh Nerv Deiat Im I P Pavlova,* **60**(3), 352-63.

Russell, V., Allie, S. & Wiggins, T. (2001). Increased noradrenergic activity in prefrontal cortex slices of an animal model for attention-deficit hyperactivity disorder - The spontaneously hypertensive rat. *Behavioural Brain Research,* **117**, 69-74.

Russell, W. R., Duncan, S. H., Scobbie, L., Duncan, G., Cantlay, L., Calder, A. G., Anderson, S. E. & Flint, H. J. (2013). Major phenylpropanoid-derived metabolites in the human gut can arise from microbial fermentation of protein. *Mol Nutr Food Res,* **57**(3), 523-35.

Schousboe, A. & Waagepetersen, H. S. (2007). GABA: Homeostatic and pharmacological aspects. *Gaba and the Basal Ganglia: From Molecules to Systems,* **160**, 9-19.

Spohn, S. N. & Mawe, G. M. (2017). Non-conventional features of peripheral serotonin signalling - the gut and beyond. *Nat Rev Gastroenterol Hepatol,* **14**(7), 412-420.

Stefanko, D. P., Barrett, R. M., Ly, A. R., Reolon, G. K. & Wood, M. A. (2009). Modulation of long-term memory for object recognition via HDAC inhibition. *Proc Natl Acad Sci U S A,* **106**(23), 9447-52.

Stickel, F., Droz, S., Patsenker, E., Bögli-Stuber, K., Aebi, B. & Leib, S. L. (2009). Severe hepatotoxicity following ingestion of Herbalife nutritional supplements contaminated with Bacillus subtilis. *J Hepatol,* **50**(1), 111-7.

Trachsel, J., Briggs, C., Gabler, N. K., Allen, H. K. & Loving, C. L. (2019). Dietary Resistant Potato Starch Alters Intestinal Microbial Communities and Their Metabolites, and Markers of Immune Regulation and Barrier Function in Swine. *Frontiers in Immunology,* **10**.

Trulson, M. E., Eubanks, E. E. & Jacobs, B. L. (1976). Behavioral evidence for supersensitivity following destruction of central serotonergic nerve terminals by 5,7-dihydroxytryptamine. *Journal of Pharmacology and Experimental Therapeutics,* **198**(1), 23-32.

Tsai, J. S., Lin, Y. S., Pan, B. S. & Chen, T. J. (2006). Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochemistry,* **41**(6), 1282-1288.

Ueno, S., Lin, A., Nikolaeva, N., Trudell, J. R., Mihic, S. J., Harris, R. A. & Harrison, N. L. (2000). Tryptophan scanning mutagenesis in TM2 of the GABA(A) receptor alpha subunit: effects on channel gating and regulation by ethanol. *British journal of pharmacology,* **131**(2), 296-302.

Unger, M. M., Spiegel, J., Dillmann, K. U., Grundmann, D., Philippeit, H., Bürmann, J., Faßbender, K., Schwiertz, A. & Schäfer, K. H. (2016). Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat Disord,* **32**, 66-72.

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., Cabrera, S. M., McDonough, C. B., Brindle, P. K., Abel, T. & Wood, M. A. (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J Neurosci,* **27**(23), 6128-40.

Verbeke, K. A., Boobis, A. R., Chiodini, A., Edwards, C. A., Franck, A., Kleerebezem, M., Nauta, A., Raes, J., van Tol, E. A. & Tuohy, K. M. (2015). Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr Res Rev,* **28**(1), 42-66.

Wang, J. J., Lee, C. L. & Pan, T. M. (2003). Improvement of monacolin K, gamma-aminobutyric acid and citrinin production ratio as a function of environmental

conditions of Monascus purpureus NTU 601. *J Ind Microbiol Biotechnol,* **30**(11), 669-76.

Yang, S. Y., Lü, F. X., Lu, Z. X., Bie, X. M., Jiao, Y., Sun, L. J. & Yu, B. (2008). Production of gamma-aminobutyric acid by Streptococcus salivarius subsp. thermophilus Y2 under submerged fermentation. *Amino Acids,* **34**(3), 473-8.

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

Zagajewski, J., Drozdowicz, D., Brzozowska, I., Hubalewska-Mazgaj, M., Stelmaszynska, T., Laidler, P. M. & Brzozowski, T. (2012). Conversion L-tryptophan to melatonin in the gastrointestinal tract: the new high performance liquid chromatography method enabling simultaneous determination of six metabolites of L-tryptophan by native fluorescence and UV-VIS detection. *J Physiol Pharmacol,* **63**(6), 613-21.

Chapter 3

An *in vitro* batch culture system to explore the impact of pre and probiotics on microbial community and metabolites

Abstract

Many important neurotransmitters are produced in the intestine and linked with the gut brain axis. The aim of this study was to determine the impact of pre and probiotics on the gut bacterial community and to determine whether these enhance neurotransmitter production. *In vitro* batch culture fermentation systems were conducted at pH values appropriate for proximal and distal colon. Samples were collected for bacterial enumeration (fluorescent *in situ* hybridisation combined with flow cytometry), neurotransmitter production (liquid chromatography mass spectrometry) and short chain fatty acids (gas chromatography) analysis. Both pre and probiotics were observed to enhance bacterial community, neurotransmitter and short chain fatty acids production under the proximal and distal conditions. It seems that bacterial growth and SCFA concentrations were greater at the more distal pH, while often NTs levels were improved at the proximal pH.

Keywords: Gut brain axis; gut microbiota; prebiotics; probiotics; psychobiotics; serotonin; GABA; dopamine; norepinephrine; epinephrine; neurotransmitters

1.Introduction

The human gut microbiota is immense and contains about three million different genes with great genetic potential (Borre *et al*., 2014). These gut microbial genes are responsible for expanding the genetic catalog of mammalian hosts, many of which encode enzymes that perform biochemical functions. The gut microbiota contributes to expanding biotransformation possibilities, as such diverse compounds can be processed in the host. The potential microbial metabolic functions are aligned with a variety of substrates that enter the gut, resulting in large amounts of metabolites, these include molecules of essential to the host such as neurotransmitters (NTs).

The gut-brain axis (GBA) is a bidirectional communication system and an integrative physiological network between the gastrointestinal tract (GIT) and the brain. GBA involves different communication pathways: (1) immune; (2) endocrine (hypothalamic-pituitary-adrenal axis); (3) autonomic nervous system (vagus nerve) (Mayer, 2011; Borre *et al*., 2014). The vagus nerve (VN) is the main nerve of the autonomic nervous system and seems essential for mediating the influence of gut microbiota on neurophysiological functions. For example, probiotic *Lactobacillus rhamnosus* intervention of mice was reported to lead to reduced levels of stress-induced corticosterone anxiety and depression related behavior, however, when this test was conducted in vagotomised mice there was no relief in depressive or anxiety-like behaviors, indicating that the behavioral characteristics of this bacterial strain are depend on gut brain signalling through the VN (Bravo *et al*., 2011). Therefore, it can be seen that the VN is one of the communication routes responsible for conveying information such as gut microbiota-derived NTs from the intestine to the brain (Bonaz *et al*., 2018; Latorre *et al*., 2016).

The gut microbiota has been related to the production of not only gut metabolites (including short chain fatty acids) but also gut microbial-derived NTs including serotonin (5-HT), gamma-aminobutyric acid (GABA) and catecholamines (CA) categorised by dopamine (DA), norepinephrine (NE) and epinephrine (EPI) (Walls *et al*., 2009; Clarke *et al*., 2014; Lyte, 2011). NTs are regarded as chemical messengers that influence a variety of both psychological and physical functions including appetite, mood, fear, sleep, heart rate, and anxiety (Wong *et al*., 2003; Borre *et al*., 2014; Byrne *et al*., 2016).

Nutrients, such as amino acids, are key components in the synthesis and modulation of NTs. Individual NTs have their own required substrate (NTs precursor) that can be obtained from the diet (Shishov et al., 2009). For example, foods rich in tryptophan include hazelnut and banana (Feldman *et al*., 1987; Lavizzari *et al*., 2006). Studies have indicated that consuming foods high in tryptophan results in greater 5-HT synthesis (Atkinson *et al*., 2006; Haleem, 2012). In turn, acute and chronic tryptophan deficiency caused by malnutrition has been indicated to negatively impact 5-HT production in the brain (Patrick and Ames, 2014; Haleem, 2012). Tryptophan first forms hydroxytryptophan via tryptophan hydroxylase (TPH) and then finally forms 5-HT via decarboxylase (Berger *et al*., 2009). The capacity to synthesize 5-HT from tryptophan is widely conserved among mammals and several bacterial species (Bailey *et al*., 2011). In mammals, 5-HT is involved in several roles, including modulation of host behaviour, impacting GI motility and also influencing bone remodelling and erythrocyte health (Spohn and Mawe, 2017). DA, NE and EPI are derived from aromatic amino acid tyrosine. DA is the central and first NT to be formed, and tyrosine and tyrosine hydroxylase (TH) and other cofactors gradually convert tyrosine to DA. DA is further governed by enzymes and cofactors to form NE which subsequently forms EPI (Shiman *et*

al., 1971; Magro *et al*., 2002). The roles of CAs in host physiology include maintaining gut integrity (Meddings and Swain, 2000) and affecting host motivational behaviour as well as decision-making (Terbeck *et al*., 2016).

GABA in the gut can be produced by gut microbiota or ingested probiotics that could utilise the non-essential amino acid dietary monosodium glutamate (MSG) to form GABA (Lyte, 2011). It is clear that dietary nutrients not only have a role in shaping the human gut microbiota composition but also support the synthesis of NTs. However, the dietary level of consumption of MSG could be considered because there has been report of potential negative health associations of MSG in humans (including weakness, numbness and heart palpitations) (Kwok, 1968).

There have been studies exploring the potential role of gut microbiome on host NTs, their related pathways with outcomes for behaviour and host physiology. For instance, compared to specific pathogen free (SPF) mice, germ-free mice have decreased 5-HT receptors and reduced circulating serotonin in hippocampus and accompanied by altered anxiety-like behaviour (Neufeld *et al*., 2011). Some studies have reported altered concentrations of NTs in GF (germ free) mice following supplementation with defined gut bacteria (Sampson *et al*., 2016; Yano *et al*., 2015). NTs can be isolated from several members of gut bacteria such as DA and 5-HT produced by *Escherichia coli* (Tsavkelova *et al*., 2000; Shishov *et al*., 2009; Strandwitz *et al*., 2019) and strains of *Bifidobacterium* and *Lactobacillus* can be regarded as natural residents of the intestine with a positive impact on GABA production (Siragusa *et al*., 2007; Barrett *et al*., 2012; Pokusaeva *et al*., 2017; Strandwitz *et al*., 2019). The gut

microbiota is considered to be a modulator of NTs levels through the gut-brain axis (VN pathway), and the variation in the gut microbiota associated with the communication in gut-microbiota-brain axis has been implicated physiologically and psychologically including within neurologic, immunologic and psychiatric conditions (Mayer, 2011; Collins *et al*., 2012; Foster and Neufeld, 2013). Neurological diseases such as depression (decreased 5-HT and CAs), Parkinson disease (PD) (decreased DA), insomnia and anxiety (decreased GABA) are linked to deficits in certain NTs (Collins *et al*., 2012; Logan and Jacka, 2014; Desbonnet *et al*., 2015; O'Mahony *et al*., 2015; Sugama and Kakinuma, 2016). Therefore, in humans, the interaction between gut microbiota variation and levels of NTs remain to be fully explored although mice studies have provided promising evidence.

Psychobiotics include beneficial bacteria, that when ingested in appropriate amounts, yield health benefit in patients with psychiatric illness, as a subset of probiotic, these bacteria can result in elevated levels of neuroactive compounds such as 5-HT and GABA, which act on the GBA (Dinan and Cryan, 2013). Subsequently, the definition of psychobiotics has been expanded to include prebiotics, which support the growth of intrinsic commensal gut bacteria (Sarker *et al*., 2016). The bacteria most commonly utilised as probiotics are Gram-positive *Lactobacillus* and *Bifidobacterium* genera, both of which have potential effects on psychological and physiological conditions such as improving anxiety, depression and appetite levels (Burnett *et al*., 2011; Mayer *et al*., 2014). Few studies have examined the psychophysiological impacts of prebiotics. Soluble fibre fructo-oligosaccharides (FOS) are a nutritional source for *Bifidobacterium*, and promote its activity and proliferation in the gut (Savignac *et al*., 2013).

The aim of the current experiment was to investigate gut microbial fermentations of probiotics and prebiotics and explore whether pre and probiotics could support NT production. As pH seems a key factor in the production of NTs like GABA pH appropriate to the proximal (pH 5.4-5.6) and distal (pH 6.7-6.9) regions were modelled. *In vitro* pH-controlled faecal batch culture were inoculated with faecal samples from healthy female volunteers to determine if pre and probiotics within a faecal community could result in increased levels of NTs with the absence of host cells.

2.Materials and methods

2.1. Faecal sample preparation

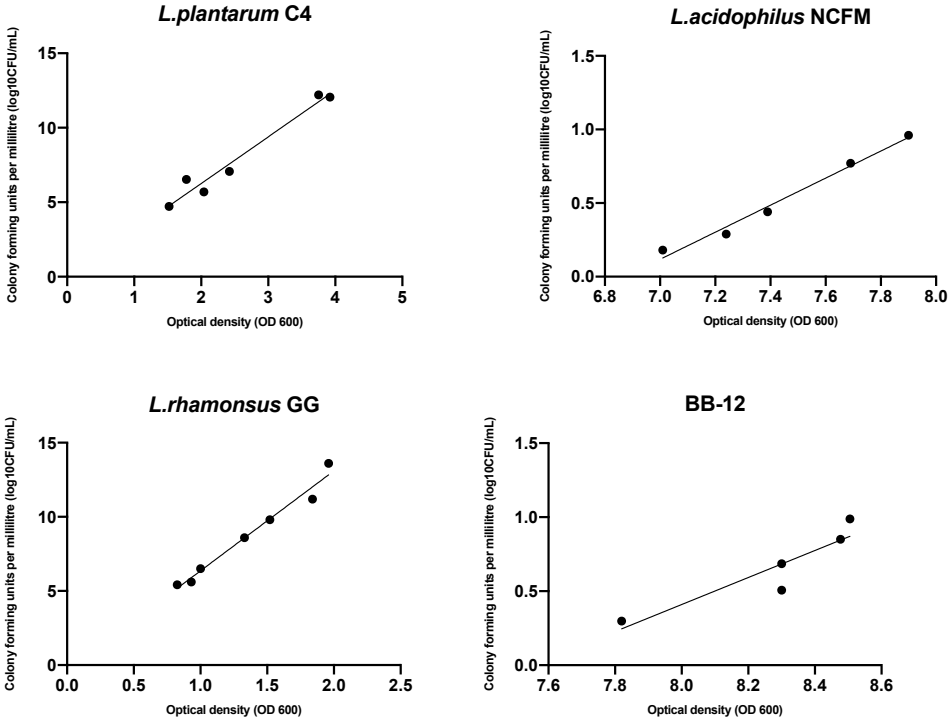
Faecal samples were collected from 8 healthy female donors (age range 21-35 years) who were free from known gastrointestinal disorders, who had not taken antibiotics for 3 months or prebiotic/probiotic supplement in the last 2 weeks before faecal donation. Volunteers deposited a fresh faecal sample (less than 3h) into an anaerobic collection pot (<1% O_2 and 9–13% CO_2) (AnaeroJarTM 2.5 L and AnaeroGemTM, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK). Collected sample was diluted with phosphate-buffered saline (PBS) 10% (w/v) (pH 7.4) and then homogenised in a stomacher (Stomacher 400, Seward, West Sussex, UK) for 2 min at 240 paddle beats per min.

2.2. Treatments bacterial strains and culture conditions

The probiotic yeast *Saccharomyctes boulardii (S. boulardii)* (OptiBac Probiotics Ltd., Hampshire, UK) product contained 5 x 10^9 cfu live culture powder in each capsule. The prebiotic FOS (Orafti ® P95) was obtained from BENEO (Orafti ® P95, Tienen, Belgium).

Bifidobacterium animalis subsp. *lactis* BB-12 (BB-12), *Lactobacillus acidophilus* NCFM (NCFM) (Danisco Brazil, Cotia), *Lactobacillus plantarum* C4 (*L. plantarum*) and *Lactobacillus rhamnosus* GG (ATCC 53103) were freeze dired and stored at -80°C in 15% (w/v) glycerol Cryobank cryogenic beads (Prolab Diagnostics, UK) prior to use. The strains were grown under anaerobic conditions at 37°C in an anaerobic chamber (MG1000, 10% CO_2, 10% H_2 and 80% N_2, Don Whitley Scientific LTD, Shipley, West Yorkshire, UK) in de Man-Rogosa-Sharpe (MRS) (Oxoid Ltd, Basingstoke, Hampshire, UK) broth supplemented with 0.05% (w/v) cysteine (MRS-C, Sigma, St. Louis, MO), respectively. The probiotics were grown at different dilutions in triplicate for 48h to establish a growth curve of optical density (OD_{600}) against colony forming units per millilitre (Log_{10}CFU/mL) to confirm a dilution factor for the bacteria to obtain 5×10^8CFU/mL (Fig. 1). One day prior to inoculating the fermentation vessels, strains were grown in the above conditions for 24 h then on the experimental day 5 $x10^8$ CFU probiotic were obtained from standard curve and OD reading, the MRS supernatant was removed by centrifuging for 10 mins at 5000 x *g*, and resuspended in 1 mL PBS (anaerobic phosphate buffered saline 1 M, pH 7.4) prior to adding to fermentation vessels.

Figure 1. The growth curve of probiotics *L. plantarum* C4, *L. acidophilus* NCFM, *L. rhamonsus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) correlating the colony forming units per milliliter.



The vessels were maintained at 37°C via a circulating water bath, the pH of basal nutrient media was maintained at the range of 5.4-5.6 (proximal large intestine) and 6.7-6.9 (distal large intestine) using pH electrodes, respectively. Four volunteer samples were used per pH set (8 volunteers used overall). The fermentation experiments were run for 48 hours and they were continuously stirred throughout the experiment. Fermentation vessels were inoculated with 15mL of 10% faecal slurry with a total working volume of 150 mL (1% final concentration). These vessels were prepared as follows. Each vessel received 135 mL autoclaved basal medium (Peptone water 2 g, yeast extract 2 g, NaCl 0.1 g, K_2HPO_4 0.04 g, KH_2PO_4 0.04 g, $MgSO_4.7H_2O$ 0.01 g, $CaCl_2.6H_2O$, 0.01 g, $NaHCO_3$ 2 g, Tween 80 2 mL, haemin 0.05 g, Vitamin K 10µL, L-cysteine HCL 0.5 g , bile salt 0.5 g , potato starch 7.5 g, tryptone 4.5 g per litre) (Sigma, St. Louis, MO), additionally, 10 mg each of L-Tryptophan, L-Glutamic acid monosodium salt hydrate and L-Tyrosine (0.067 mg/mL) were added to the

vessels to provide NTs precursors, each fermentation vessel was incubated overnight in anaerobic conditions (oxygen-free nitrogen at a rate of 15 mL/min). The following day the prebiotic and probiotic bacteria were added to the vessels as outlined below.

Vessel 1: Blank (no treatment added)

Vessel 2: *L. plantarum* C4 (5×10^8 CFU/mL)

Vessel 3: *L. rhamnosus* GG (5×10^8 CFU/mL)

Vessel 4: *L. acidophilus* NCFM (5×10^8 CFU/mL)

Vessel 5: FOS (1.5g)

Vessel 6: *Saccharomyces boulardii* (5×10^8 CFU/mL)

Vessel 7: *Bifidobacterium animalis* subsp. *lactis* BB-12 (5×10^8 CFU/mL)

2.3 Preparation of the Samples for NT, SCFA and Bacterial Community Analysis

During fermentation, samples were collected from each vessel over a series of time points (0, 6, 24 and 48 h) A 1 mL sample was collected and centrifuged at 13,000 × *g* for 10 min then stored at −20 °C for NTs and short chain fatty acids (SCFAs) analysis. For bacterial community analysis, a 750 µL supernatant of fermentation fluid was centrifuged at 13,000 × *g* for 5 min. The pellet was then resuspended in 375 µL filtered 0.1 M PBS and fixed by 1125 µL filtered paraformaldehyde (PFA 4% w/v) for 4 to 8 h at 4 °C. The sample was washed twice with 1 mL PBS to remove PFA and resuspended in filtered 600 µL ethanol-PBS (1:1, w/v). The samples were kept at −20 °C prior to FISH analysis.

2.4 In Vitro Enumeration of Bacterial Population by Flow-Fluorescent In Situ Hybridisation (FISH)

The bacterial population was analysed using fluorescent *in situ* hybridisation coupled to flow cytometry (FISH-FCM) (BD Accuri™ C6 Plus, Basingstoke, United Kingdom), detecting at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software. Samples were removed from storage at - 20 °C. After defrosting and vortexing for 10 sec permeabilisation steps were conducted using 500 µL 0.1M PBS added to 75 µL fixed samples and centrifuged at 13,000 x *g* for 3 min. The pellets were resuspended in 100 µL of TE-FISH buffer (Tris-HCl 1 M pH 8, EDTA 0.5 M pH 8, filtered distilled water, 0.22 µm pore size filter with the percentage of 10:10:80) containing lysozyme solution (1 mg/ml of 50,000 U/mg protein) and incubated for 10 min in the dark at room temperature and then centrifuged at 13,000 x *g* for 3 min. Pellets were washed with 0.1 M 500 µL PBS and then washed with 150 µL hybridisation buffer (0.9 M NaCl, 0.2 M Tris/HCl pH 8.0, 30% formamide, ddH_2O, 0.01% sodium dodecyl sulphate) and centrifuged at 13,000 x *g* for 3 min. Pellets were then resuspended in 1 mL of hybridisation buffer, homogenised and 50 µL with 4 µL of different probes aliquoted into Eppendorf tubes (1.5 mL) were incubated at 36°C overnight. Differences in bacterial populations were quantified with oligonucleotide probes aimed to target specific regions of 16S rRNA. The individual probes used (Eurofins, Wolverhampton, UK) in this study are shown in Table 1. Non-EUB and EUB338-I-II-III were linked to fluorescence Alexa 488 at the 5' end, and group specific probes were linked to fluorescence Alexa 647. Non-EUB and EUB338 were linked to Alexa 647 at the 5' end as controls to adjust threshold. 4 µL of EUB338-I-II-III was added together with 4 µL specific probes. 125 µL of hybridisation buffer was added to each Eppendorf tube after incubation samples were vortexed and centrifuged (13,000 x *g*, 3 min). Supernatants were removed and pellets were washed with 175 µL washing buffer solution (0.064 M NaCl, 0.02M Tris-HCl (pH 8.0), 0.5

M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate, 956.2 µL of ddh_2O), vortexed and incubated at 38°C in a heating block for 20 min to remove non-specific binding of the probe. Afterwards samples were centrifuged (13,000 x *g*, 3 min) and supernatants removed. Pellets were resuspended in an appropriate volume of PBS on the basis of FCM load. Number of bacteria were then calculated through determination of FCM reading and PBS dilution.

Table 1. Oligonucleotide probes used in the study for bacterial populations by fluorescent *in situ* hybridisation.

Probe name	**Sequence (5' to 3')**	**Target species**	**Reference**
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner *et al*., 1993)
Eub338I +	GCTGCCTCCCGTAGGAGT	Most Bacteria	(Daims *et al*., 1999)
Eub338II +	GCAGCCACCCGTAGGTGT	*Planctomycetales*	(Daims *et al*., 1999)
Eub338III +	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	(Daims *et al*., 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al*., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Harmsen *et al*., 2000)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz *et al*., 1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale*	(Franks *et al*., 1998)

		group *(Clostridium* cluster XIVa and XIVb)	
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker *et al*., 2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen *et al*., 2000)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker *et al*., 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(Hold *et al*., 2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	(Devereux *et al*., 1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group *(Clostridium* cluster I and II)	(Franks *et al*., 1998)

2.5. NTs analysis by Liquid chromatography mass spectrometry (LCMS)

2.5.1. Reagents and chemicals

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

Separate standard stock solutions (10000 ng/mL) of five analytes including 5-HT, DA, GABA, NE and Epinephrine (EPI) were individually prepared in HPLC water. A 1000 ng/mL mixed standard solution containing the five 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 10 levels: 1, 10, 50, 100, 250, 500, 750, 1000, 2500, 5000 ng/mL. Samples were removed from storage at -20 °C. A 400 µL sample of gut model fluid supernatant was collected in a centrifuge tube filter (Sigma-Aldrich, 0.22 µm, Polypropylene) and then centrifuged at 13,000 X *g* for 10 min at 4 °C (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) and the supernatant remained. 200 µL of HPLC water (blank), calibration standard samples and gut model samples were placed in a 96-well plate.

2.5.2. LCMS system

Samples were measured using online Nexera LC System coupled to LCMS-8050 triple quadrupole (QQQ) mass spectrophotometer (Shimadzu, Kyoto, Corporation, Japan). Data was processed using LabSolutions LCMS version 5.65 software.

2.5.3. Liquid chromatography (LC) conditions

The chromatographic separation of analytes was obtained from Discovery HS F5-3 column (2.1 mmI.D. x 150 mmL., 3 um partice size, Sigma-Aldrich Co Ltd, P/N 567503-U). The mobile phase consisted of 0.1% formic acid in water (mobile A) and 0.1% formic acid in acetonitrile (mobile B). For the entire analysis, the flow rates of both mobile phases were 0.25 mL/min, and autosampler temperature maintained at a constant temperature of 4°C. The

gradient elution programs were as follows: B conc. 25% (5 min) ➔ 35% (11min) ➔ 95% (15 min) ➔ 95% (20 min) ➔ 0% (20.01- 25 min).

2.5.4. Mass spectrometry (MS) conditions

The LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using 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. The MRM transitions of the native, stable isotopes, retention times and other conditions are shown in Table 2.

Table 2. Optimal conditions of LC-MS/MS used for the quantification of DA, 5-HT, NE, EPI and GABA in faecal supernatant.

Compounds name	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Classification
5-HT	177.10	160.10	10.527	Amino acid derivative
DA	154.10	91.05	8.078	Amino acid derivative
NE	170.10	152.15	4.988	Catecholamine
EPI	184.10	166.10	7.164	Catecholamine

GABA	104.10	87.05	3.690	Organic acid

Each analyte of ionization polarity is (+).

2.5.5. Quantification of samples

A linear calibration curve was generated based on the detected signal proportional to the concentration of the analyte. Briefly, results validation was performed following published procedures (Toxicology SwGfF, 2013). Good linearity with R^2 greater than 0.98 was obtained across the set calibration in the range from 1 ng/mL to 5000 ng/mL for each of the analytes, with accuracy within 100 % ± 20 %. Quantification of samples was determined by calibration with five analytes including 5-HT, DA, EPI, GABA and NE.

Standard calibration concentration and samples of chromatogram peaks were quantified using the software (Requires LabSolutions LCMS version 5.65) following manual inspection.

2.6. Short Chain and Branched Chain Fatty Acid Analysis by Gas Chromatography

The concentration of SCFA was determined by Gas chromatography (GC) as previously described by Richardson et al. (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 × 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 chromatograph (Agilent, Cheshire, UK) using an HP-5ms (L × I.D. 30 m × 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 by 5 °C and held at 190 °C for 30 min. Helium was the carrier gas (flow rate, 1.7 mL/min, head pressure, 133 KPa). Peak areas were integrated using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK). SCFA production was quantified by single-point internal standard method as described by Liu et al.(Liu *et al*., 2016) Peak areas of the standard (acetate, butyrate, propionate, valerate, iso-valerate and iso-butyrate) were used to calculate the response factors for each organic acid with respect to the internal standard.

2.7. Statistical Analyses

All statistical analyses used IBM SPSS version 27 (IBM Corp., USA). The FISH-FCM, NTs and SCFAs production were analysed using one way mixed ANOVA to (1) compare different test substrates and time points; (2) the production/utilisation amount of bacterial community, NTs and SCFAs level at 6, 24 and 48h (compared to baseline) were tested between pH 5.5 and 6.9 conditions, a *post hoc* Tukey HSD (Honestly Significant Difference) test was performed using to determine significant differences between blank and substrates at

respective time point. repeated measures ANOVA was performed to further analyse the data from the same vessel at the multiple time points. Statistical analysis was accepted at $p < 0.05$ for all analyses. Graphs were made using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). FISH results are reported as mean ± RSD. Mean of log transformed data is a geometric mean in log, so the SD reported is therefore the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

3. Results

3.1. Bacterial Enumeration

Under the different pH conditions and treatments, changes in bacterial compositions during batch culture fermentations are reported in Figure 2.

Effect of FOS on bacterial growth

Compared to time 0, fermentation of FOS resulted in significantly increased total bacterial at pH 6.9, time 6 h (7.33 to 8.83 $\log_{10}$ cells/mL, $p < 0.05$). FOS fermentation also resulted in significantly increased total bacterial counts in both pH 5.5 and 6.9 conditions at 24 h (8.07 to 8.57 $\log_{10}$ cells/mL, $p < 0.01$ and 7.33 to 8.70 $\log_{10}$ cells/mL, $p < 0.05$). Compared to time 0, both under the pH of 5.5 and 6.9 conditions, a significant increase in *Bifidobacterium* upon fermentation of FOS was observed at 24 h (7.08 to 8.40 $\log_{10}$ cells/mL, $p < 0.01$ and 6.57 to 8.49 $\log_{10}$ cells/mL, $p < 0.01$) and 48 h (7.08 to 7.74 $\log_{10}$ cells/mL, $p < 0.05$ and 6.57 to 8.03 $\log_{10}$ cells/mL, $p < 0.01$). Compared to time 0, FOS resulted in significantly increased *Roseburia* at pH 6.9 (5.57 to 6.81 $\log_{10}$ cells/mL, $p < 0.01$) and significantly decreased in the pH of 5.5 (6.52 to 5.13 $\log_{10}$ cells/mL, $p < 0.01$) at 24 h. Compared to the blank vessel, *Bifidobacterium* significantly increased at all time points (Blank vessel: 7.81 $\log_{10}$ cells/mL

and FOS vessel: 8.49 $\log_{10}$ cells/mL at 6 h, $p < 0.05$; Blank vessel: 7.44 $\log_{10}$ cells/mL and FOS vessel: 7.91 $\log_{10}$ cells/mL at 24 h, $p < 0.05$; Blank vessel: 7.59 $\log_{10}$ cells/mL and FOS vessel: 8.03 $\log_{10}$ cells/mL at 48 h, $p < 0.05$).

Effect of *L. acidophilus* NCFM on bacterial growth

When *L. acidophilus* NCFM was included in the fermentation vessel compared to time 0, at pH 6.9, both *Bifidobacterium* and Lactic acid bacteria group significantly increased at time 6, 24 and 48 (*Bifidobacterium* 0 to 6 h: 6.78 to 7.65 $\log_{10}$ cells/mL, $p < 0.05$; 0 to 24 h: 6.78 to 7.51 $\log_{10}$ cells/mL, $p < 0.05$; 0 to 48 h: 6.78 to 7.93 $\log_{10}$ cells/mL, $p < 0.05$; Lactic acid bacteria group 0 to 6 h: 5.32 to 6.58 $\log_{10}$ cells/mL, $p < 0.05$; 0 to 24 h: 5.32 to 6.51 $\log_{10}$ cells/mL, $p < 0.01$; 0 to 48 h: 5.32 to 5.74 $\log_{10}$ cells/mL, $p < 0.05$). *Bacteroides* significantly increased at time 6 when compared to time 0 at pH 6.9 condition (6.47 to 7.85 $\log_{10}$ cells/mL, $p < 0.05$). At pH 5.5 condition, Lactic acid bacteria significantly increased at 6 h when compared to blank vessel (blank vessel: 5.51 $\log_{10}$ cells/mL and *L. acidophilus* vessel: 6.38 $\log_{10}$ cells/mL, $p < 0.05$).

Effect of *L. plantarum* C4 on bacterial growth

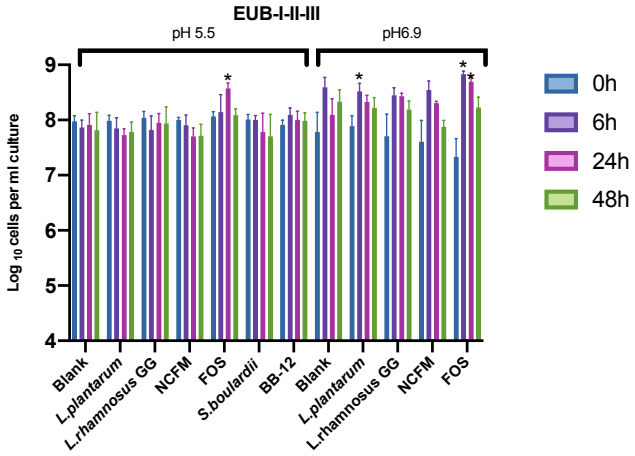
When *L. plantarum* C4 was included in the fermentation vessel compared to time 0, under pH 6.9 conditions, there was a significant increase in total bacterial count from 7.89 to 8.52 $\log_{10}$ cells/mL ($p < 0.05$) at 6 h, there was also a significant increase in *Bifidobacterium* (time 0 to 6 h: 7.05 to 7.80 $\log_{10}$ cells/mL increase, $p < 0.05$), Lactic acid bacteria (time 0 to 6 h: 6.03 to 6.72 $\log_{10}$ cells/mL increase, $p < 0.05$; time 0 to 24 h: 6.03 to 6.50 $\log_{10}$ cells/mL increase, $p < 0.05$); *Bacteroides* (time 0 to 6 h: 6.89 to 7.72 $\log_{10}$ cells/mL increase, $p < 0.01$), *Roseburia* (time 0 to 6 h: 6.03 to 6.72 $\log_{10}$ cells/mL increase, $p < 0.05$), *Atopobium* (time 0 to 6 h: 6.06 to 7.30 $\log_{10}$ cells/mL increase, $p < 0.001$; time 0 to 24 h: 6.06 to 6.78 $\log_{10}$ cells/mL increase,

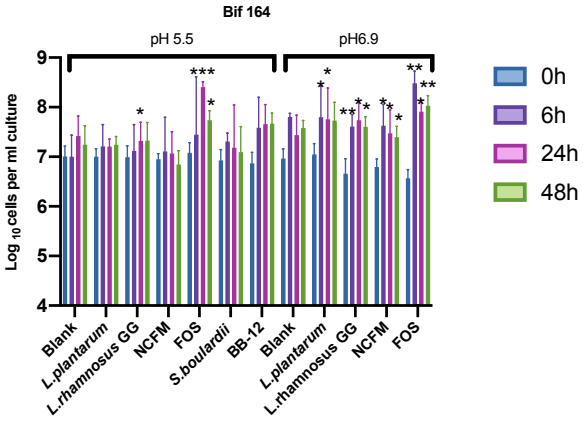
$p < 0.05$; time 0 to 48 h: 6.06 to 6.77 $\log_{10}$ cells/mL increase, $p < 0.01$). *C. histolyticum* group significantly increased at pH 6.9 (5.63 to 6.69 $\log_{10}$ cells/mL, $p < 0.05$) and significantly decreased at pH 5.5 (5.88 to 4.95 $\log_{10}$ cells/mL, $p < 0.05$) at 6 h when compared to time 0. The amounts of bacterial community were not significantly different to the blank.

