Investigating the Molecular, Metabolic, and Cognitive Effects of a Prebiotic in Psychosis

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A thesis submitted for the degree of Doctor of Philosophy

Michaelmas 2018

Abstract

Psychosis patients with lifetime exposure to antipsychotic medications often develop severe cardio-metabolic disorders and worsening cognitive impairments, therefore adjunct therapies that mitigate or attenuate these conditions would greatly enhance patient care. In recent years, the intestinal microbiome has emerged as an exciting area of research where gut microbial communities could influence cognitive and metabolic processes. However, experimental evidence of the molecular interactions between the gut and the brain as well as the translational potential into humans is currently unavailable.

The main objective of this thesis was to investigate the molecular, metabolic and cognitive effects of the prebiotic Bimuno™ galacto-oligosaccharide (B-GOS®) in psychosis. This was accomplished by establishing an olanzapine-induced weight gain rodent model and exploring the anti-obesity and pro-cognitive effects following daily prebiotics supplementation. Not only was increased levels of N-methy-D-aspartate receptor (NMDAR) subunit GluN1 observed, concomitant administration of prebiotics with olanzapine attenuated weight gain and increased circulating levels of acetate, a major product of prebiotics fermentation. The elevation of acetate imparts epigenetic changes involving the addition or removal of acetyl groups on histone molecules, therefore, an epigenetic effect of prebiotics was explored by comparing with direct sodium acetate administration. The prebiotic B-GOS® decreased brain activity of histone deacetylase (HDAC) and increased expression of HDAC-1 and HDAC-3, neither effects replicated by the supplementation of sodium acetate. Sodium acetate also did not attenuate olanzapine-induced weight gain and did not alter the expression of central NMDAR subunits suggesting that the therapeutic potential of the prebiotics B-GOS® may be mediated by alternative mechanisms. In psychosis patients after 12 weeks of B-GOS® supplementation, no significant changes on obesity-related anthropometric indices or serum immune biomarkers were observed. However, global cognitive functioning was beneficially affected and may have been driven by improvements in executive functioning, an NMDAR-dependent cognitive domain.

The findings reported in this thesis provides preliminary support for the clinical potential of B-GOS® to address cognitive impairments and weight gain in psychosis, and provides further precedent for microbiome-based strategies at large. Additional exploration of the indirect effects of other short-chain fatty acids and the involvement of the immune system will be required to confirm and extend the findings presented in this thesis.

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This thesis is dedicated to
my loving parents, and
especially my brother

If I have seen further it is by
standing on the shoulders of giants.

— Sir Isaac Newton (1642 - 1727)

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This dissertation has been completed as a part of an remarkable experience within the dreaming spires of Oxford, and would not have been possible without the intellectual and financial contribution from several important individuals.

Personal

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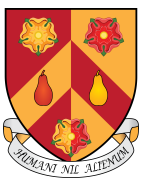
Institutional

This thesis was carried out at the Department of Psychiatry, University of Oxford from October 2015 to December 2018. It was financed by the Clarendon Fund, for without it, I would not have been able to carry out my research. It has been an honour to be a part of this scholarship community which went beyond the financial contributions.

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Lastly, I would like to honour the hundreds of little lives that have taught us more than we could have imagined. Without rodents, mice and other laboratory animals, many scientific breakthroughs would not have been possible, including findings reported in this thesis.





Declarations

This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except as specified in the text.

I state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Oxford or any other University of similar institution.

I state that each data chapter within this thesis has been submitted or is in the process of being submitted for publication. Accepted and in submission manuscripts are stated at the beginning of each chapter. All submitted/published papers are related directly to my field of study, and have been written whilst holding the status of a student for the DPhil.

In accordance with the guidelines, this thesis does not exceed 50,000 words, and it contains less than 150 figures.

Abstract

Psychosis patients with lifetime exposure to antipsychotic medications often develop severe cardio-metabolic disorders and worsening cognitive impairments, therefore adjunct therapies that mitigate or attenuate these conditions would greatly enhance patient care. In recent years, the intestinal microbiome has emerged as an exciting area of research where gut microbial communities could influence cognitive and metabolic processes. However, experimental evidence of the molecular interactions between the gut and the brain as well as the translational potential into humans is currently unavailable.

The main objective of this thesis was to investigate the molecular, metabolic and cognitive effects of the prebiotic Bimuno™ galacto-oligosaccharide (B-GOS®) in psychosis. This was accomplished by establishing an olanzapine-induced weight gain rodent model and exploring the anti-obesity and pro-cognitive effects following daily prebiotics supplementation. Not only was increased levels of N-methy-D-aspartate receptor (NMDAR) subunit GluN1 observed, concomitant administration of prebiotics with olanzapine attenuated weight gain and increased circulating levels of acetate, a major product of prebiotics fermentation. The elevation of acetate imparts epigenetic changes involving the addition or removal of acetyl groups on histone molecules, therefore, an epigenetic effect of prebiotics was explored by comparing with direct sodium acetate administration. The prebiotic B-GOS® decreased brain activity of histone deacetylase (HDAC) and increased expression of HDAC-1 and HDAC-3, neither effects replicated by the supplementation of sodium acetate. Sodium acetate also did not attenuate olanzapine-induced weight gain and did not alter the expression of central NMDAR subunits suggesting that the therapeutic potential of the prebiotics B-GOS® may be mediated by alternative mechanisms. In psychosis patients after 12 weeks of B-GOS® supplementation, no significant changes on obesity-related anthropometric indices or serum immune biomarkers were observed. However, global cognitive functioning was beneficially affected and may have been driven by improvements in executive functioning, an NMDAR-dependent cognitive domain.

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List of Abbreviations

5-HT2AR . . . . . serotonin 2A receptor
5-HT2CR . . . . . serotonin 2C receptor
ACC . . . . . acetyl co-enzyme A carboxylase
AMPK . . . . . AMP-activated protein kinase
ANOVA . . . . . Analysis of Variance
AUC . . . . . area under the curve
AWERB . . . . . Animal Welfare and Ethical Review Body
B2M β-2-microglobulin
BACS . . . . . Brief Assessment of Cognition in Schizophrenia
BBB . . . . . blood-brain barrier
BDNF . . . . . brain-derived neurotropic factor
B-GOS® . . . . . Bimuno^{TM} galacto-oligosaccharide
BMI . . . . . body mass index
CNS . . . . . central nervous system
COS . . . . . chitosan-oligosaccharide
CRP . . . . . C reactive protein
ddH_2O . . . . . deionised water
EDTA . . . . . ethylenediaminetetraacetic acid
ELISA . . . . . Enzyme-Linked Immunosorbent Assays
FDA . . . . . Food and Drug Administration
FOS . . . . . frutcto-oligosacchairde
GLP-1 . . . . . glucagon-like protein-1
GLP-1R . . . . . GLP-1 receptor
GOS . . . . . galacto-oligosaccharide
GPCR . . . . . G Protein Coupled receptor
GTA . . . . . glyceryl triacetate

H1R . . . . . histamine H1 receptor
HAT . . . . . histone acetyltransferases
HDAC . . . . . histone deacetylase
HDACi . . . . . HDAC inhibitors
HPA . . . . . hypothalamic-pituitary-adrenal
i.p. . . . . . intra-peritoneal
IL-6 . . . . . interleukin-6
IL-6R . . . . . interleukin-6 receptor
IL-8 . . . . . interleukin-8
ISHH . . . . . In-Situ Hybridization Histochemistry
LPS . . . . . lipopolysaccharide
LTP . . . . . long term potentiation
M1 . . . . . muscarinic receptor 1
M3 . . . . . muscarinic receptor 3
MCT-1 . . . . . monocarboxylate transporter 1
NAD . . . . . nicotinamide adenine dinucleotide
NMDAR . . . . . N-Methyl-D-aspartate receptor
NPY . . . . . neuropeptide Y
PAMPs . . . . . pathogen-associated molecular patterns
PVDF . . . . . polyvinylidene difluoride
QPCR . . . . . quantitative polymerase chain reaction
RCT . . . . . randomized controlled trial
RIPA . . . . . radioimmunoprecipitation assay
SCFA . . . . . short-chain fatty acid
SEM . . . . . standard error of the mean
TNF-α . . . . . tumour necrosis factor alpha
WAT . . . . . white adipose tissue
WHR . . . . . waist to hip ratio

ex nihilo nihil fit

Nothing can be made from nothing, we must all stand on our forefathers to better ourselves and the world around us. Tackling the challenges of the world today will require that more than ever.

— Parmenides of Elea's *De Rerum Natura*

1

Literature Review

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1.1 Introduction

The burden of schizophrenia is increasing globally. Current estimations reveal 21 million people worldwide are living with schizophrenia, and this count is expected to rise in parallel to population growth and aging [1]. Patients, regardless of sex, continue to experience decreased life expectancy of up to two decades [2] and can expect no significant difference in mortality as in- or outpatients [3]. Current frontline antipsychotic medications remain to be the most effective way of managing schizophrenia by suppressing positive psychiatric symptoms and decreasing the frequency of oscillation between relapse and remission [4]. However, prolonged use of certain antipsychotics may exacerbate negative and cognitive symptoms, particularly memory and executive functioning [5]. These cognitive impairments may be the most disabling aspect of the disorder and have been reported to not improve over time [6]. To further hamper recovery, significant weight gain (i.e., 7% increase) has been robustly documented with certain antipsychotics, even within the first few months of treatment [7]. Not only does weight gain often surface as a major driver of patient non-adherence, but it is a prelude to secondary health conditions (e.g., metabolic syndrome, cardiovascular disorders) which often escalates. These cardio-metabolic effetcs in combination with the perpetuity of negative symptoms and cognitive decline, the road to recovery is arduous and somewhat unattainable for many patients. Therefore, identification of interventions,

adjunctive or alternative, that attenuates cognitive impairments and weight gain without disrupting the therapeutic actions of antipsychotic medications which limits relapse, would greatly enhance patient care.

At the time of conception of this thesis, an understanding of the potential role that the intestinal microbiome may play in influencing cognition and metabolism was limited. Burgeoning interest arose from a cross-sectional comparison which hypothesized a unique taxonomic signature could be attributed to schizophrenia. This study characterized microbial genera in patients with schizophrenia and healthy matched controls and reported significantly higher levels of *lactobacillaceae* [8], a family from the Firmicutes phylum commonly found in the human enteric microbiome. This elevation was positively associated with severity of psychotic symptoms, and negatively with global assessment of functioning, implicating high abundnace of *lactobacillaceae* could be detrimental to health. Consistent with this finding, the oropharyngeal environment in patients with schizophrenia, compared to healthy controls, also showed over-representation of *lactobacillaceae*, though associations with symptomatology were not explored in these studies [9, 10]. Provided that the composition of microbial communities in the oropharynx may broadly reflect populations further downstream of the human digestive tract [11], it is then conceivable that changes in relative microbial abundance in the oropharynx may also be associated with schizophrenia symptom severity and cognitive functioning

as reported previously [8]. However, *lactobacilli* have generally been associated with beneficial health effects for the host [12] suggesting that the observed elevation in psychosis patients are the therapeutic result of antipsychotic use or a homoeostatic response to the disorder. This hypothesis has been supported by a recent cross-sectional study that proposed an unique taxonomic signature is associated with obesity. More specifically, in two independent cohorts higher relative abundance levels of bacterium within the Firmicutes phylum including *Bacilli* and *Clostridia*, and a reduction in the abundance of members in the *Bacteroidetes* phyum was reported [13]. Since Bacteroides and Firmicutes are the two major phyla that have been estimated to represent approximately 90% of the gut microbiome [14], it is conceivable that aberrant proportions may be associated with disease states. Although an obese taxonomic signature has not been replicated or supported in recent meta-analyses [15, 16], the involvement of the gut microbiome in metabolism and cognition highlights the need for further exploration in this field.

1.2 Brief History of Humans and Microbes

The gut microbiome has emerged as an attractive therapeutic target largely owing to the wide range of potential health benefits that could result by manipulating the bacterial composition through relatively simple and cost-effective interventions. In order to appreciate the pharmacological advancements made in this area, much of our understanding of the role of the gut microbiome is attributed to germ-free animals

and their unique physiology and behaviour phenotypes. These rodents exhibit a distinctve set of behavioural patterns including learning and memory impairments [17], significantly decreased locomotor activity and social cognition [18–20], decreased anxiety [18, 19, 21, 22] as well as resistance to high-fat diet-induced metabolic dysfunctions such as weight gain, insulin resistance and glucose intolerance [23]. Some of the phenotypes exhibited by germ-free rodents may appear to be beneficial (e.g., resistance to weight gain, decreased anxiety), however, they represent abnormal physiological developments that may lead to detrimental consequences. For instance, decreased anxiety may reduce the organisms' level of alertness to danger. Similarly, resistance to high-fat diet-induced weight gain may suggest a compromised lipid metabolism, which is a significant source of energy extraction that may jeopardize the overall health of the host. These studies in germ-free rodents have implicated intestinal microbial communities to be necessary for maintaining host health and likely exerts a strong contribution to metabolic processes.

1.2.1 The Gut Microbiome

In efforts of curating the intestinal microbiome population to favour beneficial bacteria, anti-microbial findings have led to the prolific belief in health conscious micro-environments that daily consumption of species such as *bifidobacterium* and *lactobacilli* would elicit physiological benefits. While strains of *bifidobacterium*, which are naturally found in humans, have been demonstrated to inhibit the growth

of pathogenic bacteria such as *Escherichia coli* [24, 25] and *lactobacillus* is considered a beneficial bacteria due to its anti-fungal effects, possibly through acidification of the colon allowing it to out-compete with pathogenic species [26], these protective demonstrations of anti-pathogenic characteristics are somewhat narrow evidence to be compelling for human ingestion, particularly in the present day. Nonetheless, these observations led to the further understanding of the role of the gut microbiome.

Under normal physiology, the enteric microflora functions symbiotically by maintaining the intestinal epithelial barrier and synthesizing essential vitamins, amino acids, and neurotransmitters from the fermentation of food ingredients that are indigestible by human enzymes. These metabolites have developed into a pervasive hypothesis of how changes in the intestines may influence peripheral and central physiology where neurotransmitters may act on the vagus nerve or small compounds may travel via the vascular system. This will be further detailed in section 1.5. Compounds can be purified or synthetically created into consumable supplements that are known as psychobiotics provided that they exhibit beneficial effects on the brain when ingested [27]. Any compound can exhibit a psychobiotic profile if it results in health benefits to psychiatric patients when ingested in adequate amounts. In contrast, high fibre diets which conferes beneficial effects for the intestinal environment, have been excluded from the psychobiotics definition as no experimental evidence is available to suggested

that they exhibit a psychobiotic profile. These psychobiotic compounds have expanded from exclusively encompassing probiotics (i.e., live organisms) to include prebiotics (i.e., non-digestible carbohydrates) and synbiotics (a combination of both probiotics and prebiotics) [28, 29].

1.2.2 Prebiotics

Although the definition and scope of prebiotics has undergone several revisions in the past [30, 31], at the time of writing this thesis, the term prebiotics have been expanded to include any substrates that are selectively utilized by host microorganisms to confer health benefits for the host [32]. These substrates are typically long-chain molecules that are non-digestible by human enzymes due to the -α or -β linkages between monosaccharide units (i.e., glucose, fructose, galactose) in this context. Current prebiotic research has focused on two major chemical groups, galactans (galactooligosaccharides (GOS)) and fructans (fructooligosaccharides (FOS) and inulin). In brief, FOS can be chemically categorized into short chain FOS and inulin-derived FOS (Figure 1.1). Short chain FOS is produced by fructose anabolism on to glucose molecules whereas inulins are fructose-based products and tends to have a greater degree of polymerization. The glycosidic linkages which contributes to the complexity of the prebiotic structure requires non-human enzymes for catabolism and metabolism, therefore ingested prebiotics often reach the colon undigested. Enzymes such as glycoside hydrolases, polysaccharide lyases

and carbohydrate esterases are responsible for the uptake and degradation of oligosaccharide structures within bacterial populations in the gut [33].

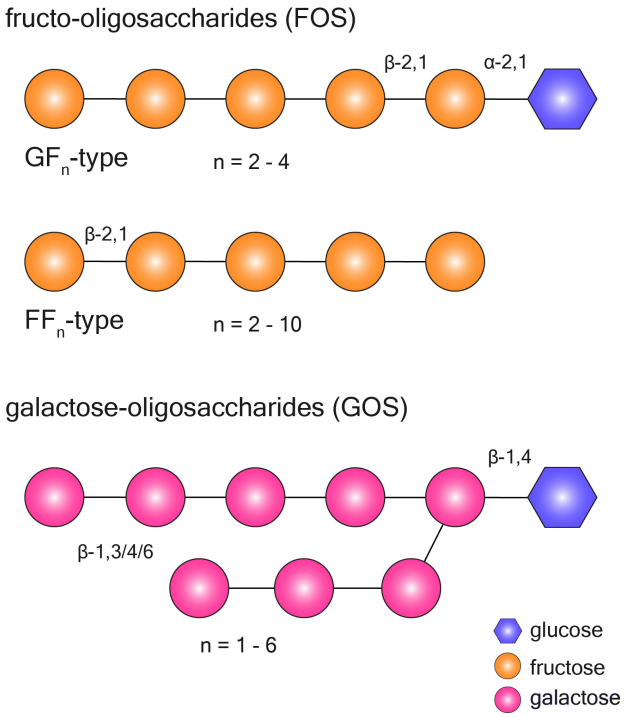


Figure 1.1: The chemical architecture of FOS and GOS based prebiotics. FOS typically contains a high degree of fructose molecules (FFn-type) or anchored with a glucose (GFn-type) molecule in a α-glycosidic linkage. GOS-based fibres typically contains a high degree of β-glycosidic linkages with variations in the polymerization structure anchored by a galactose molecule. n represents the number of repeating molecules

It is important to highlight that these substrates (prebiotics) are found naturally found within the mammalian diet, and can also be commercially available as dietary supplements in their purified form. Since the human intestine is known to be populated by millions of bacterial species, it is conceivable that there are several beneficial bacteria and enhancing the growth of more than one species may broaden the anticipated health benefits. Since indigenous commensal genus such as *lactobacilli* and *bifidobacteria* are believed to exert positive health benefits on

their host, significant amount of research has been focused on the abundance of members with these families. Prebiotics are utilized by microbes for energy in a metabolic process called fermentation and results in the production of short chain fatty acids (SCFAs) as well as other microbial products that may act locally to maintain the gut integrity or can be absorbed into the periphery and influence downstream pathways. Interestingly, specific phyla have been found to produce specific SCFAs wherein *Bacteroidetes* have been reported to synthesize acetate and proprionate, while *Firmicutes* mainly produces butyrate as a by-product [34]. Ingestion of prebiotics have been shown to proliferate the abundance of beneficial phyla. Infants who recieved daily formula containing up to 4g of an inulin/FOS mixture had higher abundance of *bifidobacteria* in their stools [35]. Similar findings have been reported in healthy adults where inulin (5-34g/day) or FOS (5-20g/day) supplementation consistently demonstrate positive bifidogenic shifts in the colonic microbiota [36]. Notably, the bifidogenic potential of prebiotics varies depending on which prebiotic is used. The studies in infants implied that GOS/FOS were the preferred fermentation substrates of *bifidobacteria*, whereas inulin and maltodextrin were relatively poor substrates for *bifidobacterium* metabolism. Even at a high dose of 10g/day, a few studies found that long chain inulin could not produce bifidogenic effects in the gut microbiome [37].