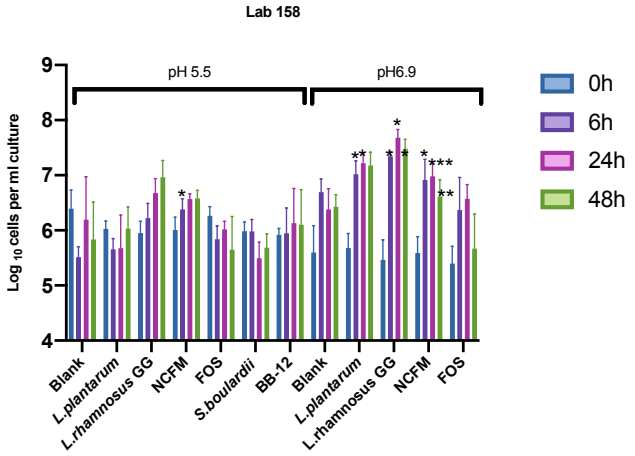
Effect of *L. rhamonsus* GG on bacterial growth

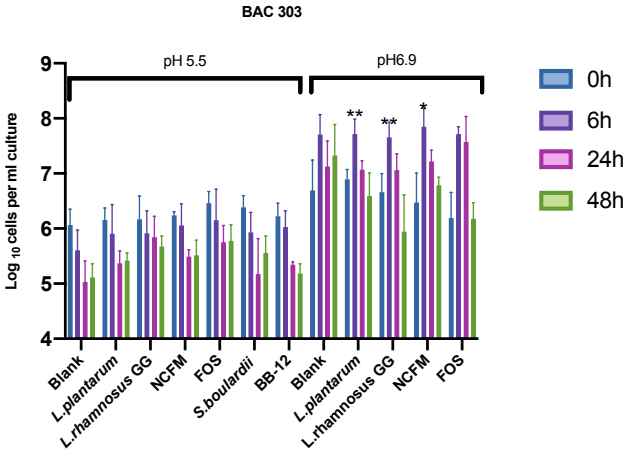
Compared to time 0, under both pH 5.5 and 6.9 conditions, *L. rhamonsus*. GG significantly increased *Bifidobacterium* at 24 h, from 6.99 to 7.32 $\log_{10}$ cells/mL, $p < 0.05$ and 6.89 to 7.47 $\log_{10}$ cells/mL, $p < 0.05$, respectively. At pH 6.9 there was a significant increase in *Bifidobacterium* (time 0 to 6 h: 6.89 to 7.61 $\log_{10}$ cells/mL increase, $p < 0.01$; time 0 to 48 h: 6.89 to 6.92 $\log_{10}$ cells/mL increase, $p < 0.05$), *Bacteroides* (time 0 to 6 h: 6.47 to 7.85 $\log_{10}$ cells/mL increase, $p < 0.05$). Compared to blank vessel, under the condition of pH 6.9, there was significantly increased Lactic acid bacteria at times 6, 24 and 48 h (time 6 h: blank vessel: 6.69 $\log_{10}$ cells/mL and *L. rhamonsus* GG vessel: 7.34 $\log_{10}$ cells/mL, $p < 0.05$; time 24 h: blank vessel: 6.38 $\log_{10}$ cells/mL and *L. rhamonsus* GG vessel: 7.68 $\log_{10}$ cells/mL, $p < 0.05$; time 48 h: blank vessel: 6.42 $\log_{10}$ cells/mL and *L. rhamonsus* GG vessel: 7.48 $\log_{10}$ cells/mL, $p < 0.01$), and significantly increased *Atopobium* (time 24 h: blank vessel: 6.45 $\log_{10}$ cells/mL and *L. rhamonsus* GG vessel: 7.06 $\log_{10}$ cells/mL, $p < 0.05$; time 48 h: blank vessel: 6.47 $\log_{10}$ cells/mL and *L. rhamonsus* GG vessel: 7.00 $\log_{10}$ cells/mL, $p < 0.05$).

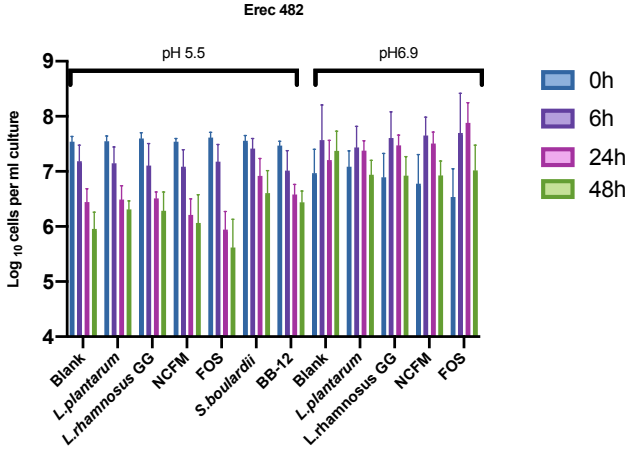
Figure 2. Bacterial groups detected by FISH-FCM (Log_{10} cells /mL) following baseline (0 h), 6 h 24 h and 48 h with the administration of *L. plantarum* C4 (*L. plantarum*), *L. rhamnosus* GG, *L. acidophilus* NCFM (NCFM), FOS, *Saccharmyces boulardii* (*S. boulardii*) and *Bifidobacterium animalis subsp. lactis* BB-12 (BB-12) within a pH controlled *in vitro* faecal (1%) batch culture system. Results are reported as Means ± SD. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h 24 h and 48 h are indicated. Results are reported as Means ± RSD. Mean of log transformed data is a geometric mean in log, so the SD reported is in fact the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

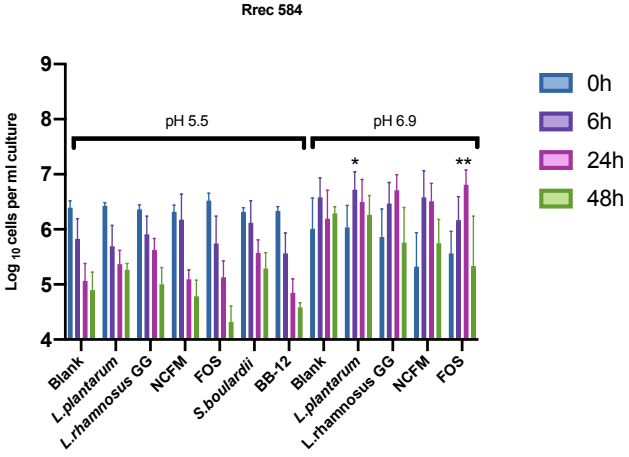

EUB-I-II-III
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

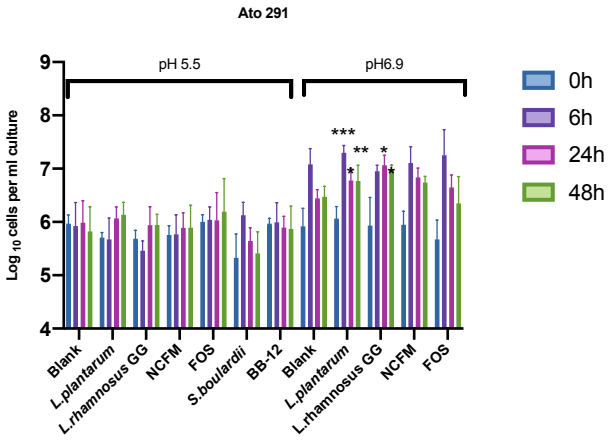

Bif 164
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

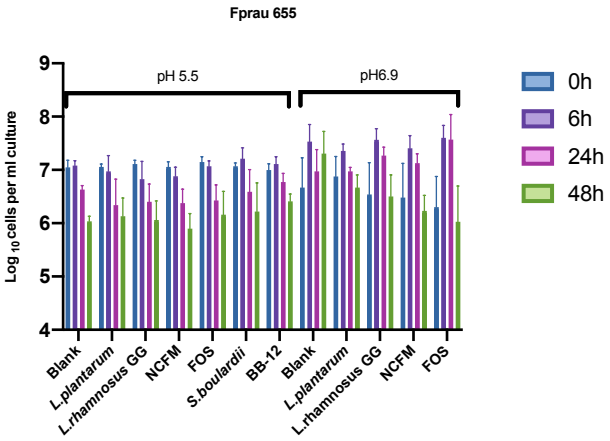

Lab 158
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

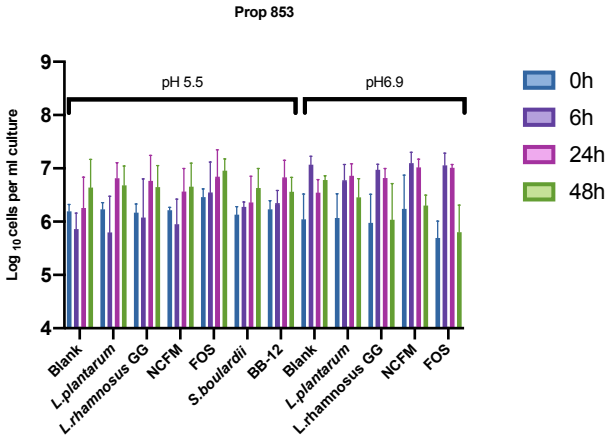

BAC 303
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

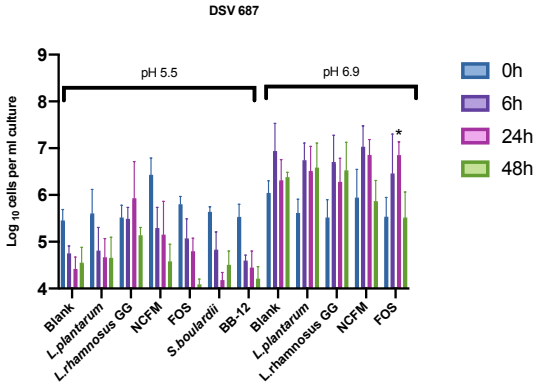

Erec 482
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

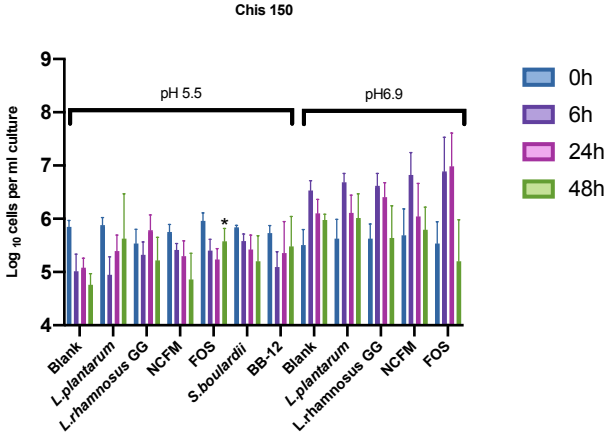

Rrec 584
pH 5.5
pH 6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12


Ato 291
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12


Fprau 655
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12


Prop 853
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12


DSV 687
pH 5.5
pH 6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12


Chis 150
pH 5.5
pH6.9
Log 10 cells per ml culture
0h
6h
24h
48h
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12

3.2. NT production

Under the different pH conditions and treatments, changes in NTs concentrations during batch culture fermentations are reported in Figure 3.

Effect of precursors and carbohydrate on NT production

In the pH 5.5 blank vessel, amino acids were present along with potato starch and tryptone as a carbohydrate and protein source respectively, and these stimulated the NE production at 6 h (from 65.12 to 232.36 ng/mL, $p < 0.01$) and led to a trend for increased DA levels (from 23.98 to 60.94 ng/mL, $p < 0.10$).

Effect of *L. plantarum* C4 on NT production

The levels of NTs were not significantly different to the blank. There was a significant increase in EPI at 6 h within pH of 5.5 condition (from 0 to 8.86 ng/mL, $p < 0.05$).

Effect of *L. acidophilus* NCFM on NT production

The levels of NTs were not significantly different to the blank. 5-HT production significantly increased at 6 h within pH 5.5 (from 5.74 to 12.81 ng/ml, $p < 0.05$) and 48 h at pH 6.9 (from 0 to 1.26 ng/mL, $p < 0.05$). EPI production was significantly elevated at time 6 h, pH 5.5 condition (1.75 to 4.24 ng/mL, $p < 0.05$).

Effect of *L. rhamonsus* GG on NT production

The levels of NTs were not significantly different to the blank. NE production was significantly enhanced within pH 5.5 condition at 6 h (from 80.33 to 194.66 ng/mL, $p < 0.05$). *L. rhamnosus* GG significantly increased in EPI at time 24 h within pH 6.9 condition (1.08 to 1.34 ng/mL, $p < 0.05$).

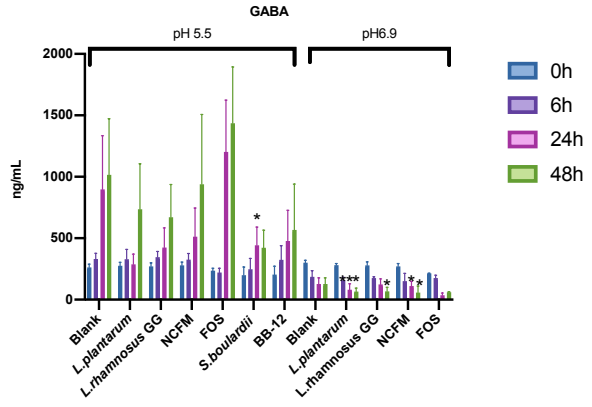
Effect of FOS on NT production

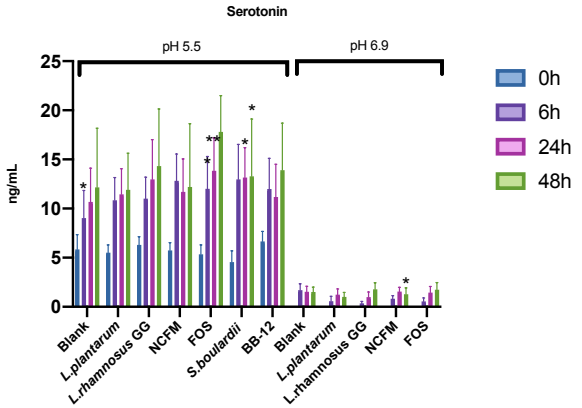
The levels of NTs were not significantly different to the blank. FOS fermentation resulted in significantly higher amount 5-HT, at 6 h (5.34 to 12.02 ng/ml, $p < 0.05$) and at 24 h (5.34 to 13.84 ng/ml, $p < 0.01$), both pH 6.9 condition when compared to the baseline. Through the whole fermentation, FOS could not support NE growth within pH 6.9 condition, However, FOS led to NE increased trending ranging from 44.74 to 260.36 ng/mL in the pH 5.5 condition.

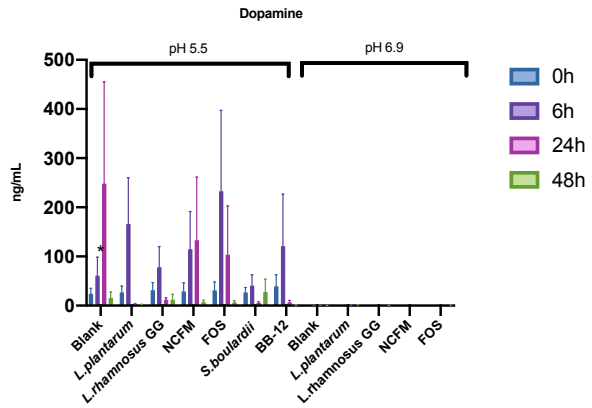
Effect of *S. boulardii* on NT production

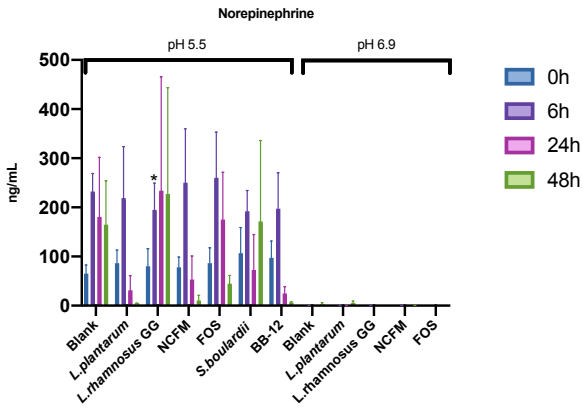
The levels of NTs were not significantly different to the blank. Compared to the time 0, GABA amounts were significantly increased at 24 h (from 198.58 to 442.0 ng/ml, $p < 0.05$), pH of 5.5 condition. 5-HT significantly increased at 24h (from 4.55 to 12.97 ng/ml, $p < 0.05$) and 48h (from 4.55 to 13.16 ng/ml, $p < 0.05$) when compared to time 0 within pH of 5.5 condition.

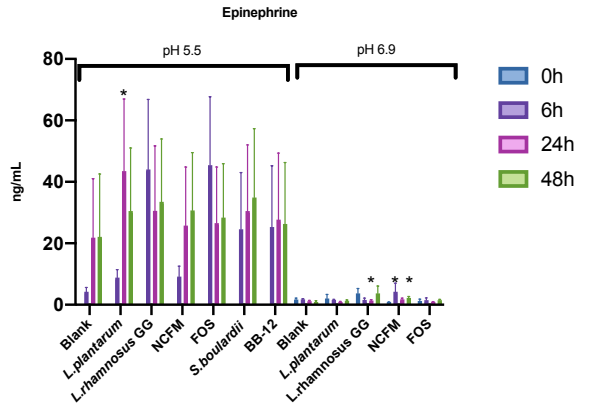
Figure 3. GABA, 5-HT, DA, NE and EPI concentration detected by LCMS (ng/mL) following baseline (0 h), 6 h 24 h and 48 h with the administration of *L. plantarum* C4 (*L. plantarum*), *L. rhamnosus* GG, *L. acidophilus* NCFM (NCFM), FOS, *Saccharmyces boulardii* (*S. boulardii*) and *Bifidobacterium animalis subsp. lactis* BB-12 (BB-12) within a pH controlled *in vitro* faecal (1%) batch culture system. Results are reported as Means ± SD. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h 24 h and 48 h are indicated.


GABA
pH 5.5
pH6.9
ng/mL
2000
1500
1000
500
0
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12
0h
6h
24h
48h


Serotonin
pH 5.5
pH 6.9
ng/mL
25
20
15
10
5
0
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12
0h
6h
24h
48h


Dopamine
pH 5.5
pH 6.9
ng/mL
500
400
300
200
100
0
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12
0h
6h
24h
48h


Norepinephrine
pH 5.5
pH 6.9
ng/mL
500
400
300
200
100
0
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12
0h
6h
24h
48h


Epinephrine
pH 5.5
pH 6.9
ng/mL
80
60
40
20
0
Blank
L.plantarum
L.rhamnosus GG
NCFM
FOS
S.boulardii
BB-12
0h
6h
24h
48h

3.3. SCFA production

Under the different pH conditions and treatments, changes in SCFA concentrations during batch culture fermentations are reported in Figure 4.

Effect of *L. plantarum* C4 on SCFA production

Compared to the blank vessel, there was no significant difference of SCFA production observed. Compared to the baseline, there was significantly increased acetate production at 24 h (pH 5.5: 0 to 7.80 mM, $p < 0.05$ and pH 6.9: 2.22 to 35.59 mM) and 48 h (pH 5.5: 0 to 9.53 mM, $p < 0.05$ and pH 6.9: 2.22 to 44.04 mM, $p < 0.05$) under the both pH 5.5 and 6.9 conditions.

Effect of *L. rhamonsus* GG on SCFA production

The SCFA amounts were not significantly different to the blank. Compared to baseline, acetate production significantly increased at 6 h (0 to 8.20 mM, $p < 0.01$) within pH 5.5 condition. At the pH 6.9, *L. rhamonsus* GG resulted in significantly increased acetate at all time points when compared to the baseline (from 17.3 to 18.81 mM at 6 h, $p < 0.01$; from 1.73 to 25.46 mM at 24 h, $p < 0.05$; from 17.3 to 33.10 mM at 48 h, $p < 0.001$). Compared to baseline, butyrate amounts were significantly increased at 48 h (from 0.14 to 8.06 mM, $p < 0.05$).

Effect of *L. acidophilus* NCFM on SCFA production

The SCFA amounts were not significantly different to the blank. Acetate production was significantly increased at all time points (from 2.20 to 17.16 mM at 6 h, $p < 0.05$; from 2.20 to 31.79 mM at 24h, $p < 0.001$; from 2.20 to 34.01 mM at 48h, $p < 0.01$) within pH 6.9 condition. Butyrate amounts were significantly increased at 24h (0.11 to 5.29 mM, $p < 0.01$) and 48h (0.11 to 5.26 mM, $p < 0.05$) when compared to baseline within pH 6.9 condition.

Effect of FOS on SCFA production

Acetate (blank: 14.63mM and FOS: 48.88mM, $p < 0.05$) amounts were significantly different to the blank at 48 h within pH 5.5 condition. Acetate production was significantly increased at 6 h (blank:21.45 mM and FOS: 58.47 mM, $p < 0.05$) when compared to blank vessel under pH 6.9 condition. Compared to the baseline, FOS resulted in significantly increased acetate at 24 h and 48 h at pH 5.5 (from 0 to 57.38 mM at 24h, $p < 0.05$; from 0 to 48.88 mM at 48h, $p < 0.05$), and resulted in significantly increased acetate at all time points within pH 6.9 (from 1.96 to 58.47 mM at 6 h, $p < 0.05$; from 1.96 to 70.02 mM at 24 h, $p < 0.05$; from 1.96 to 70.76 mM at 48h, $p < 0.05$). At the pH 6.9 condition, a significant increase in butyrate upon fermentation of FOS was observed at 6 h (from 0.13 to 2.13, $p < 0.05$) when compared to the time 0.

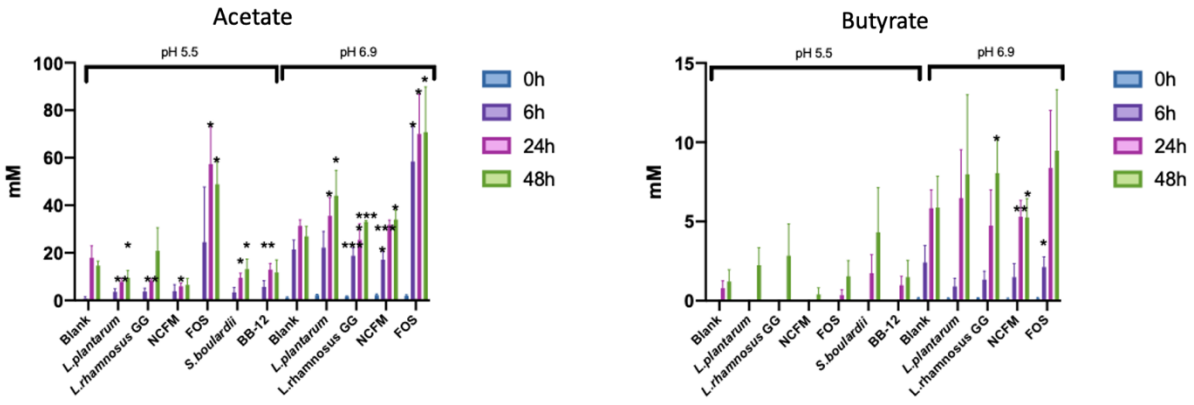
Effect of *S. boulardii* on SCFA production

The amounts of SCFAs were not significantly different to the blank. At pH 5.5 condition, compared to the baseline, acetate significantly increase at 24 h (0 to 9.57 mM, $p < 0.05$) and 48 h (0 to 13.20 mM, $p < 0.05$)

Effect of BB-12 on SCFA production

The SCFA amounts were not significantly different to the blank. Compared to time 0, BB-12 resulted in significantly increased acetate at 24 h (0 to 12.99 mM, $p < 0.01$) within pH 5.5 condition.

Figure 4. Acetate and butyrate detected by GC (mM) following baseline (0 h), 6 h 24 h and 48 h with the administration of *L. plantarum* C4 (*L. plantarum*), *L. rhamnosus* GG, *L. acidophilus* NCFM (NCFM), FOS, *Saccharmyces boulardii* (*S. boulardii*) and *Bifidobacterium animalis subsp. lactis* BB-12 (BB-12) within a pH controlled *in vitro* faecal (1%) batch culture system. Results are reported as Means ± SD. Significant differences in each vessel * $p < 0.05$; ** $p < 0.01$ between 0 h, 6 h 24 h and 48 h are indicated.



3.4. Changes in bacterial/NT/SCFA levels between fermentation pH conditions

Compared to baseline (time 0), there were significant differences between the change in bacterial numbers, NTs and SCFA levels at 8, 24 and 48 h between the pH values (pH 5.5 and pH 6.9) these are reported in Table 2.

Table 2. The change in bacteria, NT and SCFA levels were compared between pH 5.5 and pH 6.9 conditions, only bacterial group, NT and SCFA with significant differences at time 6 to 0 (T_6-T_0), time 24 to 0 (T_{24}-T_0) and time 48 to 0 (T_{48}-T_0) are displayed (mean of the data of pH 5.5 and pH 6.9 experiments and standard deviations; n 4, respectively), significantly increased (+) or decreased (-) compared to time 0.

Bacterial group (Log10 cells per ml culture)	Time (h)	pH 5.5 Mean	SD	pH 6.9 Mean	SD	*P* value
***L. plantarum* C4 treatment**						
EUB	T_6-T_0	-0.14	0..28	0.63	0.34	0.014
	T_{24}-T_0	-0.07	0.16	0.44	0.34	0.05
BIF	T_6-T_0	0.21	0.33	0.75	0.47	0.019
LAB	T_6-T_0	-0.37	0.47	1.34	0.37	0.011
	T_{48}-T_0	0.007	0.71	1.49	0.23	0.048
BAC	T_{24}-T_0	-0.13	0.50	0.17	0.48	0.004
EREC	T_6-T_0	-0.40	0.25	0.35	0.68	0.05
	T_{24}-T_0	-0.27	0.38	0.29	0.33	0.001
	T_{48}-T_0	-1.24	0.18	-0.14	0.24	0.001

RREC	T_6-T_0	-0.73	0.69	0.68	0.35	0.005
DSV	T_6-T_0	-0.80	1.18	1.13	0.73	0.01
	T_{24}-T_0	-0.50	1.47	0.90	0.88	0.02
CHIS	T_6-T_0	-0.92	0.88	1.05	0.52	0.003
	T_{24}-T_0	0.03	0.76	0.49	0.65	0.05
***L. rhamnosus* GG treatment**						
BIF	T_6-T_0	0.13	0.36	0.95	0.39	0.025
	T_{24}-T_0	0.33	0.14	1.07	0.33	0.0215
	T_{48}-T_0	0.33	0.47	0.94	0.48	0.05
BAC	T_6-T_0	-0.25	0.21	1.00	0.33	0.0006
EREC	T_6-T_0	-0.49	0.20	0.71	0.50	0.02
	T_{24}-T_0	-1.09	0.32	0.58	0.76	0.03
RREC	T_6-T_0	-0.45	0.51	0.61	0.93	0.02
FPRAU	T_6-T_0	-0.28	0.64	1.02	0.88	0.05
	T_{24}-T_0	-0.71	0.56	0.72	1.22	0.05
DSV	T_6-T_0	-0.64	1.11	1.09	1.25	0.04
CHIS	T_6-T_0	-0.21	1.02	0.99	0.93	0.04
***L. acidophilus* NCFM treatment**						
BAC	T_6-T_0	-0.24	0.83	1.37	0.91	0.03
EREC	T_{24}-T_0	-1.33	0.57	0.73	0.73	0.04
DSV	T_{24}-T_0	-1.28	1.24	0.91	1.71	0.04
CHIS	T_6-T_0	-0.13	0.50	1.13	1.19	0.02
FOS treatment						
BAC	T_6-T_0	-0.31	0.71	1.52	0.58	0.003
RREC	T_6-T_0	-0.78	0.73	0.60	1.08	0.02
CHIS	T_6-T_0	-0.56	0.60	1.36	1.20	0.038
	T_{24}-T_0	-0.72	0.60	1.45	1.08	0.015
NTs (ng/mL)	Time (h)	pH 5.5 Mean	SD	pH 6.9 Mean	SD	***P*** value
***L. plantarum* C4 treatment**						
GABA	T_{24}-T_0	5.05	129.61	-200.00	108.99	0.01
Norepinephrine	T_{48}-T_0	-83.22	54.96	4.83	8.91	0.05
Epinephrine	T_6-T_0	8.86	5.21	-0.42	2.53	0.02
***L. rhamnosus* GG treatment**						
Dopamine	T_{48}-T_0	-19.39	11.39	0.27	0.32	0.038
Norepinephrine	T_6-T_0	114.32	60.70	0.42	0.42	0.03
***L. acidophilus* NCFM treatment**						
Norepinephrine	T_{48}-T_0	-67.51	33.12	0.31	0.41	0.02
FOS treatment						
GABA	T_{48}-T_0	1201.21	895.72	-152.65	4.08	0.05

Norepinephrine	T_6-T_0	167.23	59.37	0.34	0.40	0.01
Serotonin	T_6-T_0	6.68	4.95	0.54	0.61	0.029
Serotonin	T_{24}-T_0	0.54	0.61	1.45	1.07	0.005
SCFAs (mM)	Time (h)	pH 5.5 Mean	SD	pH 6.9 Mean	SD	***P*** value
***L. plantarum* C4 treatment**						
Acetate	T_{24}-T_0	2.47	2.92	33.37	15.73	0.036
***L. rhamnosus* GG treatment**						
Acetate	T_6-T_0	3.71	2.77	17.08	6.52	0.05
***L. acidophilus* NCFM treatment**						
Acetate	T_6-T_0	3.87	5.45	14.96	7.40	0.015
Acetate	T_{24}-T_0	6.00	2.64	29.60	4.78	0.0006
Acetate	T_{48}-T_0	6.55	5.34	31.81	8.71	0.024
Butyrate	T_{24}-T_0	0	0	5.18	2.01	0.014
Butyrate	T_{48}-T_0	0.41	0.82	5.15	2.27	0.007

It seems that bacteria growth and SCFA concentrations were greater at the more distal pH, while often NTs levels were improved at the lower pH.

4. Discussion

Studies have focused on the effect of pre and probiotics on the GBA(Liu *et al*., 2015). This study was undertaken to assess the ability of intestinally derived microbiota to produce NTs and whether this process was further supported by pre and probiotics in pH- and volume-controlled batch culture fermentations. Overall, the main findings were that the pre and probiotics significantly prolonged the increase in NTs concentrations under both conditions of pH 5.5 and pH 6.9 when compared to blank vessel, simulation of the proximal and distal colon, respectively. Most of the bacterial community had a greater increase observed under the more distal conditions. Whilst more proximal conditions (pH 5.5) tended to lead to higher amounts of NTs when compared to the more neutral conditions.

The synthesis of neuroactive compounds such as dopamine and serotonin are able to produce reversible psychological and physiological changes in mice similar to those found in autism spectrum disorders (ASDs)(Wang *et al*., 2011b). Gut bacteria such as bifidobacteria and lactobacilli are reported to produce a wide range of metabolites, which may act as a basis for probiotic function (Deguchi *et al*., 1985; Conly and Stein, 1992). A further metabolite produced by intestinal lactobacilli and bifidobacteria that may also be linked to desirable host effects is NT such as GABA. It has been suggested that microbially produced GABA in the gut may influence the brain-gut– microbiome axis (Bienenstock *et al*., 2010), an emerging concept in health said to be crucial for maintaining homoeostasis (Bonaz and Sabate, 2009; Cryan and O'Mahony, 2011). The amount of GABA observed throughout the whole fermentation of the current study showed opposite trends under the pH 5.5 and 6.9 conditions. The condition of pH 6.9 led to reduced levels of GABA, confirming finding of the previous chapter. At pH 5.5 GABA production was evident with increased concentrations observed over time. This aligns with an observation of Barrett et al., who observed levels of GABA produced in the pH-controlled faecal fermentations were far lower than in non-pH-controlled screening synthetic media, most likely due to the high pH (pH 6.8) of the faecal fermentations (Barrett *et al*., 2012). The synthesis of GABA in microorganisms is mainly affected by pH which confirmed by many studies (Komatsuzaki *et al*., 2005; Tsai *et al*., 2006; Yang *et al*., 2008). GABA is converted by amino acid glutamate by the enzyme glutamate decarboxylase (GAD)(Ueno, 2000). It has been previously suggested that optimum pH for enzyme GAD activity is pH 5.0 (Komatsuzaki *et al*., 2005), indeed the current study support these findings.

Catecholamine biosynthesis starts with tyrosine hydroxylase (TH) and catalyses the conversion of tyrosine to DOPA, which is the initial and rate-limiting step in the biosynthesis

of catecholamines such as DA, NE and EPI (Nagatsu *et al*., 1964; Ikeda *et al*., 1965). This current study demonstrated differences in catecholamine biosynthesis between pH 5.5 and 6.9, this is in agreement with the previous finding which confirmed the TH activity is affected by pH, optimally operating under acidic conditions (optimum pH 5.4) but no activity under neutral conditions (Fujisawa and Okuno, 2005). Enteric nerves synthesize DA, NE and EPI, but lack phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts NE into EPI (Costa *et al*., 2000). Bacterial enzyme β-glucuronidase have been observed to play a critical role in converting host NE and DA from a biologically-inactive to a biologically-active form (Asano *et al*., 2012). The finding of low conversion of NE to EPI has been varied among different treatment, fermentation time condition used in this study. This was especially seen for treatment FOS and *L. plantarum*, probably due to the inhibition of β-glucuronidase activity (de Preter *et al*., 2008; Arenahalli Ningegowda and Siddalingaiya Gurudutt, 2012). In agreement with these findings, decreased production of DA and NE have been observed upon fermentation of *L. plantarum* and FOS vessel in the current study, NE and DA synthesis were blocked induced by decreased β-glucuronidase activity inhibited by *L. plantarum* and FOS administration.