While significant microbiome research has been conducted using probiotic and

synbiotic interventions [38], the research focus of this thesis have been addressed using a prebiotic, and more specifically, a galacto-oligosaccharide formulation of prebiotics produced by Clasado Ltd called Bimuno^{TM} galacto-oligosaccharide (B-GOS$^{®}$).

1.3 Schizophrenia Pharmacotherapy and Current Limitations

First generation antipsychotic agents have relied on the co-administration of anticholinergic agents to manage the extrapyramidal side effects, despite the likelihood of further cognitive impairments, exacerbating tardive dyskinesia and potentially psychotic delirium [39]. Although second generation antipsychotics, which shifts the dopamine-centred pharmacology towards serotonin have addressed these effects, they have not spared the developement of cognitive impairments. In a small six month pilot study, reducing the dose of risperidone and olanzapine by 50% in patients with stable psychosis resulted in greater improvements in global cognitive scores [40], suggesting that at least, high doses of these antipsychotics may impede cognitive performance. While the intestinal microbiome has been implicated in cognition and psychological well-being, it remains to be determined whether these exacerbations are the result of direct interaction with the microbiome.

Long term use of second generation antipsychotic agents, such as olanzapine [41] and risperidone [42], have resulted in orexigenic phenotypes that are potentially dependent the gut microbiome. This drug-microbiome relationship was demon-

strated in rats where the co-administration of olanzapine and an antibiotic cocktail (which negatively influences the gut by limiting the growth of several microflora) ameliorated drug-induced weight gain [43], highlighting that an intact microbiome is necessary for drug-associated weight gain. A recent report has suggested that an unique taxonomic signature involving reduced species richness and diversity in the intestines is associated with higher body mass index (BMI) [13], thereby further suggesting that antipsychotic agents may negatively alter the microbiome to favour weight gain and other adverse effects. However, there is considerable debate as to whether antipsychotic agents would possess antimicrobial activity to influence the microbiome. A few studies have highlighted antipsychotic medications to be associated with potential anti-commensal [44] and anti-microbial [41] effects, though the reverse has also been reported where minimum concentrations of risperidone and olanzapine was unable to inhibit the growth of pathogeic bacteria [45]. It is interesting that antibiotics which slightly modify the gut microbiota and had similar antibacterial activity to second generation antipsychotic agents did not result in metabolic dysfunctions [43, 46]. Nonetheless a recent review has suggested that antipsychotic-related microbiome alterations may result in observed weight gain and metabolic disturbances, and are likely to involve inflammatory mechanisms and suppression of resting metabolic rate [47]. Together this highlights the necessity of further studies to confirm antipsychotic use in modulating the intestinal microbiota,

and metabolic effects (e.g., shifting energy expenditure to weight gain) at large.

1.3.1 Neurocognitive Impairments

Neurocognitive functioning has surfaced as a core feature of schizophrenia where more than 80% of patients exhibit significant impairments, even at first episode of illness [48]. One hypothesis that has gained significant traction to elucidate these impairments is the hypofunctioning of the N-methyl-D-aspartate receptor (NMDAR), a glutamate ionotropic receptor. Under normal physiological conditions, activation of the NMDAR's is a major trigger of long term potentiation (LTP) at synapses within hippocampal subregions, a fundamental cellular activity often interpreted as a reliable correlate for learning and memory [49]. It follows that the inhibition of hippocampal LTP induction using pharmacological agents (e.g., amino-phosphono-valeric acid [50]) or gene targeting techniques (e.g., NMDA subunit gene knockouts [51]) results in impaired spatial learning. It follows that cognitive improvements are observed upon restoration of NMDAR activity.

The NMDAR is composed of two obligatory GluN1 subunits and two regulatory GluN2A and/or GluN2B subunits that forms an ion channel usually blocked by a magnesium cation. Upon membrane depolarization and the concomitant binding of glutamate, the major excitatory neurotransmitter, and a co-agonist (glycine or D-serine), calcium ions are permitted to flux into the neuron. The NMDAR signalling complex is regulated at the glycine site by the endogenous antagonist

kyrenuric acid, whose synthesis is regulated by inflammatory cytokines. While members within the interferon family (e.g., γ, β, α) have been accepted as inducers of the kynurenine pathway, there is emerging evidence suggesting that inflammatory cytokines, interleukin (IL) -11β and IL-6 may also increase levels of kyrenuric acid. This is particularly significant in schizophrenia pathophysiology, as activating the kynurenine pathway can result in depletion of tryptophan, a primary amino acid precursor of serotonin as well as production of neuroactive metabolites that can influence the regulation of dopamine and glutamate [52].

Glutamate hypofunctioning largely stems from three major observations. The initial insight originated from the ability of NMDAR antagonists and dissociated anesthetics, such as phencyclidine [53] and ketamine [54–56], to recreate the full range of psychotic symptoms including negative, cognitive and physiological features in healthy volunteers. This led to deeper cell and molecular examinations that revealed similarities between persistent pharmacological blockade of the NMDAR and tissue from patients with schizophrenia [57]. Lastly, and perhaps most profoundly, the enhancement of NMDAR functioning using exogenous compounds reduced negative symptoms and variably improved cognitive impairments in psychosis patients [58, 59]. However, preliminary trials examining a direct glutamate-based pharmacotherapy have not revealed encouraging results, likely owing to modest potency, poor penetration through the blood-brain barrier (BBB), and the

development of tolerance over time [60, 61]. Notably, positive allosteric regulation of glutamate receptors has been proposed as potential therapeutic targets with promising subunit selectivity [62].

In light of these obstacles, the intestinal microbiome becomes a more interesting therapeutic target. Proof-of-concept studies have convincingly demonstrated a gut-brain axis. Most compellingly, a GOS-based prebiotic was demonstrated to increase attentional vigilance to positive stimuli, a psychological measure of optimism, and decrease waking cortisol levels, a broad indicator of stress in healthy volunteers [63]. Since the prebiotic used in this study was previously documented to enhance the proliferation of microbes, that do not express pathogenic molecules, such as *Bifidobacterium* and *Lactobacillus* [64], it is reasonable to attribute the improvement in psychological well-being to the prebiotic intervention. Furthermore, the same prebiotic induced psychological and neurocognitive benefits in rodents, where authors observed phenotypes such as significantly lowered levels of lipopolysaccharide (LPS)-induced anxiety [65], and improved cognitive flexibility, a critical executive function [66]. The gut microbiota has also been recently implicated in maintainence of BBB integrity and microglial maturity [67], which are common cellular markers of neuroinflammation. These cellular morphologies of neuroinflammation (e.g., activated astrocytes/microglial) are implicated in psychosis [68] suggesting that microbial-derived acetate may elicit central effects in addition

to NMDAR expression levels.

The serotonin 2A receptor (5-HT2AR) is a crucial aspect of antipsychotic pharmacology and is often attributed to the therapeutic profile of olanzapine. In a recent study, a prebiotic administration in rodents could normalize LPS-induced increase in 5-HT2AR expression, as well as other NMDAR subunits [65], implicating possible pro-cognitive effects of gut-based interventions. Interestingly, the immune system may be a mechanistic link as central levels of serotonin receptors are affected by systemic inflammation. LPS-induced inflammation can result in elevated levels of cortical 5-HT2AR transcripts as well as hippocampal serotonin release [69]. The therapeutic feasibility of prebiotics in schizophrenia and antipsychotic pharmacology, would be contingent on whether the fermentation of dietary fibres could interfere with the absorption of medication and/or affinity for their central receptors.

1.3.2 Antipsychotic-Induced Weight Gain

Unfortunately, advances in pharmacology has not been successful in replicating the antipsychosis efficacy of clozapine and olanzapine without the high propensity for weight gain, leaving patients with only a healthy diet and exercise as the main tools to combat obesity. Realizing the difficulty of adopting this strategy on a relatively sedentary and under-motivated clinical population, identifying molecular pathways of antipsychotic weight gain may inspire alternative approaches to drug-related weight gain, and the obesity epidemic at large. Research has shown that the

negative effects of obesity goes beyond cardiovascular and metabolic physiology to include detrimental effects on brain structure and function [70], furthering the potential escalations that can result from obesity.

Table 1.1: Liklihood of weight gain in commonly used antipsychotics. Adapted from recent systematic review of weight gain [71]. [a]Significantly greater increase in weight at >38 weeks, when compared with <6 weeks period in both antipsychotic previously prescribed and naïve groups in meta-analysis [72]. [b]Significant weight gain seen in antipsychotic naïve group even <6 weeks in the meta-analysis by [72]. [c]Weight neutral with duration of antipsychotic use in the meta-analysis [72]. [d]No significant difference in weight when compared with placebo in multiple treatment meta-analysis [73].

Antipsychotic	Propensity for weight gain
Clozapine	High
Olanzapine	High[a,b]
Chlorpromazine	Moderate
Quetiapine	Moderate[b]
Risperidone	Moderate[b]
Paliperidone	Moderate
Aripiprazole	Low[c]
Amisulpride	Low[c]
Asenapine	Low
Haloperidol	Low[d]
Ziprasidone	Low[c,d]
Lurasidone	Low[d]

While there are many proposed causes of antipsychotic induced weight gain this thesis will focus on the neurobiological mechanisms. Variations in binding affinities, particularly at the serotonin 2C receptor (5-HT2CR) [74, 75] could explain the differential weight gain profiles of antipsychotic agents. The serotonin receptor family is considered to be largely responsible for the therapeutic actions of olanzapine [74], a medication with one of the highest weight gain propensities [7]. While the mechanisms remains unclear, olanzapine-induced hyperphagia and weight gain was

ameliorated in female *htr2c*-null C57BL/6 mice treated with lorcaserin, a 5-HT2CR agonist [76]. Similarly, binding affinity for the histamine receptor H1 (H1R) has been reported to be the strongest predictor of antidepressant associated weight gain [77]. Chronic administration of centrally acting H1R antagonists have been shown to induce weight gain [78], likely involving a potent orexigenic neurotransmitter, neuropeptide Y (NPY) and AMP-activated protein kinase (AMPK) pathways. NPY signals through the hypothalamic Y1-5 receptors and has been implicated to attenuate olanzapine-induced weight gain in rats [79]. In this study, olanzapine induced significant upregulation of H1R and NPY expression and increased total food intake wherein co-administration with β-histines, a histaminergic H1R agonist restored normal expression, implicating the activation of H1R in attenuating olanzapine associated weight gain.

The hypothalamic-pituitary-adrenal (HPA) axis is a cellular network that plays an important role in regulating feeding [80], and to a lesser extent cognitive function [81]. Hypothalamic inflammation can disrupt leptin/ghrelin homeostasis, which are major appetite regulating factors. The expression of leptin by white adipocytes is largely due to a homoeostatic response to increased fat deposition as an attempt to regulate body weight. It follows that patients on antipsychotic medications with a high propensity for weight gain, such as clozapine and olanzapine, exhibit high levels of both leptin and inflammatory cytokines [82, 83]. Although

leptin/ghrelin homeostasis is relevant to appetite regulation, circulating levels of these proteins appear to be more related to BMI, rather than antipyschotic exposure [84]. This highlights other metabolic biomarkers such as C-peptide, glucose-dependent insulinotropic polypeptide, and adipsin may provide richer insights to antipsychotic weight gain, and the quandary of weight gain at large.

1.4 The Immune System

There are two interesting inflammatory biomarkers in the context of the microbiome and schizophrenia, interleukin-6 (IL-6) and C Reactive Protein (CRP). Elevated levels of both cytokines have been reported as a risk factor for schizophrenia [85, 86] and a potential predictor for first-episode and acute exacerbations of psychosis [87]. Similarly, elevated levels of serum IL-6 have been positively associated with illness duration [88], depressive symptoms [89] and cognitive impairments [90]. Increased levels of serum IL-6 may contribute to metabolic dysfunctions since downstream signaling proteins, such as soluble glycoprotein 130 and soluble IL-6 receptor, have been significantly elevated in patients with metabolic syndrome [91]. In parallel, elevated levels of CRP appears to be associated with higher BMI [92–94] and lower cognitive performance in schizophrenia [95]. This suggests that therapeutic strategies that could decrease levels of serum CRP, and IL-6, are relevant for further exploration. Recent meta-analyses have supported prebiotics [96] as well as probiotics [97] to have significant effects in reducing serum CRP concentrations.

These findings in conjunction with antiobesity observations from antibiotics [43, 98], compounds that also limit the growth of pathogenic bacteria in the gut, further implicates the involvement of the immune system in the gut-brain axis.

Neuroinflammation as a result of systemic inflammation is a prevalent theory in neurodegenerative disorders, and is also applicable to the gut-brain axis. Aberrations in the composition of the microbiome may increase gut permeability enabling LPS, and other pathogen-associated molecular patterns (PAMPs) into the systemic circulation [99, 100]. LPS, among other pathogen-derived molecules, stimulate pattern recognition receptors on immunomodulatory cells causing them to increase cytokine production [98]. These cytokines may interact with elements of the endocannabinoid system to modulate adipogenesis, leading to obesity [101]. Studies feeding high fat diets to rodents for the purposes of studing weight gain have often reported decreased gut-vascular permeability and metabolic distress, such as elevated cytokines and markers of oxidative stress [100]. This is in-keeping with the antiobesity profile of prebiotics where daily supplementation have resulted in decreased levels of circulating cytokines [102] as well as restoration of intestinal integrity through increasing expression of α-occludin and JAM-A [99], two transmembrane tight junction proteins that regulate intestinal permeability. Together these findings implicate aberrations in the intestinal microbiome may jeopardise the integrity of the intestinal barrier, leading to the activation of the immune system.

An inflammatory response may also result from the activation of pattern recognition receptors at the luminal membrane. Authors have posited that prebiotics may physically out-compete or displace PAMPs in activating these receptors to exert anti-inflammatory actions [28, 38]. In a similar line of reasoning, beneficial microbes whose growth are replenished by dietary fibres or probiotics may serve as physical barriers that can block pathogenic bacteria from activating pattern recognition receptors such as toll-like receptors, C-type lectins and inflammasomes [28, 38]. While the microflora and gut immune receptors interface harbours many unanswered questions, the main focus of this thesis remains on the impact of gut-related changes to peripheral inflammation on brain neurochemistry.

Interestingly, insights from germ-free mice have implicated the intestinal microflora to be significant in the hydroxylation of tryptophan into serotonin [103]. This pathway likely occurs in the enterochromaffin cells which are resident serotonin producing cells found along the intestinal epithelium. Although the receptors that enable bacteria to regulate serotonin expression remains to be identified, enterochromaffin cells have been found to modulate serotonin sensitive primary afferent nerve fibers, thereby allowing a possible pathway to communicate directly with the central nervous system [104]. Notably, neurotransmitters are not the only bacterial-derived products that allow the gut to communicate with the brain as discussed in detail in the next section 1.5.

1.5 Signalling Pathways Between the Gut Microbiota and the Brain

Several critical molecules have been eluded to affecting cognition and metabolism, this section discusses the potential pathways that have been proposed to facilitate the gut-brain communication.

1.5.1 The Vagus Nerve

The vagus nerve is the clear frontrunner in terms of gut-brain mechanisms as it physically connects the abdominal cavity to the brain, and is vital for afferent input from the periphery. This connection was given firm footing in prebiotic research from vagotomised mice studies. In one report, neurochemical and behavioural benefits that were observed with prebiotics supplementation was no longer detected in mice with a severed vagus nerve [106]. Interestingly, short term vagal nerve stimulation evoked anti-depressive effects [107, 108], implicating that changes in intestinal microflora, which can lead to vagal nerve stimulation, may beneficially alter neurochemistry involved in depression. Since direct vagal nerve stimulation has already been linked to an anti-inflammatory effect (reduction of pro-inflammatory cytokines [109]), it follows that anti-obesity and pro-cognitive effects of psychobiotics can be conferred, at least in part, by the vagus nerve. Nonetheless, examining the microbial-vagal nerve interface is beyond the scope of this thesis. While Figure 1.2 denotes the all the proposed mechanisms, future discussions will focus on the effect

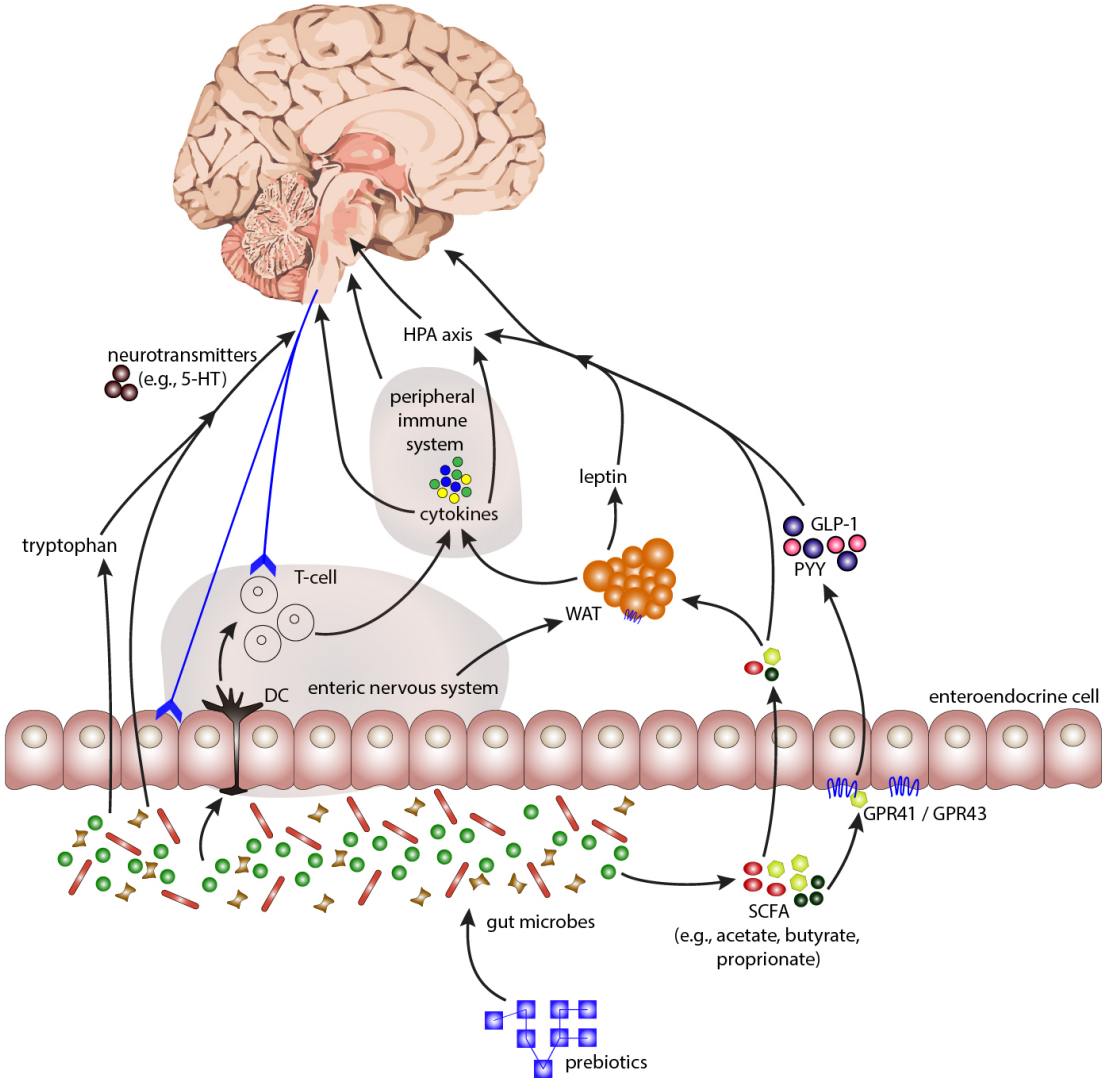


Figure 1.2: Overview of Possible pathways for the effects of prebiotics in the brain. Prebiotics are fermented by gut microbes, which result in the production of SCFAs, mainly acetate/butyrate/proprionate. These SCFAs may interact with free fatty acid receptors expressed on the luminal membrane of the intestines, or are transported into circulation through other uptake mechanisms. Once in the circulation, interactions with peripheral tissues such as white adipose tissue (WAT) may result in the production of inflammatory cytokines or appetite regulating hormones including leptin. The role of leptin in the immunomodulation and its influence on satiety via the HPA axis is somewhat understood. In parallel, the gut microbes may interact directly with the immune system through cellular interactions with members within the enteric nervous system. As a result, low grade systemic inflammation may result, potentially seeding neuroinflammation. Upon utilization of prebiotics, gut microbes produce tryptophan, which is a precursor to the serotonin neurotransmitter. It is estimated that 90% of the bodys' serotonin is produced in the gut. Whether these neurotransmitters innervate the vagus nerve or other mechanisms to influence the brain remains to be explored. Figure adapted from [105].

of SCFAs and the involvement of the immune system to the extent of circulating cytokines. Interestingly, some bacteria in the intestines are able to synthesize neurotransmitters and neuropeptides where estimates of over 90% of the body's serotonin is produced in the gut [110].