This study illustrated the amount of 5-HT varied substantially among faecal donors, the timing of fermentation and pH conditions. A study indicated faecal samples of endogenous 5-HT content per unit weight of dried faeces was 0.09–14.13 ng/mg for pregnant and post-delivery women and 0.30–9.93 ng/mg (Hirabayashi *et al*., 2020), indicating that current pre and probiotics can boost microorganism serotonin amount within a physiologically relevant range. 5-HT has a wide range of physiological properties, and there are many links between alterations in serotonergic system and disease. For instance, eating disorders and children with irritable bowel syndrome (IBS) or ASD have been reported to have a dysregulated

serotonergic system (Gauthier *et al*., 2014). Accumulated studies indicate the gut microbiome is a critical component in the modulation of the brain, for behaviour generally tryptophan metabolism and the serotonergic system appear to be a key component (Haleem, 2017).

In the current study, *S. boulardii* led to significantly increased GABA, 5-HT and acetate. A previous mouse study showed that *S. boulardii* could inhibit gut dysbiosis resulting in decreased intestinal and brain inflammation and oxidative stress thus protecting hippocampal neuronal damage (Roy Sarkar *et al*., 2021). This could be because probiotics by supporting the microbiota can lead to the enhancement of NTs and SCFA levels. The therapeutic impact of these changes remains to be determined, as the low NTs levels would be found in some defined GBA diseases, although such changes may help to improve gut symptoms and mental health.

Reduced faecal SCFA concentrations have been confirmed in gut-brain axis related disorders where brain physiology and behaviour are altered, including autism spectrum disorders (Liu *et al*., 2019) anorexia nervosa (Morita *et al*., 2015) and Parkinson's disease (Unger *et al*., 2016). FOS has a bifidogenic effect under the both proximal and distal conditions, therefore significantly increasing acetate levels within the in vitro batch culture fermentations, this makes sense as acetate is produced by *Bifidobacterium* (Bouhnik et al., 1996; Bouhink et al., 1999). FOS has been studied extensively for its bifidogenic capacity (Gibson et al., 1995; Bouhnik et al., 1996; Bouhnik et al., 1999). This finding matches with well the in vitro three stages continuous fermentation system (Liu et al., 2021). The FOS having the greatest impact on SCFA levels was also expected due to the additional carbohydrates available from this intervention.

The current study highlighted that FOS has effective prebiotic activity under the both proximal and distal conditions, in terms of increasing bifidobacteria this also coincided with increased 5-HT production, possibly due to stimulating NT producing bacteria activity in the gut. A preclinical study on FOS indicated benefits on motor, cognitive and gut symptoms in a mouse model of PD (Perez-Pardo *et al*., 2017). Several commensal organisms and probiotics have been reported to produce NTs, for example, GABA by *Bifidobacterium* and *Lactobacillus* genera (Barrett *et al*., 2012; Pokusaeva *et al*., 2017); 5-HT by *Lactobacillus* (Ouml *et al*., 2012) and *Faecalibacterium prausnitzii (Martín et al., 2015)*.

Amongst the other treatments analysed by FISH and LCMS, the results of this study showed that *L. acidophilus* NCFM stimulated the growth of *Bifidobacterium, Lactobacillus/Enterococcus, Bacteroides,* 5-HT and EPI. Indeed, this finding could be of benefit to the host, as *L. acidophilus* has been observed to have anxiolytic effects within *in vivo* and mice studies (Akkasheh *et al*., 2016; Colica *et al*., 2017). NTs are reduced to deficient levels in the disease, and their upregulation is included in most drug treatments currently available for Alzheimer's disease. Findings by the current study may provide a novel insight for GBA connection as pre and probiotics have been observed to support NT production. Therefore, observations from the current study show FOS and probiotics support the growth of beneficial bacteria at the same time as enhancing NT levels. This further shows that under physiological relevant conditions the microbiota are able to produce NTs and this production can be enhanced by FOS and probiotics.

5. Conclusions

Overall, the addition of pre and probiotics may help to enhance beneficial bacterial metabolism and NT production. This study quantified five NT levels in both proximal and distal colon faecal supernatant. The NTs content varied substantially through the fermentation time, but enhanced levels were observed in the more acidic pH range, suggesting more proximal production of NTs.

References

(2013). Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol,* **37**(7), 452-74.

Akkasheh, G., Kashani-Poor, Z., Tajabadi-Ebrahimi, M., Jafari, P., Akbari, H., Taghizadeh, M., Memarzadeh, M. R., Asemi, Z. & Esmaillzadeh, A. (2016). Clinical and metabolic response to probiotic administration in patients with major depressive disorder: A randomized, double-blind, placebo-controlled trial. *Nutrition,* **32**(3), 315-320.

Arenahalli Ningegowda, M. & Siddalingaiya Gurudutt, P. (2012). In vitro fermentation of prebiotics by Lactobacillus plantarum CFR 2194: selectivity, viability and effect of metabolites on β-glucuronidase activity. *World J Microbiol Biotechnol,* **28**(3), 901-8.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y. & Sudo, N. (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *Am J Physiol Gastrointest Liver Physiol,* **303**(11), G1288-95.

Atkinson, W., Lockhart, S., Whorwell, P. J., Keevil, B. & Houghton, L. A. (2006). Altered 5-Hydroxytryptamine Signaling in Patients With Constipation- and Diarrhea-Predominant Irritable Bowel Syndrome. *Gastroenterology,* **130**(1), 34-43.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun,* **25**(3), 397-407.

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

Berger, M., Gray, J. A. & Roth, B. L. (2009). The expanded biology of serotonin. *Annu Rev Med,* **60**, 355-66.

Bienenstock, J., Forsythe, P., Karimi, K. & Kunze, W. (2010). Neuroimmune aspects of food intake. *International Dairy Journal,* **20**(4), 253-258.

Bonaz, B., Bazin, T. & Pellissier, S. (2018). The Vagus Nerve at the Interface of the Microbiota-Gut-Brain Axis. *Frontiers in Neuroscience,* **12**.

Bonaz, B. & Sabate, J. M. (2009). [Brain-gut axis dysfunction]. *Gastroenterologie clinique et biologique,* **33 Suppl 1**, S48-58.

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

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

Burnett, S., Sebastian, C., Cohen Kadosh, K. & Blakemore, S. J. (2011). The social brain in adolescence: evidence from functional magnetic resonance imaging and behavioural studies. *Neurosci Biobehav Rev,* **35**(8), 1654-64.

Byrne, C. S., Chambers, E. S., Alhabeeb, H., Chhina, N., Morrison, D. J., Preston, T., Tedford, C., Fitzpatrick, J., Irani, C., Busza, A., Garcia-Perez, I., Fountana, S., Holmes, E., Goldstone, A. P. & Frost, G. S. (2016). Increased colonic propionate reduces anticipatory reward responses in the human striatum to high-energy foods. *Am J Clin Nutr,* **104**(1), 5-14.

Clarke, S. F., Murphy, E. F., O'Sullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., Hayes, P., O'Reilly, M., Jeffery, I. B., Wood-Martin, R., Kerins, D. M., Quigley, E., Ross, R. P., O'Toole, P. W., Molloy, M. G., Falvey, E., Shanahan, F. & Cotter, P. D. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut,* **63**(12), 1913-20.

Colica, C., Avolio, E., Bollero, P., Costa de Miranda, R., Ferraro, S., Sinibaldi Salimei, P., De Lorenzo, A. & Di Renzo, L. (2017). Evidences of a New Psychobiotic Formulation on Body Composition and Anxiety. *Mediators Inflamm,* **2017**, 5650627.

Collins, S. M., Surette, M. & Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nature Reviews Microbiology,* **10**(11), 735-742.

Conly, J. M. & Stein, K. (1992). The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis. *Prog Food Nutr Sci,* **16**(4), 307-43.

Costa, M., Brookes, S. J. & Hennig, G. W. (2000). Anatomy and physiology of the enteric nervous system. *Gut,* **47 Suppl 4**(Suppl 4), iv15-iv26.

Cryan, J. F. & O'Mahony, S. M. (2011). The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol Motil,* **23**(3), 187-92.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H. & Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol,* **22**(3), 434-44.

de Preter, V., Vanhoutte, T., Huys, G., Swings, J., Rutgeerts, P. & Verbeke, K. (2008). Baseline microbiota activity and initial bifidobacteria counts influence responses to prebiotic dosing in healthy subjects. *Aliment Pharmacol Ther,* **27**(6), 504-13.

Deguchi, Y., Morishita, T. & Mutai, M. (1985). Comparative Studies on Synthesis of Water-soluble Vitamins among Human Species of Bifidobacteria. *Agricultural and Biological Chemistry,* **49**(1), 13-19.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D., Cotter, P. D., Dinan, T. G. & Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun,* **48**, 165-73.

Devereux, R., Kane, M. D., Winfrey, J. & Stahl, D. A. (1992). Genus- and Group-Specific Hybridization Probes for Determinative and Environmental Studies of Sulfate-Reducing Bacteria. *Systematic and Applied Microbiology,* **15**(4), 601-609.

Dinan, T. G. & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil,* **25**(9), 713-9.

Feldman, J. M., Lee, E. M. & Castleberry, C. A. (1987). Catecholamine and serotonin content of foods: effect on urinary excretion of homovanillic and 5-hydroxyindoleacetic acid. *J Am Diet Assoc,* **87**(8), 1031-5.

Foster, J. A. & Neufeld, K. A. M. (2013). Gut-brain: how the microbiome influences anxiety and depression. *Trends in Neurosciences,* **36**(5), 305-312.

Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998). Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol,* **64**(9), 3336-45.

Fujisawa, H. & Okuno, S. (2005). Regulatory mechanism of tyrosine hydroxylase activity. *Biochemical and Biophysical Research Communications,* **338**(1), 271-276.

Gauthier, C., Hassler, C., Mattar, L., Launay, J.-M., Callebert, J., Steiger, H., Melchior, J.-C., Falissard, B., Berthoz, S., Mourier-Soleillant, V., Lang, F., Delorme, M., Pommereau, X., Gerardin, P., Bioulac, S., Bouvard, M. & Godart, N. (2014). Symptoms of depression and anxiety in anorexia nervosa: Links with plasma tryptophan and serotonin metabolism. *Psychoneuroendocrinology,* **39**, 170-178.

Haleem, D. J. (2012). Serotonin neurotransmission in anorexia nervosa. *Behav Pharmacol,* **23**(5-6), 478-95.

Haleem, D. J. (2017). Improving therapeutics in anorexia nervosa with tryptophan. *Life Sci,* **178**, 87-93.

Harmsen, H. J., Wildeboer-Veloo, A. C., Grijpstra, J., Knol, J., Degener, J. E. & Welling, G. W. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Appl Environ Microbiol,* **66**(10), 4523-7.

Hirabayashi, Y., Nakamura, K., Sonehara, T., Suzuki, D., Hanzawa, S., Shimizu, Y., Aizawa, T., Nakamura, K., Tamakoshi, A. & Ayabe, T. (2020). Analysis of Serotonin in Human Feces Using Solid Phase Extraction and Column-Switching LC-MS/MS. *Mass spectrometry (Tokyo, Japan),* **9**(1), A0081-A0081.

Hold, G. L., Schwiertz, A., Aminov, R. I., Blaut, M. & Flint, H. J. (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. *Appl Environ Microbiol,* **69**(7), 4320-4.

Ikeda, M., Levitt, M. & Udenfriend, S. (1965). HYDROXYLATION OF PHENYLALANINE BY PURIFIED PREPARATIONS OF ADRENAL AND BRAIN TYROSINE HYDROXYLASE. *Biochem Biophys Res Commun,* **18**, 482-8.

Komatsuzaki, N., Shima, J., Kawamoto, S., Momose, H. & Kimura, T. (2005). Production of γ-aminobutyric acid (GABA) by Lactobacillus paracasei isolated from traditional fermented foods. *Food Microbiology,* **22**(6), 497-504.

Kwok, R. H. (1968). Chinese-restaurant syndrome. *N Engl J Med,* **278**(14), 796.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. & Welling, G. W. (1995). Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microbiol,* **61**(8), 3069-75.

Latorre, R., Sternini, C., De Giorgio, R. & Greenwood-Van Meerveld, B. (2016). Enteroendocrine cells: a review of their role in brain-gut communication. *Neurogastroenterology and Motility,* **28**(5), 620-630.

Lavizzari, T., Teresa Veciana-Nogués, M., Bover-Cid, S., Mariné-Font, A. & Carmen Vidal-Carou, M. (2006). Improved method for the determination of biogenic amines and polyamines in vegetable products by ion-pair high-performance liquid chromatography. *J Chromatogr A,* **1129**(1), 67-72.

Liu, X., Cao, S. & Zhang, X. (2015). Modulation of Gut Microbiota-Brain Axis by Probiotics, Prebiotics, and Diet. *J Agric Food Chem,* **63**(36), 7885-95.

Liu, Y., Gibson, G. R. & Walton, G. E. (2016). An In Vitro Approach to Study Effects of Prebiotics and Probiotics on the Faecal Microbiota and Selected Immune Parameters Relevant to the Elderly. *PLoS One,* **11**(9), e0162604.

Logan, A. C. & Jacka, F. N. (2014). Nutritional psychiatry research: an emerging discipline and its intersection with global urbanization, environmental challenges and the evolutionary mismatch. *J Physiol Anthropol,* **33**(1), 22.

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

Magro, F., Vieira-Coelho, M. A., Fraga, S., Serrão, M. P., Veloso, F. T., Ribeiro, T. & Soares-da-Silva, P. (2002). Impaired synthesis or cellular storage of norepinephrine, dopamine, and 5-hydroxytryptamine in human inflammatory bowel disease. *Dig Dis Sci,* **47**(1), 216-24.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of

the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology (Reading),* **142 (Pt 5)**, 1097-1106.

Martín, R., Miquel, S., Chain, F., Natividad, J. M., Jury, J., Lu, J., Sokol, H., Theodorou, V., Bercik, P., Verdu, E. F., Langella, P. & Bermúdez-Humarán, L. G. (2015). Faecalibacterium prausnitzii prevents physiological damages in a chronic low-grade inflammation murine model. *BMC microbiology,* **15**, 67-67.

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut-brain communication. *Nature Reviews Neuroscience,* **12**(8), 453-466.

Mayer, E. A., Knight, R., Mazmanian, S. K., Cryan, J. F. & Tillisch, K. (2014). Gut Microbes and the Brain: Paradigm Shift in Neuroscience. *Journal of Neuroscience,* **34**(46), 15490-15496.

Meddings, J. B. & Swain, M. G. (2000). Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. *Gastroenterology,* **119**(4), 1019-28.

Nagatsu, T., Levitt, M. & Udenfriend, S. (1964). TYROSINE HYDROXYLASE. THE INITIAL STEP IN NOREPINEPHRINE BIOSYNTHESIS. *J Biol Chem,* **239**, 2910-7.

Neufeld, K. M., Kang, N., Bienenstock, J. & Foster, J. A. (2011). Reduced anxiety-like behaviour and central neurochemical change in germ-free mice. *Neurogastroenterol Motil,* **23**(3), 255-64, e119.

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

Ouml, Zo, Ul, F., Kuley, E., Ouml, Zo, Ul, Y., Ouml, Zo, Ul & lyas (2012). The Function of Lactic Acid Bacteria on Biogenic Amines Production by Food-Borne Pathogens in Arginine Decarboxylase Broth. *Food Science and Technology Research,* **18**(6), 795-804.

Patrick, R. P. & Ames, B. N. (2014). Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism. *Faseb j,* **28**(6), 2398-413.

Perez-Pardo, P., Kliest, T., Dodiya, H. B., Broersen, L. M., Garssen, J., Keshavarzian, A. & Kraneveld, A. D. (2017). The gut-brain axis in Parkinson's disease: Possibilities for food-based therapies. *Eur J Pharmacol,* **817**, 86-95.

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., Matsunami, R. K., Lugo, M., Major, A., Mori-Akiyama, Y., Hollister, E. B., Dann, S. M., Shi, X. Z., Engler, D. A., Savidge, T. & Versalovic, J. (2017). GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterol Motil,* **29**(1).

Richardson, A. J., Calder, A. G., Stewart, C. S. & Smith, A. (1989). Simultaneous Determination of Volatile and Non-Volatile Acidic Fermentation Products of Anaerobes by Capillary Gas-Chromatography. *Letters in Applied Microbiology,* **9**(1), 5-8.

Roy Sarkar, S., Mitra Mazumder, P., Chatterjee, K., Sarkar, A., Adhikary, M., Mukhopadhyay, K. & Banerjee, S. (2021). Saccharomyces boulardii ameliorates gut dysbiosis associated cognitive decline. *Physiol Behav,* **236**, 113411.

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., Challis, C., Schretter, C. E., Rocha, S., Gradinaru, V., Chesselet, M. F., Keshavarzian, A., Shannon, K. M., Krajmalnik-Brown, R., Wittung-Stafshede, P., Knight, R. & Mazmanian, S. K. (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell,* **167**(6), 1469-1480.e12.

Sarker, S. A., Sultana, S., Reuteler, G., Moine, D., Descombes, P., Charton, F., Bourdin, G., McCallin, S., Ngom-Bru, C., Neville, T., Akter, M., Huq, S., Qadri, F., Talukdar, K., Kassam, M., Delley, M., Loiseau, C., Deng, Y., El Aidy, S., Berger, B. & Brüssow, H. (2016). Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage

Preparations: A Randomized Trial in Children From Bangladesh. *EBioMedicine,* **4**, 124-37.

Savignac, H. M., Corona, G., Mills, H., Chen, L., Spencer, J. P. E., Tzortzis, G. & Burnet, P. W. J. (2013). Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. *Neurochemistry International,* **63**(8), 756-764.

Shiman, R., Akino, M. & Kaufman, S. (1971). Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J Biol Chem,* **246**(5), 1330-40.

Shishov, V. A., Kirovskaia, T. A., Kudrin, V. S. & Oleskin, A. V. (2009). [Amine neuromediators, their precursors, and oxidation products in the culture of Escherichia coli K-12]. *Prikl Biokhim Mikrobiol,* **45**(5), 550-4.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R. & Gobbetti, M. (2007). Synthesis of gamma-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Applied and environmental microbiology,* **73**(22), 7283-7290.

Spohn, S. N. & Mawe, G. M. (2017). Non-conventional features of peripheral serotonin signalling - the gut and beyond. *Nat Rev Gastroenterol Hepatol,* **14**(7), 412-420.

Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J. & Lewis, K. (2019). GABA-modulating bacteria of the human gut microbiota. *Nature Microbiology,* **4**(3), 396-403.

Sugama, S. & Kakinuma, Y. (2016). Loss of dopaminergic neurons occurs in the ventral tegmental area and hypothalamus of rats following chronic stress: Possible pathogenetic loci for depression involved in Parkinson's disease. *Neurosci Res,* **111**, 48-55.

Terbeck, S., Savulescu, J., Chesterman, L. P. & Cowen, P. J. (2016). Noradrenaline effects on social behaviour, intergroup relations, and moral decisions. *Neurosci Biobehav Rev,* **66**, 54-60.

Tsai, J. S., Lin, Y. S., Pan, B. S. & Chen, T. J. (2006). Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochemistry,* **41**(6), 1282-1288.

Tsavkelova, E. A., Botvinko, I. V., Kudrin, V. S. & Oleskin, A. V. (2000). Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. *Dokl Biochem,* **372**(1-6), 115-7.

Ueno, H. (2000). Enzymatic and structural aspects on glutamate decarboxylase. *Journal of Molecular Catalysis B: Enzymatic,* **10**(1), 67-79.

Walker, A. W., Duncan, S. H., McWilliam Leitch, E. C., Child, M. W. & Flint, H. J. (2005). pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Appl Environ Microbiol,* **71**(7), 3692-700.

Wallner, G., Amann, R. & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry,* **14**(2), 136-43.

Walls, A. B., Heimbürger, C. M., Bouman, S. D., Schousboe, A. & Waagepetersen, H. S. (2009). Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. *Neuroscience,* **158**(1), 284-292.

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T. & Conlon, M. A. (2011). Low relative abundances of the mucolytic bacterium Akkermansia muciniphila and Bifidobacterium spp. in feces of children with autism. *Applied and environmental microbiology,* **77**(18), 6718-6721.

Wong, C. G., Bottiglieri, T. & Snead, O. C., 3rd (2003). GABA, gamma-hydroxybutyric acid,

and neurological disease. *Ann Neurol,* **54 Suppl 6**, S3-12.
Yang, S. Y., Lü, F. X., Lu, Z. X., Bie, X. M., Jiao, Y., Sun, L. J. & Yu, B. (2008). Production of gamma-aminobutyric acid by Streptococcus salivarius subsp. thermophilus Y2 under submerged fermentation. *Amino Acids,* **34**(3), 473-8.
Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K. & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell,* **161**(2), 264-276.

Chapter 4

Using an *in vitro* model of the large intestine to model the microbial community in anorexia nervosa – a methodological approach

Abstract

Anorexia nervosa (AN) is a psychiatric disease with devastating physical consequences. Alterations of gut microbiota may play a role in AN through perturbations of the gut brain axis. Some studies have observed differences in the faecal microbiota of patients with AN compared to healthy controls, but results in different clinical states are heterogeneous. Diet seems on modification of the gut microbiota, and its associated metabolites. In this study a three stage gut model system that models the luminal microbiology of the large intestine was performed to identify relationships between diet and gut microbiota. Media was modified to provide a nutrient basis more appropriate to AN to determine the impact this has on the microbiota. Microbiome datasets from studies were pooled and considered focusing on Phylum, Family and Genus levels in AN's gut microbiota compared to healthy controls (HC). Next-Generation Sequencing (NGS), flow cytometry combined with fluorescence in situ hybridisation and gas chromatography showed that AN intestinal microbiota and metabolites were significantly altered at every taxonomic level and there were significant changes in composition and diversity of the gut microbiota following healthy control (HC) and AN medium. A nutrient-deficient diet resulted in reduced neurotransmitter levels, reduced butyrate-producing bacteria, anorexigenic and anxiogenic protein-producing bacteria, and all of them could be potential hallmark in AN.

These findings maybe important in determining how nutritional changes drive large intestine microbiota changes with a drastically altered diet. Furthermore, the model system could be useful in assessing appropriate gut-brain targeted treatments.

Keywords Anorexia nervosa; Dysbiosis; Restricted diet; Gut-brain axis; 16S metagenomics analysis.

1. Introduction

Anorexia nervosa (AN) is a psychiatric disease with devastating physical consequences, with a pathophysiological mechanism still to be elucidated (Eddy *et al*., 2008; Mehler and Brown, 2015). Alterations of gut microbiota may play a role in AN through perturbation of the gut brain axis (Godart *et al*., 2002; Kaye *et al*., 2004; Fernandez-Aranda *et al*., 2007). Restricting-type anorexics (R-AN) lose weight by dieting and exercising (Association, 2013). Diet seems to have an impact not only on modification of the gut microbiota composition and richness but also on recovery in patients with AN (Baxter *et al*., 2019). Metagenomics studies on AN have provided insight into how diet and nutrition can alter the gut microbiome making it a possible to understand more about this environmental factor of the disease (Kaelin and McKnight, 2013).

Within the gastrointestinal (GI) tract there are differences in physiology, digestion, flow rate, substrate availability, host secretions, pH, and oxygen tension between different anatomical regions (Flint et al., 2012). The gut microbiome is mainly present in the colon and the human gut microbiome ecosystem is characterised by the unique diversity with about 1000 microbial species encoded by genes (microbiomes). Studies of the human microbiome have revealed

significant individual variability, which indicates that even healthy individuals have significant differences in microbial composition (Qin *et al*., 2010). The large intestine is characterised by a slow flow rate (0.4 mL/min) (Tottey *et al*., 2017), pH ranges from acidic to neutral, and this space is dominated by specialised obligate anaerobes. Important differences in the intestinal environment occur between proximal, transverse and distal regions and more locally between the intestine cavity and surface (Flint *et al*., 2012). Healthy human intestinal microbiota is dominated by bacteria from two phyla-Firmicutes (including the genera *Lactobacillus*, *Clostridium*, and *Enterococcus*) and Bacteroidetes (including *Bacteroides*), which account for over 90% of the known genetic categories of the intestinal system (Eckburg *et al*., 2005). Other bacteria such as representatives from the phylum Actinobacteria (*Bifidobacterium*), Proteobacteria (*Escherichia coli*), Fusobacteria, Verrucomicrobia, and Cyanobacteria are present to a lesser extent (Qin *et al*., 2010). However, the proportion in their representation differs on an individual basis and depends on many external factors. Differences in the bacteria within the intestine has been observed in a variety of diseases including AN (Roubalova *et al*., 2020). For example, The Firmicutes / Bacteroidetes (F/B) ratio is higher in obese people compared to lean people, and tends to reduce with weight loss (Sweeney and Morton, 2013; Koliada *et al*., 2017), indeed, lower levels of Firmicutes have been reported in AN (Mack *et al*., 2016; Borgo *et al*., 2017). Additionally, AN pathophysiology also involves autoimmune component; it may be related to some gut bacterial antigens, such as Caseinolytic protease B (ClpB) anorexigenic bacterial protein produced by Enterobacteriaceae (Fetissov and Hökfelt, 2019). CIpB may be able to stimulate Peptide YY (PYY) production, thus supressing appetite by impacting on hormone secretion and satiety (Tennoune *et al*., 2014).

To better study the microbial community in AN different microbial tools can be considered. A three-stage continuous culture system was validated by Macfarlane, Macfarlane and Gibson in 1998 (Macfarlane *et al*., 1998). This system was designed to mimic nutritional and physiochemical conditions of microbiota in the colon, and was determined by measuring the human large intestine contents obtained from sudden death victims (Macfarlane *et al*., 1998). The main substrates available to microorganisms growing in the large intestine are dietary fibres, proteins, oligosaccharides and peptides that evade digestion in the small intestine, as well as a variety of host-derived substances, including mucins, pancreatic secretions and exfoliated epithelial cells (Macfarlane and Gibson, 1994; Macfarlane GT, 1995). Originally the constituents of culture medium were determined on the basis of caecal content. The flow of carbohydrate into the colon from the end of small intestine can potentially change the dynamics of the bacterial community and metabolites in the colon, particularly SCFAs (Flint *et al*., 2012). By manipulating this media it then might be possible to study how the microbial community might react in the presence of different nutrients. Studying this microbial community could be of great importance, as the GI tract can respond to changes in nutrient environment in the lumen and regulate the neuron and hormone signals of the gastrointestinal tract to help regulate appetite and food intake (Murphy and Bloom, 2004). Therefore, in the following study a media approximated to anorexic dietary intake was designed for use in a three-stage continuous culture system to enable the activities of intestinal bacteria to be established under these nutrient-deficit conditions. This *in vitro* three-stage continuous culture system was used to determine the impact of these large dietary changes on the gut microbial community under physiologically relevant conditions. Therefore, the aim of the current study: (1) to establish an anorexic three-stage continuous culture gut model system based on nutrient intake and gastrointestinal transit times in AN patients. (2) compare changes observed within our system to current data of AN patients.

2. Materials and methods

2.1. Dietary intake in comparison with healthy and Restricting-AN

Restricting-AN is based on individuals with the restricting subtypes of AN, where the illness is characterised by dietary restriction in the absence of recurrent episodes of regular binge eating and purging. To establish an R-AN gut model system and the medium concentration was modelled on the real gut dietary conditions of AN individuals. Dietary intake of healthy adults was obtained from British Nutrition Foundation (BNF) guideline (British Nutrition Foundation, 2016). This information was contrasted with the gut model media to determine the likelihood of different nutrient components to reach the large intestine. Following this, the data of Jauregui et al. (2009); Raatz et al. (2015) was used to determine a restrictive dietary intake associated with AN (restricted diet =12, quartile 1) (Jauregui Lobera and Bolanos Rios, 2009; Raatz *et al*., 2015)(Table 1). The diets were compared and a new media developed based on the differences between the macronutrient and micronutrient content of the diets, relative to the media (Table 2).

Table 1. Energy and nutrient intake in individuals with AN on restricted diet and comparison with British Nutrition Foundation (BNF) recommendations for estimated Average Requirements for adults (restricting-AN patients clinically and biological characteristics).

Daily dietary composition		**BNF value**	**Restricting AN diet** (n=12)	**Sites of absorption**
Energy intake	**Kcal**	2175	736	-
Protein intake	g	50	33	Ileum and colon

Carbohydrate	g	260	110	-
Dietary fibre	g	30	12	Colon
Fat	g	70	17	Ileum
Potassium	mg	3500	2600	Ileum and colon
Chloride	mg	2500	1825	Duodenum and colon
Sodium	mg	1600	1168	Colon
Magnesium	mg	270	227	Duodenum
Phosphorus	mg	550	607	Jejunum
Calcium	mg	700	545	Jejunum
Iron	mg	14.8	7.9	Duodenum
Vitamin K	µg	-	-	Ileum and colon
Vitamin B12	µg	1.5	2.2	Ileum
Thiamin	mg	0.8	0.704	Jejunum
Riboflavin	mg	1.1	1.1	Jejunum
Niacin	mg	13	8.6	Duodenum
Folate	µg	200	234	Duodenum

Vitamin B6	mg	1.2	0.9	Jejunum
Vitamin A	mg	600	503	Ileum
Vitamin C	mg	40	70	Ileum
Vitamin E	mg	3	2.7	Ileum
Vitamin D	µg	10	1.7	Ileum
Selenium	µg	60	39	Duodenum
Zinc	mg	7	5.4	Jejunum
The UK set of Dietary Reference Values (DRVs), including Estimated Average Requirements (EARs) and Reference Nutrients Intakes (RNIs). In this study 18-60 years old female group were considered that macronutrients requirements are based on EARs, micronutrients and protein requirements are based on RNIs.				

Table 2. Nutrients assessment. Healthy and R-AN gut model medium development

	Nutrients	Comparison % (HC Versus R-AN)	Representative medium	Healthy gut model medium (g/L) SS1	Anorexic medium (g/L) SS2
M	Carbohydrate (g)	42%	Starch	5 g	2.1 g
A C R O	Protein (g)	66%	Peptone water Tryptone Yeast extract Casein	5 g 5 g 4.5 g 3 g	3.3 g 3.3 g 2.97 g 1.98 g

NUTRIENTS					
	Dietary fibre (g)	40%	Guar Gum Inulin Pectin Arabinogalactan Xylan	1 g 1 g 2 g 2 g 2 g	0.4 g 0.4 g 0.8 g 0.8 g 0.8 g
MICRONUTRIENTS	Potassium (mg)	76%	KCI	4.5 g	3.28 g
	Chloride (mg)	73%	NaCI	4.5 g	3.28 g
	Sodium (mg)	73%	$NaHCO_3$	1.5 g	1.095 g
	Magnesium (mg)	84%	$MgSO_4 \cdot 7H_2O$	1.25 g	1.05 g
	Phosphorus (mg)	110%	KH_2PO_4 K_2HPO_4	0.5 g 0.5 g	0.55 g 0.55 g
	Calcium (mg)	78%	$CaCl_2 \cdot 6H_2O$	0.15 g	0.117 g
	Iron (mg)	53%	Hemin $FeSO_4.7H_2O$	0.5 g 0.005 g	0.0265 g 0.000795 g
	Vitamin K (µg)	68%	Vitamin K	10 µL	6.68uL

Additionally, each medium contained: 0.8 g L-cystine HCI, 1 mL Tween 80, 4 mL resazurin solution (0.025 g/100 mL, pH 7), 4 g mucin (Porcine gastric type III), 0.4 g bile salts per litre gut model, representing human secretions determined within original gut model media.

2.2. Three-staged continuous culture system

A three-stage continuous culture system was set up in sequence simulating the proximal (V1, 80 mL, pH = 5.5), transverse (V2, 100 mL, pH = 6.2) and distal colon (V3, 120 mL, pH = 6.8) comprising of a scaled-down version of three regions of the GI tract. The system was maintained under anaerobic conditions by supplying N_2 (15mL/min) and holding a continuous temperature of 37°C via a circulating water bath. The systems were inoculated to give a final concentration of 6% faecal slurry donated from four healthy female donors (age range 25-43 years). The faecal donors had not taken antibiotics within 6 months of the experiment and were not regular consumers of prebiotic or probiotic supplements. Collected faecal samples were placed in anaerobic jars (AnaeroJarTM 2.5L, Basingstoke, UK, Oxoid Ltd) with anaerobic sachets (AnaeroGen, Oxoid) and used within 2 hours of production. To prepare the faecal sample a 1 in 5 (wt:v) faecal slurry with PBS (anaerobic phosphate buffered saline; 0.1mol/l; pH 7.4) was homogenised in a stomacher (Stomacher 400, Seward, West Sussex, UK) for 2 minutes (240 paddle beats/min). Faecal slurry at 6% after inoculated into each vessel. The experiment was conducted 4 times with a different faecal donor for each run. Following inoculation, the system was run for 24h as a batch culture to allow the bacteria to multiply within the vessels. After this the flow was started, with a retention time appropriate to anorexic patients (Kamal *et al*., 1991) when considering the operating volume (300 mL) and anorexic retention time (64h, flow rate 4.68 mL/h) of the gut model system. The first steady state (SS1), when equilibrium was reached, was after 512 h (8 full volume turnovers) using standard gut model media (HC feeding). A second steady state (SS2) was achieved after a further 512 h, this was using AN media (AN feeding). Each steady state was confirmed by assessing the stabilisation of the short chain fatty acids over 3 consecutive days.

2.3. Preparation of samples collection, processing, and storage

Samples were taken following the fermentation of healthy control media and dietary restricted media (anorexic) from proximal, transverse and distal vessels at SS1 and SS2. 1 mL gut model fluid was centrifuged in a micro centrifuge Eppendorf tube (1.5 mL) at 13000 x *g* for 10 min the pellet was stored at -20 °C for future DNA extraction.

1 mL of gut model fluid was centrifuged in a micro centrifuge Eppendorf tube (1.5 mL) at 13,000 x g for 10 min and supernatant was stored at –20 °C prior to SCFA/BCFA analysis. For total bacterial community analysis, a 750 µL supernatant of gut model fluid was centrifuged at 13,000× *g* for 5 min. The pellet was then resuspended in 375 µL filtered 0.1 M PBS and fixed by 1125 µL filtered paraformaldehyde (PFA 4% *w*/*v*) for 4 to 8 h at 4 °C. The sample was washed twice with 1 mL PBS to remove PFA and resuspended in filtered 600 µL ethanol-PBS (1:1, *w*/*v*). The samples were kept at –20 °C prior to fluorescence in situ hybridisation flow cytometry (FISH-FCM) analysis. The method used to analyse FISH-FCM and SCFA/BCFA production are reported in detail elsewhere (Liu *et al*., 2021).