1.5.2 Short Chain Fatty Acids

When prebiotics are utilized by the intestinal microbiome, fermentation results in the production of SCFAs which are 2- to 5-carbon weak acids including acetate (C2), propionate (C3), butyrate (C4) and valerate (C5). These molecules are able to enter circulation and influence the expression and function of cells and tissues downstream [96]. Although the surface receptors mediating the uptake of SCFAs from blood into tissue have not yet been characterised, it seems likely that G protein-coupled receptors (GPRs) are involved. GPR-41/43 can be activated by all the major SCFAs, acetate, butyrate and proprionate [111] whose concentrations have been estimated respectively as 100-150umoL/L, 1-3umoL/L and 4-5umoL/L in healthy adult human peripheral blood. The ubiquitous expression of GPR-41/43 implicates substantial potential for these short chain carboylic acids to influence host physiology. While GPR-41 has been documented predominately in adipose tissue and GPR-43 in immune cells including monocytes and neutrophils [112], these findings further suggest a metabolic and cognitive effect of prebiotics. Furthermore, SCFAs-induced leptin production in adipocytes may be mediated by GPR-41 since

increased expression of GPR-41 has been found in the blood leukocytes of obese and type-II diabetes patients [111]. These findings implicate a possible satiety reducing mechanism as leptin is known to be an orexigenic hormone. Nonetheless, GPR-41/43 associated release of GLP-1, PYY and other peptides that regulate appetite and energy homeostasis remains to explored.

A highly contested area in SCFAs research is the identification of a specific role of a single fatty acid. In order for SCFAs to enter the peripheral circulation and influence brain physiology, it must be transported past the gut epithelium and survive drainage from the hepatic portal vein. In this view, significant reductions in butyrate (relative to acetate and propionate) have been reported across the gut epithelium, and significant reduction in propionate relative to acetate was observed across the liver [113]. These findings advocate acetate to be the predominant SCFA found in circulation. Several additional findings have supported this observation as butyrate is known to be the preferred energy source of coloncytes leading to sequesteration of the butyrate effect to the colon [114]. Nonetheless, butyrate has received substantial recognition as a key mediator of host-microbial crosstalk. It has been proposed that low circulating concentrations of butyrate is indicative of rapid metabolism by the peripheral tissues, rather than the existence of a concentration gradient [115]. Furthermore, butyrate has epigenetic implications as it inhibits histone deacetylases (HDACs) at peak plasma concentrations of 10.5-17.7mM [116], a therapeutic strategy

that has shown encouraging results in neurodegenerative disorders [117]. Similarly, epigenetic implications of acetate supplementation have already been reported [118] and restoration of cognitive behavioural impairments associated with NMDAR hypofunctioning could be rescued by oral administration of glyceryl triacetate (GTA) [119], suggesting a pivotal role of this SCFA in neurophysiology. Attributing a specific SCFA to the beneficial outcomes does not undermine the possibility that both acetate and butyrate act in concert since a SCFA mix restored microglial immaturity and malformation in the central nervous system (CNS) [120].

1.5.3 Anti-inflammatory Potential

Microglia are the resident immune cells of the CNS, mediating neuroimmune response by producing cytokines and acting as macrophages, thus it follows that they are a critical cellular marker for neurodegeneration, including psychosis [121]. The finding that SCFAs are essential for maturation and function of microglia [120] could imply an anti-inflammatory property of prebiotics. This profile has been reported in rodents after eight weeks of daily supplementation with a FOS based prebiotic [122]. Several outcomes were measured in this Alzheimer mouse model including significant decreases in serum inflammatory cytokines, attenuation of oxidative stress in spinal cord sections, and amelioration of brain tissue swelling and neuronal apoptosis. Similar observations were also demonstrated in a SOD193A transgene mice, a widely used model of amyotrophic lateral sclerosis [123]. In

this study, a ten week oral administration of 2% GOS delayed mortality by two weeks, likely due to the attenuation of motor neuron degeneration and reduced neuroinflammation as characterised by decreased inflammatory markers (i.e., iNOS and TNF-α) as well as activated astrocytes and microglia. Mice supplemented with GOS further exhibited protective cellular effects by shifting the oposing actions between anti-apoptotic and pro-apoptotic factors towards cell survival. Another oligosaccharide based prebiotic was shown to normalize increased hippocampal DNA oxidation marker 8-oxo-2'-deoxyguanosine as well as pro-inflammatory markers (i.e., TNF-α and IL-1β) [124]. Together these findings demonstrate a strong anti-oxidant and anti-inflammatory effect of oligosaccharides, and prebiotics in general.

1.5.4 Epigenetic Mechanisms

Epigenetic effects of SCFAs were briefly alluded to previously in chapter 1.5.3. The predominant pathway involves the enzyme equilibrium between HDAC and histone acetyltransferases (HAT), which together broadly regulates gene transcription. When this reaction equilibrium between HATs and HDACs is disturbed, transcriptional dysfunctions may result in phenotypes including unregulated cell proliferation (i.e., cancer). Therefore, the inhibition of HDAC or basal level maintenance of HAT that can promote the expression of prosurvival genes has become an attractive therapeutic option

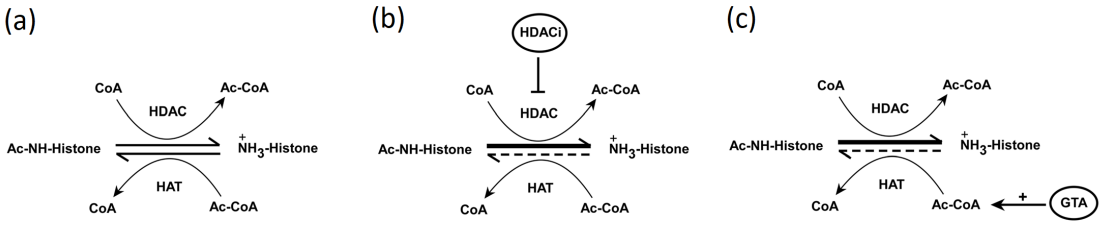


Figure 1.3: Histone acetylation homeostasis maintained by reversible actions of HAT and HDAC activities. (a) Normal cellular functions require preservation of pre-programmed histone acetylation signature by concomitant actions of HATs and HDACs. Undesirable activation of HDAC leading to the sifting of the equilibrium from acetylated form (Ac-NH2-histone) towards hypoacetylated (+NH3-histone) form of histone and that could be restored by two ways, either (b) using HDAC inhibitors or (c) using HAT activators or acetate supplementation using glyceryl triacetate (GTA)-like acetate donors facilitating acetylation catalysis by HAT perhaps by augmentation of cellular acetyl-CoA (AcCoA) levels. Figure adapted from [125].

Histone acetylation is one of many post-translational modifications that can be made onto nucleosome structures to alter the propensity of a gene to be transcribed or suppressed. The addition of an acetyl moiety on histone complexes weakens the electrostatic attraction between positively charged lysine histone tails and the negatively charged phosphate DNA backbone. The resulting structural alteration favours transcription as the local chromatin region is 'opened'thereby allowing transcriptional machinery (e.g., factors, activators, repressors, polymerases etc.) to bind. Reversely, the hydrolytic removal of acetyl groups allow the nucleosome to return to it's 'closed' state, preventing transcription. This epigenetic process is regulated by the opposing actions of HAT/HDAC, which respectively adds and removes acetyl groups from N-terminal lysine/arginine residues, and together maintains histone aceylation homeostasis. There are multiple ways to manipulate the reaction equilibrium including alteration of HDAC and HAT activity using HDAC

inhibitors (HDACi) or HAT activators. Manipulating the pool of reactants and products may also influence HAT/HDAC kinetics. Fermentation of prebiotics increase cellular pool of acetate, and subsequently acetyl-CoA, the necessary substrate of HAT thereby promoting gene transcription. Notably, acetyl-coA is carboxylated by acetyl-coA carboxylase (ACC) into malonyl-coA which plays a pivotal role in fatty acid metabolism via the citric acid cycle [126], implicating a role in nutrition metabolism and potentially antipsychotic weight gain.

A Brief Structural and Functional Overview

There are at least 18 members in the HDAC family and is categorized into four (1-4) classes based on sequence homology to *Saccharomyces cerevisiae*. Class I HDACs (HDAC-1, -2, -3, and -8) are nuclear proteins ubiquitously expressed in most tissues and cell lines and have a compact structure that is homologous to yeast RPD3. Class II HDACs are subdivided into IIa (HDAC-4, -5, -7 and -9 and its splice variant MITR) and IIb (HDAC6 and HDAC10) and share protein sequence homology to to yeast HDAC-1 protein. Class III HDACs are nicotinamide adenine dinucleotide (NAD) dependent enzymes and class IV which consists of only HDAC-11. The coalesced action of HDAC-1 and HDAC-2 appears to be critical to normal neuronal development where a double-knock out results in gross structural brain damage in a mice model [127].

Schizophrenia-like phenotypes such as impaired synaptic plasticity, short-term

memory and prepulse inhibition of the startle response have resulted from the overexpression of HDAC-1 [128], and to a lesser extent HDAC-2 [129]. As a result, compounds that inhibit HDAC, which could alter the expression of 2-20% of genes, have been shown to exert beneficial therapeutic potential in disorders associated with memory impairment [130]. Furthermore, down-expression of the gamma-aminobutyric acid neurotransmisson system, which is strongly implicated in psychosis, may result from hypermethylation of promoters and potential therapeutic strategies using HDACi, such as valproate, has been suggested to restore expression [131]. Interestingly, studies have shown that valporate enhances the clinical efficacy of clozapine or sulpiride but not with haloperidol or olanzapine [132], highlighting epigenetic mechanisms as a potential operative in antipsychotic pharmacology.

1.6 Overview of Data Chapters

At the time of conception of this thesis, an understanding of the potential role that the microbiota may play in influencing cognition and behaviour was limited. The findings presented herein have been designed to investigate whether a prebiotic intervention that has previously been shown to alter the intestinal microbiota architecture could be used to attenuate olanzapine-induced weight gain and promote pro-cognitive functioning. The findings are also discussed to support, or refute, potential mechanisms involved in the gut-brain axis. The preclinical chapters detail investigations in a well-established female rat model of olanzapine-induced weight

gain and explores the feasibility and neurobiological effects of co-administering prebiotics and exogenous acetate. This investigation had implicated the SCFA acetate as a critical mediator of the observed effects thus mechanistic details were further examined by comparing the epigenetic potential of the prebiotic with sodium acetate supplementation. Together, the experimental findings in the animal model composed a prelude to the clinical application of the prebiotic where a cross-over placebo-controlled study in patients with psychosis was conducted. The major clinical outcomes focused on changes in anthropometric indices and cognitive measures. The secondary outcome involved specific serum biomarkers to evaluate the involvement of the immune system in the gut-brain communication. The section below details the overall scientific questions and specific aims of each chapter in efforts of guiding the readers.

1.6.1 Chapter Specific Research Questions & Aims

Chapter 2: Can olanzapine-induced weight gain be attenuated by a prebiotic and how does this prebiotics influence NMDAR expression?

- To establish a rodent model of olanzapine-induced weight gain at a clinically relevant dose
- To examine whether the administration of the prebiotic Bimuno^{TM} galacto-oligosaccharide (B-GOS$^{®}$) could attenuate olanzapine-induced weight gain without compromising the central pharmacological action of the drug

- To evaluate whether B-GOS® influenced the actions of olanzapine on cortical and hippocampal NMDAR subunit proteins and transcripts
- To confirm the influence of B-GOS® and olanzapine on the concentration of plasma inflammatory biomarkers and SCFA acetate
- To examine putative mechanisms in the periphery by characterizing expression levels of ACC, GRP-43, and specific faecal gut microbial genera

Chapter 3: Is acetate sufficient to confer the metabolic and central profile of the prebiotic?

- Determine the individual influences of B-GOS® and sodium acetate intake on HDAC and HAT activities in the rat brain, and the expression of HDAC encoding genes
- Examined whether sodium acetate ingestion would prevent olanzapine-induced weight gain as we have reported for B-GOS®
- Examined the effect of sodium acetate supplementation on the abundance of selected gut bacterial genera which are altered by B-GOS®

Chapter 4: Can antipsychotic associated weight gain in patients with schizophrenia be attenuated by a prebiotic?

- To investigate whether anthropometric measurements of psychosis patients are influenced by the prebiotic B-GOS® administration

- To document the change in serum levels of inflammatory markers (CRP and IL-6) and the SCFA acetate
- To characterize the correlations between anthropometric measurements and serum markers at baseline, before and after B-GOS® supplementation

Chapter 5: Can cognitive impairments in patients with schizophrenia be attenuated by prebiotic supplementation?

- To examine whether cognitive improvements can be observed after B-GOS® supplementation in patients with psychosis
- To determine whether the prebiotic B-GOS® influences psychological well-being
- To characterize the correlations between cognitive tasks/domains and serum markers at baseline, before and after B-GOS® supplementation

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

The first woman to be awarded a Nobel Prize in 1903 for her contributions to physics, and again awarded the 1911 Nobel Prize for her contributions to chemistry. Her reflection above advocates that although we may fear the unknown, it should not limit our desire to understand the unknown.

— Marie Curie (1867 - 1934)

2

Prebiotic Attenuation of Olanzapine-Induced Weight Gain in Rats

The majority of data in this chapter has been published in the *Journal of Translational Psychiatry* "Prebiotic attenuation of olanzapine-induced weight gain in rats: analysis of central and peripheral biomarkers and gut microbiota" [133]

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Abstract

Olanzapine is an effective antipsychotic agent, however, due to its high propensity to confer weight gain, many psychosis patients experience difficulty with tolerance and adherence. Metabolic dysfunction in obese subjects can be attenuated by the prebiotic, B-GOS® and rodent studies have shown that central NMDAR and serotonin receptors, which are relevant to psychosis, can also be altered. The work presented here examined whether B-GOS® influenced body weight, central NMDA and serotonin receptors in a female model of olanzapine-induced weight gain at a clinically relevant dose. Putative mechanistic details were provided by levels of circulating acetate, IL-1β, IL-8 and TNF-α, liver acetyl co-enzyme A carboxylase (ACC), white adipose tissue (WAT), acetate receptor GPR-43, and specific faecal bacteria genera. Adult male and female Sprague-Dawley rats were administered a B-GOS® (0.5g/kg/day) solution or water for 21 days, and received a single, daily, intraperitoneal (i.p.) injection of olanzapine or saline on days 8-21. The intake of the prebiotic significantly attenuated olanzapine-induced weight gain only in

female rats thus further molecular studies in male rats were excluded from this chapter. The anorexigenic effect of B-GOS® was observed without changes to the frontal cortex 5-HT2AR blockade. Cortical GluN1 levels were elevated by olanzapine in the presence of B-GOS®. Plasma acetate concentrations increased following B-GOS® or olanzapine administration alone, but reduced when prebiotic and drug were administered in combination. This pattern was paralleled by hepatic ACC mRNA expression. The abundance of WAT GPR-43 mRNA was reduced by olanzapine, only in the absence of B-GOS®. Co-administration of B-GOS® and olanzapine also elevated plasma TNF-α, which is reported to influence lipid metabolism. Finally, B-GOS® elevated faecal *Bifidobacterium Spp.* and reduced some bacteria in the Firmicutes phylum, whilst olanzapine treatment either alone or with the prebiotic, was without effect. These data suggest that inclusion of B-GOS® as an adjunct to olanzapine treatment in schizophrenia may prevent weight gain and have benefits on cognitive function in psychosis. The role of acetate in these effects requires further investigation.

2.1 Introduction

Olanzapine is a second-generation antipsychotic agent with high efficacy in reducing the positive symptoms seen in psychosis patients, resulting in decreased tendency of relapse [4]. However, significant weight gain is a prominent side effect of second-generation antipsychotic medications, particularly clozapine and olanzapine robustly

conferring more than 7% increase in body weight in patients within the first year of treatment [7, 72]. Rapid weight gain decreases the tolerability of the medication and also serves as a prolusion to secondary health concerns including metabolic syndrome and cardiovascular disease [134], both of which are major drivers of morbidity and mortality in psychotic illnesses. Olanzapine-induced weight gain is not the only clinical obstacle, but cognitive impairments, which may be exacerbated by olanzapine [40], have been consistently observed in patients with schizophrenia, even at first episode of illness [48]. Therefore, an adjunctive intervention that reduces antipsychotic-mediated weight gain without compromising the antipsychotic effect of the medication, whilst potentially improving the cognitive deficits seen in schizophrenia, would be therapeutically beneficial.

2.1.1 Neuropharmacology of Olanzapine

The variation in binding affinities to dopaminergic and serotoninergic receptors represent a key pharmacological parameter that differentiates first and second generation antipsychotic medications and these affinity profiles are often attributed to their clinical effects [74]. In rodents, chronic olanzapine administration resulted in reduced binding densities of central 5-HT2AR and 5-HT1AR [135], and to a lesser extent 5-HT2CR [136]. This effect is considered important for the therapeutic actions of olanzapine on both positive and negative symptoms of schizophrenia, although the mechanisms underlying cognitive impairments, and how they are

affected by olanzapine, is not well understood. One pervasive hypothesis is the hypofunctioning of glutamate NMDAR, an ionotropic receptor that is composed of two obligatory GluN1 subunits and two regulatory GluN2A and/or GluN2B subunits, and in some cells, GluN3A.

Using NMDAR antagonists, studies have demonstrated that optimal NMDAR function is crucial for healthy neurodevelopment, learning and memory in rodents, and partial blockage induces a range of psychiatric and neurocognitive symptoms in healthy individuals [53]. Reductions in NMDAR levels in rodent brains have been reported following olanzapine administration, which may contribute to the development of cognitive impairments [137–139]. In these studies, repeated administration of subclinical doses (<2-4mg/kg) were sufficient to investigate cognitive deficits and antipsychotic-induced metabolic dysfunctions in male rats [43, 140]. However, it has been well documented that olanzapine-induced weight gain is sex-dependent in rodents where only female rats exhibit increased weight [140–142]. Furthermore, the half-life of olanzapine in rats is significantly shorter than in humans (i.e., 2.5-3hrs compared to the human average of 30hrs) suggesting that in order to achieve optimal occupancy of central receptors, doses above 7.5mg/kg are required, with full saturation achieved at 10mg/kg [143, 144]. Together, this implies that female rodent models at clinically relevant doses are required for translational research.

2.1.2 The Gut-Brain Communication

Recent studies have shown that augmenting intestinal microflora influences central glutamate neurotransmission, host body weight, metabolism, and systemic inflammation. Following 21 days of olanzapine treatment, faecal microbiota analysis in female rats revealed that significant weight gain was paralleled with significant shifts in phyla composition including increased abundance of Firmicutes, reductions in Bacteriodetes, as well as reduced diversity of Actinobacteria and Proteobacteria [43, 140]. Since germ-free mice have provided the precedent that a unique taxonomic signature may underlie diet-induced obesity [23, 145], a potential strategy to attenuate or ameliorate drug-induced weight gain could be through the intestinal microbiome.

The microflora may influence weight gain through the immune system. Circulating cytokines, in particular TNF-α, have recently been shown to influence processes in lipid metabolism [146] and appetite suppression [147]. Notably, suppression of energy expenditure due to a decreased rate of non-aerobic resting metabolism has also been attributed to shifts in the intestinal phylum composition in a female mice model of risperidone-induced weight gain [42], as well as in children [46]. The administration of probiotics (live cultures of beneficial bacteria) and prebiotics (dietary fibres that grow indigenous good bacteria) in obese and overweight adults resulted in corrections of key metabolic parameters (e.g., body weight, BMI) and serum markers (e.g., cytokines, acetate) [102, 147].

Fermentation of B-GOS® by microbes produces SCFAs including acetate, butyrate, and propionate [148, 149], which can act locally to maintain the intestinal barrier or distally to influence host physiology through fatty acid receptors GPRs [34]. In the brain, acetate uptake by glial cells has been proposed to occur in an NMDAR-dependent process [150]. This is consistent with previous observations where acetate supplementation elevated brain NMDAR subunit levels [66] and rescued hypofunctioning in rodents [119]. Interestingly, peripheral acetate may function as an appetite suppressant via central homeostatic mechanisms [151], or through increased hepatic levels of acetyl co-enzyme A carboxylase (ACC), the rate limiting enzyme of fatty acid synthesis, as seen in rats treated with olanzapine, following antibiotic administration [43]. Furthermore, reduced expression of the acetate binding receptor, GPR-43, in adipose tissue has been associated with high fat diet mediated weight gain in mice, and supplementation with SCFA cocktail, including acetate alone, restores GPR-43 abundance to control levels [152]. Together, the above observations suggest that prebiotic fermentation by gut microbiota may offer a safe and promising approach to improve the metabolic health of schizophrenia patients treated with atypical antipsychotics, and may have additional effects on central NMDAR function.

2.1.3 Specific Objectives

The aim of this study was therefore to:

- Establish a rodent model of olanzapine-induced weight gain at a clinically relevant dose
- Examine whether the administration of the prebiotic B-GOS® could attenuate olanzapine-induced weight gain without compromising the central pharmacological action of the drug
- Evaluate whether B-GOS® influenced the actions of olanzapine on cortical and hippocampal NMDAR subunit proteins and transcripts
- Confirm the influence of B-GOS® and olanzapine on the concentration of plasma inflammatory biomarkers and SCFA acetate
- Explore putative mechanisms in the periphery by characterizing expression levels of ACC, GRP-43, and specific faecal gut microbial genera

2.2 Materials & Methods

Clasado BioSciences Ltd., UK, supplied commercially available, purified B-GOS® powder. Custom DNA oligomers were purchased from Eurofins Genomics, UK. Olanzapine was purchased from Oxford Pharmacy Stores UK, and dissolved at 10mg/ml in sterile saline solution (0.9% NaCl) adjusted to pH 6.5 with concentrated hydrochloric acid.

Antibodies used in immunoblotting included receptor subunits anti-GluN1 (1:5000, Millipore), anti-GluN2A (1:5000, Millipore), anti-GluN2B (1:5000, Millipore), 5-HT2AR (1:1000, Abcam), 5-HT1AR (1:1000, Abcam) and anti-β-Actin

(1:1000000, Sigma) as a housekeeping protein. Horseradish peroxidase-conjugated goat anti-rabbit (1:10000, Bio-Rad) and goat anti-mouse (1:5000, Biorad) were used as secondary antibodies.

2.2.1 Animals

Male and female adult Sprague-Dawley rats (n=24), 220-250g (6-8wks), were housed three per cage with rodent chow and water *ad libitum* on a 12hr light/dark cycle (21±1°C, humidity 50±5%). All procedures were performed in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and associated Home Office guidelines. The local Animal Welfare and Ethical Review Body (AWERB) at the University of Oxford approved the procedures specific to this study.

Drug and Prebiotic Administration

Rodents were randomly assigned to one of four treatment groups: saline/water, saline/B-GOS®, olanzapine/water and olanzapine/B-GOS®. Depending on group, rats were provided with water or water supplemented with B-GOS® (approx. 0.5g/kg/day) for one week. This was followed by two weeks of daily i.p. injection of olanzapine (10mg/kg) or saline, during which water or B-GOS® administration continued. This dose of olanzapine was chosen because of its relevance to clinical use and i.p. injection of the drug is a standard route of delivery to rodents to ensure all animals receive the same dose, and is consistent with other olanzapine studies [43, 140]. Quantity of fluid intake B-GOS® concentration at 17g/L and

weight gain were monitored daily. Weights were normalised to the day of olanzapine administration in order to model the olanzapine effect and were expressed as percentage weight gain (i.e., [weight – weight on olanzapine administration] / weight on olanzapine administration).

Plasma and Tissue Collection

Twenty four hours after the final injection, animals were humanely culled by cervical dislocation and decapitated to obtain trunk blood, which was collected in ethylenediaminetetraacetic acid (EDTA) coated 2ml eppendorf tubes. All blood samples were centrifuged at maximum speed on a benchtop centrifuge for 5min. Top plasma phase was pipetted into a fresh eppendorf tube and frozen on dry ice. Whole brains were removed and bisected into left and right hemispheres on ice. One half was flash frozen in isopentane on dry ice for cryosectioning, while the other was dissected to obtain frontal cortex and hippocampal tissues for immunoblotting. All plasma and tissue samples were stored at -80°C until analysed.

2.2.2 Immunoblotting

Immunoblotting was carried out as previously described [64]. In brief, frozen brain tissues were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, UK) containing 0.1% (v/v) protease inhibitor cocktail. Protein concentrations of tissue homogenates were determined using Bradford's protein assay (Bio-Rad laboratories, CA, USA). Total protein (5-50µg) were loaded into a 4-20%

Mini-Protean® TGX™ precast Gel (Bio-Rad) and separated with SDS-PAGE in 1X running Buffer (25mM Tris base, 250mM Glycine, 0.1% SDS, diH_2O). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane overnight by electroblotting in 1X transfer buffer (25mM Tris base, 192mM Glycine, 20% methanol, diH_2O). The membrane blots were reactivated in methanol and incubated for 1hr at room temperature in blocking buffer (0.1% PBS-Tween20 with 5% skim milk). Primary and secondary antibodies were prepared in 0.1% PBS-T with 2% milk and added to blots sequentially for 1hr and 40mins respectively, separated by three 20min washes in Tris-buffered saline with PBS-T. Immunoreactivity was visualized with enhanced chemiluminescence (Amersham ECL Prime) and exposure to Hyperfilm (Amersham) at various time points. Densitometric analysis was performed using AlphaImager3400 to quantify relative protein expression using film exposures below the saturation point. β-actin was used as an internal control to normalize the optical density values in each sample.

2.2.3 In-Situ Hybridization Histochemistry

Fourteen micron sagittal sections (lateral 2.90mm, bregma 0.48mm, interaural 9.48mm) of frozen rat brain hemispheres were collected on glass slides (3sections/slide) and subjected to prehybridization treatment using an established In-Situ Hybridization Histochemistry (ISHH) protocol as previously described [153]. Oligodeoxyribonucleotide cDNA probes complementary to: GluN1 (bases 746-780,

NM008169.1), GluN2A (bases 1642-1676, NM008170.2) or GluN2B (bases 1758-1792, NM010350.2) were 3'-end labelled with [^{35}S]-dATP using terminal deoxynucleotidyl transferase (Promega, UK). Probes were then diluted in hybridization buffer, pipetted onto the tissue sections (1x106cpm/section), cover slipped and then incubated for over 16hrs at 34°C in lidded Perspex trays lined with filter paper soaked with SSC/50% formamide, four times. Posthybridization washes included two SSC rinses at room temperature to remove coverslips, three times in 0.5x SSC for 20min at 55°C, and two times in 0.5x SSC for 30min at room temperature. Slides were rinsed in diH_2O, dried and exposed to X-ray film (Kodak, Biomax MS) for 7-28 days with ^{14}C microscales. Average grey densities in the frontal cortex, and dentate gyrus, CA1 and CA3 subfields of the hippocampus in the three sections from each group were measured using computer assisted image analysis. Densities were calibrated to ^{35}SnCi/g tissue equivalents using commercial ^{14}C microscales.

2.2.4 Enzyme-linked Immunosorbent Assay

Levels of circulating inflammatory markers and acetate in plasma were measured using commercially available assay kits. All samples were assayed in duplicate and protocols were carried out according to manufacturers' instructions. Rat IL-1β and TNF-α Quantikine Enzyme-Linked Immunosorbent Assays (ELISA) were purchased from Bio-techne, UK; and interleukin-8 (IL-8) Sandwich ELISA kit from 2BScientific, UK. Colorimetric acetate assay kit was purchased from Abcam, UK.

2.2.5 Quantitative Polymerase Chain Reaction

RNA Extraction of Hepatic and White Adipose Tissue

Total RNA was extracted from the remaining fragment of cortical tissue using Tri-Reagent according to manufacturers' instructions, and reverse-transcribed to cDNA, using a commercial kit (Life Technologies, UK). The SYBR Green methodology (Power SYBER, Life Technologies, UK) was applied to amplify mRNA encoding ACC and GPR-43 in liver and WAT cDNA preparations, respectively, using previously published primer sets. Quantitative Polymerase Chain Reaction (QPCR) was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). All QPCR data are presented as fold change relative to saline controls and calculated using the $2\text{-}^{\Delta\Delta}$Ct relative quantitation method [154]. β-2-microglobulin (B2M) was used as the housekeeping gene.

Gut Microflora Analysis

The abundance of 16S rDNA encoding specific microbial genera and total bacteria in faecal pellets were analysed using QPCR and previously published primers [155]. Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) after mechanical disruption. The concentrations of isolated DNA were quantified spectrophotometrically by measuring absorbance at 260nm. The SYBR Green methodology (Power SYBER, Life Technologies, UK) was used to amplify 20ng DNA on a 7900HT Fast Real Time PCR System (Applied Biosystems, US).

Duplicate samples were held at 95°C for 10min and subjected to 40 cycles of denaturation (95°C for 15sec) and annealing/extension (60°C for 60sec). These QPCR data were also calculated using the $2^{-\Delta\Delta}$Ct relative quantitation method [154] and reported as fold-changes relative to the saline/water group.

2.2.6 Statistical Analyses

Statistical analyses were performed using SPSS version 22. Normal distribution of data was confirmed using Shapiro-Wilk test. All data are expressed as mean±standard error of the mean (SEM) unless otherwise stated. Body weight data was analysed with two-way repeated measures analysis of variance (ANOVA), comparing time (day), treatment (saline, olanzapine), and diet (water, B-GOS®), followed by *post hoc* bonferroni correction when significance was reached. Western blot optical density ratios, mRNA abundance and plasma changes were analysed using two-way ANOVA with bonferroni correction. Microbial data were analysed with non-parametric Kruskall-Wallis test, and Mann-Whitney U *post hoc* comparisons. The level of significance was set at 0.05 and p-values and F-statistics are reported as exact values.

2.3 Results

2.3.1 Rodent Olanzapine-Induced Weight Gain is Gender Dependent

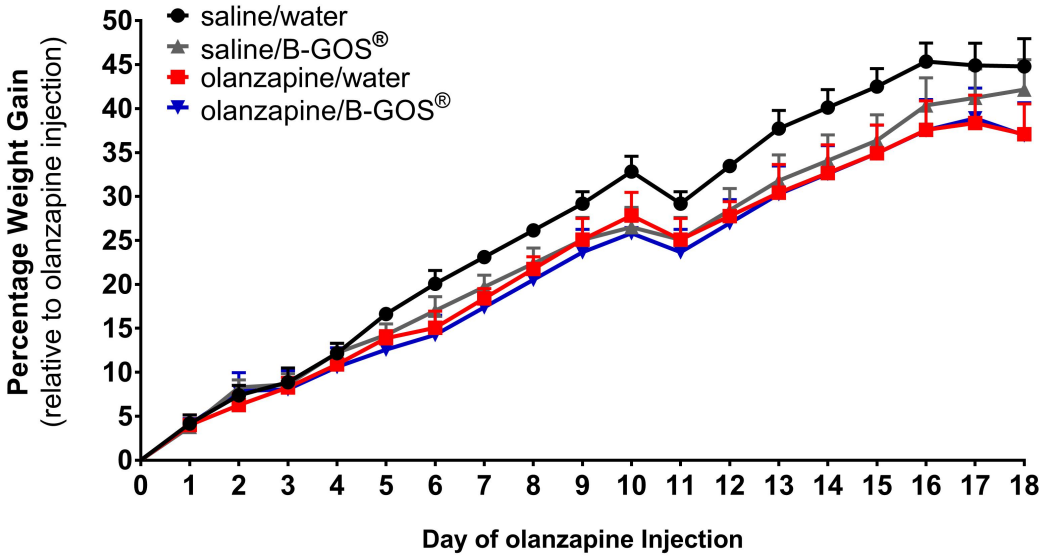
Male Rats

Ingestion of B-GOS® for one week prior to, and throughout a two week i.p.) olanzapine administration, at a clinically relevant dose, did not influence body weight in male rats overall (Figure 2.1). The olanzapine/water rats gained 8.6% less weight than saline/water controls (45.5% vs 34.9%; $p>0.05$), which was similar to the prebiotic fed groups (saline/B-GOS® 43.1% vs. olanzapine/B-GOS® 36.6%). Overall, repeated measures ANOVA did not reveal significant treatment or diet effects.

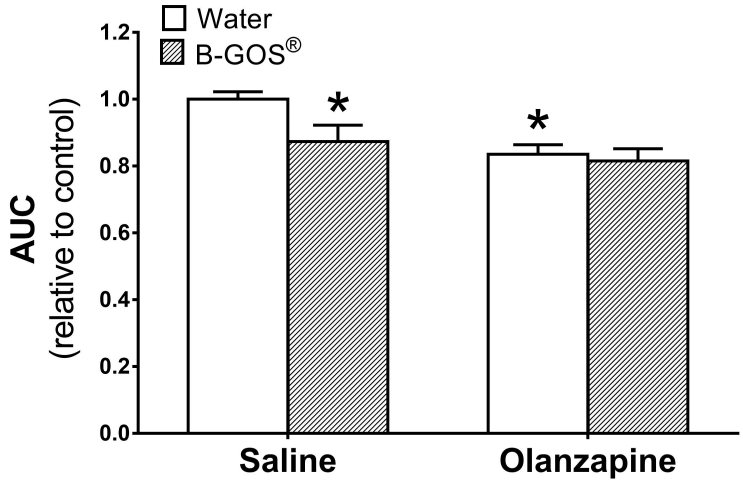
Analysis of the area under the curve (AUC) revealed a significant overall treatment effect ($F_{1,20}=9.857$; $p=0.005$), and a strong overall diet trend ($F_{1,20}=4.214$; $p=0.053$). *Post hoc* analysis suggests that these overall effects was driven by the reduction in weight when olanzapine was administered (saline/water vs. olanzapine/water, $F_{1,20}=6.288$; $p=0.021$), confirming that an olanzapine-induced weight gain model cannot be replicated in male rats. The strong trend in diet was driven by the administration of the prebiotic alone (saline/water vs. saline/B-GOS®, $F_{1,20}=10.732$, $p=0.004$) which resulted in decreased weight.

Female Rats

In female rats, weight gain was observed in rodents injected with olanzapine compared to saline, and this was attenuated by intake of B-GOS® (Figure 2.2). A



(a) Percentage Weight Gain



(b) Area Under the Curve

Figure 2.1: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on weight gain in adult male rats. Animals ingested B-GOS® seven days prior to, and throughout the two-week administration of olanzapine. Percentage weight gain was calculated from the day before olanzapine administration. Results are expressed as mean±SEM for each group (n=6); *p<0.05, compared to the saline/water group.

significant time x treatment interaction ($F_{13,260}$=7.958; p<0.001) was observed, and *post hoc* analysis revealed that this was driven by weight gained by olanzapine/water rats on days 8-14 (p<0.05). There was also a significant diet x treatment interaction

($F_{1,20}$=5.102; p=0.035), where overall weight gain in olanzapine/B-GOS® rats was less than olanzapine/water animals ($F_{1,20}$=7.467; p=0.013). A time x diet x treatment effect was not observed ($F_{13,260}$=0.124; p>0.05), indicating B-GOS® did not influence overall weight gain during the two week olanzapine and saline injections.

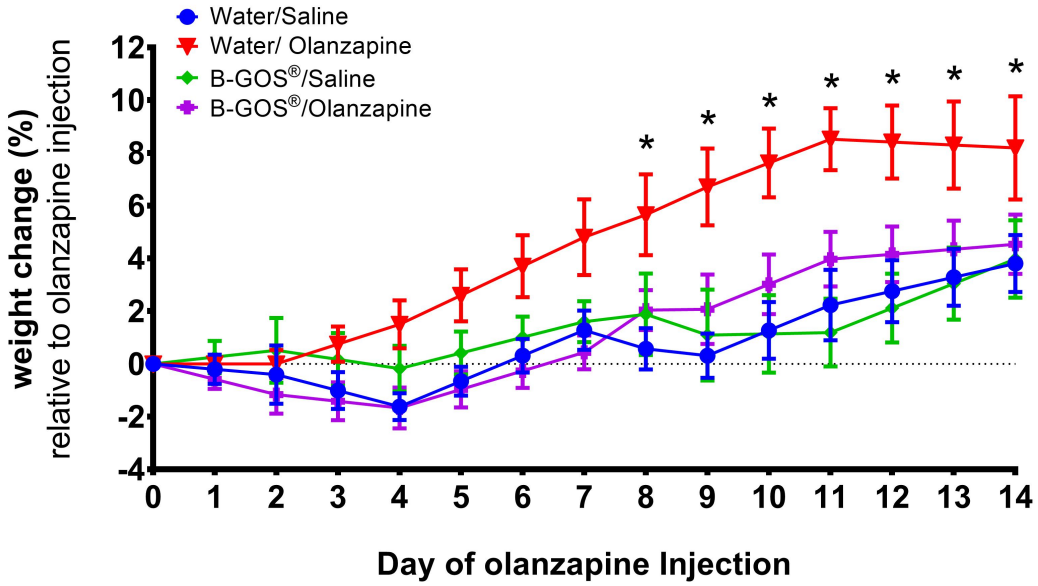
The analysis of AUC illustrates the diet x treatment interaction ($F_{1,20}$=4.629; p=0.044; Figure 2.2b). Animals in the olanzapine/water group experienced significantly greater weight gain than saline/water controls (p=0.005); this change was attenuated by B-GOS® (water/olanzapine vs. B-GOS®/olanzapine, p=0.01).