2.4. DNA extraction, quantification and qualification

DNA extraction using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) Cat. No. 51604) was performed with some modifications of a method from manufacturer's instructions. Cell pellets were defrosted on ice and washed with 0.5 mL PBS. Each pellet was resuspended in 1 mL of InhibitEX buffer, and then the suspension was transferred to a new tube with acid washed glass beads from Sigma (≤ 100 µm). These were subjected to a bead beater (FastPrep-24™ 5G) for 60 seconds and put on ice for 60 seconds. This step was repeated 3 times. After, the sample was vortexed for 1 min, centrifuged at 13000 x *g* for 1

min and 0.6 mL of supernatant was transferred to fresh tube with 25 µL of Proteinase K. This was followed by the addition of 0.6 mL of AL buffer then vortex for 15 seconds. Samples were then incubated at 70°C for 10 min and 0.6 mL of ethanol (99.9%) added to the lysate and vortexed to mix. Carefully, 0.6 mL of the lysate was added to the QIAamp spin column. The cups were closed and centrifuged at 13000 x *g* for 1 min and the tubes containing the filtrate were discarded. In a new 2 mL collection tube the previous step was repeated until all the lysate had been loaded onto the spin column. Carefully, the spin column for each tube was opened and 0.5 mL of Buffer AW1 added and centrifuged at 13000 x *g* for 1 min and filtrate discarded. Spin columns were then placed in a new 2 mL collection tube and 0.5 mL of AW2 buffer added, tubes were centrifuged for 3 min at 13000 x *g* and the collection tubes containing the filtrate discarded. In new 2 mL collection tubes, spin columns were placed and centrifuged for 3 min at 13000 x *g*. The QIAamp spin columns were then transferred into a new set of 1.5 microcentrifuge tubes and 0.03 mL of distilled water added, these were incubated at room temperature for 1 min and then centrifuged at 13000 *g* for 1 min. The microcentrifuge tubes with the filtrate were discarded and the spin column placed into a new 1.5 mL microcentrifuge tube and the previous step repeated. DNA concentration of the final product was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). A 2 µL volume of sample was placed on the Nanodrop pedestal and the DNA quantity was assessed. Each sample was diluted in ddH_2O to provide a concentration within the range with 10 to 50 ng/ µL, Samples were placed in a 96-well plate and send to Eurofins Genomics Europe Shared Services (Germany, Ebersberg; project NG-27480).

2.5 16s rRNA Gene-Based Next-Generation Sequencing (NGS) and Bioinformatics

16S rRNA sequencing was carried out by Eurofins, Germany. Briefly,16S rRNA gene sequences were amplified from extracted DNA samples, characterised by amplication of V3-V4 (forward, 349F: 5'-GYGCASCAGKCGMGAAW-3'; reverse, 806R: 5'-GGACTACVSGGGTATCTAAT-3') variable region of the 16S rRNA gene by polymerase chain reaction (PCR), as previously described (Klindworth *et al*., 2012). After sequencing, de-multiplexing of the data based on the Illumina index reads was performed and raw data converted to FASTQ files. Illumina adapters were removed using the FASTP program and error correction was performed on the region where two reads overlapped. Raw paired end reads were subjected to quality filtering using software before paired-end read assembling with Fast Length Adjustment of short reads (FLASh) software (2.2.00 version). For precise Operational Taxonomic Units (OTUs) analysis, data containing sequence error (i.e., merged sequences shorter than 458 bp, raw reads with ambiguous base cells, chimeric sequences) were removed. The remaining representative reads from non-chimeric clusters were clustered de novo into OTUs (97% similarity threshold) using a Cluster Database at High Identify with Tolerance (CD-HIT) software program Version 4.6 (University of California, San Diego) based on 99% similarity accounting for PCR and sequencing errors of less than 1%. After pre-clustering (Huse *et al*., 2010), the trimmed reads were also checked and chimeric sequences were removed using an implementation of the UCHIME program (version 4.2.40).

Afterwards, taxonomic assignments were performed using the Basic Local Alignment Search Tool (BLAST) version 2.6.0+ (National Library of Medicine, Bethesda) and the following setting:(*i*)16s RNA sequences reference dataset from the Ribosomal Database Project September 2016 released; (*ii*) taxonomic classification database National Center for Biotechnology Information (NCBI 16S, Bethesda, MD, USA).

2.6. Data processing and Bioinformatics Analysis

The pooled libraries were paired-end read sequenced on a MiSeq System (Illumina Inc, San Diego, California, USA). OTU clustering and taxonomic information was converted to FASTQ files and processed using CLC Genomics Workbench version 21.0.4 and CLC Microbial Genomics Module (QIAGEN Bioinformatics) (Denmark). Sequences were first trimmed and merged and then were clustered into OTUs at 99% sequence similarity level using the Amplicon-Based OTU clustering tool. The creation of new OTUs was allowed considering 99% taxonomic similarity. The most abundant sequences were selected as representative of each cluster and then assigned to a taxonomy level using CLC Microbial Genomics default values and the SILVA Database September 2016 release.

2.7. Statistical analysis

2.7.1.16S metagenomics sequencing and analysis

Classification of 16S rRNA forward sequence reads was performed using CLC Genomics Workbench 21.0.4 version. The OTU log-fold change between healthy and anorexic conditions was statistically analysed by FDR correction and Kruskal-Wallis test. False discovery rate (FDR) was performed to correct *p*-values. miRNAs with healthy conditions versus anorexic conditions fold change (FC) > 1.5 and FDR adjusted *p*-value < 0.05 were considered significant.

2.7.2. Total bacteria and SCFA/BCFA analysis

Data from FISH-FCM and Gas chromatography (GC) were analysed with SPSS version 27 (IBM Corp., Armonk, NY, USA). Changes in total bacteria, SCFA/ BCFA production were assessed between the 2 steady states using a one-way analysis of

variance (ANOVA). Significant differences were assessed by post hoc Tukey HSD (Honestly Significant Difference) test. Statistical significance was set $p < 0.05$.

3. Results

3.1. Microbial profiles in the simulation of proximal, transverse, and distal colon (HC verses AN)

The faecal-derived microbial communities in the different segments of proximal, transverse and distal from fermentation of HC and AN media were profiled. The composition of the intestinal microbiota was significantly influenced by AN media at every taxonomic level ($p < 0.05$). When looking differences in microbiota composition between fermentation of HC and AN media, both common and unique microbial signatures of response were observed.

At phylum level, the predominant bacterial taxa were Firmicutes and Bacteroidetes followed by Actinobacteria, Proteobacteria and Fusobacteria, several taxonomic differences were observed between the microbiota of the HC verses AN in the stimulation of proximal, transverse and distal colon (Fig 1). When compared to the microbial phylum profile between fermentation of HC and AN media, Briefly, the trend of relative abundance of phylum Firmicutes and Actinobacteria were increased in the fermentation of HC media, whereas Bacteroidetes, Proteobacteria and Fusobacteria were increased in the AN model. Significant differences in Phylum levels between AN and HC are reported in Table 4. Compared to HC media, the F/B ratio were calculated observed to be reduced in proximal, transverse and distal colon in AN model (Fig 2).

The most abundant families were Bacteroidaceae, Ruminococcaceae, Lachnospiracea, Veillonellaceae, Enterobacteriaceae, Porphyromonadaceae, Bifidobacteriaceae, and Prevotellaceae, (Fig 3). At Family level, in particular, compared to AN model, Actinomycetaceae was only observed in HC distal colon, but was undetectable in AN model ($p < 0.05$). The Campylobacteraceae family was also only observed in HC model (transverse and distal colon) ($p < 0.05$ and $p < 0.01$). In contrast, it is worth noting that some families were undetectable in HC media and only significantly increased after AN media fermentation, for example, relative abundance of Methylobacteriaceae significantly increased in all vessels (proximal, $p < 0.005$; transverse, $p < 0.05$ and distal $p < 0.05$) after fermentation of AN media. The abundance of families of Fusobacteriaceae and Desulfovibrionaceae significantly increased in V1 (proximal, both $p < 0.001$) after fermentation of AN media. Families of Barnesiellaceae and Oxalobacteraceae significantly increased after fermentation on AN media in V2 (transverse, $p < 0.05$) and V3 (distal, $p < 0.05$). Dethiosulfovibrionaceae abundance significantly increased in V2 and V3 (transverse and distal, both $p < 0.05$) AN model.

The most abundant genera were *Ruminococcus*, *Lactobacillus*, *Roseburia, Faecalibacterium*, *Parabacteroides*, *Bifidobacterium*, and *Prevotella* (Fig 4). Significant differences in Phylum, Family and genus levels between AN and HC are reported in Table 4 and 5.

In genus level, some genus were only observed in the HC model, whilst the AN model was unable to support growth of these genus. On the contrary, compared to HC media, there were some genera only detectable following fermentation of AN media (Table 5).

Figure 1. Phylum-level profile. Relative abundance of common microbial taxa. Bar charts showing the relative abundance of the most represented microbial taxa, defined as having a

mean relative abundance of > 1%, in gut model of proximal, transverse, and distal following fermentation of healthy control media (HC, n=4) then restrictive AN media (AN, n=4).

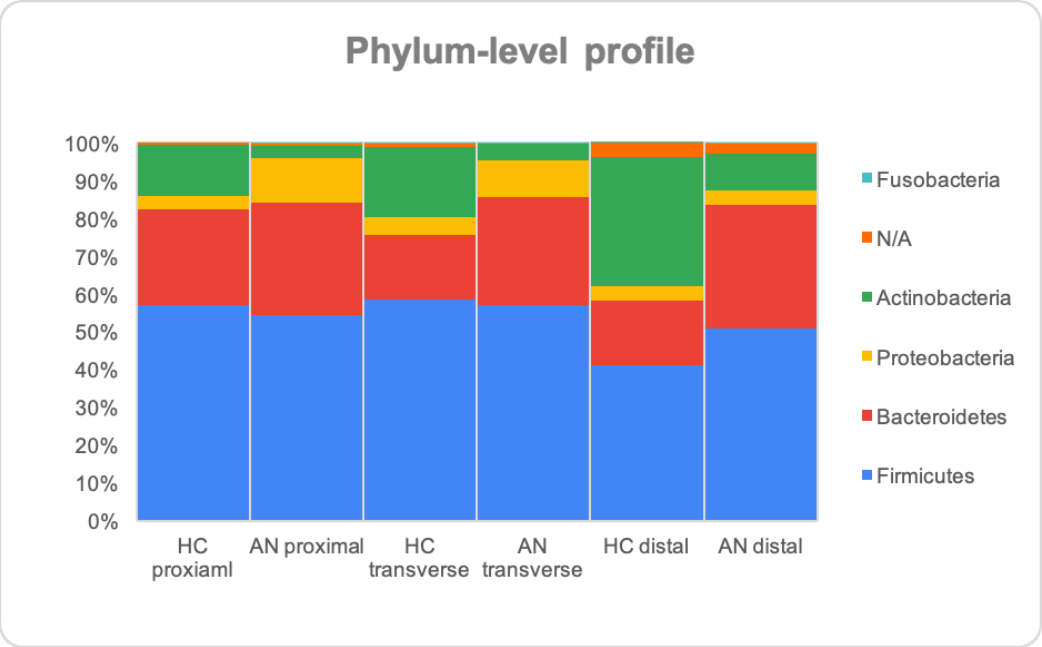


Figure 2. F/B ratio. Comparison of microbiota following fermentation of healthy and restrictive AN media. The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated following fermentation of HC and AN media in the different sites of the colonic model.

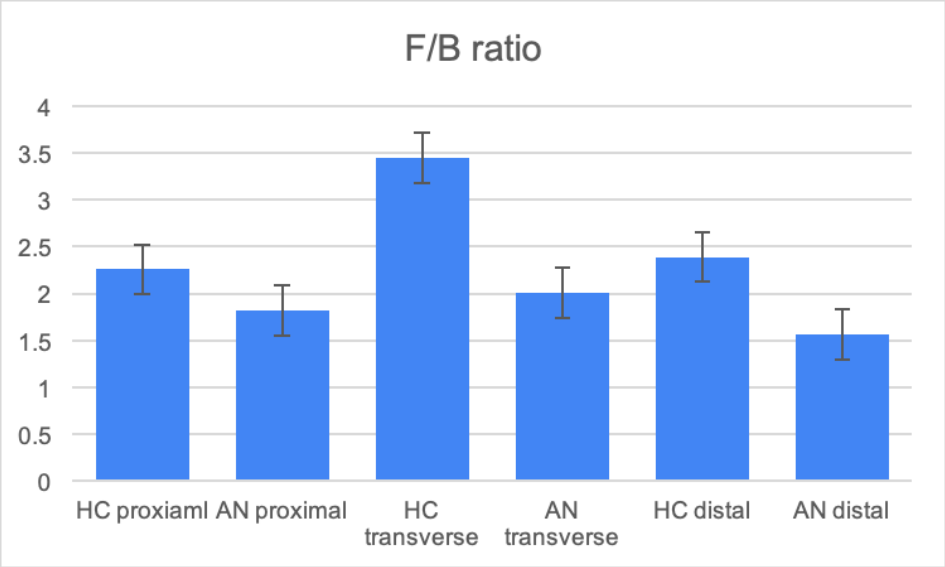


Figure 3. Family-level profiles. Relative abundance of common microbial taxa. Bar charts showing the relative abundance of the most represented microbial taxa, defined as having a mean relative abundance of > 1%, in gut model of proximal, transverse, and distal following fermentation of healthy control media (HC, n=4) then restrictive AN media (AN, n=4).

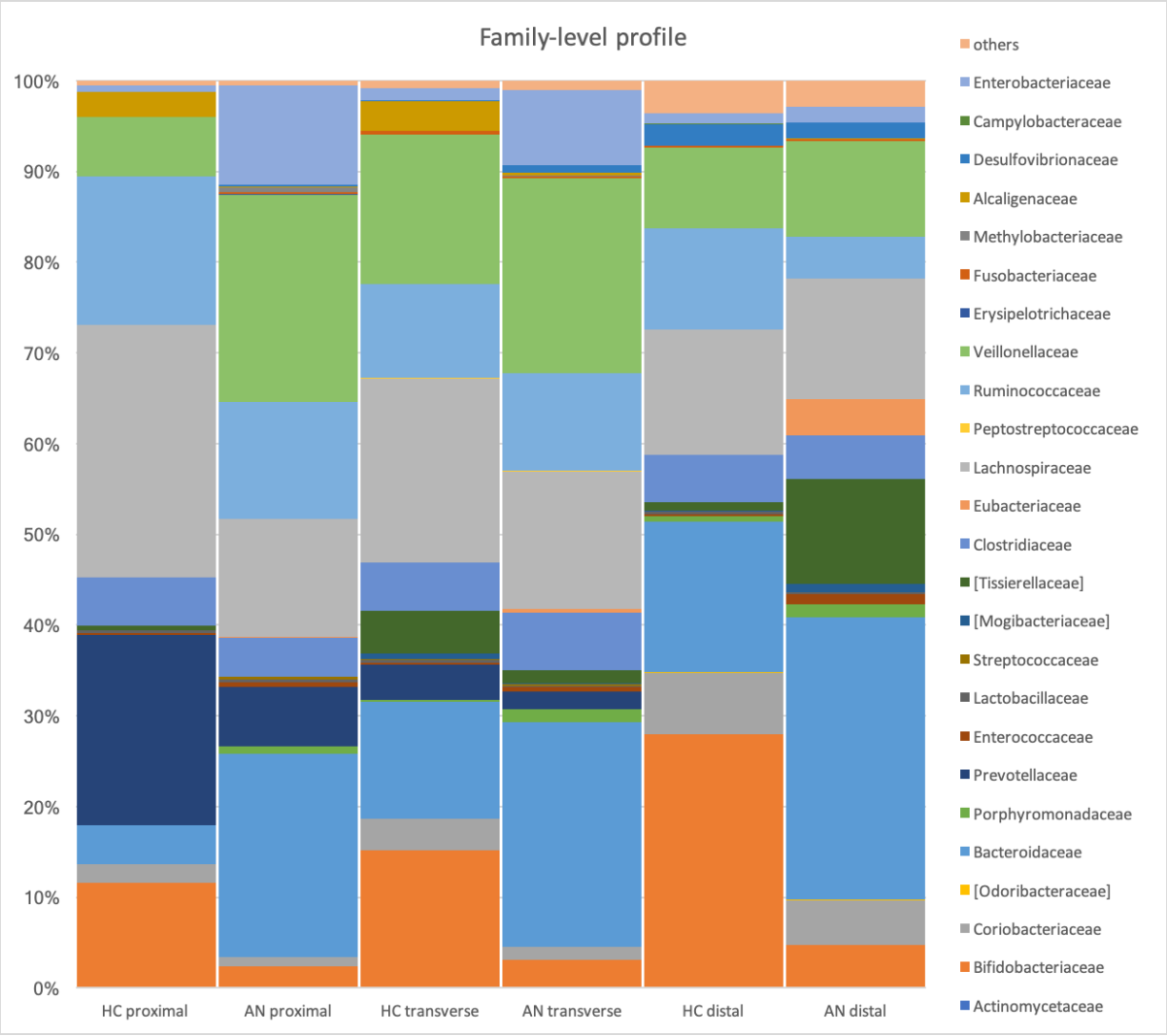


Table 4. Taxa significantly increased (+) or decreased (-) in abundance following fermentation of AN media and HC media (Data are expressed means ± standard deviation. AN, restricting AN media; HC, healthy control media).

Taxonomic level	Classification	Colon site	HC relative abundance	AN relative abundance	AN	HC versus AN *p*-value
Phylum	*Firmicutes*	Proximal	57.1±25.3	54.3±23.9	-	0.042
	Proteobacteria	Proximal	3.5±3.8	11.7±18.8	+	0.035
	Actinobacteria	Proximal	13.6±7.8	3.4±3.1	-	0.000855
		Transverse	18.6±9.0	4.6±4.8	-	0.012
		Distal	34.3±25.9	9.6±10.9	-	0.006
Family	*Eubacteriaceae*	Proximal	0.003±0.004	0.14±0.23	+	0.01
		Transverse	0.016±0.035	0.42±0.73	+	0.03
		Distal	3.35734E-05±0.0045	3.97±4.93	+	0.001
	Tissierellaceae	Transverse	4.7±11.5	1.4±1.7	-	0.05
		Distal	0.91±0.31	11.6±7.1	+	0.00008
	Mogibacteriaceae	Transverse	0.7±1.5	0.16±0.3	-	0.05
		Distal	0.13±0.11	0.94±1.0	+	0.01
	Actinomycetaceae	Distal	0.01±0.02	0	-	0.04
	Porphyromonadaceae	Proximal	0.0087±0.008688	0.76±0.76	+	0.00002
		Transverse	0.24±0.19	1.4±1.59	+	0.013
	Lactobacillaceae	Transverse	0.3±0.3	0.13±0.15	-	0.02
		Distal	0.28±0.09	0.13±0.03	-	0.005
	Desulfovibrionaceae	Proximal	0	0.156±0.20	+	0.00027
		Transverse	0.11±0.15	0.81±0.83	+	0.03
	Campylobacteraceae	Transverse	0.028±0.054	0	-	0.017
		Distal	0.13±0.19	0	-	0.003
	Enterobacteriaceae	Proximal	0.695±0.871	10.97±17.91	+	0.05
		Transverse	13.19±18.88	8.37±12.74	-	0.029
	Bifidobacteriaceae	Transverse	15.1±9.7	3.1±4.1	-	0.001
	Coriobacteriaceae	Transverse	3.5±2.4	1.5±0.8	-	0.006

Veillonellaceae	Proximal	6.62±5.87	22.90±18.75	+	0.0017
Methylobacteriaceae	Proximal	0	0.46±0.82	+	0.004
	Transverse	0	0.13±0.25	+	0.003
	Distal	0	0.001±0.002	+	0.0025
Fusobacteriaceae	Proximal	0	0.26±0.74	+	0.0007
Alcaligenaceae	Proximal	2.74±3.80	0.16±0.16	-	0.009
Dethiosulfovibrionaceae	Transverse	0	0.11±0.14	+	0.03
	Distal	0	0.087±0.05	+	0.04

Figure 4. Taxonomic differences were detected between HC medium conditions and AN medium condition using CLC workbench analysis (Corrected *p* values). Only genera with significant differences in the relative abundance between HC controls and AN condition are displayed.

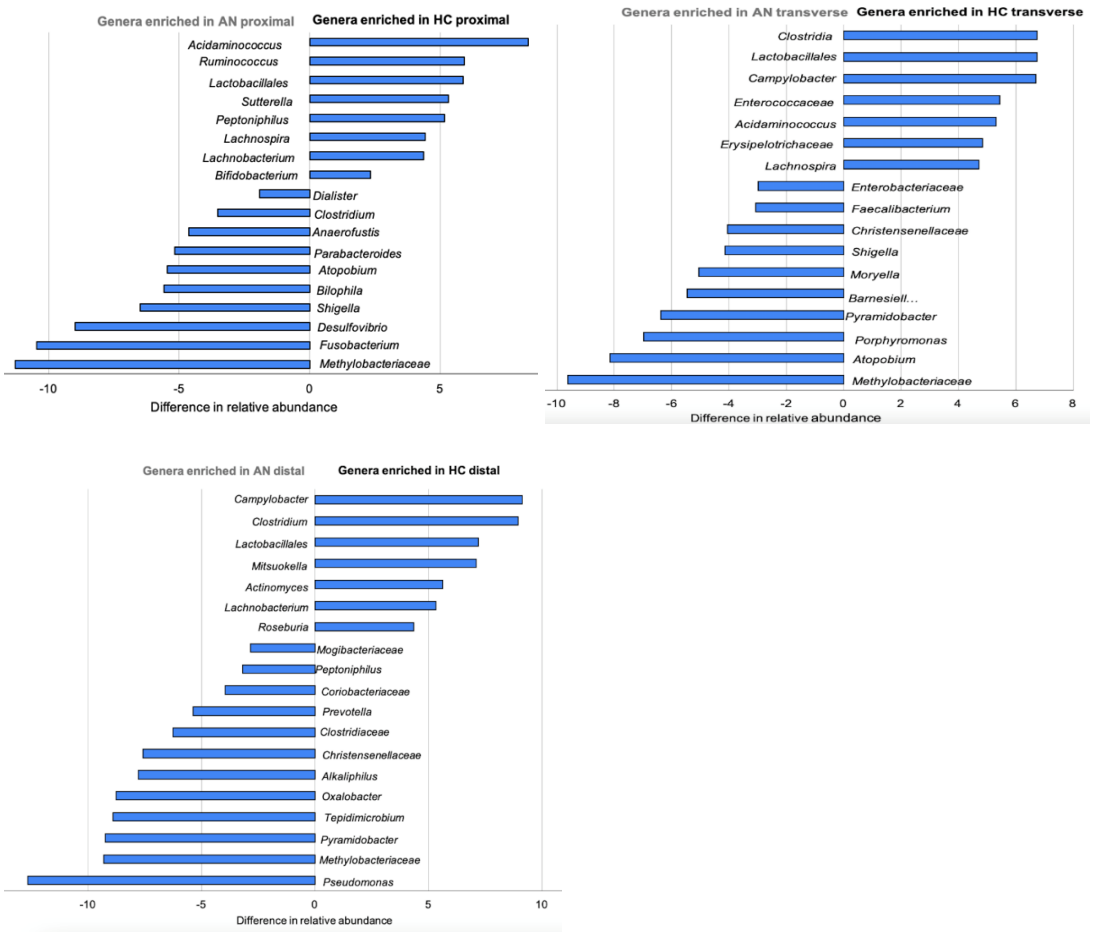


Table 5. Genus-level from each vessel V1, V2 and V3 mimicking the proximal, transverse and distal colon of in vitro colonic model. Differences between the HC and AN microbiome in the same subjects (HC microbiome versus AN microbiome), Only genera with significant differences in the relative abundance between HC and AN are displayed. Genus significantly increased (+) or decreased (-) in abundance following fermentation of AN media and HC media (Data are expressed means ± standard deviation).

Genus	**Colon site**	**Fold Change (FC)**	**HC relative abundance**	**AN relative abundance**	**Log2 (FC)**	**AN**	***P* value**
Lachnospira	Proximal	21.33	2.39±1.95	0.12±0.11	4.42	-	0.0005
	Transverse	26.14	1.86±2.05	0.08±0.08	4.71	-	9.72509E-05
Methylobacteriaceae	Proximal	-2473.24	0	0.41±0.82	-11.27	+	0.00065
	Transverse	-786.90	0	0.12±0.24	-9.62	+	0.00034
	Distal	-631.16	0	0.001±0.001	-9.30	+	0.032
Sutterella	Proximal	39.58	2.45±3.80	0.14±0.16	5.31	_	0.0014
Fusobacterium	Proximal	-1413.56	0	0.3±0.74	-10.47	+	0.0016
Parabacteroides	Proximal	-36.29	0.008±0.009	0.64±0.76	-5.18	+	0.0017
Desulfovibrio	Proximal	-508.46	0	0.12±0.20	-8.98	+	0.0024
Acidaminococcus	Proximal	331.53	0.019±0.038	0	8.37	-	0.0087
	Transverse	40.01	0.009±0.017	0	5.32	-	0.05
Atopobium	Proximal	-43.63	0.003±0.006	0.21±0.039	-5.45	+	0.01
	Transverse	-284.06	0.0005±0.001	0.36±0.71	-8.15	+	0.0003
	Distal	-1090.46	0	0.91±1.22	-10.09	+	0.007
Ruminococcus	Proximal	60.25	13.09±7.87	6.17±4.71	5.91	-	0.02
Shigella	Proximal	-90.39	0	0.014±0.028	-6.49	+	0.02
	Transverse	-17.59	0.0004±0.0009	0.01±0.01	-4.14	+	0.045
Bilophila	Proximal	-47.64	0	0.007±0.01	-5.57	+	0.02
Peptoniphilus	Proximal	35.63	0.525±0.81	0.02±0.03	5.15	-	0.029
	Distal	9.13	0.61±0.56	5.03±6.51	3.19	+	0.0046
Clostridium	Proximal	-11.43	0.12±0.18	0.94±1.23	-3.52	+	0.036
	Transverse	107.59	4.92±1.85	6.49±6.18	6.75	+	0.011
	Distal	-76.25	0.0008±0.001	0.006±0.005	-6.25	+	0.017

Bifidobacterium	Proximal	4.97	12.18±7.56	2.69±3.09	2.32	-	0.037
Dialister	Proximal	-3.78	5.16±4.90	24.65±18.75	-1.91	+	0.039
Anaerofustis	Proximal	-24.68	0	0.0055±0.004	-4.63	+	0.043
Lachnobacterium	Proximal	20.55	0.45±0.88	0.01±0.02	4.36	-	0.05
	Distal	39.94	0.11±0.12	0.0048±0.007	5.32	-	0.008
Lactobacillales	Proximal	59.16	0.009±0.0019	0	5.89	-	0.05
	Transverse	107.19	0.02±0.04	0	6.74	-	0.01
	Distal	145.54	0.09±0.13	0.0009±0.001	7.19	-	0.006
Porphyromonas	Transverse	-1887.65	0.003±0.006	0.81±1.61	-10.88	+	0.012
Campylobacter	Transverse	103.27	0.027±0.05	0	6.69	-	0.023
	Distal	554.43	0.13±0.18	0	9.11	-	0.0058
Enterobacteriaceae	Transverse	-7.95	0.25±0.35	1.83±2.95	-2.99	+	0.025
Faecalibacterium	Transverse	-8.44	3.56±6.43	5.45±9.07	-3.07	+	0.027
Pyramidobacter	Transverse	-82.50	0	0.10±0.1	-6.366	+	0.033
Christensenellaceae	Transverse	-16.64	0.001±0.001	0.03±0.06	-4.05	+	0.034
	Distal	-191.67	0.003±0.003	0.0026±0.0037	-7.58	-	0.001
Erysipelotrichaceae	Transverse	28.54	0.006±0.008	0	4.83	-	0.05
Enterococcaceae	Transverse	43.46	0.01±0.02	0	5.44	-	0.05
Barnesiellaceae	Transverse	-44.26	0	0.005±0.009	-5.46	+	0.05
Moryella	Transverse	-33.09	0.003±0.007	0.06±0.12	-5.05	+	0.05
Tepidimicrobium	Distal	-473.61	0.007±0.009	2.67±3.77	-8.88	+	0.003
*Pseudoramibacter_*	Distal	-672.80	0.003±0.004	3.46±4.90	-9.39	+	0.0034
Eubacterium							
Mitsuokella	Distal	136.52	1.12±1.57	0.018±0.026	7.09	-	0.008
Alkaliphilus	Distal	-219.59	0.0008±0.001	0.18±0.26	-7.77	+	0.015
Coriobacteriaceae	Distal	-15.54	0.04±0.05	0.08±0.08	-3.96	+	0.019
Pyramidobacter	Distal	-604.45	0	0.10±0.14	-9.24	+	0.03
Mogibacteriaceae	Distal	-7.187	0.10±0.07	0.83±1.03	-2.85	+	0.032
Oxalobacter	Distal	-433.30	0	0.07±0.09	-8.75	+	0.034
Prevotella	Distal	-41.07	0.0078±0.011	0.00097±0.0013	-5.36	-	0.038
Roseburia	Distal	20.43	0.11±0.05	0.011±0.015	4.35	-	0.04
Actinomyces	Distal	49.38	0.011±0.016	0	5.63	-	0.04

3.2. Total bacteria, SCFA and BCFA production

Changes in total bacteria, SCFA and BCFA concentrations are shown in Table 6.

Table 6. Total bacteria and SCFA/BCFA detected by FISH-FCM (Log_{10} cells/mL) and GC (mM) respectively) from each vessel V1, V2 and V3 mimicking the proximal, transverse and distal colon of within a pH controlled *in vitro* faecal (6%) colonic model. Samples were collected at SS1 (Healthy media) and SS2 (Anorexic media). Data are means ± standard deviation. n.s., not significant. Significant difference in each vessel between SS1 and SS2 are indicated. Total bacteria and SCFA/BCFA increased (+) or decreased (-) in amounts following fermentation of AN media and HC media (Data are expressed means ± standard deviation).

	Colon site	**HC**	**AN**	**AN**	***p* value**
Total bacteria	Proximal	8.50±0.12	8.16±0.05	-	0.0018
(log_{10} cells/mL)	Transverse	8.23±0.23	7.73±0.32	-	0.05
	Distal	8.00±0.09	7.28±0.40	-	0.01
Acetate (mM)	Proximal	30.02±8.59	16.79±1.96	-	0.023
	Transverse	42.64±17.17	21.31±9.98	-	n.s.
	Distal	46.83±28.44	31.94±13.40	-	n.s.
Butyrate (mM)	Proximal	28.80±3.26	16.82±5.98	-	0.012
	Transverse	37.37±6.08	17.16±9.57	-	0.011
	Distal	36.61±12.20	20.29±7.76	-	n.s.
Propionate (mM)	Proximal	17.54±9.40	14.27±13.78	-	n.s.
	Transverse	28.82±8.11	15.03±6.66	-	0.039

	Distal	27.44±10.21	19.54±9.97	-	n.s.
BCFA (mM)	Proximal	2.91±3.38	2.67±3.61	-	n.s.
	Transverse	6.11±1.64	3.08±1.86	-	0.05
	Distal	5.70±1.84	7.06±4.16	+	n.s.

4. Discussion

An *in vitro* model system represents an innovative tool that allows the dynamic microbial communities of the gastrointestinal tract to be studied, permitting adjustments to a variety of parameters, including nutrients, temperature, pH and retention time. Manipulating these factors can enable the modelling of physiologically-relevant conditions, but without the requirement for human intervention studies (Macfarlane *et al*., 1998; Williams *et al*., 2015; Nissen *et al*., 2020). In this manuscript a three stage gut model was developed investigating the effect of a restricting AN diet on the microbial community. The drastic changes in diet, modelled as a change in media led to extensive microbial differences between the healthy control media (HC) and the anorexic media (AN). Comparisons between what was observed in the AN model compared to what is known from AN individuals will be made to determine the suitability of the model system.

At the phylum level when comparing the microbiota post HC media to AN media it was observed that there was an increased relative abundance of Proteobacteria. Borgo *et al*., (2017) conducted analysis on stool samples from 15 restrictive AN patients as compared to healthy controls and also noted increased abundance of Proteobacteria in AN patients, this matches well with the current *in vitro* observations (Borgo *et al*., 2017). Indeed, high

abundance of Proteobacteria has been related to dysbiosis in hosts with metabolic or inflammatory disorders with potentially detrimental effects on gut microbiota composition and immune function (Biagi *et al*., 2010; Moon *et al*., 2018). As such this change is unlikely to be positive to the host, but could be well modelled by use of media in the current experiment.

The F/B ratio of the proximal, transverse and distal colon following the AN media was lower than following HC media fermentation. The most abundant bacterial in human gut microbiota are Firmicutes and Bacteroidetes phyla members. Borgo and co-workers (2017) also noted reduced levels of Firmicutes in AN individuals, as such, these observations fit well with the findings of the current *in vitro* trial (Borgo *et al*., 2017). It is worth noting that the F/B ratio is frequently reported to be increased in obese people compared to lean people and decreases with weight loss (Sweeney and Morton, 2013; Koliada *et al*., 2017). Thus these microbial groups are likely to be sensitive to dietary changes.

Actinobacteria is a phylum associated with maintenance of gut homeostasis (Binda *et al*., 2018) and includes *Bifidobacterium* as a key member. In 2016 Mack et al., reported the microbial profile of AN patients pre and post weight gain, as compared to healthy controls. Through this analysis it was observed that Actinobacteria was elevated in AN patients, and remained this way post weight gain (Borgo *et al*., 2017). The increase of this phylum during AN is likely to relate to the increased fibre ratio relative to other macronutrients (Grundy *et al*., 2016). In contrast within the current model system a reduction in Actinobacteria was observed following AN media fermentation. Reasons for this discrepancy could be due to the

reduced levels of FOS in the AN media, reducing the available substrate in the AN media, as such alteration in the FOS levels of the media may help to better model this. It is also worth noting that an increase in Actinobacteria has been observed in faeces of obese individuals (Turnbaugh et al., 2009). Looking in the literature, high fat seldom has an influence on levels of Actinobacteria, for example, Murphy et al., (2010) observe in mice a high fat lead to no change in this bacterial group. However, from studies of Turnbaugh et al., 2009, it is apparent that high levels of Actinobacteria can exist in obese individuals, thus this may be due to enhanced levels of carbohydrate reaching the large intestine.Therefore supporting that in environments with excessive nutrients Actinobacteria may also flourish.