2.3.2 Brain Expression of Serotonin Receptors & NMDAR Subunits

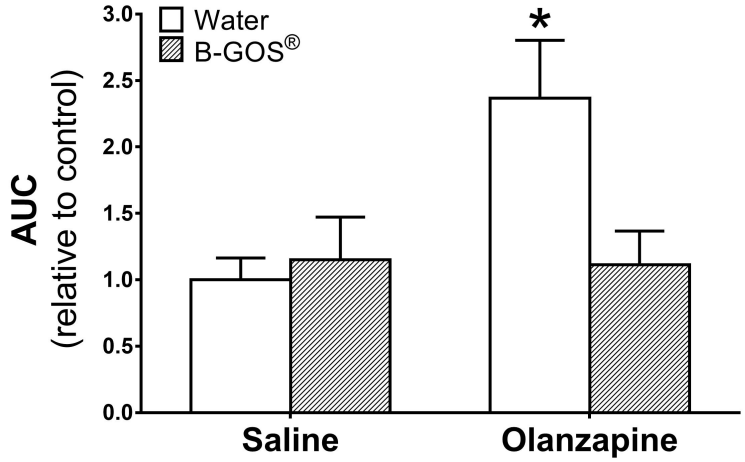
Further molecular changes were examined in the female model of olanzapine-induced weight gain. The effect of B-GOS® on the GluN subunits were also explored in male rats and included in appendix A.1 (mRNA expression A.1.1; protein levels A.1.2).

Administration of olanzapine, independent of B-GOS® supplementation, resulted in a significant effect of treatment ($F_{1,17}$=64.582; p<0.001; Figure 2.3c) on cortical 5-HT2AR protein levels where a 30% decrease was observed. No significant effects were observed in 5-HT2AR mRNA abundance (Figure 2.3d), though a diet x treatment trend ($F_{1,17}$=4.403; p=0.051) was noted. There was no significant effect of olanzapine or B-GOS® on cortical 5-HT1AR protein or mRNA (Figure 2.3a-b; p>0.05).

An overall effect of treatment ($F_{1,20}$=8.683; p=0.01) on cortical levels of GluN1



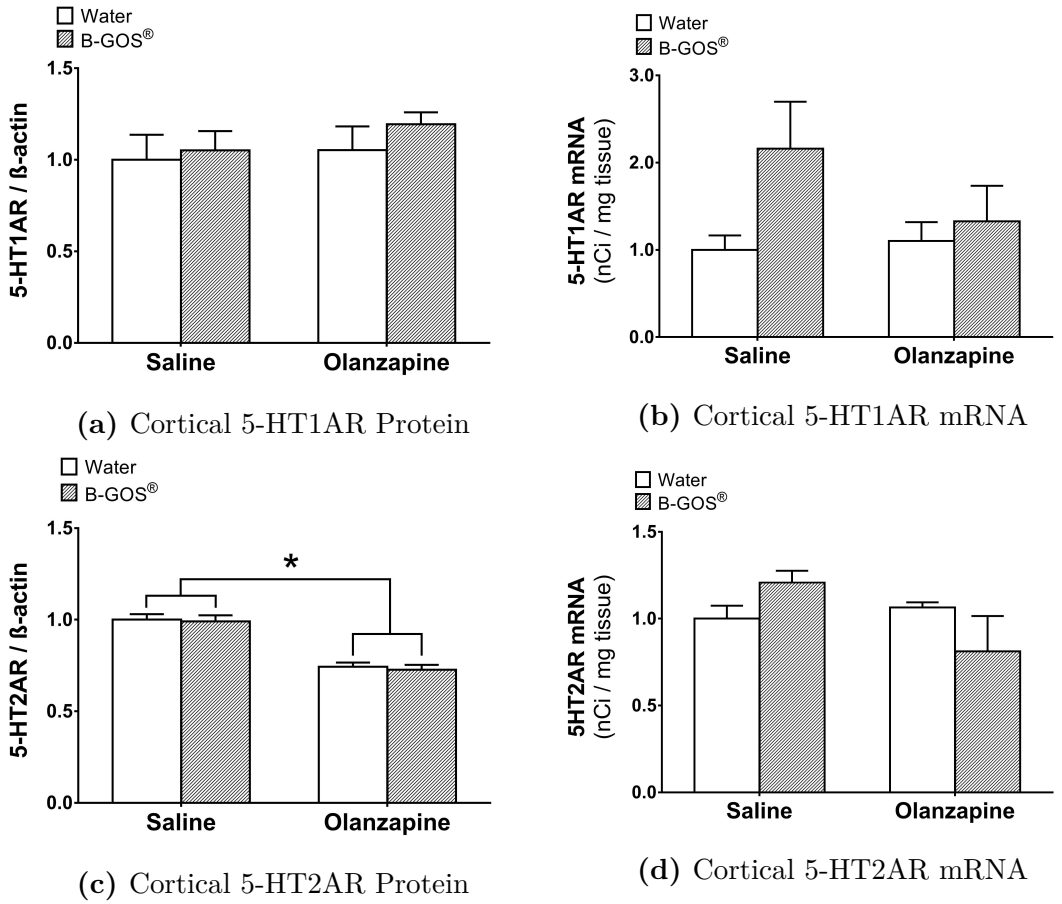
(a) Percentage Weight Gain



(b) Area Under the Curve

Figure 2.2: Effect of B-GOS® ingestion and olanzapine administration on weight gain in adult female rats. Animals ingested B-GOS® seven days prior to, and throughout the two week administration of olanzapine. (a) Percentage weight gain was calculated from the day before olanzapine administration. (b) Area under the curve analysis. Results are expressed as mean±SEM for each group (n=6); *p<0.05, compared to the saline/water group.

was detected (Figure 2.4a). *Post hoc* analysis revealed that olanzapine increased GluN1 levels in B-GOS® fed rats only (olanzapine/B-GOS® vs. olanzapine/water: +13%; $F_{1,20}$=11.723; p=0.028), whilst mRNA levels remained comparable between



(a) Cortical 5-HT1AR Protein

(b) Cortical 5-HT1AR mRNA

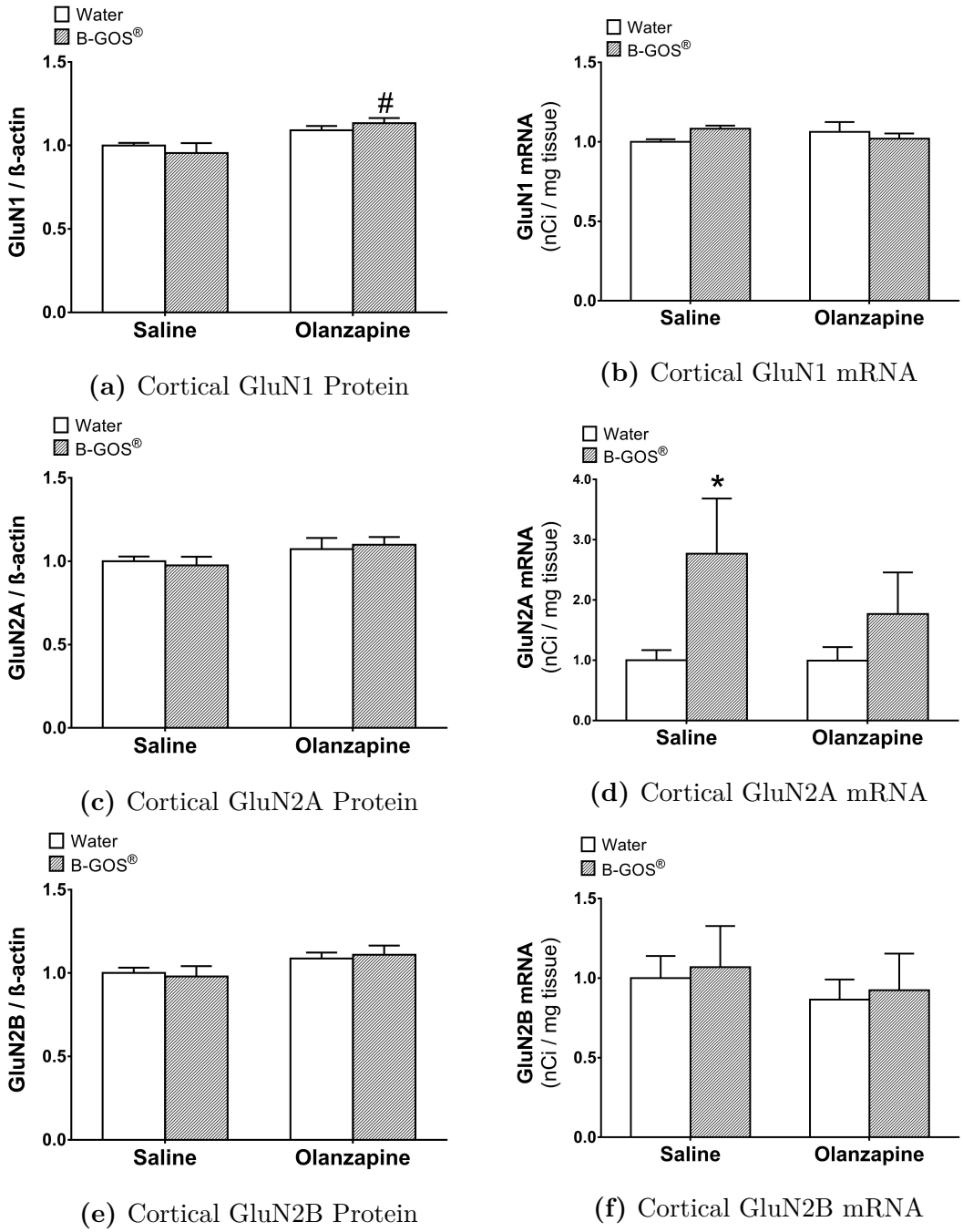
(c) Cortical 5-HT2AR Protein

(d) Cortical 5-HT2AR mRNA

Figure 2.3: Effect of B-GOS® and olanzapine administration in adult female rats on the relative levels of protein and mRNA transcript of serotonin receptors. Results are expressed as fold-change mean±SEM for each group (n=6). *p<0.05.

all groups. A similar trend was observed for GluN2B protein, though statistical significance was not reached ($F_{1,20}$=3.622; p=0.072; Figure 2.4e). Cortical GluN2A mRNA expression was significantly affected by diet ($F_{1,19}$=5.638; p=0.031); Figure 2.4C, which was mainly driven by greater transcript levels in saline/B-GOS® rats compared to the saline/water group ($F_{1,19}$=4.838; p=0.047).

In the hippocampal subfields (Table 2.1), there was a significant effect of treatment on GluN1 mRNA abundance in the CA3 ($F_{1,19}$=5.464; p=0.034), where olan-



(a) Cortical GluN1 Protein

(b) Cortical GluN1 mRNA

(c) Cortical GluN2A Protein

(d) Cortical GluN2A mRNA

(e) Cortical GluN2B Protein

(f) Cortical GluN2B mRNA

Figure 2.4: Effect of B-GOS® and olanzapine administration in adult female rats on the cortical protein and mRNA level of NMDAR subunits (a,b) GluN1, (c,d) GluN2A, and (e,f) GluN2B subunits, respectively. Results are expressed as mean±SEM for each group (n=6). $^{*}p<0.05$ compared to saline/water. $^{\#}p<0.05$ compared to saline/B-GOS®.

zapine significantly increased (+17%) levels in the absence of B-GOS® ($F_{1,19}$=4.988; p=0.040). No significant main effects of treatment or diet were observed on GluN2A,

GluN2B or 5-HT1AR protein and mRNA levels in the hippocampus.

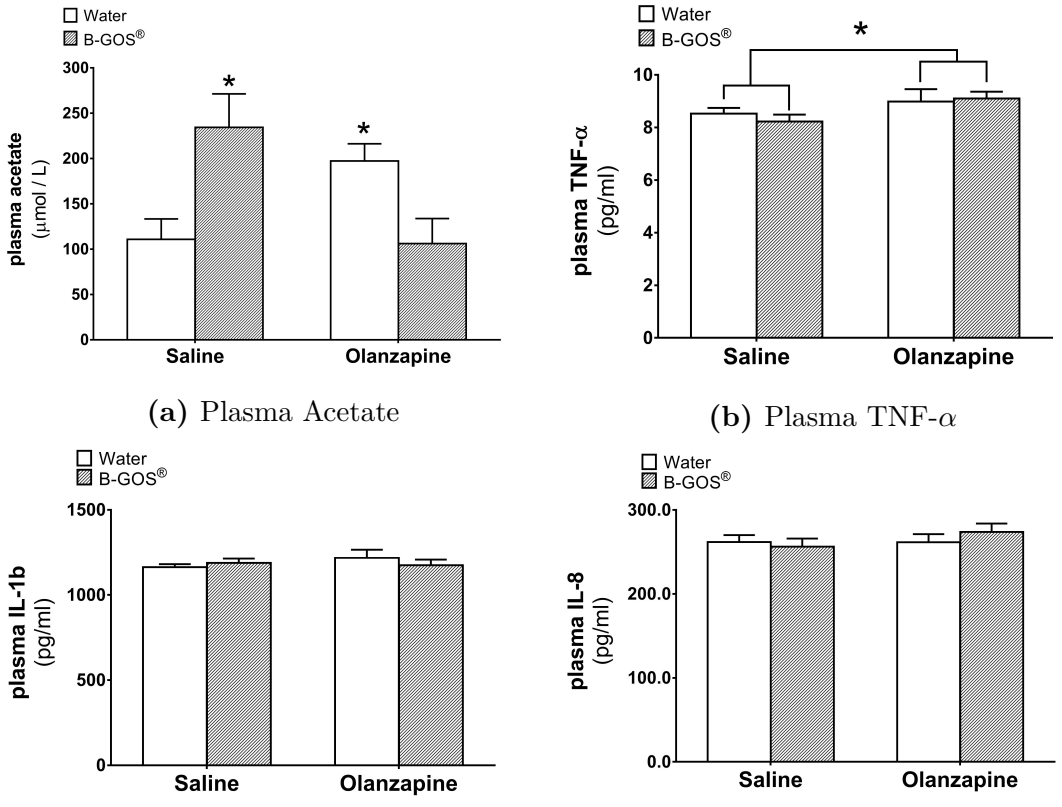
Table 2.1: Effect of Prebiotic on 5-HT1AR and NMDAR subunits in the hippocampus. Results are expressed as mean (SEM) of protein levels (protein/β-actin) or mRNA expression (nCi/mg tissue). *p<0.05 overall effect of treatment

			Saline		Olanzapine	
	Region	Parameter	Water	B-GOS®	Water	B-GOS®
5-HT1A	Hippocampus	Protein	1.28 (0.10)	1.28 (0.06)	1.14 (0.08)	1.14 (0.05)
	CA1	mRNA	518.83 (43.66)	741.59 (104.21)	522.14 (43.62)	618.16 (87.84)
	CA3	mRNA	362.51 (40.81)	545.7 (111.44)	541.09 (33.57)	457.38 (109.72)
	DG	mRNA	865.73 (55.97)	1047.54 (137.64)	981.64 (36.24)	1067.72 (143.93)
GluN1	Hippocampus	Protein	0.52 (0.02)	3.05 (1.37)	0.53 (0.01)	0.52 (0.02)
	CA1	mRNA	537.2 (37.39)	524.83 (18.20)	525.3 (16.45)	545.8 (19.59)
	CA3	mRNA	428.01 (9.50)	402.13 (23.08)	**499.55 (35.18)***	450.34 (16.32)
	DG	mRNA	421.71 (20.48)	413.49 (22.64)	457.07 (22.36)	444.53 (13.37)
GluN2A	Hippocampus	Protein	0.87 (0.02)	0.91 (0.03)	0.89 (0.03)	0.88 (0.03)
	CA1	mRNA	1889.73 (196.92)	2318.98 (223.33)	1877.17 (138.43)	2116.8 (204.93)
	CA3	mRNA	962.7 (156.67)	1087.6 (220.15)	937.25 (116.42)	1011.88 (198.56)
	DG	mRNA	1353.34 (221.58)	1584.36 (223.71)	1276.06 (116.88)	1394.5 (202.46)
GluN2B	Hippocampus	Protein	0.89 (0.02)	0.93 (0.02)	0.92 (0.02)	0.89 (0.02)
	CA1	mRNA	1823.92 (193.99)	1647.21 (174.81)	2035.27 (130.60)	1815.84 (167.53)
	CA3	mRNA	1151.33 (143.25)	960.15 (175.99)	1225.19 (112.32)	1072.1 (118.32)
	DG	mRNA	1597.51 (163.72)	1489.14 (186.00)	1828.89 (147.58)	1543.75 (135.73)

2.3.3 Circulating Acetate & Inflammatory Biomarkers

Levels of circulating SCFA acetate and inflammatory biomarkers were examined (Figure 2.5). A significant diet x treatment interaction was observed ($F_{1,20}$=15.341; p=0.001) with serum acetate, likely driven by B-GOS® significantly increasing circulating acetate concentrations by approximately two-fold (water/saline vs. B-GOS®/saline, p=0.005) as suggested by *post hoc* analysis. Similarly, the administration of olanzapine alone significantly increased circulating acetate levels by 1.78-fold (water/saline vs. water/olanzapine, p=0.037). The combined administration of both olanzapine and B-GOS®, however, decreased acetate to control levels (water/olanzapine vs. B-GOS®/olanzapine, p=0.029). A significant effect of

treatment on the concentration of TNF-α ($F_{1,19}$=4.835, p=0.037), and *post hoc* analysis revealed that this was driven by olanzapine administration increasing levels of circulating TNF-α in the presence of B-GOS® (B-GOS®/saline vs. B-GOS®/olanzapine, p=0.049). No other interactions or overall effects were observed on serum concentrations of IL-1β or IL-8.



(a) Plasma Acetate

(b) Plasma TNF-α

(c) Plasma IL-1β

(d) Plasma IL-8

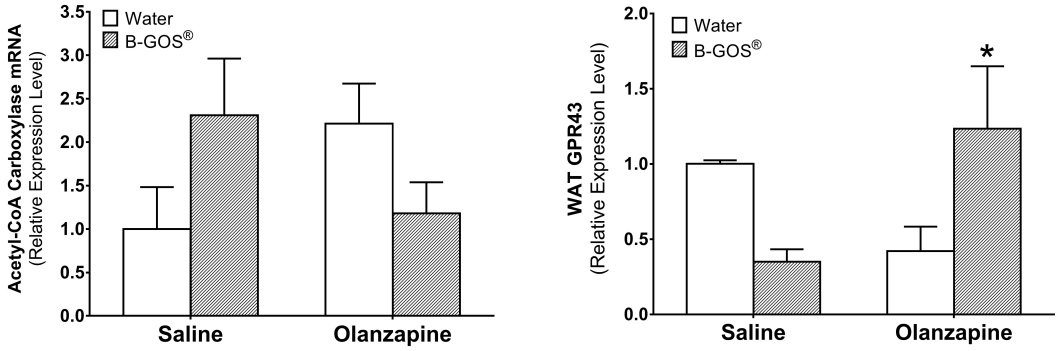
Figure 2.5: Effect of B-GOS® and olanzapine administration in adult female rats on the relative levels of circulating (a) SCFA acetate and inflammatory biomarkers (b) TNF-α (c) IL-1β and (d) IL-8. Results are expressed as mean±SEM for each group (n=6). *p<0.05 compared to saline/water. $^{\#}p$<0.05 overall effect of treatment.