The observation of significantly increased Enterobacteriaceae in the proximal and transverse regions make sense when considering AN. Enterobacteriaceae family are associated with gut inflammation which favors bacterial translocation, promoting systemic inflammation (Winter and Baumler, 2014). A study showed the role of Enterobacteriaceae family in eating disorders (EDs); mainly species of *Escherichia coli* are capable of producing small protein sequence ClpB (Breton *et al*., 2016). ClpB appears to interfere with a-melanocyte-stimulating hormone (a-MSH) involved in anxiety and satiety signalling as an appetite regulator (Adan and Vink, 2001). As such, higher levels of Enterobacteriaceae are associated with AN (Breton *et al*., 2016). Consistent with this observation, an increase in this family may be associated with a higher production of neuropeptide ClpB, which may mediate gut-brain axis communication in AN individuals. Furthermore, the observation of increased levels of Enterobacteriaceae was also seen in the Borgo study of 2017 ((Borgo *et al*., 2017).

In the current study the relative abundance of Fusobacteria was higher in all three colon

segments in the AN model compared to the HC model particular within the proximal region. Whilst this result has not been previously observed in AN, it is a noteworthy result, because Fusobacteria is more frequently cultured from Kwashiorkor, an acute form of protein-energetic malnutrition condition (Pham *et al*., 2019). Smith's study transferred faecal samples from children with kwashiorkor to germ-free mice, which led to drastic weight loss and metabolic abnormalities compared to the control group that received transplants from healthy children. This suggests that gut microbial community during starvation may play a role in body weight regulation (Smith *et al*., 2013). Although malnutrition, secondary to AN, develops more chronically, it is plausible that microbial communities selected by a low-energy gut environment may perpetuate in AN individuals, and could impact on weight loss and behaviour (van de Wouw *et al*., 2017).

An additional observation of the *in vitro* model was that Dethiosulfovibrionaceae family significantly increased in the transverse and distal colon of the AN model, whereas it remained undetectable in the HC model. Whilst not previously observed in AN, this finding is associated with weight loss as a study found that Dethiosulfovibrionaceae significantly increased in obese woman during a phase of rapid weight and body fat mass loss following bariatric surgery (Sanmiguel *et al*., 2017). The study considered the potential of the bacteria changes to be associated with changes in appetite. It is therefore possible that this group of bacteria could have a role to play in appetite in AN.

At the genus level, compared to HC media, this study observed significant decreased relative abundance of *Lactobacillales* in all three colon regions following AN media fermentation. A

study conducted by Armougoum et al. showed reduced *Lactobacillus* in AN compared to obese patients (Armougom *et al*., 2009). Thus it is likely that sufficient nutrients were not available to support the growth of this genera in this mixed microbial community.

Compared to HC media, the relative abundance of *Clostridium* significantly increased in all three colon regions following fermentation of AN media. However, if we consider the research of Borgo *et al*., 2017, a significantly reduced relative abundance of *Clostridium* was observed in AN faecal samples (Borgo *et al.*, 2017). Other studies have found increased *Clostridium* in AN, for example, a study by Queipo-Ortuno et al. observed increased *Clostridium* group IV levels in the rat model of restrictive AN with exercise (Queipo-Ortuño *et al*., 2013). A study conducted by Mack et al. (2016) reported higher levels of *Clostridium* cluster I and cluster XI in faeces of AN individuals when compared to normal-weight participants (Mack *et al*., 2016). The difference with the Borgo study is likely to be as this genera is a large diverse microbial group, so it is possible that differences in clusters between the groups have led to the different outcomes observed.

Coriobacteriaceae genus was significantly increased within AN model distal colon. It is worth noting the Mortia et al., 2015 did not observe any change in this microbial group, which could relate to the difference of the macronutrient intake within AN patients. However, Morkl et al., (2017) observed *Coriobacteriaceae* as the only enriched genus type in faecal of AN compared to other entities (Mörkl *et al*., 2018). *Coriobacteriaceae* colonise the GI tract and are implicated in bile salt conversion and lipid metabolism (Clavel *et al*., 2009). As such it is possible that this genus gains a competitive advantage with this state of limited nutrients, and is therefore able to grow in AN.

Roseburia genus identified by this study significantly reduced in the distal AN model, and this result is also confirmed by AN faecal studies (Mack et al.,2016; Borgo et al., 2017, Hanachi et al., 2019). *Roseburia* is a carbohydrate degrading bacteria and key butyrate producer (Macfarlane *et al*., 1998; Louis *et al*., 2010). Decreased levels of *Roseburia* has also been found in patients with inflammatory bowel diseases (Imhann *et al*., 2018). Low-grade inflammation and altered gut permeability have been observed in an AN animal model (Jesus *et al*., 2014; Belmonte *et al*., 2016), this could be in part driven by the reduced levels of butyrate, suggesting a putative role in AN's pathophysiology.

Compared to the HC model, the *Atopobium* genus was significantly increased in all three colon regions of the AN model. However, a study indicated that the level of the *Atopobium* genus in patients with AN is not significantly different to normal-weight participants (Mortia et al., 2015). It is worth noting that the *Atopobium* genus was enriched in major depressive disorder (MDD) and bipolar disorder (Nikolova *et al*., 2021). MDD has been reported to occur in 50–75% of female with AN (Abbate-Daga *et al*., 2011; Calugi *et al*., 2014; Thornton *et al*., 2016). Therefore the changes observed *in vitro* could also have a role to play in the gut brain axis.

Methylobacteriaceae genus was detected only in the AN model including the proximal, transverse and distal colon with decreased trend when compared to the HC model. There is no finding confirmed AN’s gut status related to *Methylobacteriaceae* level. *Methylobacteriaceae* may have a negative effect on the immunocompromised hosts as has been reported to cause infections post colonistation including symptoms of fever, bloodstream infections, peritonitis, and pneumonia (Sanders *et al*., 2000; Lai *et al*., 2011).

Another aspect worth considering is that in much AN research the number of mucin degrading bacteria is enhanced in the AN population (Mack *et al*., 2016). In the current model the mucin concentrations were the same in the HC and AN models, this is likely result in mucin then becoming a key source of substance for the bacteria. In the current model an increase in mucin utilisers in the AN model was not observed. This indicates that the concentration of mucin relative to the other nutrients needs considering, furthermore, the source of mucin being porcine may not be optimal in the model.

The current model indicated significantly decreased total bacteria in AN model including proximal, transverse and distal which is in line with finding by Morita et al (2015), who found lower total bacteria in AN's faeces when compared to healthy subjects (Morita *et al*., 2015). Previous studies that measured faecal samples in AN individuals reported decreased levels of SCFAs when compared to healthy subjects (Morita *et al*., 2015; Mack *et al*., 2016; Borgo *et al*., 2017; Speranza *et al*., 2018), likely as a mechanism to compensate for the lower energy and carbohydrate intake (Speranza *et al*., 2018). For instance, a depletion in carbohydrate-fermenting *Roseburia* could result in a decrease propionate production, concurrent with the decreased level of carbohydrate (Louis *et al*., 2010). Furthermore, studies by Bailey et al (2011) and Borgo et al (2017) observed that butyrate concentration negatively correlated with depression and anxiety scores (Bailey *et al*., 2011; Borgo *et al*., 2017). In agreement with above findings, in the current model, the levels of total bacteria and SCFA were significantly reduced in AN model when compared to HC model. The current study modelled 'anorexic dietary intake' on the microbial community and SCFA profiles, data generated were largely in line with studies on gut dysbiosis in AN individuals (Armougom *et*

al., 2009; Million *et al*., 2013; Morita *et al*., 2015; Mack *et al*., 2016).

Increased concentration of total BCFAs particularly iso-butyrate and iso-valerate have been observed in stool samples of patients with AN (Mack *et al*., 2016). In vitro batch-culture with faecal inoculation conducted by Macfarlane et al., (1992) indicated that SCFAs were the principal end products formed during the degradation of protein by gut bacteria, it was observed that approximately 30% of the protein broken down was converted to SCFAs. In the current study AN model resulted in decreased levels of BCFAs when compared to HC model, there was however a tendency for more BCFA distally, but this did not reach significance. which may response to limited protein availability in anorexic medium.

This study highlights how dietary restrictions might impact the microbial community. The *in vitro* system also provides an insight of possible differences between the HC and AN microbiome in the proximal, transverse and distal colonic regions, i.e. further up the GI tract than would normally be studied. Numerous differences were confirmed between the HC and AN microbiome within the *in vitro* model and when compared to existing *in vivo* observations. It seems that fermentation of the AN media model mimics core features of restricting dietary AN, resulting in several changes in the intestinal microbial ecosystem, some common and others that map well to changes we might expect to see in AN. This again indicates a significant impact of limited food intake on gut microbiota. There are no studies evaluating the effect of restricting anorexic dietary approaches on large intestine in AN individuals, concerning the AN microbiome, the literature focuses mainly on the role of the gut microbiota in faeces. Therefore, this discussion focussed on the distal colon as this is more likely to be similar to faeces. Most findings by this study are consistent with previous observations including gut microbiome in AN, MMD and malnutrition status, and further

confirmed that nutritional deficiency is associated with profound alterations of bacterial community structure in AN. Nevertheless, AN is a multifaceted and complex condition these models provide an innovative technological tool not restricted by ethical concerns. As such, using these models it may be possible to study likely effects of intervention on the AN gut microbiota.

5. Conclusion

This study reported that the restricting-AN diet-related microbiome differs in HC diet-related, which could also be mimicked *in vitro*. Findings by this study have shown that the R-AN diet may lead to negative potential alternation in the gut microbial community, some expected results and others unique. R-AN diet may thus manipulate the gut microbial community and alter metabolic capacity towards a configuration that might confer negative effects to the host but further research is required to extend understanding of AN compositional changes in the microbiome and the impact of a restrictive diet.

References

Abbate-DagAbbate-Daga, G., Buzzichelli, S., Amianto, F., Rocca, G., Marzola, E., McClintock, S. M. & Fassino, S. (2011). Cognitive flexibility in verbal and nonverbal domains and decision making in anorexia nervosa patients: a pilot study. *BMC Psychiatry,* **11**(1), 162.

Adan, R. A. H. & Vink, T. (2001). Drug target discovery by pharmacogenetics: mutations in the melanocortin system and eating disorders. *European Neuropsychopharmacology,* **11**(6), 483-490.

Armougom, F., Henry, M., Vialettes, B., Raccah, D. & Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. *PLoS One,* **4**(9), e7125.

Association, A. P. (2013). *Desk reference to the diagnostic criteria from DSM-5 (R)*. American Psychiatric Publishing.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun,* **25**(3), 397-407.

Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C. & Schmidt, T. M. (2019). Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. *mBio,* **10**(1).

Belmonte, L., Achamrah, N., Nobis, S., Guerin, C., Riou, G., Bole-Feysot, C., Boyer, O., Richard, V., Do Rego, J. C., Dechelotte, P., Goichon, A. & Coeffier, M. (2016). A role for intestinal TLR4-driven inflammatory response during activity-based anorexia. *Scientific Reports,* **6**.

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., Nikkïla, J., Monti, D., Satokari, R., Franceschi, C., Brigidi, P. & De Vos, W. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One,* **5**(5), e10667.

Binda, C., Lopetuso, L. R., Rizzatti, G., Gibiino, G., Cennamo, V. & Gasbarrini, A. (2018). Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Dig Liver Dis,* **50**(5), 421-428.

Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., Anselmetti, S., Scarone, S., Pontiroli, A. E., Morace, G. & Borghi, E. (2017). Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. *Plos One,* **12**(6).

Breton, J., Legrand, R., Akkermann, K., Jarv, A., Harro, J., Dechelotte, P. & Fetissov, S. O. (2016). Elevated Plasma Concentrations of Bacterial ClpB Protein in Patients with Eating Disorders. *International Journal of Eating Disorders,* **49**(8), 805-808.

British Nutrition Foundation. (2016). *Nutrition requirements*. URL: https://www.nutrition.org.uk/bnfevents/events/158-healthyliving/resources/907-nutritionrequirements.html [26 October 2019].

Calugi, S., El Ghoch, M., Conti, M. & Dalle Grave, R. (2014). Depression and treatment outcome in anorexia nervosa. *Psychiatry Res,* **218**(1-2), 195-200.

Clavel, T., Charrier, C., Braune, A., Wenning, M., Blaut, M. & Haller, D. (2009). Isolation of bacteria from the ileal mucosa of TNFdeltaARE mice and description of Enterorhabdus mucosicola gen. nov., sp. nov. *Int J Syst Evol Microbiol,* **59**(Pt 7), 1805-12.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the human intestinal microbial

flora. *Science,* **308**(5728), 1635-8.
Eddy, K. T., Dorer, D. J., Franko, D. L., Tahilani, K., Thompson-Brenner, H. & Herzog, D. B. (2008). Diagnostic crossover in anorexia nervosa and bulimia nervosa: implications for DSM-V. *Am J Psychiatry,* **165**(2), 245-50.
Fernandez-Aranda, F., Pinheiro, A. P., Tozzi, F., Thornton, L. M., Fichter, M. M., Halmi, K. A., Kaplan, A. S., Klump, K. L., Strober, M., Woodside, D. B., Crow, S., Mitchell, J., Rotondo, A., Keel, P., Plotnicov, K. H., Berrettini, W. H., Kaye, W. H., Crawford, S. F., Johnson, C., Brandt, H., La Via, M. & Bulik, C. M. (2007). Symptom profile of major depressive disorder in women with eating disorders. *Aust N Z J Psychiatry,* **41**(1), 24-31.
Fetissov, S. O. & Hökfelt, T. (2019). On the origin of eating disorders: altered signaling between gut microbiota, adaptive immunity and the brain melanocortin system regulating feeding behavior. *Current Opinion in Pharmacology,* **48**, 82-91.
Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes,* **3**(4), 289-306.
Godart, N. T., Flament, M. F., Perdereau, F. & Jeammet, P. (2002). Comorbidity between eating disorders and anxiety disorders: a review. *Int J Eat Disord,* **32**(3), 253-70.
Grundy, M. M. L., Edwards, C. H., Mackie, A. R., Gidley, M. J., Butterworth, P. J. & Ellis, P. R. (2016). Re-evaluation of the mechanisms of dietary fibre and implications for macronutrient bioaccessibility, digestion and postprandial metabolism. *British Journal of Nutrition,* **116**(5), 816-833.
Huse, S. M., Welch, D. M., Morrison, H. G. & Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ Microbiol,* **12**(7), 1889-98.
Imhann, F., Vich Vila, A., Bonder, M. J., Fu, J., Gevers, D., Visschedijk, M. C., Spekhorst, L. M., Alberts, R., Franke, L., van Dullemen, H. M., Ter Steege, R. W. F., Huttenhower, C., Dijkstra, G., Xavier, R. J., Festen, E. A. M., Wijmenga, C., Zhernakova, A. & Weersma, R. K. (2018). Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut,* **67**(1), 108-119.
Jauregui Lobera, I. & Bolanos Rios, P. (2009). Choice of diet in patients with anorexia nervosa. *Nutr Hosp,* **24**(6), 682-7.
Jesus, P., Ouelaa, W., Francois, M., Riachy, L., Guerin, C., Aziz, M., Do Rego, J. C., Dechelotte, P., Fetissov, S. O. & Coeffier, M. (2014). Alteration of intestinal barrier function during activity-based anorexia in mice. *Clinical Nutrition,* **33**(6), 1046-1053.
Kaelin, W. G., Jr. & McKnight, S. L. (2013). Influence of metabolism on epigenetics and disease. *Cell,* **153**(1), 56-69.
Kamal, N., Chami, T., Andersen, A., Rosell, F. A., Schuster, M. M. & Whitehead, W. E. (1991). Delayed gastrointestinal transit times in anorexia nervosa and bulimia nervosa. *Gastroenterology,* **101**(5), 1320-4.
Kaye, W. H., Bulik, C. M., Thornton, L., Barbarich, N., Masters, K. & Grp, P. F. C. (2004). Comorbidity of anxiety disorders with anorexia and bulimia nervosa. *American Journal of Psychiatry,* **161**(12), 2215-2221.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research,* **41**(1), e1-e1.
Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O. & Vaiserman, A. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *Bmc Microbiology,* **17**.

Lai, C.-C., Cheng, A., Liu, W.-L., Tan, C.-K., Huang, Y.-T., Chung, K.-P., Lee, M.-R. & Hsueh, P.-R. (2011). Infections Caused by Unusual Methylobacterium Species. *Journal of Clinical Microbiology,* **49**(9), 3329-3331.

Liu, L., Poveda, C., Jenkins, P. E. & Walton, G. E. (2021). An In Vitro Approach to Studying the Microbial Community and Impact of Pre and Probiotics under Anorexia Nervosa Related Dietary Restrictions. *Nutrients,* **13**(12), 4447.

Louis, P., Young, P., Holtrop, G. & Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environmental Microbiology,* **12**(2), 304-314.

Macfarlane GT, G. G. (1995). Microbiological aspects of short chain fatty acid production in the large bowel.

Macfarlane, G. T. & Gibson, G. R. (1994). Metabolic activities of the normal colonic flora. *In:* Gibson, S. A. W. (ed.) *Human Health - The Contribution of Microorganisms*. London: Springer-Verlag.

Macfarlane, G. T., Macfarlane, S. & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology,* **35**(2), 180-187.

Mack, I., Cuntz, U., Grämer, C., Niedermaier, S., Pohl, C., Schwiertz, A., Zimmermann, K., Zipfel, S., Enck, P. & Penders, J. (2016). Weight gain in anorexia nervosa does not ameliorate the faecal microbiota, branched chain fatty acid profiles, and gastrointestinal complaints. *Sci Rep,* **6**, 26752.

Mehler, P. S. & Brown, C. (2015). Anorexia nervosa - medical complications. *Journal of Eating Disorders,* **3**.

Million, M., Angelakis, E., Maraninchi, M., Henry, M., Giorgi, R., Valero, R., Vialettes, B. & Raoult, D. (2013). Correlation between body mass index and gut concentrations of Lactobacillus reuteri, Bifidobacterium animalis, Methanobrevibacter smithii and Escherichia coli. *International Journal of Obesity,* **37**(11), 1460-1466.

Moon, C. D., Young, W., Maclean, P. H., Cookson, A. L. & Bermingham, E. N. (2018). Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen,* **7**(5), e00677.

Morita, C., Tsuji, H., Hata, T., Gondo, M., Takakura, S., Kawai, K., Yoshihara, K., Ogata, K., Nomoto, K., Miyazaki, K. & Sudo, N. (2015). Gut Dysbiosis in Patients with Anorexia Nervosa. *PLoS One,* **10**(12), e0145274.

Mörkl, S., Wagner-Skacel, J., Lahousen, T., Lackner, S., Holasek, S. J., Bengesser, S. A., Painold, A., Holl, A. K. & Reininghaus, E. (2018). The Role of Nutrition and the Gut-Brain Axis in Psychiatry: A Review of the Literature. *Neuropsychobiology*, 1-9.

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., Clarke, S. F., O'Toole, P. W., Quigley, E. M., Stanton, C., Ross, P. R., O'Doherty, R. M. & Shanahan, F. (2010). Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* **59**(12), 1635-42.

Murphy, K. G. & Bloom, S. R. (2004). Gut hormones in the control of appetite. *Exp Physiol,* **89**(5), 507-16.

Nikolova, V. L., Hall, M. R. B., Hall, L. J., Cleare, A. J., Stone, J. M. & Young, A. H. (2021). Perturbations in Gut Microbiota Composition in Psychiatric Disorders: A Review and Meta-analysis. *JAMA Psychiatry,* **78**(12), 1343-1354.

Nissen, L., Casciano, F. & Gianotti, A. (2020). Intestinal fermentation in vitro models to study food-induced gut microbiota shift: an updated review. *FEMS Microbiol Lett,* **367**(12).

Pham, T.-P.-T., Tidjani Alou, M., Bachar, D., Levasseur, A., Brah, S., Alhousseini, D., Sokhna, C., Diallo, A., Wieringa, F., Million, M. & Raoult, D. (2019). Gut Microbiota Alteration

is Characterized by a Proteobacteria and Fusobacteria Bloom in Kwashiorkor and a Bacteroidetes Paucity in Marasmus. *Scientific Reports,* **9**(1), 9084.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D. & Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* **464**(7285), 59-65.

Queipo-Ortuño, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., Casanueva, F. & Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One,* **8**(5), e65465.

Raatz, S. K., Jahns, L., Johnson, L. K., Crosby, R., Mitchell, J. E., Crow, S., Peterson, C., Le Grange, D. & Wonderlich, S. A. (2015). Nutritional adequacy of dietary intake in women with anorexia nervosa. *Nutrients,* **7**(5), 3652-65.

Roubalova, R., Prochazkova, P., Papezova, H., Smitka, K., Bilej, M. & Tlaskalova-Hogenova, H. (2020). Anorexia nervosa: Gut microbiota-immune-brain interactions. *Clinical Nutrition,* **39**(3), 676-684.

Sanders, J. W., Martin, J. W., Hooke, M. & Hooke, J. (2000). Methylobacterium mesophilicum infection: case report and literature review of an unusual opportunistic pathogen. *Clin Infect Dis,* **30**(6), 936-8.

Sanmiguel, C. P., Jacobs, J., Gupta, A., Ju, T., Stains, J., Coveleskie, K., Lagishetty, V., Balioukova, A., Chen, Y., Dutson, E., Mayer, E. A. & Labus, J. S. (2017). Surgically Induced Changes in Gut Microbiome and Hedonic Eating as Related to Weight Loss: Preliminary Findings in Obese Women Undergoing Bariatric Surgery. *Psychosom Med,* **79**(8), 880-887.

Smith, M. I., Yatsunenko, T., Manary, M. J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A. L., Rich, S. S., Concannon, P., Mychaleckyj, J. C., Liu, J., Houpt, E., Li, J. V., Holmes, E., Nicholson, J., Knights, D., Ursell, L. K., Knight, R. & Gordon, J. I. (2013). Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science,* **339**(6119), 548-54.

Speranza, E., Cioffi, I., Santarpia, L., Del Piano, C., De Caprio, C., Naccarato, M., Marra, M., De Filippo, E., Contaldo, F. & Pasanisi, F. (2018). Fecal Short Chain Fatty Acids and Dietary Intake in Italian Women With Restrictive Anorexia Nervosa: A Pilot Study. *Front Nutr,* **5**, 119.

Sweeney, T. E. & Morton, J. M. (2013). The human gut microbiome: a review of the effect of obesity and surgically induced weight loss. *JAMA Surg,* **148**(6), 563-9.

Tennoune, N., Chan, P., Breton, J., Legrand, R., Chabane, Y. N., Akkermann, K., Jarv, A., Ouelaa, W., Takagi, K., Ghouzali, I., Francois, M., Lucas, N., Bole-Feysot, C., Pestel-Caron, M., do Rego, J. C., Vaudry, D., Harro, J., De, E., Dechelotte, P. & Fetissov, S. O. (2014). Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. *Translational Psychiatry,* **4**.

Thornton, L. M., Welch, E., Munn-Chernoff, M. A., Lichtenstein, P. & Bulik, C. M. (2016). Anorexia Nervosa, Major Depression, and Suicide Attempts: Shared Genetic Factors. *Suicide & life-threatening behavior,* **46**(5), 525-534.

Tottey, W., Feria-Gervasio, D., Gaci, N., Laillet, B., Pujos, E., Martin, J.-F., Sebedio, J.-L., Sion, B., Jarrige, J.-F., Alric, M. & Brugère, J.-F. (2017). Colonic Transit Time Is a

Driven Force of the Gut Microbiota Composition and Metabolism: In Vitro Evidence. *Journal of neurogastroenterology and motility,* **23**(1), 124-134.
van de Wouw, M., Schellekens, H., Dinan, T. G. & Cryan, J. F. (2017). Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite. *J Nutr,* **147**(5), 727-745.
Williams, C. F., Walton, G. E., Jiang, L., Plummer, S., Garaiova, I. & Gibson, G. R. (2015). Comparative analysis of intestinal tract models. *Annu Rev Food Sci Technol,* **6**, 329-50.
Winter, S. E. & Baumler, A. J. (2014). Dysbiosis in the inflamed intestine Chance favors the prepared microbe. *Gut Microbes,* **5**(1), 71-73.

a, G., Buzzichelli, S., Amianto, F., Rocca, G., Marzola, E., McClintock, S. M. & Fassino, S. (2011). Cognitive flexibility in verbal and nonverbal domains and decision making in anorexia nervosa patients: a pilot study. *BMC Psychiatry,* **11**(1), 162.
Adan, R. A. H. & Vink, T. (2001). Drug target discovery by pharmacogenetics: mutations in the melanocortin system and eating disorders. *European Neuropsychopharmacology,* **11**(6), 483-490.
Armougom, F., Henry, M., Vialettes, B., Raccah, D. & Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. *PLoS One,* **4**(9), e7125.
Association, A. P. (2013). *Desk reference to the diagnostic criteria from DSM-5 (R)*. American Psychiatric Publishing.
Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun,* **25**(3), 397-407.
Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C. & Schmidt, T. M. (2019). Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. *mBio,* **10**(1).
Belmonte, L., Achamrah, N., Nobis, S., Guerin, C., Riou, G., Bole-Feysot, C., Boyer, O., Richard, V., Do Rego, J. C., Dechelotte, P., Goichon, A. & Coeffier, M. (2016). A role for intestinal TLR4-driven inflammatory response during activity-based anorexia. *Scientific Reports,* **6**.
Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., Nikkïla, J., Monti, D., Satokari, R., Franceschi, C., Brigidi, P. & De Vos, W. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One,* **5**(5), e10667.
Binda, C., Lopetuso, L. R., Rizzatti, G., Gibiino, G., Cennamo, V. & Gasbarrini, A. (2018). Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Dig Liver Dis,* **50**(5), 421-428.
Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., Anselmetti, S., Scarone, S., Pontiroli, A. E., Morace, G. & Borghi, E. (2017). Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. *Plos One,* **12**(6).
Breton, J., Legrand, R., Akkermann, K., Jarv, A., Harro, J., Dechelotte, P. & Fetissov, S. O. (2016). Elevated Plasma Concentrations of Bacterial ClpB Protein in Patients with Eating Disorders. *International Journal of Eating Disorders,* **49**(8), 805-808.
British Nutrition Foundation. (2016). *Nutrition requirements*. URL: https://www.nutrition.org.uk/bnfevents/events/158-healthyliving/resources/907-nutritionrequirements.html [26 October 2019].
Calugi, S., El Ghoch, M., Conti, M. & Dalle Grave, R. (2014). Depression and treatment outcome in anorexia nervosa. *Psychiatry Res,* **218**(1-2), 195-200.

Clavel, T., Charrier, C., Braune, A., Wenning, M., Blaut, M. & Haller, D. (2009). Isolation of bacteria from the ileal mucosa of TNFdeltaARE mice and description of Enterorhabdus mucosicola gen. nov., sp. nov. *Int J Syst Evol Microbiol,* **59**(Pt 7), 1805-12.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science,* **308**(5728), 1635-8.

Eddy, K. T., Dorer, D. J., Franko, D. L., Tahilani, K., Thompson-Brenner, H. & Herzog, D. B. (2008). Diagnostic crossover in anorexia nervosa and bulimia nervosa: implications for DSM-V. *Am J Psychiatry,* **165**(2), 245-50.

Fernandez-Aranda, F., Pinheiro, A. P., Tozzi, F., Thornton, L. M., Fichter, M. M., Halmi, K. A., Kaplan, A. S., Klump, K. L., Strober, M., Woodside, D. B., Crow, S., Mitchell, J., Rotondo, A., Keel, P., Plotnicov, K. H., Berrettini, W. H., Kaye, W. H., Crawford, S. F., Johnson, C., Brandt, H., La Via, M. & Bulik, C. M. (2007). Symptom profile of major depressive disorder in women with eating disorders. *Aust N Z J Psychiatry,* **41**(1), 24-31.

Fetissov, S. O. & Hökfelt, T. (2019). On the origin of eating disorders: altered signaling between gut microbiota, adaptive immunity and the brain melanocortin system regulating feeding behavior. *Current Opinion in Pharmacology,* **48**, 82-91.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes,* **3**(4), 289-306.

Godart, N. T., Flament, M. F., Perdereau, F. & Jeammet, P. (2002). Comorbidity between eating disorders and anxiety disorders: a review. *Int J Eat Disord,* **32**(3), 253-70.

Grundy, M. M. L., Edwards, C. H., Mackie, A. R., Gidley, M. J., Butterworth, P. J. & Ellis, P. R. (2016). Re-evaluation of the mechanisms of dietary fibre and implications for macronutrient bioaccessibility, digestion and postprandial metabolism. *British Journal of Nutrition,* **116**(5), 816-833.

Imhann, F., Vich Vila, A., Bonder, M. J., Fu, J., Gevers, D., Visschedijk, M. C., Spekhorst, L. M., Alberts, R., Franke, L., van Dullemen, H. M., Ter Steege, R. W. F., Huttenhower, C., Dijkstra, G., Xavier, R. J., Festen, E. A. M., Wijmenga, C., Zhernakova, A. & Weersma, R. K. (2018). Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut,* **67**(1), 108-119.

Jauregui Lobera, I. & Bolanos Rios, P. (2009). Choice of diet in patients with anorexia nervosa. *Nutr Hosp,* **24**(6), 682-7.

Jesus, P., Ouelaa, W., Francois, M., Riachy, L., Guerin, C., Aziz, M., Do Rego, J. C., Dechelotte, P., Fetissov, S. O. & Coeffier, M. (2014). Alteration of intestinal barrier function during activity-based anorexia in mice. *Clinical Nutrition,* **33**(6), 1046-1053.

Kaelin, W. G., Jr. & McKnight, S. L. (2013). Influence of metabolism on epigenetics and disease. *Cell,* **153**(1), 56-69.

Kaye, W. H., Bulik, C. M., Thornton, L., Barbarich, N., Masters, K. & Grp, P. F. C. (2004). Comorbidity of anxiety disorders with anorexia and bulimia nervosa. *American Journal of Psychiatry,* **161**(12), 2215-2221.

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O. & Vaiserman, A. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *Bmc Microbiology,* **17**.

Lai, C.-C., Cheng, A., Liu, W.-L., Tan, C.-K., Huang, Y.-T., Chung, K.-P., Lee, M.-R. & Hsueh, P.-R. (2011). Infections Caused by Unusual Methylobacterium Species. *Journal of Clinical Microbiology,* **49**(9), 3329-3331.

Liu, L., Poveda, C., Jenkins, P. E. & Walton, G. E. (2021). An In Vitro Approach to Studying the Microbial Community and Impact of Pre and Probiotics under Anorexia Nervosa

Related Dietary Restrictions. *Nutrients,* **13**(12), 4447.
Louis, P., Young, P., Holtrop, G. & Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environmental Microbiology,* **12**(2), 304-314.
Macfarlane GT, G. G. (1995). Microbiological aspects of short chain fatty acid production in the large bowel.
Macfarlane, G. T. & Gibson, G. R. (1994). Metabolic activities of the normal colonic flora. *In:* Gibson, S. A. W. (ed.) *Human Health - The Contribution of Microorganisms*. London: Springer-Verlag.
Macfarlane, G. T., Macfarlane, S. & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology,* **35**(2), 180-187.
Mack, I., Cuntz, U., Grämer, C., Niedermaier, S., Pohl, C., Schwiertz, A., Zimmermann, K., Zipfel, S., Enck, P. & Penders, J. (2016). Weight gain in anorexia nervosa does not ameliorate the faecal microbiota, branched chain fatty acid profiles, and gastrointestinal complaints. *Sci Rep,* **6**, 26752.
Mehler, P. S. & Brown, C. (2015). Anorexia nervosa - medical complications. *Journal of Eating Disorders,* **3**.
Million, M., Angelakis, E., Maraninchi, M., Henry, M., Giorgi, R., Valero, R., Vialettes, B. & Raoult, D. (2013). Correlation between body mass index and gut concentrations of Lactobacillus reuteri, Bifidobacterium animalis, Methanobrevibacter smithii and Escherichia coli. *International Journal of Obesity,* **37**(11), 1460-1466.
Moon, C. D., Young, W., Maclean, P. H., Cookson, A. L. & Bermingham, E. N. (2018). Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen,* **7**(5), e00677.
Morita, C., Tsuji, H., Hata, T., Gondo, M., Takakura, S., Kawai, K., Yoshihara, K., Ogata, K., Nomoto, K., Miyazaki, K. & Sudo, N. (2015). Gut Dysbiosis in Patients with Anorexia Nervosa. *PLoS One,* **10**(12), e0145274.
Mörkl, S., Wagner-Skacel, J., Lahousen, T., Lackner, S., Holasek, S. J., Bengesser, S. A., Painold, A., Holl, A. K. & Reininghaus, E. (2018). The Role of Nutrition and the Gut-Brain Axis in Psychiatry: A Review of the Literature. *Neuropsychobiology*, 1-9.
Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., Clarke, S. F., O'Toole, P. W., Quigley, E. M., Stanton, C., Ross, P. R., O'Doherty, R. M. & Shanahan, F. (2010). Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* **59**(12), 1635-42.
Murphy, K. G. & Bloom, S. R. (2004). Gut hormones in the control of appetite. *Exp Physiol,* **89**(5), 507-16.
Nikolova, V. L., Hall, M. R. B., Hall, L. J., Cleare, A. J., Stone, J. M. & Young, A. H. (2021). Perturbations in Gut Microbiota Composition in Psychiatric Disorders: A Review and Meta-analysis. *JAMA Psychiatry,* **78**(12), 1343-1354.
Nissen, L., Casciano, F. & Gianotti, A. (2020). Intestinal fermentation in vitro models to study food-induced gut microbiota shift: an updated review. *FEMS Microbiol Lett,* **367**(12).
Pham, T.-P.-T., Tidjani Alou, M., Bachar, D., Levasseur, A., Brah, S., Alhousseini, D., Sokhna, C., Diallo, A., Wieringa, F., Million, M. & Raoult, D. (2019). Gut Microbiota Alteration is Characterized by a Proteobacteria and Fusobacteria Bloom in Kwashiorkor and a Bacteroidetes Paucity in Marasmus. *Scientific Reports,* **9**(1), 9084.
Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen,

T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D. & Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* **464**(7285), 59-65.

Queipo-Ortuño, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., Casanueva, F. & Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One,* **8**(5), e65465.

Raatz, S. K., Jahns, L., Johnson, L. K., Crosby, R., Mitchell, J. E., Crow, S., Peterson, C., Le Grange, D. & Wonderlich, S. A. (2015). Nutritional adequacy of dietary intake in women with anorexia nervosa. *Nutrients,* **7**(5), 3652-65.

Roubalova, R., Prochazkova, P., Papezova, H., Smitka, K., Bilej, M. & Tlaskalova-Hogenova, H. (2020). Anorexia nervosa: Gut microbiota-immune-brain interactions. *Clinical Nutrition,* **39**(3), 676-684.

Sanders, J. W., Martin, J. W., Hooke, M. & Hooke, J. (2000). Methylobacterium mesophilicum infection: case report and literature review of an unusual opportunistic pathogen. *Clin Infect Dis,* **30**(6), 936-8.

Sanmiguel, C. P., Jacobs, J., Gupta, A., Ju, T., Stains, J., Coveleskie, K., Lagishetty, V., Balioukova, A., Chen, Y., Dutson, E., Mayer, E. A. & Labus, J. S. (2017). Surgically Induced Changes in Gut Microbiome and Hedonic Eating as Related to Weight Loss: Preliminary Findings in Obese Women Undergoing Bariatric Surgery. *Psychosom Med,* **79**(8), 880-887.

Smith, M. I., Yatsunenko, T., Manary, M. J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A. L., Rich, S. S., Concannon, P., Mychaleckyj, J. C., Liu, J., Houpt, E., Li, J. V., Holmes, E., Nicholson, J., Knights, D., Ursell, L. K., Knight, R. & Gordon, J. I. (2013). Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science,* **339**(6119), 548-54.

Speranza, E., Cioffi, I., Santarpia, L., Del Piano, C., De Caprio, C., Naccarato, M., Marra, M., De Filippo, E., Contaldo, F. & Pasanisi, F. (2018). Fecal Short Chain Fatty Acids and Dietary Intake in Italian Women With Restrictive Anorexia Nervosa: A Pilot Study. *Front Nutr,* **5**, 119.

Sweeney, T. E. & Morton, J. M. (2013). The human gut microbiome: a review of the effect of obesity and surgically induced weight loss. *JAMA Surg,* **148**(6), 563-9.

Tennoune, N., Chan, P., Breton, J., Legrand, R., Chabane, Y. N., Akkermann, K., Jarv, A., Ouelaa, W., Takagi, K., Ghouzali, I., Francois, M., Lucas, N., Bole-Feysot, C., Pestel-Caron, M., do Rego, J. C., Vaudry, D., Harro, J., De, E., Dechelotte, P. & Fetissov, S. O. (2014). Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. *Translational Psychiatry,* **4**.

Thornton, L. M., Welch, E., Munn-Chernoff, M. A., Lichtenstein, P. & Bulik, C. M. (2016). Anorexia Nervosa, Major Depression, and Suicide Attempts: Shared Genetic Factors. *Suicide & life-threatening behavior,* **46**(5), 525-534.

Tottey, W., Feria-Gervasio, D., Gaci, N., Laillet, B., Pujos, E., Martin, J.-F., Sebedio, J.-L., Sion, B., Jarrige, J.-F., Alric, M. & Brugère, J.-F. (2017). Colonic Transit Time Is a Driven Force of the Gut Microbiota Composition and Metabolism: In Vitro Evidence. *Journal of neurogastroenterology and motility,* **23**(1), 124-134.

van de Wouw, M., Schellekens, H., Dinan, T. G. & Cryan, J. F. (2017). Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite. *J Nutr,* **147**(5), 727-745.

Williams, C. F., Walton, G. E., Jiang, L., Plummer, S., Garaiova, I. & Gibson, G. R. (2015).

Comparative analysis of intestinal tract models. *Annu Rev Food Sci Technol,* **6**, 329-50.
Winter, S. E. & Baumler, A. J. (2014). Dysbiosis in the inflamed intestine Chance favors the prepared microbe. *Gut Microbes,* **5**(1), 71-73.

Chapter 5

An in vitro Approach to Studying the Microbial Community and Impact of Pre and Probiotics under Anorexia Nervosa Related Dietary Restrictions

Abstract

Individuals with anorexia nervosa (AN) often suffer psychological and gastrointestinal problems consistent with a dysregulated gut microbial community. Psychobiotics have been postulated to modify microbiota and improve mental wellbeing and gut symptoms, but there is currently a lack of evidence for such approaches in AN. The aim of this study was to use an in vitro colonic model to evaluate the impact of dietary restrictions associated with AN on the intestinal ecosystem and to assess the impact of pre and probiotic intervention. Bacteriology was quantified using flow cytometry combined with fluorescence in situ hybridisation and metabolic products (including neurotransmitters) by gas chromatography (GC) and liquid chromatography mass spectrometry (LCMS). Consistent with previous research, the nutritional changes significantly reduced total microbiota and metabolites compared with healthy conditions. Pre and probiotic supplementation on restricted conditions enhanced the microbial community and modulated metabolic activity to resemble that of the healthy diet. The model system indicates that nutritional changes associated with AN can impact the microbial community, and that these changes can, at least in part, be restored through use of pre and probiotic interventions.

Keywords: anorexia nervosa; gut-brain axis; gut microbiota; neurotransmitters; psychobiotics; prebiotics; probiotics

1. Introduction

Anorexia nervosa (AN) is a serious psychiatric disorder characterised by restriction of dietary intake (typically leading to low body weight) and a distorted perception of body shape (Association, 2013). Compared to those without eating disorders (EDs), significant differences in gut microbiota composition have been demonstrated in individuals with AN, with an imbalanced relative abundance in Gram positive/Gram negative bacteria (Borgo *et al*., 2017; Kleiman *et al*., 2015; Morita *et al*., 2015; Mack *et al*., 2016). Most commensal bacterial species in gut microbiota are members of the Bacteroidetes and Firmicutes phyla. The F/B ratio is high in obese people compared to lean people, and tends to reduce with weight loss (Koliada *et al*., 2017; Sweeney and Morton, 2013); therefore the F/B ratio could be impacted by the presence of AN symptoms (Borgo *et al*., 2017; Mack *et al*., 2016). Individuals with AN have been observed to have decreased levels of SCFA-producing *Roseburia* (Borgo *et al*., 2017; Mack *et al*., 2016), and lower abundance of *Lactobacillus* compared to healthy volunteers (Armougom *et al*., 2009; Million *et al*., 2013). The number of intestinal bacteria in individuals with AN is severely reduced because of limited dietary intake, leading to a gut microbial community adapting to a starvation state (Morita *et al*., 2015; Jalanka *et al*., 2015). Indeed, reduced levels of microbial growth in a nutrient limited environment is well known (Shehata and Marr, 1971). However, such an alteration could be of importance when considering dietary restrictions in AN.

The gut microbial community has important interactions with host metabolism, affecting body weight regulation and hormonal processes, along with a direct impact on the brain and behaviour via the gut-brain axis (GBA) (Herpertz-Dahlmann *et al*., 2017; Mack *et al*., 2018;

Neuman *et al*., 2015; Seitz *et al*., 2019a; Seitz *et al*., 2019b). Indeed, research indicates a key role of gut microbiota in the regulation of behaviour, mood, gastrointestinal symptoms, nutrient metabolism, satiety and appetite, functions often altered in AN (Prochazkova *et al*., 2019). Within AN, changes in nutritional intake result in an altered microbial community that could impact GBA communication and further affect neurological function. This opens up the prospect of studying how the gut microbiota impact on the brain and whether modification of gut microbiota could be helpful in the fight against AN (David *et al*., 2014).

Gut microbiota produce short-chain fatty acids (SCFA) via saccharide fermentation. SCFA could be important molecules in AN as increased SCFA levels in faecal samples have been observed in obese and overweight people compared to AN sufferers (Morita *et al*., 2015; Fernandes *et al*., 2014; Turnbaugh *et al*., 2006), and could relate to physiological processes including the GBA and immune system (Braniste *et al*., 2014; Stilling *et al*., 2014; Sherwin *et al*., 2016). Furthermore, SCFA act as signaling molecules and are involved in regulating gut transit time, appetite, and energy homeostasis (Sherwin *et al*., 2016). Additionally, microorganisms have been reported to produce, or be involved in the production of, various neuroactive compounds such as serotonin (5-HT), dopamine (DA), and norepinephrine (NE) (Lyte, 2011). 5-HT is one of the main neurotransmitters (NTs) located in the brain, but almost 90-95% of total body 5-HT is located in the gastrointestinal tract (GIT) and is secreted via the epithelial enterochromaffin cells (ECs) of the gut, thus is involved in GBA bidirectional signaling (Gershon and Tack, 2007). A study by Yano et al (Yano *et al*., 2015) indicated that enteric bacteria such as *Streptococcus, Escherichia* and *Enterococcus* species may modulate host 5-HT biosynthesis by increasing its precursor, tryptophan, in plasma. Reduced tryptophan has been associated with anxiety and borderline personality symptoms in patients with AN and

bulimia nervosa (Kaye *et al*., 2004; Weltzin *et al*., 1991). However, serotonergic activity status can be predicated from 5-HT metabolites 5-HIAA, and long-term weight-restored patients with AN have elevated 5-HIAA in cerebrospinal fluid (Kaye *et al*., 1991).

Psychobiotics are supplements designed to modulate the gut microbiota to enhance mood, and can be in the form of beneficial bacterial supplements (probiotics) or support for positive bacteria already within the gut (prebiotics), ultimately, these must also affect bacteria–brain communication (Sarkar *et al*., 2016). The bacteria enhanced through these interventions may therefore result in elevated levels of neuroactive compounds such as 5-HT and gamma-aminobutyric acid (GABA), which act on the GBA (Dinan and Cryan, 2013; Grenham *et al*., 2011). Psychobiotics are probiotics that have shown potential effects on psychological and physiological conditions such as improving anxiety, depression and appetite levels (Mayer *et al*., 2014; Savignac *et al*., 2013). Few studies have examined the psychophysiological impact of prebiotics. Soluble fibre fructo-oligosaccharides (FOS) are a nutritional source for *Bifidobacterium*, reflected by their bifidogenic capability, thus they promote its activity and proliferation in the gut [34]. It is unknown how an 'anorexic' dietary intake might affect the microbiota, and further how pre and probiotic may impact on this.

In vitro models can be a useful tool for determining how microbial communities grow in the presence of different nutrients but without the need for human participants with greater physiologically relevant conditions including human large intestine nutrients, temperature and pH. A three-stage continuous culture system was developed by Macfarlane et al. (Macfarlane *et al*., 1998) to mimic nutritional and physiochemical conditions of microbiota in the colon. The constituents of culture medium were determined on the basis of large intestine contents with the main substrates available for microorganisms determined to be dietary fibre, proteins,

oligosaccharides and peptides that evade digestion in the small intestine, as well as a variety of host-derived substances, including mucins, pancreatic secretions and exfoliated epithelial cells (Macfarlane and Gibson, 1994; Macfarlane GT, 1995). In the current study a media approximated to anorexic dietary intake, along with host derived substances, was developed for use in a three-stage continuous culture system, enabling the activities of intestinal bacteria to be established under these nutrient-deficit conditions. The subsequent impact of pre and probiotics on this community will be investigated.

2. Materials and Methods

2.1. Three-Stage Compound Continuous Culture System

A three-stage continuous culture system was set up in sequence simulating the proximal (V1, 80 mL, pH = 5.5), transverse (V2, 100 mL, pH = 6.2) and distal colon (V3, 120 mL, pH = 6.8) comprised of a scaled-down version of three regions of the GI tract (Figure 1). The system was maintained in anaerobic conditions by supplying by N_2 (15 mL/min) and holding a continuous temperature of 37°C via a circulating water bath. The systems were inoculated to give a final concentration of 6% faecal slurry, samples were donated from four healthy female donors (age range 25-43 years). The faecal donors had not taken antibiotics within 6 months of the experiment, and were not regular consumers of prebiotic or probiotic supplements. Collected faecal samples were placed in anaerobic jars (AnaeroJarTM 2.5 L, Basingstoke, UK, Oxoid Ltd.) with anaerobic sachets (AnaeroGen, Oxoid) and used within 2 hours of production. To prepare the faecal sample a 1 in 5 (wt:v) faecal slurry with PBS (anaerobic phosphate buffered saline; 0.1 mol/l; pH 7.4) was homogenised in a stomacher (Stomacher 400, Seward, West Sussex, UK) for 2 minutes (240 paddle beats/min).

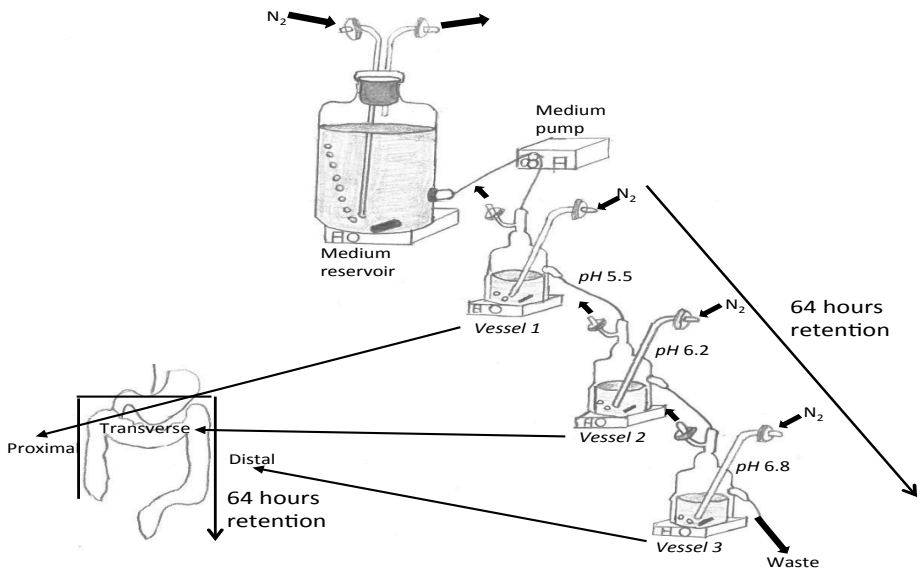


Figure 1. Schematic of gut model system indicating the retention time and the relation of the vessels to the large intestine

The experiment was conducted 4 times with a different faecal donor for each run. Following inoculation, the system was run for 24 h to allow the bacteria to multiply within the vessels. After this the flow was started, with a retention time appropriate to anorexic patients (Kamal *et al*., 1991), when considering the operating volume (300 mL) and anorexic retention time (64 h, flow rate 4.68 mL/h) of the gut model system. The first steady state (SS1), when equilibrium was reached was after 512 h (8 full volume turnovers) was reached using standard gut model media (HC feeding). A second steady state (SS2) was achieved after a further 512 h, this was using nutrient restricted media (AN feeding). This was determined by assessing the stability of the SCFA over 3 consecutive days. restricted media continued to be used along with a potential psychobiotic, FOS (1.67 g/daily) or *Saccharomyces boulardii* (5 x 10^8 cfu) treatment daily into

V1 for at least a 512 h upon which third steady state (SS3) was achieved. Samples were collected at three time points (SS1, SS2, and SS3).

2.2. Gut Model Medium Determination in HC, AN, and AN with Pre and Probiotic Feeding

AN gut model system and the medium concentration was modelled on the real gut environment conditions of AN individuals. Jauregui et al. and Raatz et al. (Jauregui Lobera and Bolanos Rios, 2009; Raatz *et al*., 2015) outlined the typical dietary intake of restrictive AN patients compared to healthy people. By considering both healthy and restrictive diets and the constituents of gut model media the nutrients (**Table 1**) likely to reach the colon were determined (Macfarlane *et al*., 1998). In terms of other components found within the media representative of human secretions, mucin and bile salts were included at the same concentration in both media.

Table 1. Nutrient assessment. AN/restricted gut model recipe was determined from comparing daily dietary nutrition requirements based on the British Nutrition Foundation (BNF) guideline (British Nutrition Foundation, 2016). Compared to the nutrient intake of individuals with the restricting subtype of AN, where the illness is characterised by dietary restriction in the absence of recurrent episodes of regular binge eating and purging (Association, 2013). Additionally, each medium contained: 0.8 g L-cystine HCl, 1 ml Tween 80, 4 ml resazurin solution (0.025 g/100 ml, pH 7), 4 g mucin (Porcine gastric type III), 0.4 g bile salts per litre gut model, representing human secretions determined within original gut model media.

	Nutrients	**BNF level**	**Quartile 1** (Restricting AN n=12)	**Comparison %**	**Representative medium**	**Healthy gut model medium (g/L) SS1**	**Anorexic medium (g/L) SS2 and SS3**
M	**Carbohydrate** (g)	260	110	42%	Starch	5 g	2.1 g

A C R O N U T R I E N T S	**Protein** (g)	50	33	66%	Peptone water Tryptone Yeast extract Casein	5 g 5 g 4.5 g 3 g	3.3 g 3.3 g 2.97 g 1.98 g
	Dietary fibre (g)	30	12	40%	Guar Gum FOS Pectin Arabinogalactan Xylan	1 g 1 g 2 g 2 g 2 g	0.4 g 0.4 g 0.8 g 0.8 g 0.8 g
M I C R O N U T R I E N T S	**Potassium (mg)**	3500	2660	76%	KCI	4.5 g	3.28 g
	Chloride (mg)	2500	1825	73%	NaCI	4.5 g	3.28 g
	Sodium (mg)	1600	1168	73%	$NaHCO_3$	1.5 g	1.095 g
	Magnesium (mg)	270	227	84%	MgSO4·7H2O	1.25 g	1.05 g
	Phosphorus (mg)	550	607	110%	KH_2PO_4 K_2HPO_4	0.5 g 0.5 g	0.55 g 0.55 g
	Calcium (mg)	700	545	78%	$CaCl2·6H_2O$	0.15 g	0.117 g
	Iron (mg)	14.8	7.9	53%	Hemin $FeSO4.7H_2O$	0.5 g 0.005 g	0.0265 g 0.000795 g
	Vitamin K (µg)	-		68%	Vitamin K	10uL	6.68uL

2.3.Treatments

Probiotic yeast *Saccharomyces boulardii* (OptiBac Probiotics Ltd., Hampshire, UK) product contained 5× 10^9 cfu live culture powder in each capsule. Prebiotic FOS (Orafti P95) was obtained from BENEO (Orafti P95, Tienen, Belgium).

2.4. Preparation of the Samples for SCFA/BCFA Analysis, NTs Analysis and Bacterial Community Analysis

Samples were taken at SS1, SS2 and SS3 time point from proximal, transverse and distal vessels. 1 mL of gut model fluid was centrifuged in a micro centrifuge Eppendorf tube (1.5 mL) at 13000 x *g* for 10 min the supernatant was stored at -20 °C prior to SCFA/BCFA analysis. A further 0.4 mL was collected and centrifuged at 13000 x *g* for 10 min then stored at -20 °C for neurotransmitter analysis.

For bacterial community analysis, a 750 µL supernatant of gut model fluid was centrifuged at 13000 x *g* for 5 min. The pellet was then resuspended in 375 µL filtered 0.1 M PBS and fixed by 1125 µL filtered paraformaldehyde (PFA 4% w/v) for 4 to 8 hours at 4 °C, sample was washed twice with 1 ml PBS to remove PFA and resuspended in filtered 600 µL ethanol-PBS (1:1, w/v), samples were kept at -20 °C prior to FISH analysis.

2.5. In vitro Enumeration of Bacterial Population by Flow-Fluorescent In Situ Hybridisation (FISH)

The bacterial population was analysed using fluorescent in situ hybridisation coupled to flow cytometry (BD Accuri™ C6 Plus, Basingstoke, United Kingdom), detecting at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software. Samples were removed from storage at -20 °C. After defrosting and vortexing for 10 sec permeabilisation steps were conducted using 500 µL 0.1 M PBS added to 75 µL fixed samples and centrifuged at 13000 x *g* for 3 min. The pellets were resuspended in 100 µL of TE-FISH buffer (Tris-HCl 1 M pH 8, EDTA 0.5 M pH 8, filtered distilled water, 0.22 µm pore size filter with the percentage of 10:10:80) containing lysozyme solution (1 mg/ml of 50,000 U/mg protein) and incubated for 10 min in the dark at room temperature and then centrifuged at 13000 x *g* for 3 min. Pellets were washed with 0.1 M 500 µL PBS and then washed with 150 µL hybridisation buffer (0.9 M NaCl, 0.2 M Tris/HCl pH 8.0, 30% formamide, ddH_2O, 0.01% sodium dodecyl sulphate) and centrifuged at 13000 x *g* for 3 min. Pellets were then resuspended in 1 mL of hybridisation buffer, homogenised and 50 µL with 4 µL of different probes aliquoted into Eppendorf tubes (1.5 mL) were incubated at 36°C overnight. Differences in bacterial populations were quantified with oligonucleotide probes aimed to target specific regions of 16S rRNA. The individuals probes used (Eurofins, Wolverhampton, UK) in this study are shown in **Table 2**. Non-EUB and EUB338-I-II-III were linked to fluorescence Alexa 488 at the 5′ end, and group specific probes were linked to fluorescence Alexa 647. Non-EUB and EUB338 were linked to Alexa 647 at the 5′ end as controls to adjust threshold. 4 µL of EUB338-I-II-III was added together with 4 µL specific probes. 125 µL of hybridisation buffer was added to each Eppendorf tube after incubation samples were vortexed and centrifuged (13000 x *g*, 3 min). Supernatants were removed and pellets were washed with 175 µL washing buffer solution (0.064 M NaCl, 0.02

M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate, 956.2 µL of ddh_2O), vortexed and incubated at 38°C in a heating block for 20 min to remove non-specific binding of the probe. Afterwards samples were centrifuged (13000 x *g*, 3 min) and supernatants removed. Pellets were resuspended in an appropriate volume of PBS on the basis of flow cytometry (FCM) load. Number of bacteria were then calculated through determination of FCM reading and PBS dilution.

Table 2. Oligonucleotide probes used in the study for bacterial populations by fluorescent in situ hybridisation.

Probe name	**Sequence (5' to 3')**	**Target species**	**Reference**
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner *et al*., 1993)
Eub338I +	GCTGCCTCCCGTAGGAGT	Most Bacteria	(Daims *et al*., 1999)
Eub338II +	GCAGCCACCCGTAGGTGT	*Planctomycetales*	(Daims *et al*., 1999)
Eub338III +	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	(Daims *et al*., 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al*., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Hermie J. M. Harmsen, 1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz *et al*., 1996)

Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale group* (*Clostridium* cluster XIVa and XIVb)	(Franks *et al*., 1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker *et al*., 2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen *et al*., 2000)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker *et al*., 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(Hold *et al*., 2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	(Devereux *et al*., 1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	(Franks *et al*., 1998)
Phasco741	TCAGCGTCAGACACAGTC	*Phascolartobacterium faecium*, *Acidaminococcus fermentans*, *Succiniclasticum ruminis*	(Harmsen *et al*., 2002)

SUBU1237	CCCTCTGTTCCGACCATT	*Burkholderia* spp.	(Stoffels *et al*., 1998)
Muc1437	CCTTGCGGTTGGCTTCAGAT	*Akkermansia muciniphila*	(Audie *et al*., 1993)

2.6. Neurotransmitter Analysis by Liquid Chromatography Mass Spectrometry (LCMS)

2.6.1. Reagents and Chemicals

HPLC Plus grade acetonitrile (≥ 99.9%) was purchased from Sigma-Aldrich. 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 gut model fluid samples. Analytical standards powder including LC-MS grade dopamine hydrochloride (99%) and L (-)-Epinephrine (99%) were purchased from Alfa Aesar (Lancashire, UK). L-Noradrenaline (98%), Gamma-Aminobutyric acid (99%) and serotonin were purchased from Sigma-Aldrich Co Ltd.

2.6.2. Stock Solutions, Calibration Standards and Sample Preparation

Separate standard stock solutions (10000 ng/ml) of five analytes including 5-HT, DA, GABA, NE and Epinephrine (EPI) were individually prepared in HPLC water. A 1000 ng/ml mixed standard solution containing the five 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 10 levels: 1, 10, 50, 100, 250, 500, 750, 1000, 2500, 5000 ng/ml. Samples were removed from storage at -20 °C. A 400 μl sample of gut model fluid supernatant was collected in a centrifuge tube filter (Sigma-Aldrich, 0.22 μm, Polypropylene) and then centrifuged at 13000 X *g* for 10 min at 4 °C (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) and the supernatant remained. 200 μl of HPLC water (Blank), calibration standard samples and gut model samples were placed in 96-well plates.

2.6.3. LCMS System

Samples were measured using online Nexera LC System coupled to LCMS-8050 triple quadrupole (QQQ) mass spectrometry (Shimadzu, Kyoto, Corporation, Japan). Data were processed using LabSolutions LCMS version 5.65 software.

2.6.4. Liquid Chromatography (LC) Conditions

The chromatographic separation of analytes was obtained from Discovery HS F5-3 column (2.1 mmI.D. x 150 mmL. 3 um particle size, Sigma-Aldrich Co Ltd., P/N 567503-U). The mobile phase consisted of 0.1% formic acid in water (mobile A) and 0.1% formic acid in acetonitrile (mobile B). For the entire analysis, the flow rates of both mobile phases were 0.25 mL/min, and the autosampler temperature at a maintained constant temperature was set to 4°C. The gradient elution programs were as follows: B conc. 25% (5 min) →35% (11 min) → 95% (15 min) → 95% (20 min) → 0% (20.01- 25 min).

2.6.5. Mass Spectrometry (MS) Conditions

The LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using 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. The MRM transitions of the native, stable isotopes, retention times and other conditions are shown in **Table 3**.

Table 3. Optimal conditions of LC-MS/MS used for the quantification of DA, 5-HT, NE, EPI and GABA in faecal supernatant.

Compounds name	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Classification
5-HT	177.10	160.10	10.527	Amino acid derivative
DA	154.10	91.05	8.078	Amino acid derivative
NE	170.10	152.15	4.988	Catecholamine
EPI	184.10	166.10	7.164	Catecholamine
GABA	104.10	87.05	3.690	Organic acid

Each analyte of ionisation polarity is (+).

2.6.6. Quantification of Samples

A linear calibration curve was generated based on the detected signal proportional to the concentration of the analyte. Briefly, results validation was performed following published procedures (Scientific Working Group, 2013). Good linearity with R^2 greater than 0.98 was obtained across the set calibration in the range from 1 ng/mL to 5000 ng/mL for each of the analytes, with accuracy within 100 % ± 20 %. Quantification of samples was determined by calibration with five analytes including 5-HT, DA, EPI, GABA and NE.

2.7. Short Chain and Branched Chain Fatty Acid Analysis by Gas Chromatography

The concentration of SCFAs was determined by Gas chromatography (GC) as previously described by Richardson et al.(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) 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 x *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 × I.D. 30 m × 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 by 5°C and held at 190 °C for 30 min. Helium was the carrier gas (flow rate, 1.7 ml/min, head pressure, 133 KPa). Peak areas were integrated using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK). SCFA production was quantified by single-point internal standard method was described by Liu et al. (Liu *et al*., 2016). Peak areas of the standard (acetate, butyrate, propionate, valerate, iso-valerate and iso-butyrate) were used to calculate the response factors for each organic acid with respect to the internal standard.

2.8. Statistical Analysis

Data from FCM-FISH, LC/MS and GC were analysed with SPSS version 27 (IBM Corp., USA). Changes in specific bacterial groups, NTs and SCFA/ BCFA production were assessed between the three steady states using a one-way analysis of variance (ANOVA). Significant differences were assessed by *post hoc* Tukey HSD (Honestly Significant Difference) test. Statistical significance was set $p < 0.05$. Analyses were performed using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). FISH results are reported as mean ± RSD. Mean of log transformed data is a geometric mean in log, so the SD reported is therefore the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

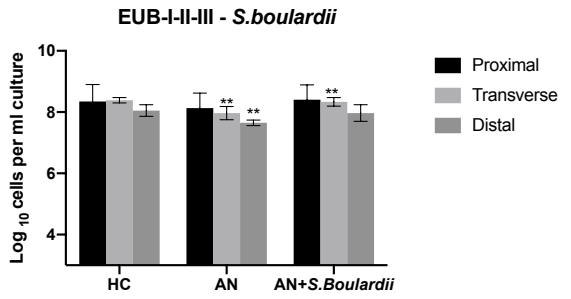
3. Results

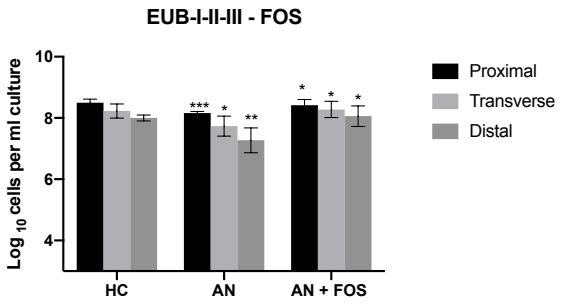
3.1. Bacterial Enumeration

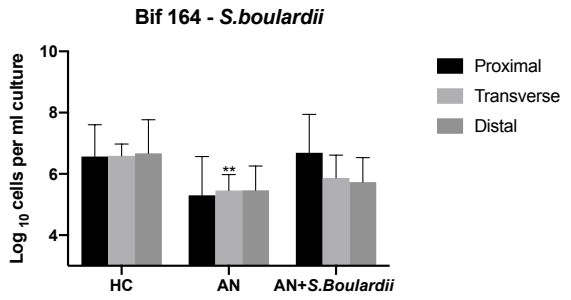
Changes in bacterial compositions in the gut model systems are reported in Figure 2. Compared to HC media (SS1), there was a reduction observed across all bacterial groups following anorexic media (SS2) in the proximal (V1), transverse (V2) and distal (V3) simulation. When compared to the HC media, the levels of total bacteria (V1, V2, V3 FOS model; V2, V3 *S. boulardii* model), *Bifidobacterium* (V1, V2, V3 FOS model; V2 *S. boulardii* model), *Bacteroide*s spp. (V2, V3 *S. boulardii* model; V2 FOS model), *Roseburia* (V1, V2, V3 FOS model; V2, V3 *S. boulardii* model), *Clostridium coccoides-Eubacterium rectale* group (V3 *S. boulardii* model), *Clostridium* cluster IX (V1, V2 FOS model; V3 *S. boulardii* model), *Faecalibacterium prausnitzii* (V1, V2, V3 FOS model), *Clostridium histolyticum* (V3 *S. boulardii* model) and *Phascolartobacterium faecium* (V2 *S. boulardii* model) following the

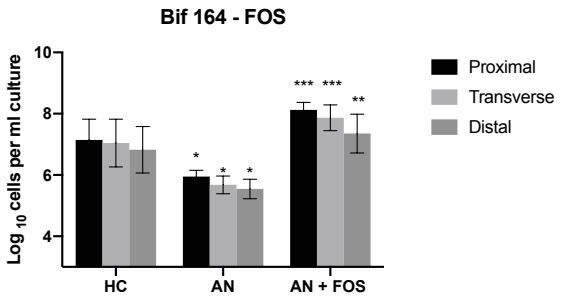
AN-media were significantly reduced (*p*-values indicated in **Figure 2**). The level of *Akkermansia muciniphila* was significantly decreased following the mimicking of AN-media proximal colon in both *S. boulardii* and FOS model (5.97 to 4.59 $\log_{10}$ cells/mL, $p < 0.05$ and 5.23 to 4.73 $\log_{10}$ cells/mL, $p < 0.05$), V2 (5.80 to 5.03 $\log_{10}$ cells/mL, $p < 0.05$) and V3 (5.48 to 5.70 $\log_{10}$ cells/mL, $p < 0.05$) in FOS model.

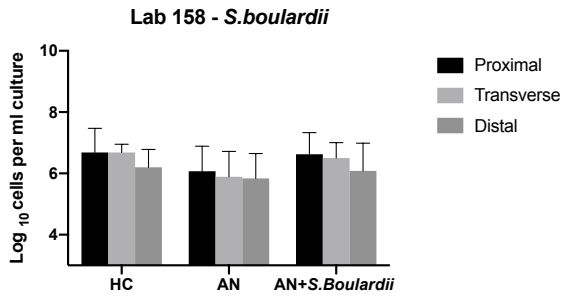
However, following the addition of FOS to anorexic media models, a significant increase of total bacteria occurred from 8.16 to 8.42 $\log_{10}$ cells/mL ($p < 0.05$), from 7.73 to 8.28 $\log_{10}$ cells/mL ($p < 0.05$) and from 7.28 to 8.06 $\log_{10}$ cells/mL ($p < 0.05$), in V1, V2 and V3, respectively. Additionally, FOS significantly increased numbers of *Roseburia* spp. from 5.49 to 7.28 $\log_{10}$ cells/mL ($p < 0.01$), from 5.16 to 7.07 $\log_{10}$ CFU/mL ($p < 0.01$) and from 4.95 to 6.57 $\log_{10}$ cells /mL ($p < 0.05$), in V1, V2 and V3, respectively. Numbers of *Bifidobacterium* were significantly increased from 5.30 to 6.68 $\log_{10}$ cells /mL ($p < 0.001$) in V1, from 5.46 to 5.87 $\log_{10}$ cells /mL ($p < 0.001$) in V2 and from 5.46 to 5.72 $\log_{10}$ cells /mL in V3 ($p < 0.01$) after FOS administration. *Bacteroides* were significantly increased from 6.72 to 7.3 $\log_{10}$ cells /mL ($p < 0.05$) and *Phascolartobacterium faecium* were significantly increased from 5.26 to 6.04 $\log_{10}$ cells /mL in V2 ($p < 0.05$) after *S. boulardii* administration within AN- media. FOS significantly increased numbers of *Desulfovibrio* and *Clostridium coccoides-Eubacterium rectale* in V1 from 4.47 to 5.62 $\log_{10}$ cells /mL ($p < 0.05$) and in V3 from 6.69 to 7.49 $\log_{10}$ cells /mL ($p < 0.05$), respectively. *S. boulardii* significantly increased numbers of *Atopobium, Clostridium histolyticum* and *Desulfovibrio* in V1 from 5.40 to 6.11 $\log_{10}$ cells /mL ($p < 0.05$), in V2 from 5.40 to 6.35 $\log_{10}$ cells /mL ($p < 0.05$) and in V3 from 4.43 to 5.28 $\log_{10}$ cells /mL ($p < 0.05$), respectively.