2.3.4 Gene Expression of SCFA Metabolising Proteins

Acetate increases the cellular pool of acetyl-coA, the physiologically relevant substrate that is taken up by ACC and feeds into the citric acid cycle. A significant diet x treatment interaction (Figure 2.6) was observed with the hepatic expression of ACC ($F_{1,22}$=5.384; p=0.032) as well as with WAT expression of SCFA receptor GPR-43 ($F_{1,22}$=7.592; p=0.014). Subsequent *post hoc* analysis of ACC mRNA did not detect a significant differences between groups (saline/water vs saline/B-GOS®; p=0.089. Olanzapine/water vs olanzapine/B-GOS®; p=0.117). The mRNA abundance of WAT GPR-43 in the olanzapine/B-GOS® group was significantly greater than in the olanzapine/water group (Figure 2.6b; p=0.026). Although B-GOS® alone reduced GPR43 mRNA to a magnitude similar to that seen in water/olanzapine animals (approx. 70%), statistical significance was not reached (water/saline vs water/B-GOS®, p=0.153).

2.3.5 Faecal Microbiota Characterization

The intake of B-GOS® increased *Bifidobacteria Spp.* compared to water/saline animals (Figure 2.7; p=0.026), corroborating previously published results in humans and rodents [65, 156]. This increase was not observed in olanzapine-injected animals (olanzapine/water vs. olanzapine/B-GOS®, p>0.05). Other microbial genera including *Escherichia/Shigella spp.*, *Coprococcus Spp.*, *Oscillibacter Spp.*, *C. Coccoides Spp.*, *Roseuria Intestinalis Cluster*, and *clostridium* XVIII cluster



(a) Hepatic ACC mRNA **(b)** WAT GPR43 mRNA

Figure 2.6: Effect of B-GOS® and olanzapine administration in adult female rats on the relative expression levels of (a) hepatic acetyl co-enzyme A carboxylase (ACC) and (b) white adipose tissue (WAT) GPR-43. Results are expressed as mean±SEM relative to saline/water controls for each group (n=6). $*p<0.05$ compared to olanzapine/water.

significantly decreased following the ingestion of B-GOS® alone. The administration of olanzapine alone did not affect faecal microbiota composition.

2.4 Discussion

This study firstly confirms that olanzapine-induced weight gain in rodents is gender dependent at the clinically relevant dose of 10mg/kg, and is consistent with previously published work [140, 141]. The levels of central serotonin receptor and NMDAR subunits in the female olanzapine-induced weight gain model after daily ingestion of the prebiotic, B-GOS® were documented. The key findings were illustrated in the female model where prebiotics feeding attenuated olanzapine-induced weight gain without reducing central 5-HT2AR levels. The study also demonstrated that olanzapine administration did not decrease NMDAR subunits gene expression, as previously reported in male rats [64]. However in the presence

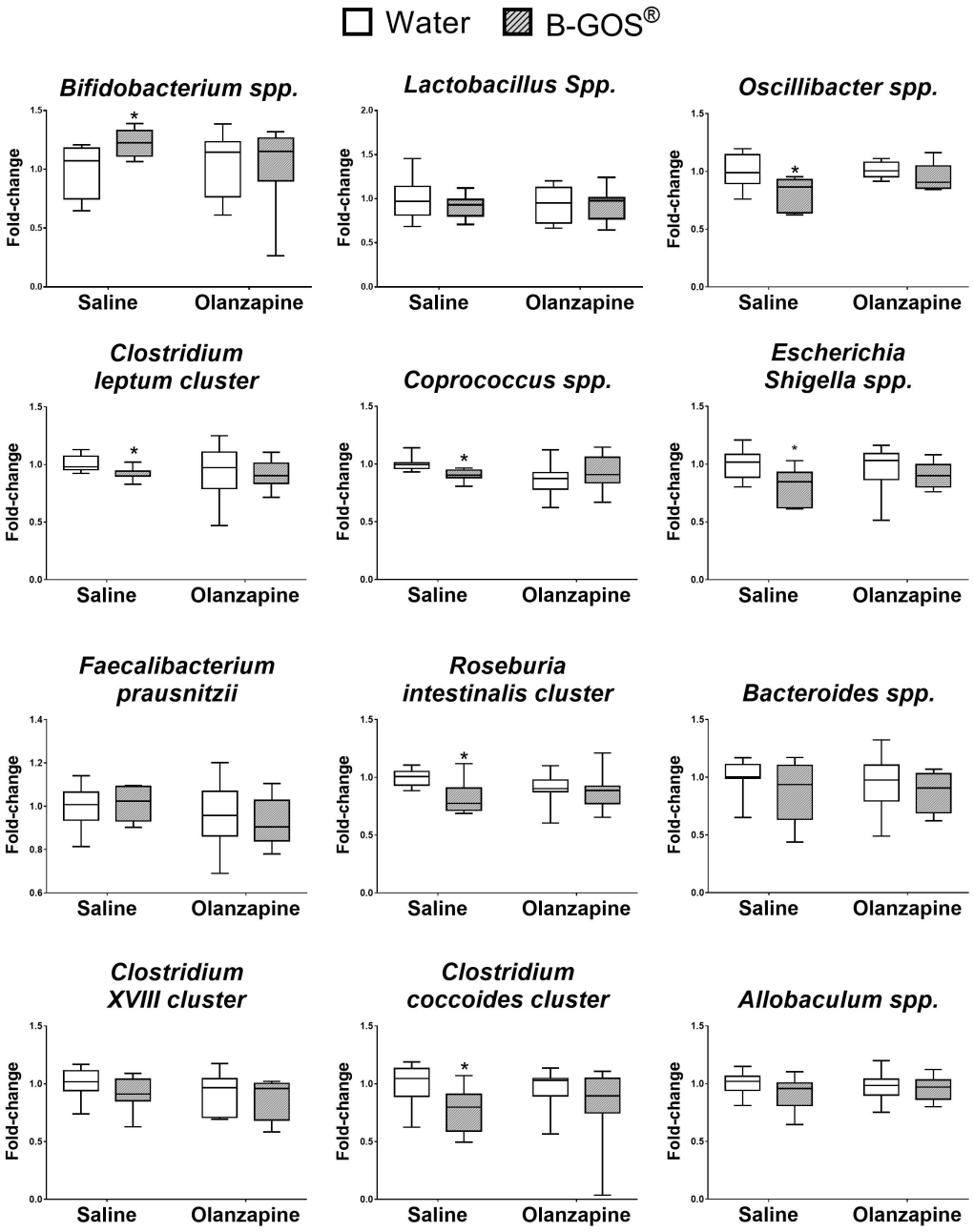


Figure 2.7: Effect of B-GOS® and olanzapine administration on the faecal bacterial composition in female rats. Animals ingested B-GOS® seven days prior to, and throughout, the two-week administration of olanzapine. Reported communities are common to the rodent and have previously been examined in relation to prebiotics [155]. Results are expressed as fold-change relative to the saline/water group, and presented with medians [first quartile to third quartile]. $^*p<0.05$, compared to the saline/water group.

of B-GOS®, increased levels of the GluN1 subunit in the frontal cortex, and GluN1 expression in the hippocampal CA3 subregion were observed. Interestingly, plasma levels of circulating acetate was increased by both B-GOS® and olanzapine alone, but was reduced to control levels upon co-administration. This elevation in acetate was confirmed to be physiologically relevant as an interaction between B-GOS® x olanzapine was found for hepatic ACC and WAT GPR-43 gene expression. Finally, olanzapine alone or with B-GOS® did not affect the abundance of some specific genera of enteric bacteria, whilst B-GOS® alone had some notable effects. Taken together, these data suggest that B-GOS® may reduce weight gain when used adjunctly with second generation antipsychotics, without affecting their central molecular actions.

2.4.1 Gender-Dependent Weight Gain

The observation that olanzapine was only able to induce weight gain in female rats is consistent with previous studies [140–142] and has the additional merit of being administered at a clinically relevant dose of olanzapine (10mg/kg). Although male rodents have not been shown to gain weight, other metabolic measures including reductions in lean muscle mass and increased adiposity are typically observed, potentially masking weight gain and resulting in the neutral change in total body weight [141]. Furthermore, inconsistent dosing regimens may also result in the observed variability as clinically relevant doses (10mg/kg) have been criticized to induce conditioned place aversion and extreme sedation [141]. While this is

potentially unethical, it more importantly undermines the validity of the observed effects in rodents as increased stress markedly impact feeding and body weight [157]. Notably there are some reports suggesting female psychosis patients are more susceptible to weight gain [158, 159], and that clozapine does not confer weight gain in rodents [160]. These findings further highlight the challenges in establishing a suitable translational model for psychosis.

2.4.2 Role of Intestinal Microflora in Weight Gain

The B-GOS®-mediated reduction in olanzapine-induced weight gain is a novel finding. A previous study demonstrated that an antibiotics cocktail could attenuate olanzapine-induced weight gain in female rats [43], and in conjunction to the observations presented here, implies that specific intestinal communities are involved in drug-induced weight gain. The main link between both investigations are the hindered actions of undesirable microbes wherein the prebiotic allows non-pathogenic species to outgrow and thereby restrict the proliferation of harmful bacteria, and antibiotics by directly eliminating all bacteria. Long-term use of antibiotics to curb medication-induced metabolic dysfunction is not a viable translational strategy as patients are likely to develop antibiotic resistance, a caveat noted by the authors of the study [43]. In comparison, the use of the prebiotics B-GOS® becomes more tantalizing as it offers a safe and natural strategy to attenuate metabolic side effects.

Studies in germ-free mice have shown that olanzapine-induced weight gain is reliant on the presence of gut microbiota [41], and in female rats, repeated administration of olanzapine increased the relative abundance of the phylum Firmicutes, but reduced Actinobacteria, Proteobacteria, and Bacteroidetes [140]. The latter observations may have been the result of specific antibiotic actions of olanzapine; *in vitro* growth of the Proteobacteria *Escherichia coli NC101*, was completely inhibited by physiological concentrations of olanzapine, whereas the proliferation of the Firmicutes strain, *Enteroccoccus faecalis OGIRF*, was only delayed by the drug but not completely repressed [41]. The findings here revealed that although B-GOS® alone increased *Bifidobacteria Spp.*, and reduced species within the Firmicutes (*coprococcus, oscillibacter, c. coccoides, roseburia intestinalis cluster, clostridium XVIII cluster*) and Proteobacteria (*Escherichia/Shigella spp.*) phyla, no antibacterial effects of olanzapine were observed. Since antipsychotics, including olanzapine, have previously been shown to exert antimicrobial effects [41, 44], the discrepancies to the findings reported in this chapter may reflect differential duration and dose of olanzapine administration, and/or the method of bacterial analysis (metagenomics vs qPCR). Nevertheless, it is worth highlighting that olanzapine attenuated the effects of B-GOS®, which suggests that the antipsychotics did influence the gut microbiota, albeit when their pre-existing levels were below those of control animals. Whether the bacteria affected by B-GOS® would proliferate

beyond control levels with a longer duration of olanzapine administration at a clinically relevant dose will have to be explored in future studies. Our data do not support the involvement of at least some bacterial species in short-term olanzapine-induced weight gain.

The analysis of plasma cytokines provide preliminary mechanistic insights. The elevation of TNF-α in olanzapine/B-GOS® rats may contribute to the reduction in olanzapine-induced weight gain as pro-inflammatory TNFα has been recognised as an appetite suppressant [161], and has a significant influence on lipid metabolism in adipose tissue. Although its levels are mildly increased in obesity [162], studies in animal models of this condition show that the activation of the TNF-α system inhibits ongoing weight gain [163]. In view of this evidence, the co-administration of B-GOS® and olanzapine leading to the release of TNF-α may have contributed to the attenuation of weight gain. Given that IL-1β and IL-8 levels remained unaltered in all groups, it is also likely that the elevation in TNF-α was not echoing an inflammatory state. However further studies are required to support this hypothesis.

2.4.3 Significance of the 5-HT2AR

The antagonism of brain 5-HT2AR by olanzapine is an important pharmacological property that may underlie its psychotropic actions. In the current study, this blockade was interpreted from a decrease in cortical 5-HT2AR levels; the intake of the prebiotic B-GOS® did not interfere with this effect, implying that central

actions of the prebiotic are independent of the serotonin system. Interestingly, olanzapine-mediated decrease in 5-HT2AR expression was not observed in male rats (appendix A.2.1) suggesting that an insufficient dose, or ineffectiveness, of olanzapine underlies the lack of increase in body weight.

Weight loss and reduced metabolic dysfunction in olanzapine-treated female rats could be achieved by the administration of the satiety hormone glucagon-like protein (GLP-1) [164, 165], which likely activates the GLP-1 receptor (GLP-1R). Recent evidence has suggested that GLP-1 may modulate feeding behaviour via the activation of cortical NMDARs [166], a beneficial effect given that NMDAR hypofunction has been postulated in the pathophysiology of schizophrenia [167]. However, GLP-1 may confound pharmacological actions of olanzapine as activation of GLP-1R increased brain 5-HT2AR [168], implying that the co-administration of GLP-1 agonists, or its analogues, with olanzapine may negate the antipsychotic action of the drug. In the present study, central GLP-1R levels were also examined but did not result in any significant changes in male or female rats (appendix A.3.)

2.4.4 NMDAR Hypofunctioning

The current study demonstrates that B-GOS® attenuates weight gain without affecting olanzapine/5-HT2AR interactions, and also that it may have beneficial effects on NMDAR-mediated brain functions, which have been consistently reported in male rodents [64, 65]. Earlier work has illustrated a two week exposure to

olanzapine (2mg/kg/day twice daily) in male rats does not result in changes to cortical NMDAR subunit levels [169]. In the present study, both male and female rats corroborates these observations at a clinically relevant dose (10mg/kg/day). However, when olanzapine was administered concomitantly with B-GOS® an increase in cortical GluN1 protein was observed. In the hippocampus, olanzapine increased the hippocampal expression of GluN1 and GluN2B in female and male rats, respectively which is in contrary to a previous study reporting reductions in hippocampal GluN1 but elevated GluN2A in a four-week administration of olanzapine at 10mg/kg/day [138]. While, it is possible that gender differences, as well as methodological techniques, may contribute to some of the inconsistent reports, it is also likely that olanzapine pharmacology may vary temporally (i.e., chronic vs. acute treatments/route of administration). Furthermore, olanzapine-mediated increased expression of cortical GluN1 in the female rats was only observed in the presence of prebiotic intake, suggesting a possible augmentation of antipsychotic effects by B-GOS®. The physiological relevance of olanzapine-induced elevation of GluN subunits in the brain, seen in both the current study and the chronic experiments, are therefore worth further examination.

Previous studies have demonstrated that following the ingestion of the prebiotic B-GOS®, higher levels of plasma acetate could be detected in rats and exogenous acetate supplemention could replicate the elevated levels of NMDAR subunits

[66], a likely mechanism for the acetate-associated correction of NMDAR induced behavioural deficits [119]. The findings here show that B-GOS® intake also increases plasma acetate in female rats, though cortical GluN2A was elevated, rather than GluN1 [64] or GluN2B [66] as reported previously. Since acetate acts as an appetite suppressant either directly [151] or via stimulation of anorectic hormones PYY and GLP-1 [170], it follows to examine whether the increase in acetate was associated with B-GOS®-mediated reduction of olanzapine-induced weight gain. Contrary to this hypothesis, the results suggests that the reduction of acetate appeared to be linked to the B-GOS®-mediated attenuation of olanzapine-induced weight gain, and that levels of this SCFA increased after olanzapine injections. These data imply that elevated acetate levels may contribute to olanzapine-mediated weight gain, and requires further investigation.

2.4.5 Relevance of Acetate in the Periphery

Feeding rats with a high fat diet can led to increased plasma acetate levels, and this has been strongly associated with metabolic dysfunction and weight gain [171]. This is consistent with the present observation where olanzapine-induced weight gain was associated with an increase in circulating acetate levels and the normalisation of body weight was accompanied by the reduction of plasma acetate. However, in the current study B-GOS® also elevated plasma acetate levels but did not increase body weight. One interpretation is that acetate does not mediate weight gain, and perhaps elevated

acetate in olanzapine-treated rats was a consequence of altered metabolism wherein the satiety-inducing action of this SCFA was compromised [151]. A precedent for this is the elevation of the anorectic hormone leptin following olanzapine administration in rodents and patients [172]. In these cases, increased leptin may have been a homeostatic response to the medication-induced metabolic disturbance that fails to initiate its appetite suppressing properties. Another complication in interpreting these findings are data showing that a high fat diet decreases acetate, and that wheat fibre supplementation restores it to normal concentrations [173]. This is in keeping with the current observation that olanzapine/B-GOS® rats have similar weights and plasma acetate to controls. The combination of prebiotic and antipsychotics also normalizes gene expression in peripheral tissues.

The parallel changes in hepatic ACC mRNA expression and plasma acetate confirms that the metabolic pathway involving this SCFA are influenced by both prebiotic and olanzapine. This is further corroborated by contrasting changes in WAT GPR-43 mRNA and plasma acetate, which is intuitive since a homeostatic response to elevations of a substrate could conceivably involve a 'downregulation' of its binding receptor. Importantly, previous work has shown that weight gain following a high fat diet is associated with reduced WAT GPR-43 expression, and the administration of acetate elevated this receptor to control levels [152]. A similar scenario was observed where the administration of olanzapine alone incurred

significantly greater body weight and lower WAT GRP-43 abundance than the B-GOS®/olanzapine group. A detailed investigation of the mechanisms underlying the effects of B-GOS® and/or olanzapine was not the focus of this study, and therefore the role of peripheral SCFA binding receptors, other SCFAs, and genes involved in lipid metabolism and their possible differential responses to prebiotic and antipsychotic treatment warrant further investigations.

Strikingly, a recent study in male mice which administered another formulation of GOS at a higher concentration and for a longer duration, showed some psychotropic effects but did not report changes in caecum acetate levels [174]. This suggests that acetate may not be a key mediator of the prebiotic effects, and that the changes observed in the current study were incidental and not related to any causal mechanisms. However, a reduction of *Bifidibacteria* and *Lactobacilli* with GOS had been demonstrated [174] which contrasts with previous findings that an increase in the former following B-GOS® ingestion had been reported [64, 65]. These discrepancies indicate that primary actions of prebiotics can be different, and emphasises that findings reported in this chapter, and thesis at large, with B-GOS® are only applicable to this specific prebiotic mixture, and cannot be extended to all formulations of GOS.

2.5 Conclusion

The current study demonstrates a strategy to reduce olanzapine-mediated weight gain in schizophrenia without compromising a key central pharmacological action of this antipsychotic. This study also demonstrated augmented NMDAR expression, supporting a potential pro-cognitive effect of the prebiotic. Possible mechanisms of the metabolic effect of prebiotics through an acetate physiology warrants further investigation.