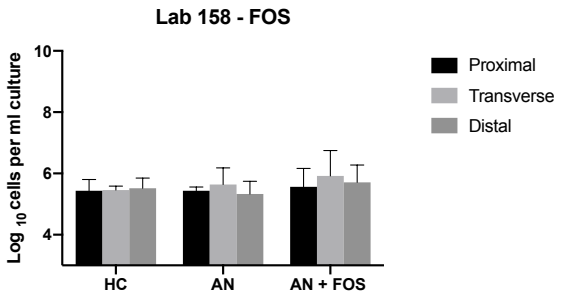

EUB-I-II-III - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

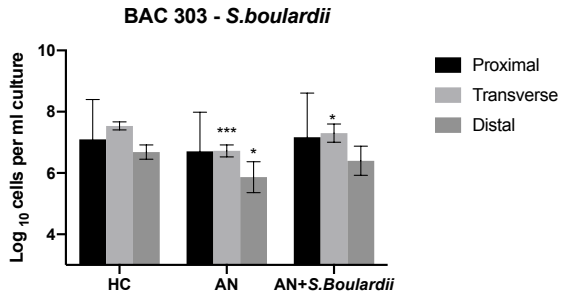

EUB-I-II-III - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN + FOS
Proximal
Transverse
Distal

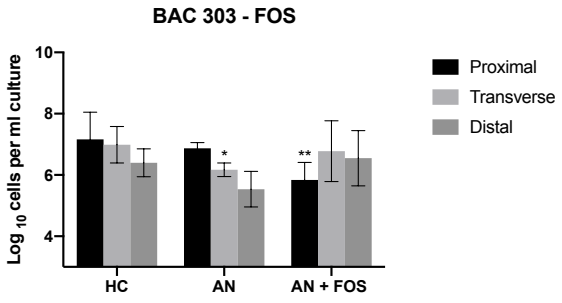

Bif 164 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

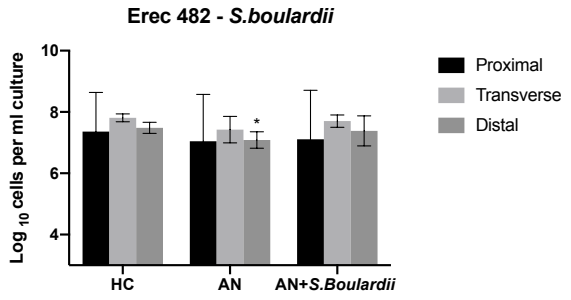

Bif 164 - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN + FOS
Proximal
Transverse
Distal

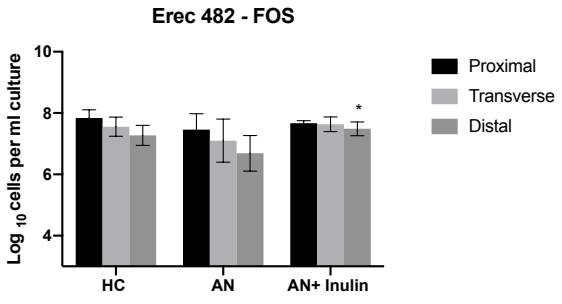

Lab 158 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

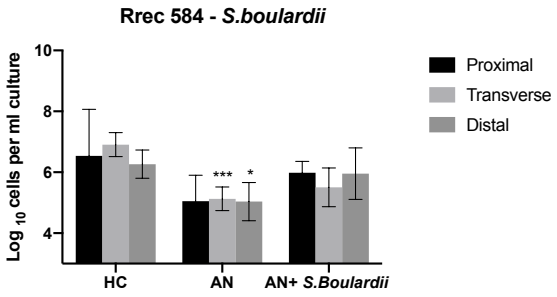

Lab 158 - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN + FOS
Proximal
Transverse
Distal

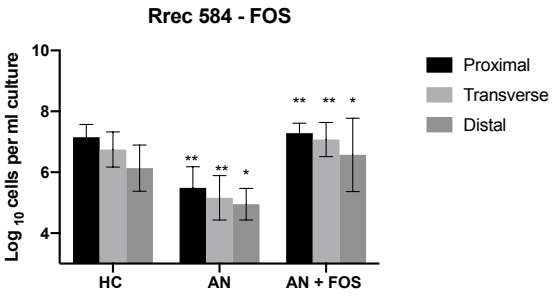

BAC 303 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

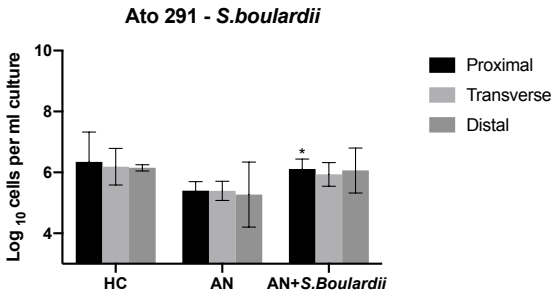

BAC 303 - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN + FOS
Proximal
Transverse
Distal

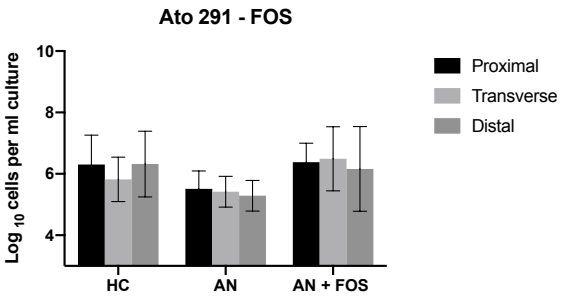

Erec 482 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

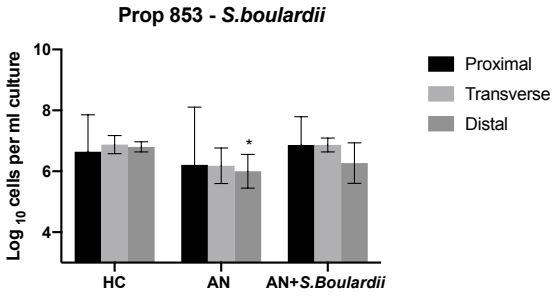

Erec 482 - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+ Inulin
Proximal
Transverse
Distal

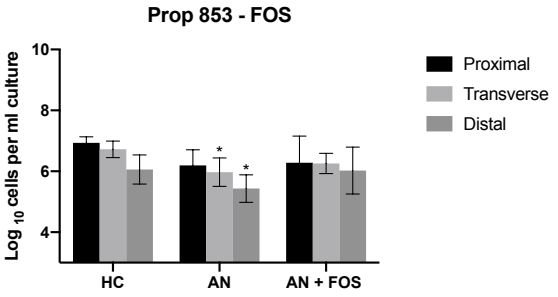

Rrec 584 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
***
*
HC
AN
AN+ S.Boulardii
Proximal
Transverse
Distal

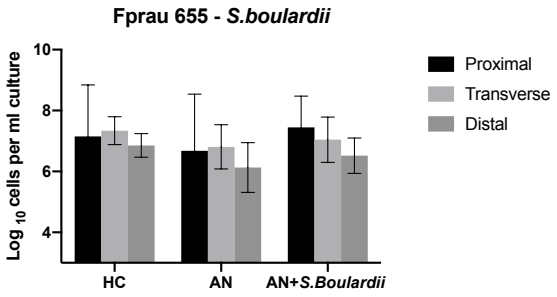

Rrec 584 - FOS
Log 10 cells per ml culture
10
8
6
4
**
**
*
**
**
*
HC
AN
AN + FOS
Proximal
Transverse
Distal

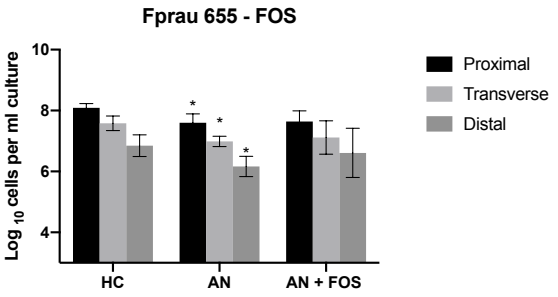

Ato 291 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
*
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal

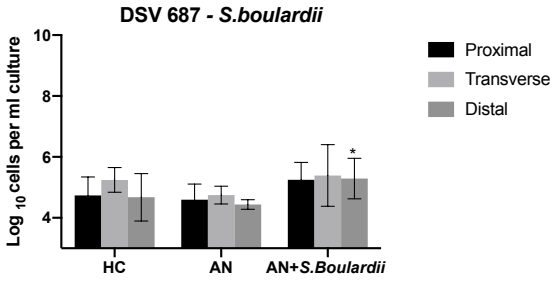

Ato 291 - FOS
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN + FOS
Proximal
Transverse
Distal

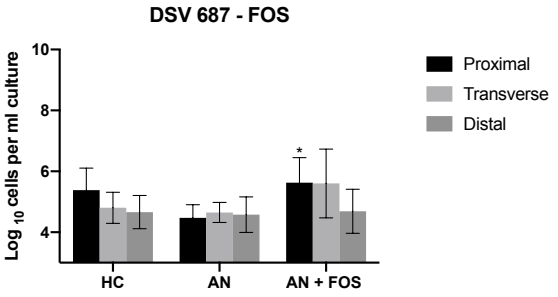

Prop 853 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
*
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal


Prop 853 - FOS
Log 10 cells per ml culture
10
8
6
4
*
*
HC
AN
AN + FOS
Proximal
Transverse
Distal


Fprau 655 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal


Fprau 655 - FOS
Log 10 cells per ml culture
10
8
6
4
*
*
*
HC
AN
AN + FOS
Proximal
Transverse
Distal


DSV 687 - S.boulardii
Log 10 cells per ml culture
10
8
6
4
*
HC
AN
AN+S.Boulardii
Proximal
Transverse
Distal


DSV 687 - FOS
Log 10 cells per ml culture
10
8
6
4
*
HC
AN
AN + FOS
Proximal
Transverse
Distal

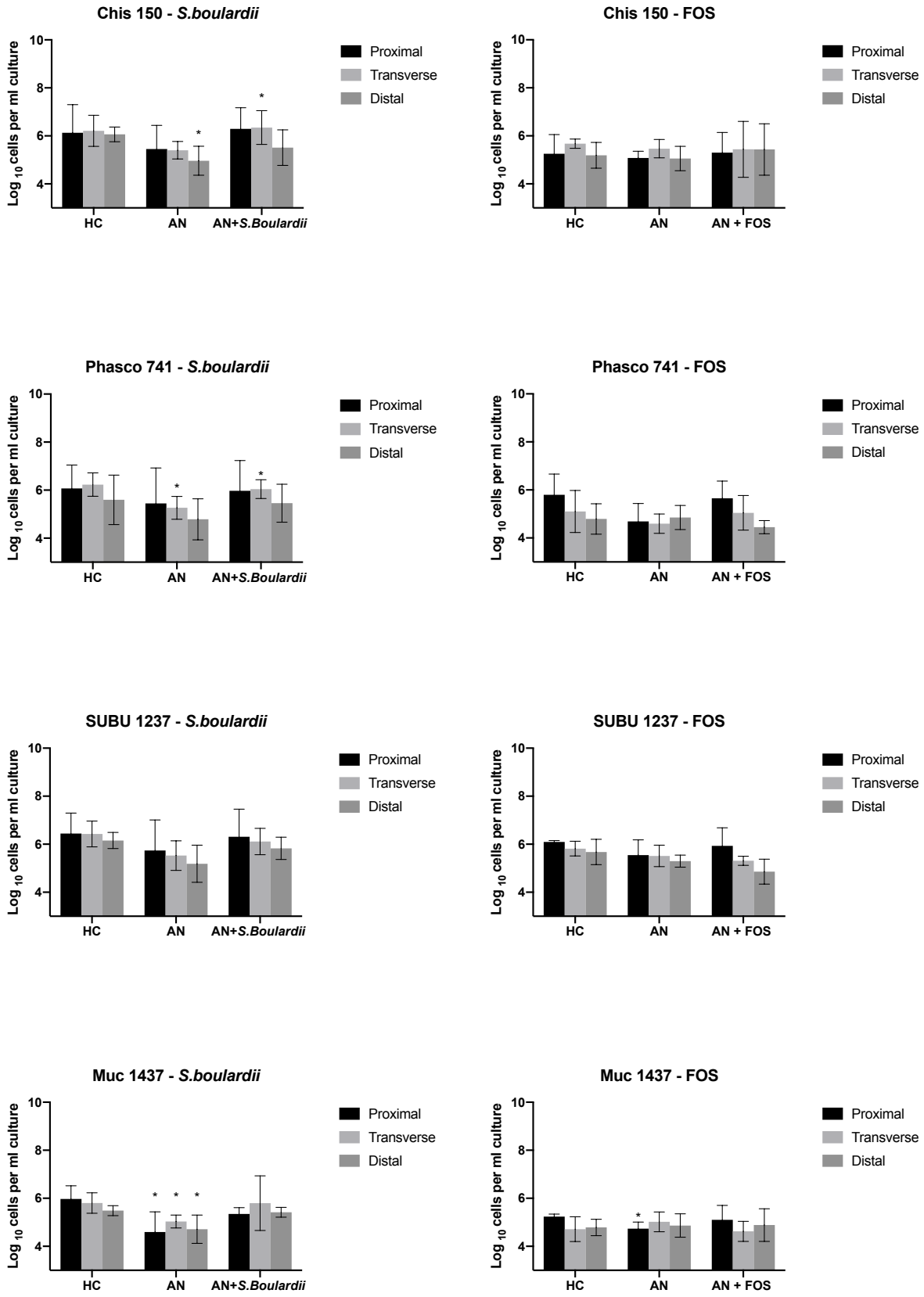


Figure 2. Bacterial groups at different steady states detected by FISH-FCM (Log_{10} cells /mL) from each vessel V1, V2 and V3 which mimicking the proximal, transverse and distal colon of in vitro colonic model. Samples were collected at SS1 (Healthy media), SS2 (Anorexic media) and SS3 (Anorexic media with the daily administration of *Saccharomyces boulardii* /FOS). Values are mean ± SD. Significant difference in each vessel * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between SS1 and SS2, SS2 and SS3 are indicated. FISH results are reported as Means ± RSD. Mean of log transformed

data is a geometric mean in log, so the SD reported is in fact the log of a ratio. Therefore RSD has been noted throughout the thesis to be clear that this is how the results are presented.

3.2. NTs production

Changes in NTs concentrations in gut model systems are reported in **Figure 3**. Compared to HC media, AN media led to a significant decrease in DA, NE and EPI concentrations in V1 in the proximal colon simulation. Additionally, when compared to HC media, AN media led to a significant decrease in EPI and NE (V2 FOS model). AN media led 5-HT and GABA to decrease in all vessels. However, DA and EPI production were significantly increased in V1 from 1.07 to 2.97 ng/ml ($p < 0.05$) and from 2.99 to 10.48 ng/ml ($p < 0.05$) after FOS administration with AN-media. Compared to AN-media, the fermentation of *S. boulardii* mediated a significant increase in production of 5-HT (from 73.45 to 97.60 ng/ml) and GABA (from 146.61 to 349.55 ng/ml) both in V1, stimulating the proximal colon (both $p < 0.05$).

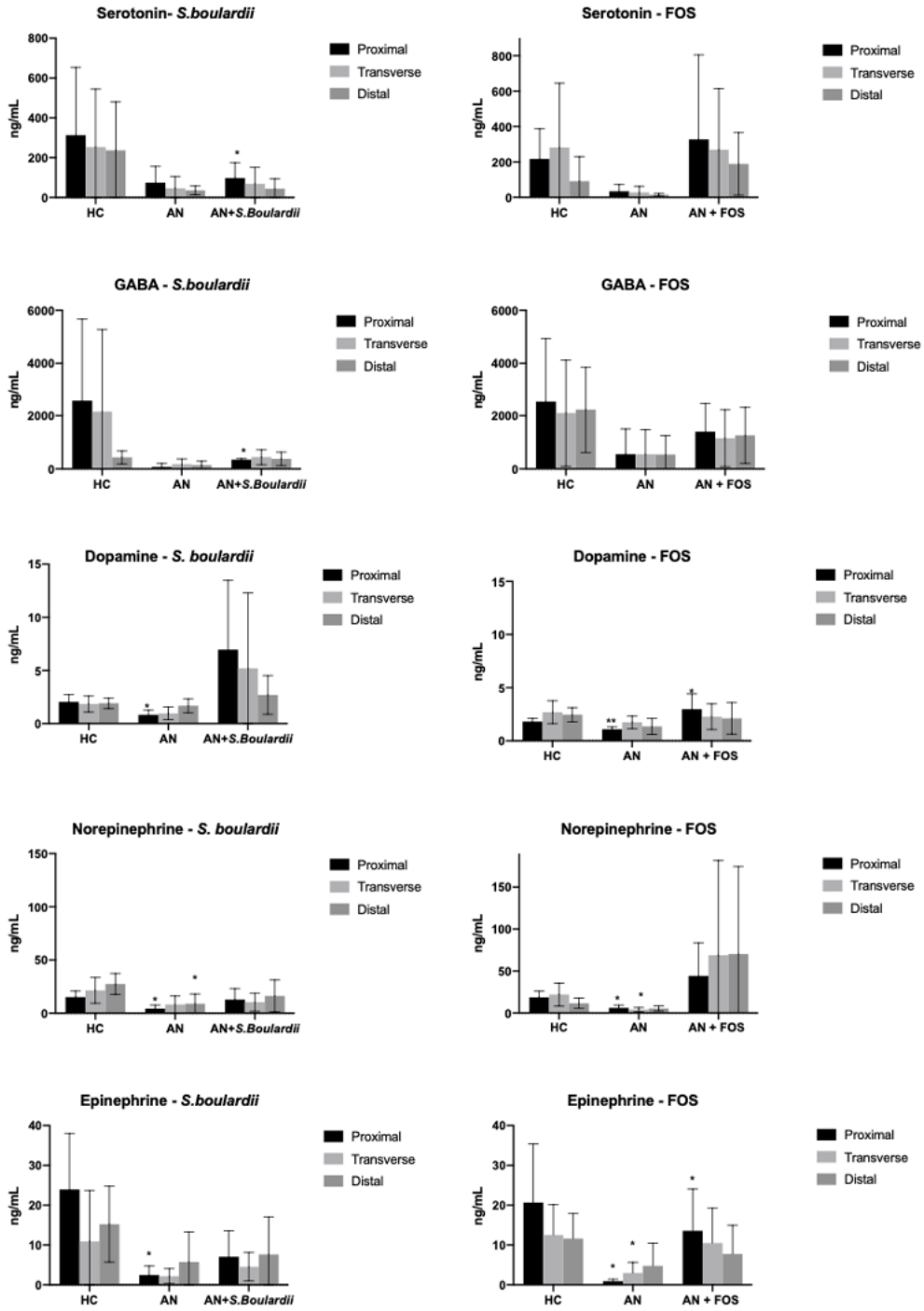


Figure 3. LCMS analysis. 5-HT, GABA, DA, NE and EPI concentration in culture broths with SS1 (healthy media), SS2 (anorexic media) and SS3 (Anorexic media with the daily administration of *Saccharomyces boulardii* /FOS recovered from V1, V2 and V3 (stimulation of proximal, transverse and distal) of in vitro colonic model systems. Results are reported as Means ± SD (ng/ml). For each measurement, significant difference in each vessel * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between SS1 and SS2, SS2 and SS3 are indicated.

3.3. SCFA and BCFA Production

Changes in SCFA and BCFA concentrations are shown in **Figure 4**. This study shows a lower concentration of acetate and butyrate following AN media in all vessels compared to HC media. Levels of acetate (V1 *S. boulardii* model; V3 FOS model, both $p < 0.05$), butyrate (V1, V2 FOS model, both $p < 0.01$), propionate (V2 *S. boulardii* model, $p < 0.05$) and BCFA (V2 *S. boulardii* model, $p < 0.05$) were significantly decreased in AN media. A higher concentration of BCFA in V3 *S. boulardii* model from 5.70 to 7.06 mM ($p = 0.57$) and V1 FOS model from 1.00 to 1.71 mM ($p = 0.59$) was observed following fermentation of AN media. Supplementation of FOS to AN gut models led to a significant increase in levels of acetate in V1, V2 and V3, simulating the proximal, transverse (both $p < 0.05$) and distal ($p < 0.001$), respectively. The fermentation of FOS mediated a significant increase in concentrations of propionate in V1 ($p < 0.01$) and butyrate in V1 and V3 ($p < 0.01$ and $p < 0.05$), simulating proximal and distal colon. FOS media led to a decrease in levels of BCFA in V1 from 1.71 to 0.31 mM ($p = 0.27$) and V2 from 3.50 to 3.03 mM ($p = 0.76$), stimulating the proximal and transverse colon.

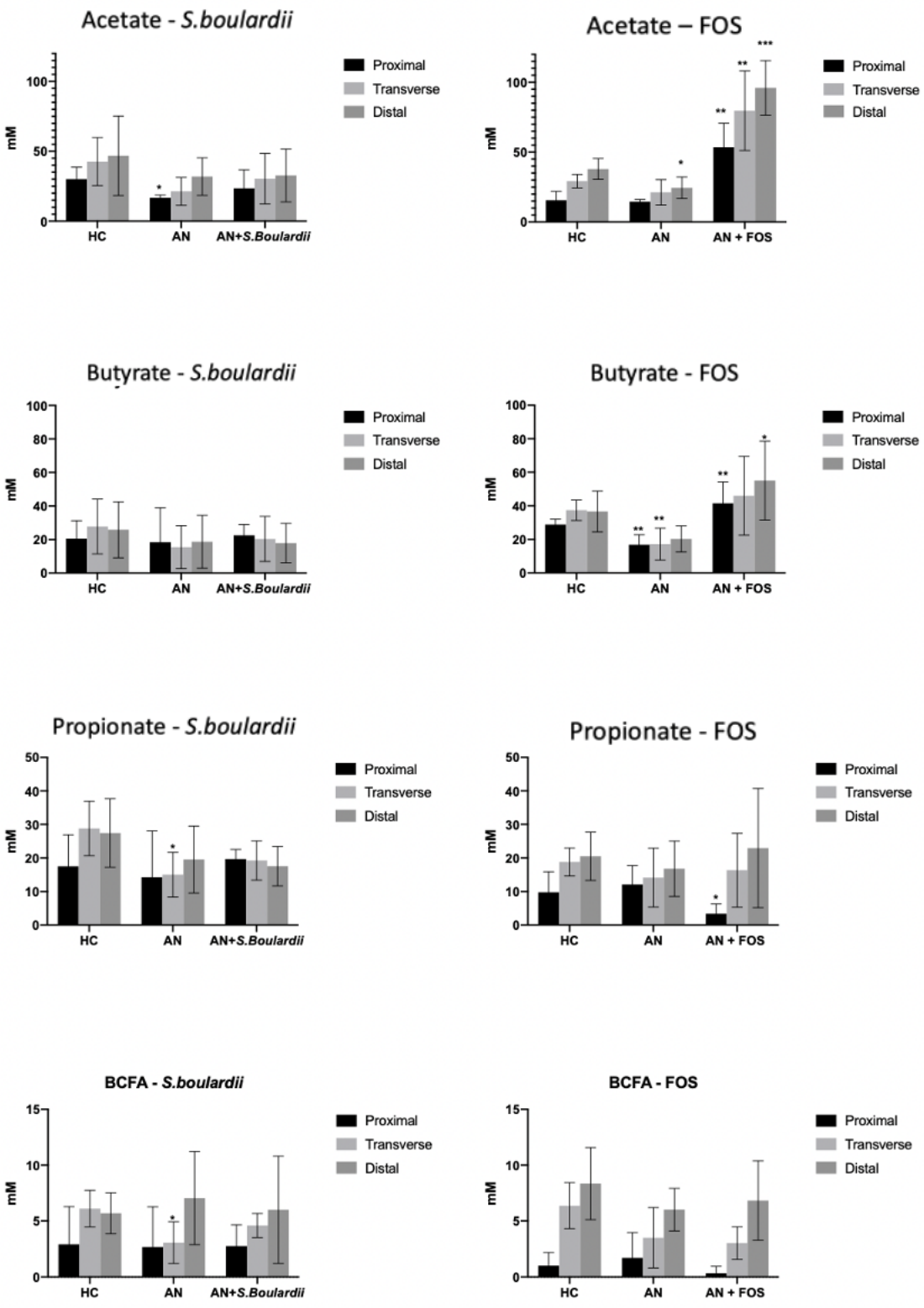


Figure 4. GC analysis. Acetate, propionate, butyrate and BCFA concentration in culture broths with SS1 (healthy media), SS2 (anorexic media) and SS3 (Anorexic media with the daily administration of *Saccharomyces boulardii* /FOS recovered from V1, V2 and V3 (stimulation of proximal, transverse and distal) of in vitro colonic model systems. Results are reported as Means ± SD (mM). For each measurement, significant difference in each vessel * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between SS1 and SS2, SS2 and SS3 are indicated.

4. Discussion

An in vitro gut model was used to explore the impact of dietary changes, as seen in AN, on the bacterial community and metabolic end products. The subsequent impact of a pre and probiotic was assessed to determine if such an intervention could enhance microbiota and metabolites. Compared to healthy-based medium, the results showed a limited gut microbial community and metabolite profile following nutrient restricted conditions. However, both pre and probiotic within these AN conditions resulted in recovery of bacterial populations and key metabolites. The data presented in this paper demonstrate this novel anorexic colonic model system and further expand on understanding the impact of starvation on the microbial community. This study has confirmed that reduced nutrients are associated with profound alterations of bacterial community structure, with reductions observed in total bacteria and several specific bacteria groups. In line with this, microbial diversity and composition in recent AN studies have reported differences to healthy and overweight individuals. However, this research goes further by linking these changes to alterations in microbial metabolites that might be pertinent for mental well-being and by exploring the outcome of experimental supplementation of pre and probiotics.

In the current study 'anorexic' intake resulted in decreased numbers of *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium prausnitzii* bacterial groups which may protect against gut mucosal barrier function abnormalities. In animal studies, induced starvation is associated with decreased mucin production which may lead to thinning of the mucosal layer and increased gut permeability known as "leaky gut" (Kelly *et al*., 2015; Petra *et al*., 2015). Such a state is linked to the immune system and GBA that can trigger the release of proinflammatory cytokines leading to higher levels of inflammation, correlated with depression (Sarkar *et al*., 2018). Those with conditions such as depression, for example, have often been reported to have increased

gut permeability which can also be affected by starvation (Seitz *et al*., 2019a; Tlaskalova-Hogenova *et al*., 2011). In the current model it is worth noting that the mucin concentrations were the same in the HC and AN models, suggesting that other factors might impact levels of these microorganisms. It is also of interest that *Akkermanisia muciniphila* has been observed to increase in AN patients, but not in the current study. This could be because in AN patients mucin starts to be a valuable substrate for gut microbiota with the reduction of several other nutrient sources; in a way that was not mimicked in the current experiment.

AN studies have observed decreased numbers of *Roseburia* (Prochazkova et al., 2019), findings supported in this in vitro study showing lower *Roseburia* and its dominant metabolites butyrate and propionate. However, a general increase in both bacterial groups after FOS and *S. boulardii* treatment was observed, suggesting that pre and probiotic administration may have a positive impact on the growth of these bacteria. SCFA are believed to have direct anti-inflammatory effects in the gut (Correa-Oliveira *et al*., 2016; Donohoe *et al*., 2011). Decreased *Roseburia* spp. abundance has been also found in patients with inflammatory bowel diseases (Imhann *et al*., 2018). In this study, both interventions increased *Roseburia* spp.. Higher levels of this microorganism may support reduced levels of inflammation and better gut barrier function, therefore having a positive influence on the GBA. As such the interventions used could provide a feasible approach in AN through positively influencing the microbial community.

Compared to HC feeding, decreased abundance of *Clostridium* spp. was observed in AN models, which coincided with decreased SCFA levels, and was in line with the findings of Borgo et al. (Borgo *et al*., 2017), who demonstrated that AN individuals' have relatively few carbohydrate fermenters, such as decreased numbers of *Clostridium*, which supports lower faecal butyrate concentration. Indeed, the introduction of the AN media led not only to large

reductions in the bacterial community, but also in the levels of SCFA and NTs, phenomena which have previously been observed as a result of dietary restriction (Genton *et al*., 2015). So far, several studies have explored faecal excretion of SCFA in AN individuals, compared to healthy individuals, noting a reduction of faecal concentrations of mainly butyrate and propionate (Borgo *et al*., 2017; Armougom *et al*., 2009; Mack *et al*., 2018). This is likely to be a result of the reduced fermentation capacity by the AN microbiota, characterised by lower levels of SCFA-producing bacteria and reduced carbohydrate levels, consistent with microbial signatures observed (Borgo *et al*., 2017). Recent studies have focused on SCFAs and their effect on the CNS. These fermentation products can cross the blood-brain barrier (BBB) and might influence early brain development (van de Wouw *et al*., 2017; Wang *et al*., 2011a). Borgo et al. (Borgo *et al*., 2017) evaluated SCFA levels in plasma finding that acetate was the only metabolite detected, suggesting transmission across the BBB. It should be considered that SCFAs act as key metabolites on peripheral tissues as a substrate for lipogenesis and acting on appetite regulation (Frost *et al*., 2014; Puertollano *et al*., 2014). SCFAs as signaling molecules could affect the GBA through a modulation of the ENS system, by stimulating gut hormones and cytokine release or directly via afferent neural pathways (Collins *et al*., 2012). Therefore, a hypocaloric diet typically characterised by low carbohydrate intake could result in lowering faecal SCFA levels in individuals with AN, likely by developing improved mechanisms in absorption and digestion of nutrients in the gut and prolonging the colonic transit time due to constipation (Genton *et al*., 2015; Boulange *et al*., 2016). The observations of SCFA changes could be of great relevance. Compared to HC and AN feeding, as the amount of starch as well as fibre may directly correlate to butyrate, acetate and propionate levels. SCFAs participate in endocrine regulation, and impact on physiological and psychological functions. SCFA are involved in regulating the expression of appetite hormones such as peptide YY (PYY) and ghrelin (Byrne *et al*., 2016). Ghrelin is known as an appetite-stimulating hormone and germ-

free mice have been observed to have significantly decreased ghrelin levels compared to conventional mice. Infusion of acetate increased both ghrelin levels and caloric intake, indicating that ghrelin expression, and thus appetite, can be enhanced through increased acetate levels (Perry *et al*., 2016). As such there are communication pathways among the gut microbiome, SCFA and anorexigenic/orexigenic hormones, that could hold potential in therapeutic feeding regimes for AN.

It is also worth considering other microbial groups that were not targeted in this study. For example, Enterobacteriaceae has been considered capable of enhanced energy extraction from the diet. Furthermore, Enterobacteriaceae has a role to play in the production of an anorexigenic bacterial protein, Caseinolytic protease B, which may be able to stimulate PYY production (Santonicola *et al*., 2019). While this microbial group has not been a focus for the current research it may also have a role to play in hormone secretion and satiety and thus warrants further study.

Studies have indicated that body mass index (BMI) and weight are positively related to both butyrate and propionate concentrations in stool samples (Borgo *et al*., 2017; Speranza *et al*., 2018) whereas butyrate levels have been observed to be negatively correlated with depression and anxiety scores. Both mice (Bailey *et al*., 2011) and human (Borgo *et al*., 2017)studies have indicated that behaviour disruption occurs alongside changes in gut microbiota. The current study also indicated that the introduction of pre and probiotic supplementation could positively impact on both the microbial community and SCFA levels without further modulating the diet. This is of great potential in regulating the impact of bacterial metabolic activity in the colon to improve gut health and mood.

This paper has demonstrated that five NTs in faecal fluid can successfully be measured by LC/MS. Only a few LC/MS methods have been described to determine plasma and urine NTs levels, and the uniqueness of gut model faecal supernatant NTs analysis and quantification may provide a clue about GBA connection. Whilst colonic cells are often considered as also required in the production of many NTs, following microbial fermentation in the absence on colonic cells all five NTs were detected to be at lower concentrations following AN-media compared with HC-media. 5-HT, DA and NE are classified by monoaminergic NTs and both human and animal studies stated that diet-induced starvation depletes central monoamines, leading to dysregulated NTs levels and receptor sensitivity (Cowen *et al*., 1996; Goodwin *et al*., 1987; Goodwin *et al*., 1990). 5-HT is synthesised from its precursor tryptophan, an essential amino acid that must be obtained through the diet (Petty et al., 1996). The lower levels of 5-HT observed in this AN model could be due to the low protein content, since tryptophan is a necessary precursor of 5-HT. A study from Prochazkova et al. (2019) (Prochazkova *et al*., 2019) found that decreased 5-HT, tryptophan and 5-HT metabolite levels in AN individuals' stool samples compared to healthy controls, and Bailer et al. suggested that AN individuals' have a reduction in serotonergic activity due to reduced dietary supplies of 5-HT precursors.

DA is involved in hedonic aspects food intake and satiety regulation, in AN, the concentration of DA metabolites in cerebrospinal fluid is decreased (Kaye *et al*., 1999). Both altered 5-HT and DA activity increase after recovery from AN (Kaye *et al*., 1999; Bailer *et al*., 2005) and, in the current study, after pre and probiotic supplementation with AN-feeding, levels of 5-HT and DA were recovered compared to AN-feeding especially with *S. boulardii* supplementation. This could suggest that pre and probiotics that target NTs (psychobiotics) may, by supporting the microbiota, lead to the enhancement of NTs levels. The therapeutic impact of these changes

remain to be determined, as the NTs levels here are lower than would be found in a host, although such changes may help to improve gut symptoms and mental health.

It is worth noting that there are several limitations associated with the use of model systems. For example, for NTs production it is often deemed necessary to have human cells present, the low levels of production that have been observed indicate the potential involvement of the microbial community in direct NTs production. In addition, whilst a limited nutrient environment has been modelled, it has not been possible to model the human secretions as they would naturally occur. To counter this, components like mucin and bile salts have been added to the media, but the amounts used may not be completely appropriate. Furthermore, AN is a multifaceted and complex condition; the idea of the model is to determine how dietary restriction might impact on the microbial community, starting from a 'healthy' bacterial population and the subsequent effect this might have on microbial metabolites. Addition of prebiotic and yeast added more sustenance to the media and therefore resulting in microbial growth. It could be argued that re-feeding would also result in such changes, but the approach of using pre and probiotics is designed to give a targeted response, using far fewer nutrients and resulting in positive changes to the microbial community and subsequently to neuro-metabolic related secretions. It is recognised that this study does not offer a replication of the in vivo bacterial community in AN, but can give an indication of the impact of drastic dietary changes on the microbial community and metabolites.

Following FOS feeding, positive metabolic alterations were observed from bacterial profiles and metabolite levels combined with increased abundance of 5-HT and GABA. These current findings indicate that prebiotics may be effective in the treatment of neurological problems including depression and anxiety. Under the *S. boulardii* administration, DA level were

increased sharply, although no research mentioned DA levels in faeces, some studies indicated DA to be found in blood (0.03 ng/ml) (Dugdale, 2021) and urine (65000 to 400000 ng/24 hrs) (Haldeman *et al*., 2021), indicating that probiotic yeast *S. boulardii* can boost microorganism NTs production within a physiological relevant range. The current data indicate potential for use of both pre and probiotics to modulate the microbiota and also factors associated with GBA.