If you know you are on the right track, if you have this inner knowledge, then nobody can turn you off. No matter what they say.

A cytogeneticist and winner of the 1983 Nobel Prize in Physiology or Medicine for her discovery of mobile genetic elements in maize, the quote above was a reflection of her greatest satisfaction in life: discovering a secret that no one else had known as the knowledge at the time was that genes could not change except by mutation.

— Barbara McClintock (1902 - 1992)

3

Prebiotic Reduction of Brain Histone Deacetylase (HDAC) Activity and Olanzapine-Mediated Weight Gain are Acetate Independent

The data presented in this chapter has been published in the *Journal of Neuropharmacology* "Prebiotic Reduction of Brain Histone Deacetylase (HDAC) Activity and Olanzapine-Mediated Weight Gain are Acetate Independent" [175].

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Abstract

Prebiotic intake is one intervention that can elicit a wide range of potential health benefits by manipulating the microflora composition. The previous chapter in this thesis demonstrated the prebiotic B-GOS® attenuates olanzapine-induced weight gain, potentially through mechanisms involving acetate, the major SCFA that is produced upon fermentation. The present study investigates the individual influences of the prebiotic B-GOS® and sodium acetate intake on brain activity of HAT/HDAC, cortical and hippocampal expression of members within the HDAC family and NMDAR subunits, as well as their potential in attenuating olanzapine-induced weight gain. Significant B-GOS®-induced epigenetic effects was observed, particularly in decreasing HDAC activity and hippocampal HDAC-4 expression, though elevated levels of mRNA were observed in cortical HDAC-1 and HDAC-3. Sodium acetate supplementation did not result in significant changes to cortical HDAC expression and activity. Furthermore, significantly decreased HAT activity was observed and increased hippocampal levels of HDAC-3 and HDAC-4. Sodium

acetate did not attenuate olanzapine-induced weight gain and no significant changes in microbial genera populations were detected in faecal pellets. Together the present study suggests that acetate intake as a way to augment cellular pools of acetyl-coA to influence epigenetic processes does not echo the metabolic effects of the prebiotic B-GOS®, and only partially influence central enzymes. This highlights that the gut-brain axis communication likely relies on mechanisms additional to SCFA actions where future studies should examine the concerted effects of endocrine or immune metabolites.

3.1 Introduction

Rats supplemented the prebiotic B-GOS® was previously demonstrated to prevent olanzapine-induced weight gain [133] (and see chapter 2) and improve cognitive flexibility [66]. The fermentation of B-GOS® yields significant amount of acetate, propionate and butyrate [148] and enters the brain via the circulation [66]. The elevation of brain acetate imparts epigenetic changes, which involve the addition or removal of acetyl groups on histone molecules bound to genomic DNA. Increased HDAC activity reduces acetyl groups on histones, which decreases DNA unwinding thereby limiting gene expression. Conversely, HAT adds acetyl groups on to histones, which promotes DNA uncoiling and elevates gene transcription [176]. The changes in central gene expression previously reported [65, 66, 177] in response to prebiotic feeding, may have been the result of downstream effects of bacteria-derived acetate

on epigenetic mechanisms. In this view, gene transcription can be promoted by direct inhibition of HDAC or activation of HAT, or other indirect ways that increase the cellular substrate of acetyl-CoA (Figure 1.3).

HDAC inhibitors have been evaluated as potential therapeutic strategies in several neurodegenerative and neurodevelopmental cognitive disorders [117, 178]. In psychiatry, the SCFAs sodium butyrate [179] and valproate [131] have been shown to confer therapeutic benefits for depression, anxiety, and schizophrenia. However compounds including valproic acid, vorinostat and sodium butyrate have inconclusive antipsychosis activity and confers potential toxicity to the host [125]. Therefore, enriching the cellular pool of acetyl-CoA using exogenous acetate carrier like GTA or sodium acetate to restore acetyl-histone homeostasis can potentially reverse hypoacetylation.

The therapeutic potential of acetate has been advocated by research on neurodegenerative disorders such as Canavan's disease [180, 181], where oral acetate improved motor function in a genetic model of the disorder, and in studies of behavioural phenotypes associated with glutamate receptor NMDAR blockade, showing that acetate attenuated cognitive deficits [119]. The latter study has particular clinical relevance given that NMDAR hypofunction has been implicated in the pathophysiology of schizophrenia [137–139]. Region specific increase in NMDAR subunits have been observed following B-GOS® feeding and acetate supplementation

in rats [64, 66, 133], further illustrating that prebiotics, and possibly prebiotic-derived acetate, may mediate the central effects of these carbohydrates.

B-GOS® attenuation of olanzapine-induced weight gain may be mediated by acetate as direct supplementation has been shown to suppress food intake [151], conceivably counter-balancing increased energy consumption following olanzapine administration [143]. In this regard, the production of anorexigenic gut hormones could also explain why a prebiotic that grows bacteria has the same effect on olanzapine-induced weight gain as antibiotic administration [140] and germ-free status [41]. Elevated secretion of anorectic gut hormones have been reported following increased colonic acetate concentrations [182], in germ-free mice [183] as well as with some spectrum antibiotics [184]. Together these observations suggest that raising relevant hormone concentrations or removing pathogenic gut bacteria are two approaches common to prebiotics, antibiotics as well as germ-free conditions that can alleviate antipsychotic-mediated weight gain. Therefore, it is reasonable to further investigate whether the intake of acetate alone would have the same central and metabolic effects as B-GOS®.

3.1.1 Specific Objectives

The aim of this study was therefore to:

- Determine the individual influences of B-GOS® and sodium acetate intake on HDAC and HAT activities in the rat brain, and the expression of HDAC

encoding genes

- Examined whether sodium acetate ingestion would prevent olanzapine-induced weight gain as we have reported for B-GOS®
- Examined the effect of sodium acetate supplementation on the abundance of selected gut bacterial genera which are altered by B-GOS®

3.2 Materials & Methods

Custom DNA oligomers and olanzapine were purchased and prepared as described in Chapter 2.2. Sodium acetate was purchased from Sigma-Aldrich. Nuclear extraction and HDAC activity kit was obtained from EpiQuik. HAT activity kit and Colorimetric acetate assay kit was purchased from Abcam, UK.

3.2.1 Animals

Female adult Sprague-Dawley rats (n=48), 220-250g (6-8wks), were housed and all procedures were approved as previously described in section 2.2.1.

Prebiotics & Sodium Acetate Administration

The administration of B-GOS® and olanzapine to rats was previously described in chapter 2.2.1, and comprised of four different treatment groups: saline/water, saline/B-GOS®, olanzapine/water, olanzapine/B-GOS®. Similarly, rodents treated with sodium acetate supplementation with olanzapine were randomly assigned to four treatment groups: saline/water, acetate/saline, water/olanzapine, and

acetate/olanzapine. Sodium acetate was administered to rats via drinking water (approx. 0.5g/kg/day; 5mg/mL) [151]. Animal body weight and fluid intake were monitored daily; sodium acetate concentrations were adjusted accordingly to maintain desired dose. This method of acetate delivery is consistent with studies with B-GOS® [133], and avoids stress to animals incurred by the intra-gastric gavage, a method required to administer GTA [118]. Furthermore, the authors of the latter study have stated that GTA is a lipid complex thus has greater brain penetration than acetate itself. However, SCFAs arising from the gut are not conjugated to larger lipid molecules, and so GTA would not provide a true physiological representation of acetate arising from the bacterial fermentation of dietary carbohydrates.

All animals received a two week, daily i.p. injection of olanzapine (10mg/kg) or saline, during which water or supplement (B-GOS® or sodium acetate) administration continued. Weights were normalised to the first day of olanzapine administration, and expressed as percentage weight gain (i.e., [weight – weight on acetate administration] / weight on acetate administration).

Plasma and Tissue Collection

Twenty-four hours after the final injection, animals were humanely culled by cervical dislocation and decapitated to obtain trunk blood, which was collected in heparin coated 2ml eppendorf tubes. All blood samples were centrifuged, pipetted into a fresh eppendorf tube and frozen on dry ice as in chapter 2.2.1. Whole brains

were removed, bisected and the hippocampus and cortex dissected out from one hemisphere. All cortical regions and hippocampus from the other hemisphere were dissected from the mid-brain and basal ganglia as one piece of tissue. These regions were selected because prebiotic and probiotic intake significantly influences gene expression therein, and they are neuroanatomical regions that underlie the specific behaviours that are influenced by these supplements [64, 66, 106]. All plasma and tissue samples were stored at -80°C until analysed.

3.2.2 Plasma Acetate

Levels of circulating SCFA acetate was measured as detailed in section 2.2.4.

3.2.3 Nuclear Extraction of Brain Homogenate

Frozen peripheral half brain tissue, which excludes the frontal cortex and hippocampus, were used for nuclear protein extraction. Extraction was carried out to manufacturers instructions EpiQuik^{TM} Nuclear Extraction Kit I (Epigentek, Cat. No. OP-0002). Briefly, 30mg of tissue was gently homogenised with steel beads in extraction buffer for 30min, vortexing at 3min intervals. The homogenate was centrifuged and the resulting supernatant was measured using Bradford's protein assay (Bio-rad laboratories, CA, USA). These samples were referred to as 'brain homogenate'. The specific quantification of HDAC1-4 mRNAs was chosen as their central expression are influenced by acetate intake, and/or they modulate NMDA receptor expression [185, 186].

HDAC activity

HDAC enzyme activity was evaluated using commercial colorimetric assay kit EpiQuik^{TM} from Epigentek (Farmingdale, NY, Cat. No P-4002). Active HDAC's in 5ug nuclear extracts hydrolyze acetylated histone substrates captured on strip wells. Assay was carried out using manufacturers instructions. In brief, specific antibodies to unacetylated substrates recognize and can be colorimetrically quantified through an ELISA-like reaction. HDAC activity is inversely proportional to the amount of the undeacetylated histone substrates.

HAT activity

HAT activity was evaluated using commercial colorimetric assay kit from Abcam (Cat. No. ab65352). Assay was carried out using manufacturers instructions. In brief, a free form of Co-enzyme A (Co-A) is released upon HAT acetylation of peptide substrates and serves as an essential co-enzyme for NADH production. Reducing soluble tetrazolium dye with NADH turns the solution from yellow to purple enabling spectrophotometric measurements at 440nm.

3.2.4 Quantitative Polymerase Chain Reaction

RNA Extraction of Brain Regions

Total RNA was extracted from isolated cortical and hippocampal tissue using Tri-Reagent according to manufacturers' instructions, and reverse-transcribed to cDNA, using a commercial kit (Life Technologies, UK). The SYBR Green methodology

(Power SYBER, Life Technologies, UK) was applied to amplify mRNA encoding HDAC-1-4 using previously published primer sets. QPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). All QPCR data are presented as fold change (relative to β-2-microglobulin, B2M) which were calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method [154].

Gut Microflora Analysis

The abundance of 16S DNA encoding specific microbial genera and total bacteria in faecal pellets were analysed using QPCR and previously published primers [155]. Bacterial DNA was extracted using a silica based extraction method. In brief, 20mg of faecal sample was suspended in a 6M guanidine hydrochloride and mechanically homogenized using steel beads. After centrifugation, the supernatant was combined with 10% silica-dioxide solution, mixed, and centrifuged. Supernatant was washed using sodium-ethanol solution (28mL 100% EtOH, 12mL dH_2O, 240µL 5M NaCl). Samples were dried and re-suspended in water. The concentrations of isolated DNA were quantified spectrophotometrically by measuring absorbance at 260nm. The SYBR Green methodology (Power SYBER, Life Technologies, UK) was used to amplify 20ng DNA on a 7900HT Fast Real-Time PCR System (Applied Biosystems, US). Duplicate samples were held at 95°C for 10min and subjected to 40 cycles of denaturation (95°C for 15sec) and annealing/extension (60°C for 60sec). These QPCR data were also calculated using the $2^{-\Delta\Delta Ct}$ relative quantification method

[154] and reported as fold-changes relative to the saline/water group. This was to verify whether acetate production in the gut, through prebiotic fermentation, contributes to changes in the levels of gut bacteria.

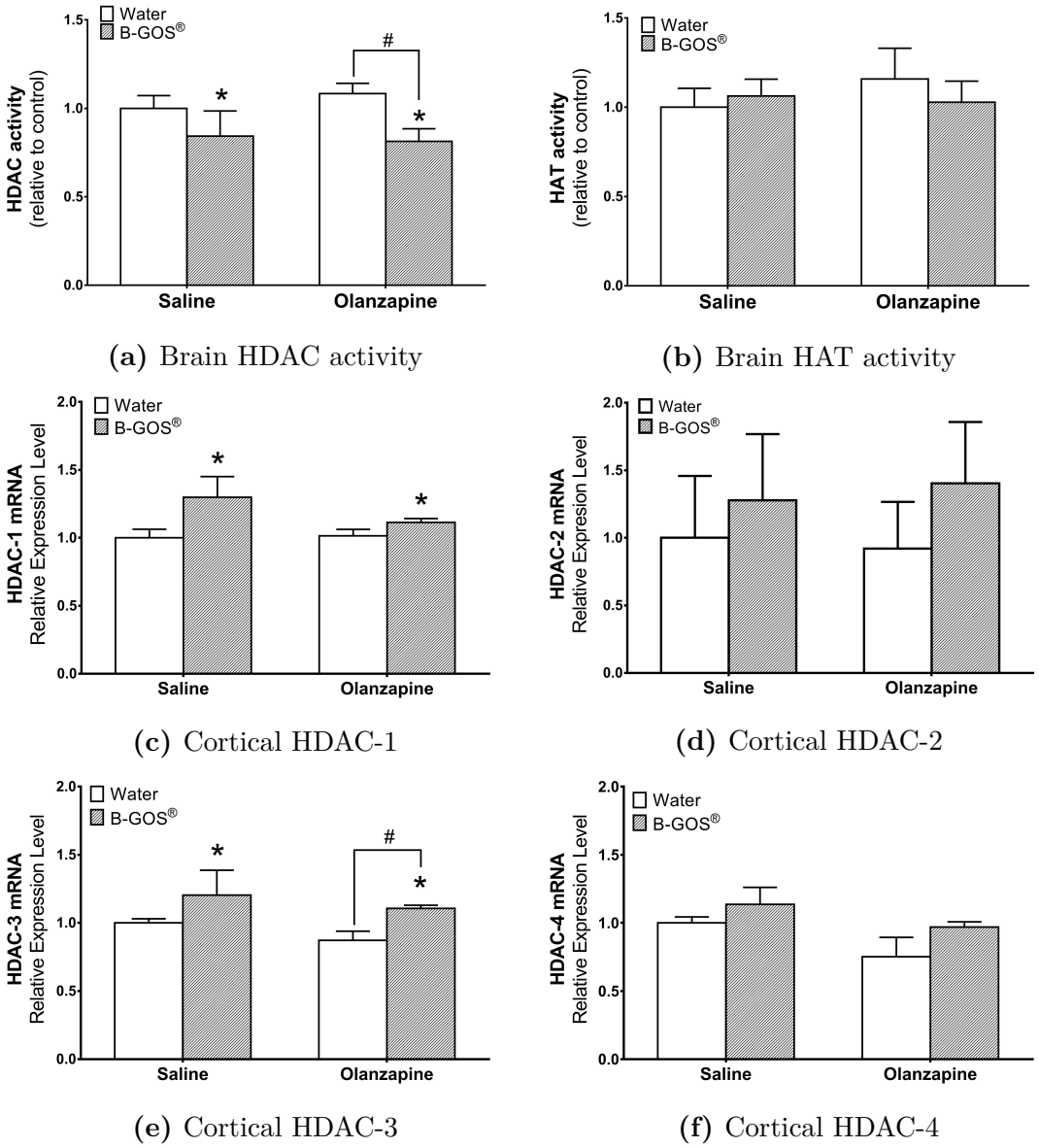
3.2.5 Statistical Analyses

Statistical analyses were performed using SPSS, version 22. Normal distribution of data was confirmed using Shapiro-Wilk test. All data are expressed as mean±SEM. Body weight and microbial data were analysed as described in chapter 2.2.6. HDAC/HAT activities, mRNA abundance and plasma changes were analysed using two-way ANOVA with bonferroni correction. The level of significance was set at 0.05 and p-values and F-statistics are reported as exact values.

3.3 Results

3.3.1 Brain HDAC Activity & Expression Following Prebiotics

Brain HDAC activity was characterized after B-GOS® administration (Figure 3.1a), and a significant overall effect of diet was observed ($F_{1,19}$=5.974; p=0.028). This effect was likely driven by the reduction by B-GOS® in olanzapine injected animals ($F_{1,14}$=4.777; p=0.046), and to a lesser contribution from saline injected animals ($F_{1,14}$=1.161; p>0.05). No significant changes were observed for HAT activity in these samples (Figure 3.1b).



(a) Brain HDAC activity

(b) Brain HAT activity

(c) Cortical HDAC-1

(d) Cortical HDAC-2

(e) Cortical HDAC-3

(f) Cortical HDAC-4

Figure 3.1: Effect of B-GOS® and olanzapine administration in adult female rats on the brain activity of (a) HDAC (b) HAT and cortical expression levels of (c) HDAC-1 (d) HDAC-2 (e) HDAC-3 and (f) HDAC-4. Results are expressed as mean±SEM for each group (n=6). $^{*}p<0.05$ overall effect of diet. $^{\#}p<0.05$ compared to olanzapine/water.

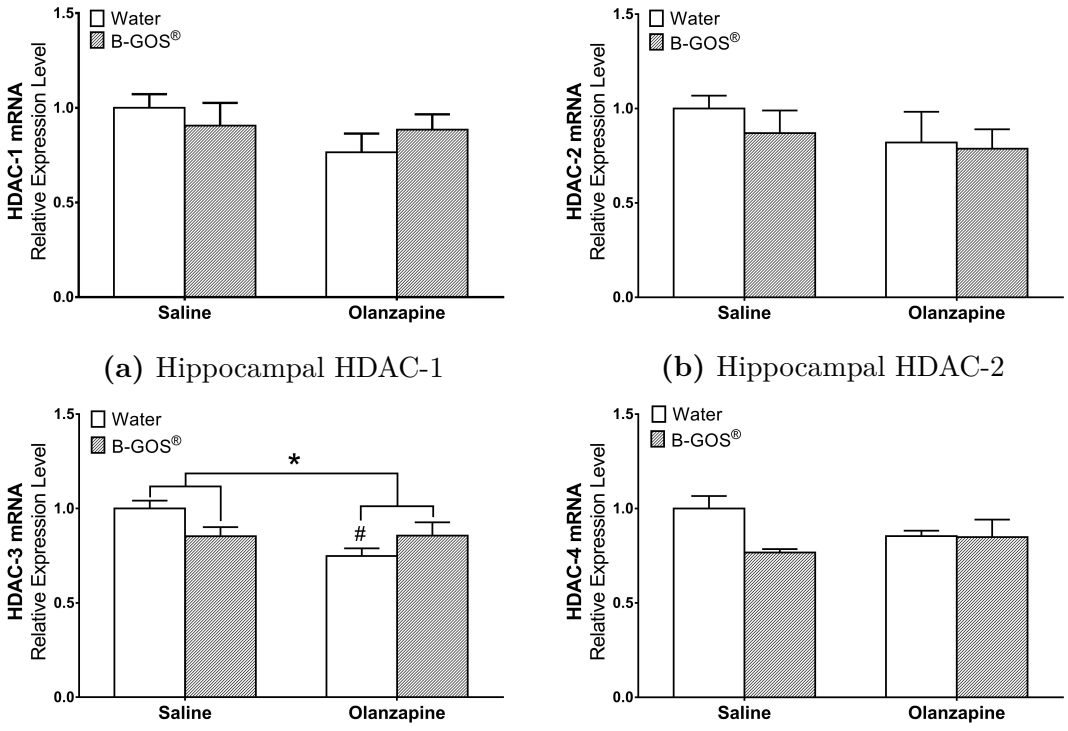
There was a significant overall effect of diet on cortical levels of HDAC-1 ($F_{1,18}$=5.396; p=0.032) and HDAC-3 ($F_{1,18}$=6.745; p=0.018) mRNA (Figure 3.1c,d). No specific direction was revealed in *post hoc* analysis though a moderate trend between saline/water vs. saline/B-GOS® was noted ($F_{1,18}$=4.065; p=0.059). A

significant 26% increase in HDAC-3 gene transcript levels was detected between rats treated olanzapine/water and olanzapine/B-GOS® ($F_{1,18}$=10.696; p=0.004). No significant effect of treatment or diet, or interactions, was observed for HDAC-2 and HDAC-4 (Figure 3.1e,f).