DA, EPI and NE, three primary catecholamines derived from the presence of tyrosine, an amino acid found in dietary proteins. DA is further degraded into EPI and NE. All three NTs play a crucial role in maintaining the GI tract, including nutrient absorption, innate immune function and gut motility (Mittal *et al*., 2017). For example, EPI regulates smooth muscle relaxation and colonic motor function (Hirst and Silinsky, 1975). Compared with HC feeding, the level of catecholamines in AN feeding was significantly reduced in the current study. This may be due to the lack of L-tyrosine to support catecholamine synthesis, implying that AN individuals suffer from dysregulated NTs, mood disorder and GI tract disorders. FOS administration on AN feeding has a potential effect on the catecholamines production; and appeared to aid its synthesis.

5. Conclusions

This in vitro study examined the impact dietary restriction associated with AN on the gut microbial community and its metabolites. This model is not an exact replication of the in vivo bacterial community in AN but it does highlight the close link between reduced dietary intake, bacterial community and gut homeostasis status. The pre and probiotics show potential as psychobiotics, as administration showed promising and positive results in that might represent an approach to positively supporting the microbiota, metabolites and neuroactive compounds, in a way that could be of benefit to an AN host.

References

(2013). Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. J Anal Toxicol, 37(7), 452-74.

Armougom, F., Henry, M., Vialettes, B., Raccah, D. & Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. PLoS One, 4(9), e7125.

Association, A. P. (2013). *Desk reference to the diagnostic criteria from DSM-5 (R)*. American Psychiatric Publishing.

Audie, J. P., Janin, A., Porchet, N., Copin, M. C., Gosselin, B. & Aubert, J. P. (1993). Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. J Histochem Cytochem, 41(10), 1479-85.

Bailer, U. F., Frank, G. K., Henry, S. E., Price, J. C., Meltzer, C. C., Weissfeld, L., Mathis, C. A., Drevets, W. C., Wagner, A., Hoge, J., Ziolko, S. K., McConaha, C. W. & Kaye, W. H. (2005). Altered brain serotonin 5-HT1A receptor binding after recovery from anorexia nervosa measured by positron emission tomography and [carbonyl11C]WAY-100635. Arch Gen Psychiatry, 62(9), 1032-41.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun, 25(3), 397-407.

Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., Anselmetti, S., Scarone, S., Pontiroli, A. E., Morace, G. & Borghi, E. (2017). Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. Plos One, 12(6).

Boulange, C. L., Neves, A. L., Chilloux, J., Nicholson, J. K. & Dumas, M. E. (2016). Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Medicine, 8.

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

British Nutrition Foundation. (2016). Nutrition requirements. URL: https://www.nutrition.org.uk/bnfevents/events/158-healthyliving/resources/907-nutritionrequirements.html [26 October 2019].

Byrne, C. S., Chambers, E. S., Alhabeeb, H., Chhina, N., Morrison, D. J., Preston, T., Tedford, C., Fitzpatrick, J., Irani, C., Busza, A., Garcia-Perez, I., Fountana, S., Holmes, E., Goldstone, A. P. & Frost, G. S. (2016). Increased colonic propionate reduces anticipatory reward responses in the human striatum to high-energy foods. Am J Clin Nutr, 104(1), 5-14.

Collins, S. M., Surette, M. & Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. Nature Reviews Microbiology, 10(11), 735-742.

Correa-Oliveira, R., Fachi, J. L., Vieira, A., Sato, F. T. & Vinolo, M. A. (2016). Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunology, 5(4), e73.

Cowen, P. J., Clifford, E. M., Walsh, A. E., Williams, C. & Fairburn, C. G. (1996). Moderate dieting causes 5-HT2C receptor supersensitivity. Psychol Med, 26(6), 1155-9.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H. & Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and

evaluation of a more comprehensive probe set. Syst Appl Microbiol, 22(3), 434-44.
David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J. & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505(7484), 559-63.
Devereux, R., Kane, M. D., Winfrey, J. & Stahl, D. A. (1992). Genus- and Group-Specific Hybridization Probes for Determinative and Environmental Studies of Sulfate-Reducing Bacteria. Systematic and Applied Microbiology, 15(4), 601-609.
Dinan, T. G. & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol Motil, 25(9), 713-9.
Donohoe, D. R., Garge, N., Zhang, X., Sun, W., O'Connell, T. M., Bunger, M. K. & Bultman, S. J. (2011). The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab, 13(5), 517-26.
Dugdale, D. C. (2021). catecholamine blood test.

Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T. M. & Comelli, E. M. (2014). Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. Nutr Diabetes, 4, e121.

Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998). Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol, 64(9), 3336-45.
Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J. R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E. & Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nat Commun, 5, 3611.
Genton, L., Cani, P. D. & Schrenzel, J. (2015). Alterations of gut barrier and gut microbiota in food restriction, food deprivation and protein-energy wasting. Clin Nutr, 34(3), 341-9.
Gershon, M. D. & Tack, J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. Gastroenterology, 132(1), 397-414.
Goodwin, G. M., Cowen, P. J., Fairburn, C. G., Parry-Billings, M., Calder, P. C. & Newsholme, E. A. (1990). Plasma concentrations of tryptophan and dieting. BMJ, 300(6738), 1499-500.
Goodwin, G. M., Fairburn, C. G. & Cowen, P. J. (1987). Dieting changes serotonergic function in women, not men: implications for the aetiology of anorexia nervosa? Psychol Med, 17(4), 839-42.
Grenham, S., Clarke, G., Cryan, J. F. & Dinan, T. G. (2011). Brain-gut-microbe communication in health and disease. Frontiers in Physiology, 2.
Haldeman, C., Turley, K. & Foley, M. (2021). Catecholamines (Urine). URL: https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=167&contentid=catecholamines_urine [26 October 2021].
Harmsen, H. J., Raangs, G. C., He, T., Degener, J. E. & Welling, G. W. (2002). Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl Environ Microbiol, 68(6), 2982-90.

Harmsen, H. J., Wildeboer-Veloo, A. C., Grijpstra, J., Knol, J., Degener, J. E. & Welling, G. W. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. Appl

Environ Microbiol, 66(10), 4523-7.
Hermie J. M. Harmsen, P. E. F. S. G. W. W. (1999). A 16S rRNA-targeted Probe for Detection of Lactobacilli and Enterococci in Faecal Samples by Fluorescent In Situ Hybridization. Microbial Ecology in Health and Disease, 11(1), 3-12.
Herpertz-Dahlmann, B., Seitz, J. & Baines, J. (2017). Food matters: how the microbiome and gut-brain interaction might impact the development and course of anorexia nervosa. European Child & Adolescent Psychiatry, 26(9), 1031-1041.
Hirst, G. D. & Silinsky, E. M. (1975). Some effects of 5-hydroxytryptamine, dopamine and noradrenaline on neurones in the submucous plexus of guinea-pig small intestine. J Physiol, 251(3), 817-32.
Hold, G. L., Schwiertz, A., Aminov, R. I., Blaut, M. & Flint, H. J. (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Appl Environ Microbiol, 69(7), 4320-4.
Imhann, F., Vich Vila, A., Bonder, M. J., Fu, J., Gevers, D., Visschedijk, M. C., Spekhorst, L. M., Alberts, R., Franke, L., van Dullemen, H. M., Ter Steege, R. W. F., Huttenhower, C., Dijkstra, G., Xavier, R. J., Festen, E. A. M., Wijmenga, C., Zhernakova, A. & Weersma, R. K. (2018). Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. Gut, 67(1), 108-119.
Jalanka, J., Salonen, A., Salojarvi, J., Ritari, J., Immonen, O., Marciani, L., Gowland, P., Hoad, C., Garsed, K., Lam, C., Palva, A., Spiller, R. C. & de Vos, W. M. (2015). Effects of bowel cleansing on the intestinal microbiota. Gut, 64(10), 1562-8.
Jauregui Lobera, I. & Bolanos Rios, P. (2009). Choice of diet in patients with anorexia nervosa. Nutr Hosp, 24(6), 682-7.
Kamal, N., Chami, T., Andersen, A., Rosell, F. A., Schuster, M. M. & Whitehead, W. E. (1991). Delayed gastrointestinal transit times in anorexia nervosa and bulimia nervosa. Gastroenterology, 101(5), 1320-4.
Kaye, W. H., Bulik, C. M., Thornton, L., Barbarich, N., Masters, K. & Grp, P. F. C. (2004). Comorbidity of anxiety disorders with anorexia and bulimia nervosa. American Journal of Psychiatry, 161(12), 2215-2221.
Kaye, W. H., Frank, G. K. & McConaha, C. (1999). Altered dopamine activity after recovery from restricting-type anorexia nervosa. Neuropsychopharmacology, 21(4), 503-6.
Kaye, W. H., Gwirtsman, H. E., George, D. T. & Ebert, M. H. (1991). Altered Serotonin Activity in Anorexia-Nervosa after Long-Term Weight Restoration - Does Elevated Cerebrospinal-Fluid 5-Hydroxyindoleacetic Acid Level Correlate with Rigid and Obsessive Behavior. Archives of General Psychiatry, 48(6), 556-562.
Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G. & Hyland, N. P. (2015). Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. Frontiers in cellular neuroscience, 9, 392-392.
Kleiman, S. C., Watson, H. J., Bulik-Sullivan, E. C., Huh, E. Y., Tarantino, L. M., Bulik, C. M. & Carroll, I. M. (2015). The Intestinal Microbiota in Acute Anorexia Nervosa and During Renourishment: Relationship to Depression, Anxiety, and Eating Disorder Psychopathology. Psychosomatic Medicine, 77(9), 969-981.
Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O. & Vaiserman, A. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. Bmc Microbiology, 17.
Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. & Welling, G. W. (1995). Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol, 61(8), 3069-75.

Liu, Y., Gibson, G. R. & Walton, G. E. (2016). An In Vitro Approach to Study Effects of Prebiotics and Probiotics on the Faecal Microbiota and Selected Immune Parameters Relevant to the Elderly. PLoS One, 11(9), e0162604.

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

Macfarlane GT, G. G. (1995). Microbiological aspects of short chain fatty acid production in the large bowel.

Macfarlane, G. T. & Gibson, G. R. (1994). Metabolic activities of the normal colonic flora. In: Gibson, S. A. W. (ed.) Human Health - The Contribution of Microorganisms. London: Springer-Verlag.

Macfarlane, G. T., Macfarlane, S. & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microbial Ecology, 35(2), 180-187.

Mack, I., Cuntz, U., Grämer, C., Niedermaier, S., Pohl, C., Schwiertz, A., Zimmermann, K., Zipfel, S., Enck, P. & Penders, J. (2016). Weight gain in anorexia nervosa does not ameliorate the faecal microbiota, branched chain fatty acid profiles, and gastrointestinal complaints. Sci Rep, 6, 26752.

Mack, I., Penders, J., Cook, J., Dugmore, J., Mazurak, N. & Enck, P. (2018). Is the Impact of Starvation on the Gut Microbiota Specific or Unspecific to Anorexia Nervosa? A Narrative Review Based on a Systematic Literature Search. Current Neuropharmacology, 16(8), 1131-1149.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology (Reading), 142 (Pt 5), 1097-1106.

Mayer, E. A., Knight, R., Mazmanian, S. K., Cryan, J. F. & Tillisch, K. (2014). Gut Microbes and the Brain: Paradigm Shift in Neuroscience. Journal of Neuroscience, 34(46), 15490-15496.

Million, M., Angelakis, E., Maraninchi, M., Henry, M., Giorgi, R., Valero, R., Vialettes, B. & Raoult, D. (2013). Correlation between body mass index and gut concentrations of Lactobacillus reuteri, Bifidobacterium animalis, Methanobrevibacter smithii and Escherichia coli. International Journal of Obesity, 37(11), 1460-1466.

Mittal, R., Debs, L. H., Patel, A. P., Nguyen, D., Patel, K., O'Connor, G., Grati, M., Mittal, J., Yan, D., Eshraghi, A. A., Deo, S. K., Daunert, S. & Liu, X. Z. (2017). Neurotransmitters: The Critical Modulators Regulating Gut-Brain Axis. J Cell Physiol, 232(9), 2359-2372.

Morita, C., Tsuji, H., Hata, T., Gondo, M., Takakura, S., Kawai, K., Yoshihara, K., Ogata, K., Nomoto, K., Miyazaki, K. & Sudo, N. (2015). Gut Dysbiosis in Patients with Anorexia Nervosa. PLoS One, 10(12), e0145274.

Neuman, H., Debelius, J. W., Knight, R. & Koren, O. (2015). Microbial endocrinology: the interplay between the microbiota and the endocrine system. Fems Microbiology Reviews, 39(4), 509-521.

Perry, R. J., Peng, L., Barry, N. A., Cline, G. W., Zhang, D., Cardone, R. L., Petersen, K. F., Kibbey, R. G., Goodman, A. L. & Shulman, G. I. (2016). Acetate mediates a microbiome-brain-beta-cell axis to promote metabolic syndrome. Nature, 534(7606), 213-7.

Petra, A. I., Panagiotidou, S., Hatziagelaki, E., Stewart, J. M., Conti, P. & Theoharides, T. C.
(2015). Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. Clin Ther, 37(5), 984-95.

Prochazkova, P., Roubalova, R., Dvorak, J., Tlaskalova-Hogenova, H., Cermakova, M., Tomasova, P., Sediva, B., Kuzma, M., Bulant, J., Bilej, M., Hrabak, P., Meisnerova, E., Lambertova, A. & Papezova, H. (2019). Microbiota, Microbial Metabolites, and Barrier Function in A Patient with Anorexia Nervosa after Fecal Microbiota Transplantation. Microorganisms, 7(9).

Puertollano, E., Kolida, S. & Yaqoob, P. (2014). Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. Curr Opin Clin Nutr Metab Care, 17(2), 139-44.

Raatz, S. K., Jahns, L., Johnson, L. K., Crosby, R., Mitchell, J. E., Crow, S., Peterson, C., Le Grange, D. & Wonderlich, S. A. (2015). Nutritional adequacy of dietary intake in women with anorexia nervosa. Nutrients, 7(5), 3652-65.

Richardson, A. J., Calder, A. G., Stewart, C. S. & Smith, A. (1989). Simultaneous Determination of Volatile and Non-Volatile Acidic Fermentation Products of Anaerobes by Capillary Gas-Chromatography. Letters in Applied Microbiology, 9(1), 5-8.

Santonicola, A., Gagliardi, M., Guarino, M. P. L., Siniscalchi, M., Ciacci, C. & Iovino, P. (2019). Eating Disorders and Gastrointestinal Diseases. Nutrients, 11(12).

Sarkar, A., Harty, S., Lehto, S. M., Moeller, A. H., Dinan, T. G., Dunbar, R. I. M., Cryan, J. F. & Burnet, P. W. J. (2018). The Microbiome in Psychology and Cognitive Neuroscience. Trends Cogn Sci, 22(7), 611-636.

Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F. & Burnet, P. W. J. (2016). Psychobiotics and the Manipulation of Bacteria-Gut-Brain Signals. Trends in Neurosciences, 39(11), 763-781.

Savignac, H. M., Corona, G., Mills, H., Chen, L., Spencer, J. P. E., Tzortzis, G. & Burnet, P. W. J. (2013). Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. Neurochemistry International, 63(8), 756-764.

Seitz, J., Belheouane, M., Schulz, N., Dempfle, A., Baines, J. F. & Herpertz-Dahlmann, B. (2019a). The Impact of Starvation on the Microbiome and Gut-Brain Interaction in Anorexia Nervosa. Frontiers in Endocrinology, 10.

Seitz, J., Trinh, S. & Herpertz-Dahlmann, B. (2019b). The Microbiome and Eating Disorders. Psychiatric Clinics of North America, 42(1), 93-+.

Shehata, T. E. & Marr, A. G. (1971). Effect of nutrient concentration on the growth of Escherichia coli. J Bacteriol, 107(1), 210-6.

Sherwin, E., Rea, K., Dinan, T. G. & Cryan, J. F. (2016). A gut (microbiome) feeling about the brain. Current Opinion in Gastroenterology, 32(2), 96-102.

Speranza, E., Cioffi, I., Santarpia, L., Del Piano, C., De Caprio, C., Naccarato, M., Marra, M., De Filippo, E., Contaldo, F. & Pasanisi, F. (2018). Fecal Short Chain Fatty Acids and Dietary Intake in Italian Women With Restrictive Anorexia Nervosa: A Pilot Study. Front Nutr, 5, 119.

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

Stoffels, M., Amann, R., Ludwig, W., Hekmat, D. & Schleifer, K. H. (1998). Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. Appl Environ Microbiol, 64(3), 930-9.

Sweeney, T. E. & Morton, J. M. (2013). The human gut microbiome: a review of the effect of

obesity and surgically induced weight loss. JAMA Surg, 148(6), 563-9.
Tlaskalova-Hogenova, H., Stepankova, R., Kozakova, H., Hudcovic, T., Vannucci, L., Tuckova, L., Rossmann, P., Hrncir, T., Kverka, M., Zakostelska, Z., Klimesova, K., Pribylova, J., Bartova, J., Sanchez, D., Fundova, P., Borovska, D., Srutkova, D., Zidek, Z., Schwarzer, M., Drastich, P. & Funda, D. P. (2011). The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol, 8(2), 110-20.
Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444(7122), 1027-31.
van de Wouw, M., Schellekens, H., Dinan, T. G. & Cryan, J. F. (2017). Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite. J Nutr, 147(5), 727-745.
Walker, A. W., Duncan, S. H., McWilliam Leitch, E. C., Child, M. W. & Flint, H. J. (2005). pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol, 71(7), 3692-700.
Wallner, G., Amann, R. & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry, 14(2), 136-43.
Wang, B., Koga, K., Osuga, Y., Cardenas, I., Izumi, G., Takamura, M., Hirata, T., Yoshino, O., Hirota, Y., Harada, M., Mor, G. & Taketani, Y. (2011). Toll-like receptor-3 ligation-induced indoleamine 2, 3-dioxygenase expression in human trophoblasts. Endocrinology, 152(12), 4984-92.
Weltzin, T. E., Fernstrom, M. H., Hansen, D., Mcconaha, C. & Kaye, W. H. (1991). Abnormal Caloric Requirements for Weight Maintenance in Patients with Anorexia and Bulimia-Nervosa. American Journal of Psychiatry, 148(12), 1675-1682.
Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K. & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell, 161(2), 264-276.

Chapter 6

General discussion and future perspectives

General discussion

The etiology of EDs are multifaceted and complex; due to the presence of predisposing, precipitating, and perpetuating factors that allow the onset and maintenance of the disorders (Culbert *et al*., 2015). There are no effective treatments for AN and chronicity is high (Steinhausen, 2009). However, gut microbiota dysbiosis has previously been linked with psychological function and mental well-being including anxiety and depression, both of which are commonly comorbid with AN (Rogers *et al*., 2016). As such, the gut–brain axis is of particular interest in understanding the psychopathology of AN. In recent decades increase in knowledge has been gathered about the influence of the gut microbiota on various aspects of human health including brain health and behaviour regulation (Cryan *et al*., 2019). Gut microbiota composition is characterised by individual variability and it could be shaped by macronutrients and micronutrients (Rinninella *et al*., 2019; Yang *et al*., 2020). It is clear that diet is one of the most major factors shaping the gut microbiota, having a marked impact on gut microbial diversity, abundance and metabolic capacity of specific microbes (Wu *et al*., 2011; Moles and Otaegui, 2020; Redondo-Useros *et al*., 2020). However, whether and how dietary pattern affects the brain via its impact on the gut microbiota is only now becoming to gain more attention, with an increasing emphasis on the role of dietary pattern in maintaining optimal mental health status (Marx *et al*., 2017; Mörkl *et al*., 2018; Adan *et al*., 2019). Animals studies stating the potential of nutritional interventions on the microbiome and GBA communication have led to advancements in understanding of the role of diet in this bidirectional communication (Arumugam *et al*., 2011; Chassaing *et al*., 2015; Agus *et al*.,

2016). Alterations in gut microbiota composition can be due partly to the microorganisms established in the food we eat, for example in probiotic products. In this body of work (four experiments), dietary components/dietary pattern and how this impacts on gut microbiota has been studied along with a focus on metabolites to determine a potential impact on host behaviour/brain processes related neuroactive compounds. This research therefore enhance knowledge on potential psychobiotics treatments as well as increasing etiology AN understanding through the use of *in vitro* approaches.

In initial work conducted by the gut microbial community did not produce non-detectable amounts of NTs. However, only basal media was used in the absence of amino acids, the precursors of NTs. Thus, in Chapter 2, potato starch and tryptone were added to the basal media to provide a carbohydrate and protein source, additionally dietary NTs precursors (tryptophan, tyrosine and monosodium glutamate) were investigated with faecal microbiota of healthy female volunteers, using an *in vitro* batch culture fermentation system under physiologically relevant conditions. FISH was used to assess gut microbiota composition and microbial metabolic activities were analysed by LCMS and GC. NTs precursors were shown to enhance the microbial community, increased NTs and SCFA concentrations. Therefore, from this study it was determined that *in vitro* models of the human gut could be appropriate tools for studying NT production. Therefore, to determine if this production could be enhanced fermentations were carried out including prebiotics and probiotics in the batch systems (pH 5.5 and pH 6.9), as a simulation of proximal and distal colon (Chapter 3). This research resulted in significantly increased beneficial bacteria and NTs levels, such as 5-HT and DA, following both pre and probiotic fermentation. As such, these products are crucial for developing novel psychobiotics for treatment in cognitive conditions and could support

AN, especially considering gut microbiota previously correlated with gastrointestinal and behavioural problems in AN (Borgo *et al*., 2017). An *in vitro* batch culture is acceptable system for short period of incubation, to record the efficacy of substrates, gut microbial changes and metabolic activity. However, these systems do not enable the continuous supply of nutrients or to remove waste from the vessels; as a result bacteria will enter the stationary phase due to rapid progression and nutrients depleted, and also batch culture system only mimicking one colon part (Macfarlane *et al*., 1992). In chapter 3, both pH 5.5 and pH 6.9 *in vitro* batch culture systems were with the same fermentation media, this experiment was to explore neurotransmitter production at different pH values, but it is better to consider the nutrient gradient in digestive system (form proximal to distal). Furthermore, to appropriately study AN a model was designed based on dietary patterns to explore anorexic drastic dietary pattern changes could impact the microbial community using a continuous three-stage colonic fermentation model system (Chapter 4). This system was applied to better monitor microbial community changes in different colon segments through the modified nutrients gradient and gastrointestinal transit time in R-AN individuals. The idea of this chapter was to validate the anorexic gut environment conditions on the basis of typical dietary intake of R-AN compared to standard gut model media, with media content calculated based on micronutrients and macronutrients available within gut content. The faecal microbiota began form a 'healthy' gut environment and the experiment subsequently subsequnely modelled 'anorexic' gut nutrients. 16S rRNA sequencing identified differences between the healthy and anorexic diet-based microbiomes. This chapter developed R-AN diet-based gut model system (anorexic gut model media and retention time) and confirmed that nutritional deficiency is associated with profound alternations of microbiome and also varies in different colon segments. R-AN diet-based gut environment conditions drove significant changes in AN related gut microbiome including Enterobacteriaceae, *Lactobacillus, Roseburia, Clostridium*

and *Coriobacteriaceae*. The data of this study is in line with the current database of AN patients. Our informative findings may be important in determining how compositional changes in colon microbiota harbour GBA related trait biomarkers for AN individuals. This is the first time such a model has been developed, and this could aid research in the field, especially when considering a GBA component to AN.

Results from previous chapters showed promising effects of pre and probiotics at manipulating the microbiota and neuro-related factors. Whilst it was apparent that AN related dietary restrictions had a negative impact faecal bacterial community and metabolic activity. Hence, an *in vitro* approach to studying the microbial community and impact of pre and probiotics under AN related dietary restrictions was employed (Chapter 5). In this chapter, the effects of R-AN diet and pre and probiotic were investigated in order to understand the link between reduced dietary intake, with pre and probiotics impacting on bacterial community and gut homeostasis restoration. The gut microbiota can be modulated by modifying dietary components, introducing psychobiotics (endogenous factors that influence the gut microbiota via the pre and probiotics with bacterially mediated positive effects on mental health (Houghton *et al*., 2016). The experiment was conducted whilst encorporating the new model system. The model highlighted that nutritional changes associated with AN impact the microbial community, and that these changes can, at least in part, be restored through the use of pre and probiotic interventions. Additionally, significant differences in neurotransmitter levels were reported at AN media (SS2), confirming previous studies in AN individuals (Prochazkova *et al*., 2019), and emphasising the major hypothesis that diet has an important role in shaping gut microbiota and pre and probiotic could reshape gut microbial community whilst impacting on neurotransmitter pathways.

Overall, all experiments found bacterial and homeostasis status changes with nutrients and pre/probiotic supplementation. Furthermore, with gut model system, neurotransmitters levels and SCFAs were significantly recovered within a physiological relevant range with both *S. boulardii* and FOS intervention. The bacterial metabolites observed, SCFA and NTs, play a crucial role in maintaining nutrient absorption, gut motility, mood, cognitive function and act as appetite regulators. Furthermore, SCFA support gut barrier function. So, overall this research strongly highlight the potential for pre and probiotic intervention to be used to restore microbial disbalance caused by drastic diet changes, whilst supporting GBA.

Limitations

This research has limitations that should be taken into consideration. In chapter 2, 3 and 5, although NTs original of faecal supernatant can successfully be measured by LC/MS, it would normally be necessary to have human cells present; the low levels of production that have been observed indicate the potential involvement of the microbial community in direct neurotransmitter production. In chapter 2 and 3, batch culture inoculation was only conducted by healthy female volunteers, samples rather than AN samples, which is limited to exploring the true physiology and gut status of AN due to lack of comparison with HC and AN differences. However, the modelling was introduced to try to overcome this hurdle.

In chapter 4 and 5, whilst a limited nutrient environment has been modelled, it has not been possible to model the human secretions as they would naturally occur as, AN is a multifaceted and complex condition. The idea of the model was to determine how dietary restriction might impact the microbial community, starting from a 'healthy' bacterial population and studying effects this might have on microbial metabolites. It is recognised

that this study does not offer a replication of the *in vivo* bacterial community in AN, for example *Akkermanisia muciniphila* has been observed to increase in AN patients by previous research, but not in the this model system. This could be because in AN patients mucin starts to be a valuable substrate for gut microbiota with the reduction of several other nutrient sources in a way that was not mimicked in the current experiment. Furthermore, within the gut model system, the only variable parameter of nutrients composition was evaluated between HC and AN media impact on microbiota and homeostasis status, but it is necessary to consider another variable parameter of gut transit time effects on microbial composition and metabolic functions as delayed gastrointestinal transit times in AN have been indicated contribute to EDs by causing the patient to feel bloated, thereby exacerbating fear of fatness which may reflexly inhibit gastric emptying (Kamal *et al*., 1991).

Future work

Much more research needs to be done in AN associated GBA. Future work should focus on the importance of AN clinical samples relation to the specific considerations of subject population. Combined with the current research, future study will recruit patients with AN and request faecal samples for analysis by *in vitro* colonic system and explore how the microbiota in individuals with AN behave with different treatments and subsequently analyse gut microbial inhabitants and their metabolites. This allows differences in microbial function to be observed, along with ways to optimise this community to be explored.

Apart from faecal samples, blood, saliva and urine could be collected, NTs levels can be accurately analysed by these samples with blood testing showing the level of circulating hormones. Compared to the blood samples, salivary testing indicates what is actually being

utilised by the tissues, and NTs circulate in the blood and are filtered out by the kidneys via the urine, both urine and saliva collection are non-invasive to measure NTs levels accurately and may hold an important part in AN research of GBA. Combined with previous literature review, appetite hormones are biomarkers for AN individuals, appetite-regulating hormone also interact with the gut microbiota in shaping appetite and metabolic status (Alcock *et al.*, 2014; Torres-Fuentes *et al.*, 2015; Fetissov, 2017). Appetite hormone are therefore required to analyse a potential therapeutic method for treatment of metabolic disorder and food intake.

Further, an *in vivo* AN intervention study is suitable for this project to gain a deeper insight into the psychobiotics mechanistic effect of GBA in AN individuals. Accumulated psychobiotics intervention studies have suggested that potential correlation between alternations in the gut microbiota and psychopathological symptoms in patients with GBA related mental disorders (Akbari *et al*., 2016; Akkasheh *et al*., 2016; Majeed *et al*., 2018). Therefore it would be interesting to better understand how pre and probiotics modulate GBA in connection to AN individuals, correlation between physiological and psychological outcomes, such as gut metabolic activity and depression/anxiety levels; appetite hormone and behaviour correlation, GI symptoms score such as constipation and bloating linked to psychobiotics administration.

Overall, this is a fascinating research area. This thesis marks the beginning of many potential experiments, exploring the role of the GBA in AN and finding a possible microbiota based solution.

References

Adan, R. A. H., van der Beek, E. M., Buitelaar, J. K., Cryan, J. F., Hebebrand, J., Higgs, S., Schellekens, H. & Dickson, S. L. (2019). Nutritional psychiatry: Towards improving mental health by what you eat. Eur Neuropsychopharmacol, 29(12), 1321-1332.

Agus, A., Denizot, J., Thévenot, J., Martinez-Medina, M., Massier, S., Sauvanet, P., Bernalier-Donadille, A., Denis, S., Hofman, P., Bonnet, R., Billard, E. & Barnich, N. (2016). Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation. Sci Rep, 6, 19032.

Akbari, E., Asemi, Z., Kakhaki, R. D., Bahmani, F., Kouchaki, E., Tamtaji, O. R., Hamidi, G. A. & Salami, M. (2016). Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. Frontiers in Aging Neuroscience, 8.

Akkasheh, G., Kashani-Poor, Z., Tajabadi-Ebrahimi, M., Jafari, P., Akbari, H., Taghizadeh, M., Memarzadeh, M. R., Asemi, Z. & Esmaillzadeh, A. (2016). Clinical and metabolic response to probiotic administration in patients with major depressive disorder: A randomized, double-blind, placebo-controlled trial. Nutrition, 32(3), 315-320.

Alcock, J., Maley, C. C. & Aktipis, C. A. (2014). Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. Bioessays, 36(10), 940-9.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H. B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E. G., Wang, J., Guarner, F., Pedersen, O., de Vos, W. M., Brunak, S., Doré, J., Antolín, M., Artiguenave, F., Blottiere, H. M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K. U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Mérieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S. D. & Bork, P. (2011). Enterotypes of the human gut microbiome. Nature, 473(7346), 174-80.

Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., Anselmetti, S., Scarone, S., Pontiroli, A. E., Morace, G. & Borghi, E. (2017). Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. Plos One, 12(6).

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E. & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. Nature, 519(7541), 92-6.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., Codagnone, M. G., Cussotto, S., Fulling, C., Golubeva, A. V., Guzzetta, K. E., Jaggar, M., Long-Smith, C. M., Lyte, J. M., Martin, J. A., Molinero-Perez, A., Moloney, G., Morelli, E., Morillas, E., O'Connor, R., Cruz-Pereira, J. S., Peterson, V. L., Rea, K., Ritz, N. L., Sherwin, E., Spichak, S., Teichman, E. M., van de Wouw, M., Ventura-Silva, A. P., Wallace-Fitzsimons, S. E., Hyland, N., Clarke, G. & Dinan, T. G. (2019). The Microbiota-Gut-Brain Axis. Physiol Rev, 99(4), 1877-2013.

Culbert, K. M., Racine, S. E. & Klump, K. L. (2015). Research Review: What we have learned

about the causes of eating disorders - a synthesis of sociocultural, psychological, and biological research. Journal of Child Psychology and Psychiatry, 56(11), 1141-1164.
Fetissov, S. O. (2017). Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. Nat Rev Endocrinol, 13(1), 11-25.
Houghton, D., Stewart, C. J., Day, C. P. & Trenell, M. (2016). Gut Microbiota and Lifestyle Interventions in NAFLD. International Journal of Molecular Sciences, 17(4), 447.
Kamal, N., Chami, T., Andersen, A., Rosell, F. A., Schuster, M. M. & Whitehead, W. E. (1991). Delayed gastrointestinal transit times in anorexia nervosa and bulimia nervosa. Gastroenterology, 101(5), 1320-4.
Macfarlane, G. T., Gibson, G. R. & Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. J Appl Bacteriol, 72(1), 57-64.
Majeed, M., Nagabhushanam, K., Arumugam, S., Majeed, S. & Ali, F. (2018). Bacillus coagulans MTCC 5856 for the management of major depression with irritable bowel syndrome: a randomised, double-blind, placebo controlled, multi-centre, pilot clinical study. Food & Nutrition Research, 62.
Marx, W., Moseley, G., Berk, M. & Jacka, F. (2017). Nutritional psychiatry: the present state of the evidence. Proceedings of the Nutrition Society, 76(4), 427-436.
Moles, L. & Otaegui, D. (2020). The Impact of Diet on Microbiota Evolution and Human Health. Is Diet an Adequate Tool for Microbiota Modulation? Nutrients, 12(6).
Mörkl, S., Wagner-Skacel, J., Lahousen, T., Lackner, S., Holasek, S. J., Bengesser, S. A., Painold, A., Holl, A. K. & Reininghaus, E. (2018). The Role of Nutrition and the Gut-Brain Axis in Psychiatry: A Review of the Literature. Neuropsychobiology, 1-9.
Prochazkova, P., Roubalova, R., Dvorak, J., Tlaskalova-Hogenova, H., Cermakova, M., Tomasova, P., Sediva, B., Kuzma, M., Bulant, J., Bilej, M., Hrabak, P., Meisnerova, E., Lambertova, A. & Papezova, H. (2019). Microbiota, Microbial Metabolites, and Barrier Function in A Patient with Anorexia Nervosa after Fecal Microbiota Transplantation. Microorganisms, 7(9).
Redondo-Useros, N., Nova, E., González-Zancada, N., Díaz, L. E., Gómez-Martínez, S. & Marcos, A. (2020). Microbiota and Lifestyle: A Special Focus on Diet. Nutrients, 12(6), 1776.
Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A. & Mele, M. C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms, 7(1), 14.
Rogers, G. B., Keating, D. J., Young, R. L., Wong, M. L., Licinio, J. & Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. Molecular Psychiatry, 21(6), 738-748.
Steinhausen, H. C. (2009). Outcome of Eating Disorders. Child and Adolescent Psychiatric Clinics of North America, 18(1), 225-+.
Torres-Fuentes, C., Schellekens, H., Dinan, T. G. & Cryan, J. F. (2015). A natural solution for obesity: bioactives for the prevention and treatment of weight gain. A review. Nutr Neurosci, 18(2), 49-65.
Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H. Z., Bushman, F. D. & Lewis, J. D. (2011). Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science, 334(6052), 105-108.
Yang, Q., Liang, Q., Balakrishnan, B., Belobrajdic, D. P., Feng, Q.-J. & Zhang, W. (2020). Role of Dietary Nutrients in the Modulation of Gut Microbiota: A Narrative Review. Nutrients, 12(2), 381.