No significant overall effect of diet or treatment were observed for hippocampal mRNA levels of HDAC-1 and HDAC-2 (Figure 3.2a,b). A significant diet x treatment interaction was observed in the hippocampal levels of HDAC-3 ($F_{1,20}$=5.955; p=0.024), likely driven by the administration of olanzapine only ($F_{1,19}$=11.710; p=0.003). This decrease likely contributed to the overall effect of treatment (Figure 3.2c) where olanzapine reduced HDAC-3 mRNA when all unsupplemented and supplemented animals were considered ($F_{1,20}$=5.756; p=0.026. Supplementation with B-GOS® alone incurred a 20% decrease in HDAC-3 mRNA, but did not survive *post hoc* analysis ($F_{1,19}$=3.951; p=0.061). Moderate trends in diet ($F_{1,20}$=3.971; p=0.060) and diet x treatment interaction ($F_{1,20}$=3.6.36; p=0.071, Figure 3.2d) were observed for mRNA levels of HDAC-4, despite its reduction with B-GOS® alone.

3.3.2 Sodium Acetate on Plasma Acetate & Brain HDAC

Circulating levels of acetate were increased following either sodium acetate supplementation or the repeated injection of olanzapine (Figure 3.3). There were no significant overall effects of diet or treatment, but a diet x treatment interaction was observed ($F_{1,20}$=10.191; p=0.005). *Post hoc* analyses highlight significant increases



(a) Hippocampal HDAC-1 **(b)** Hippocampal HDAC-2

(c) Hippocampal HDAC-3 **(d)** Hippocampal HDAC-4

Figure 3.2: Effect of B-GOS® and olanzapine administration in adult female rats on the hippocampal gene expression of HDAC members 1-4. Results are expressed as mean±SEM for each group (n=6). $^{*}p<0.05$ overall effect of treatment. $^{\#}p<0.05$ compared to water/saline.

in serum acetate as the result of both acetate ($F_{1,20}$=7.510; p=0.013) and olanzapine ($F_{1,20}$=8.224 p=0.010) administration, but not their co-administration (Figure 3.3).

There was no significant effects of diet, treatment or a diet x treatment interaction on the activity of HDAC in the brain homogenate following acetate supplementation (Figure 3.4a). The activity of HAT (Figure 3.4b) was significantly decreased by sodium acetate as indicated by an overall effect of diet ($F_{1,23}$ =6.501; p=0.019). No significant changes in the cortical expression of HDAC1-4 was detected.

No significant changes to the expression of HDAC-1 and HDAC-2 were observed

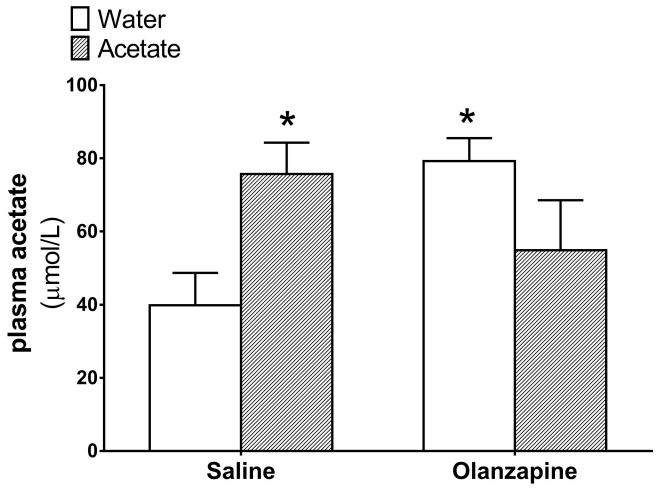


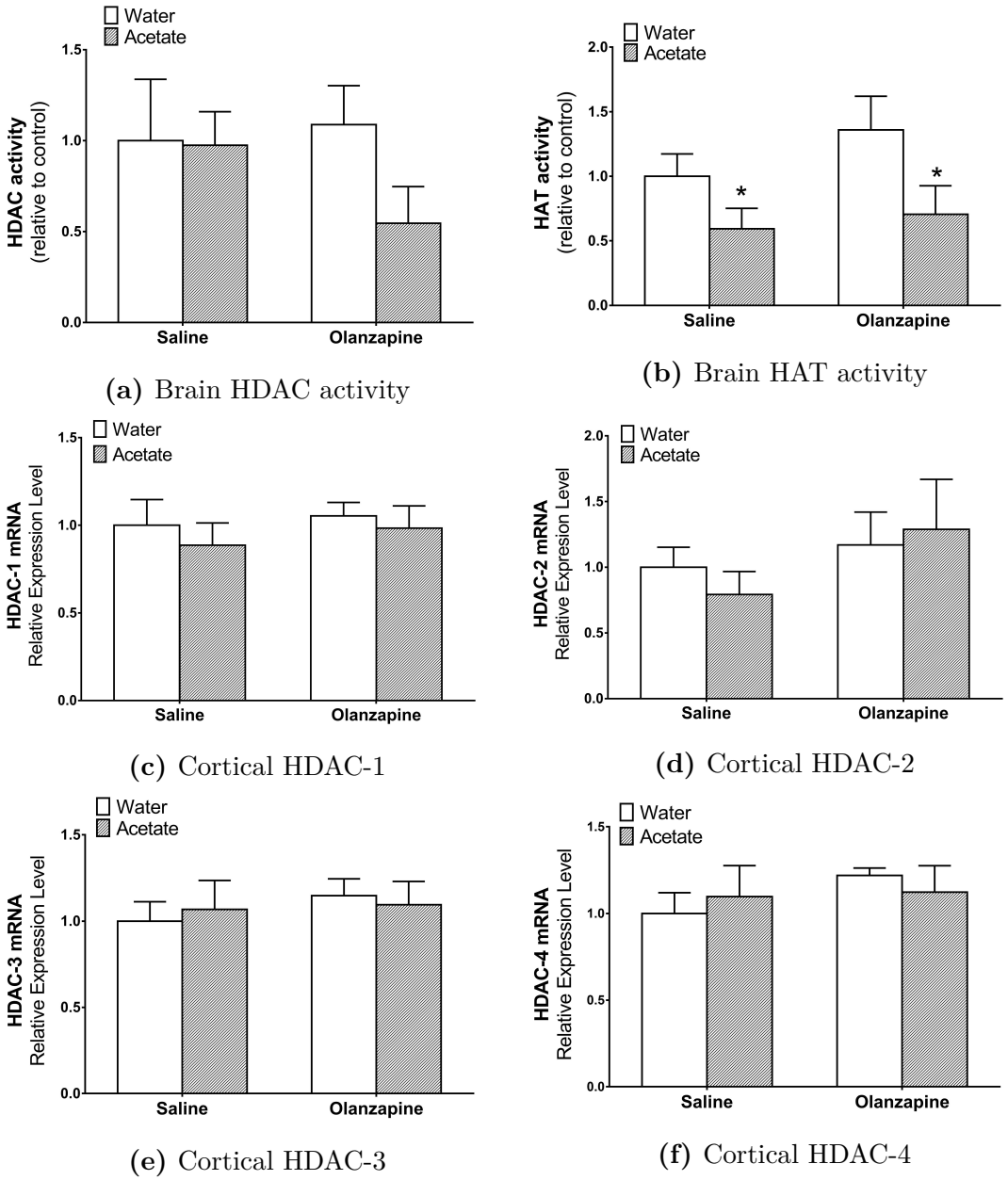
Figure 3.3: Effect of sodium acetate (500mg/kg) and olanzapine administration in adult female rats on the levels of circulating plasma acetate. Results are expressed as mean±SEM for each group (n=6). *p<0.05 compared to water/saline.

(Figure 3.5). A significant treatment x diet interaction was observed in mRNA expression of HDAC-3 ($F_{1,20}$=5.076; p=0.038) and HDAC-4 ($F_{1,20}$=7.336; p=0.015). Hippocampal HDAC-3 mRNA levels were significantly elevated by acetate ($F_{1,17}$=5.438; p=0.032) or olanzapine ($F_{1,17}$=7.757; p=0.013) supplementation only, but when co-administered, this increase returned to baseline. This effect was paralleled in HDAC-4 gene expression (Figure 3.5d) where a significant increase was observed when acetate ($F_{1,17}$=5.236; p=0.035) and olanzapine ($F_{1,17}$=5.766; p=0.028) were administered alone, but the effects was negated when administered together.

3.3.3 Sodium Acetate Does Not Attenuate Weight Gain

A significant time x treatment interaction ($F_{5,100}$=4.289; p=0.004) as well as a time x diet (acetate) interaction ($F_{14,280}$=3.658; p=0.031) on total body weight was found (Figure 3.6a). No overall interaction between treatment x diet was observed

suggesting that both olanzapine and acetate influenced body weight independently throughout the two week period. A time x diet x treatment interaction was not



(a) Brain HDAC activity

(b) Brain HAT activity

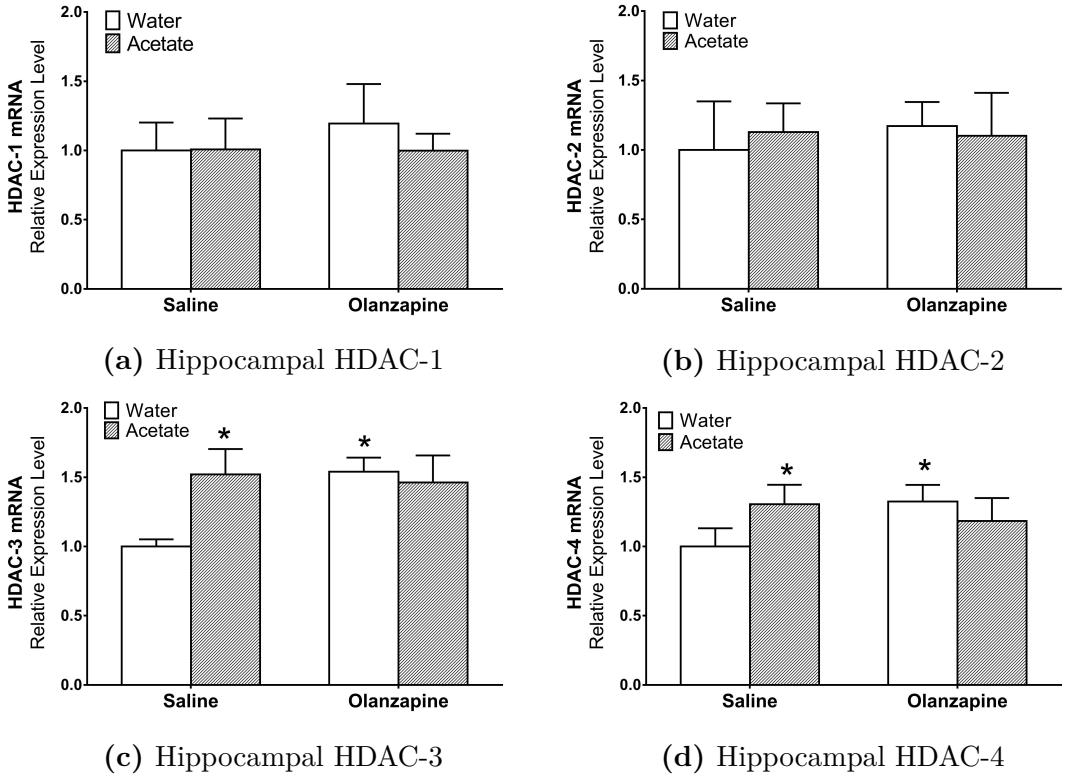
(c) Cortical HDAC-1

(d) Cortical HDAC-2

(e) Cortical HDAC-3

(f) Cortical HDAC-4

Figure 3.4: Effect of acetate (500mg/kg) and olanzapine administration in adult female rats on the enzyme activity of (a) HDAC and (b) HAT as well as expression levels of HDAC members 1-4 in the brain (excluded pre-frontal cortex, hippocampus and brain stem). Results are expressed as mean±SEM for each group (n=6). *$p<0.05$ compared to saline/water.



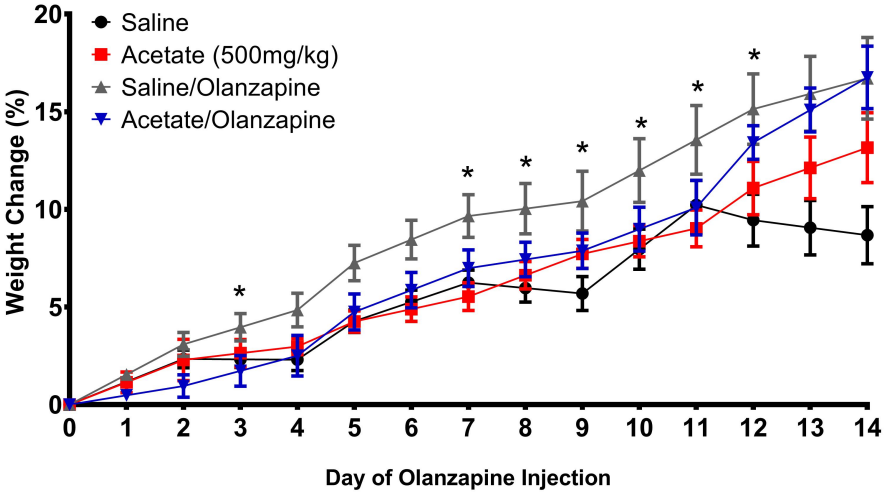
(a) Hippocampal HDAC-1 **(b)** Hippocampal HDAC-2

(c) Hippocampal HDAC-3 **(d)** Hippocampal HDAC-4

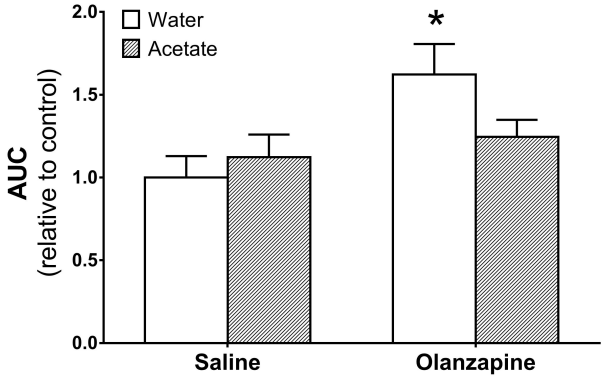
Figure 3.5: Effect of acetate (500mg/kg) and olanzapine administration in adult female rats on the hippocampal gene expression of HDAC-1-4. Results are expressed as mean±SEM for each group (n=6). *p<0.05 compared to water/saline.

observed. Corroborating this observation there was an overall effect of treatment ($F_{14,280}$=7.027; p=0.015) but no overall effect of diet on body weight likely driven by the differences in weight between water/saline and water/olanzapine groups; particularly from day seven (p<0.05).

The AUC analyses (Figure 3.6b) further confirmed these findings where an overall effect of treatment ($F_{14,280}$=6.923; p=0.016) was observed, driven by the difference between water/saline and water/olanzapine groups ($F_{14,280}$=9.654=0.006).



(a) Sodium Acetate on Total Body Weight



(b) Area Under the Curve

Figure 3.6: Effect of acetate supplementation (500mg/kg/day) and olanzapine administration in adult female rats. (a) Percentage weight gain was calculated from the day before olanzapine administration. (b) Area under the curve analysis. Results are expressed as mean±SEM for each group (n=6). *p<0.05 compared to water/saline.

3.3.4 Cortical & Hippocampal NMDAR Subunit Expression

Daily supplementation of sodium acetate did not significantly alter mRNA expression of NMDAR subunits GluN1, GluN2A, GluN2B or GluN2C in the hippocampus or frontal cortex (Table 3.1). Notably, a treatment trend was observed in the hippocampal expression of GluN2A ($F_{1,17}$=3.886; p=0.065).

Table 3.1: Expression of NMDAR Subunits in Frontal Cortex and Hippocampus after sodium acetate supplementation. Results are expressed as mean±SEM. *$p<0.05$ compared to saline/water.

		Saline		Olanzapine	
Region	Subunit	Water	Acetate	Water	Acetate
Hippocampus	GluN1	1.00 ± 0.392	1.12 ± 0.143	1.63 ± 0.372	1.23 ± 0.206
	GluN2A	1.00 ± 0.273	0.91 ± 0.068	1.05 ± 0.118	1.57 ± 0.163
	GluN2B	1.00 ± 0.325	1.33 ± 0.228	1.83 ± 0.45	1.31 ± 0.239
	GluN2C	1.00 ± 0.309	0.87 ± 0.178	0.740 ± 0.13	0.61 ± 0.122
Frontal Cortex	GluN1	1.00 ± 0.139	1.10 ± 0.168	1.13 ± 0.133	1.21 ± 0.076
	GluN2A	1.00 ± 0.142	1.23 ± 0.115	1.15 ± 0.091	1.22 ± 0.086
	GluN2B	1.00 ± 0.146	1.23 ± 0.122	1.18 ± 0.159	1.27 ± 0.091
	GluN2C	1.00 ± 0.280	1.29 ± 0.358	0.95 ± 0.115	1.11 ± 0.164

3.3.5 Faecal Microbiota Characterization

The intake of sodium acetate did not significantly alter bacterial populations (Figure 3.7) including *Escherichia/Shigella spp.*, *Coprococcus Spp.*, *Oscillibacter Spp.*, *C. Coccoides Spp.*, *Roseuria Intestinalis Cluster*, and *clostridium XVIII cluster*. The administration of olanzapine did not affect faecal microbiota. The total bacterial load in each sample (16S DNA abundance) also remained constant between groups.

3.4 Discussion

In the present study, the central epigenetic effect of a daily, oral administration of either the prebiotic, B-GOS®, or sodium acetate in rats was compared. Further investigations evaluated whether the intake of acetate alone, like B-GOS® [133], could attenuate olanzapine-induced weight gain. The administration of B-GOS® inhibited central HDAC activity and increased cortical HDAC-1 and HDAC-3 expression. However, the ingestion of sodium acetate inhibited HAT, not HDAC,

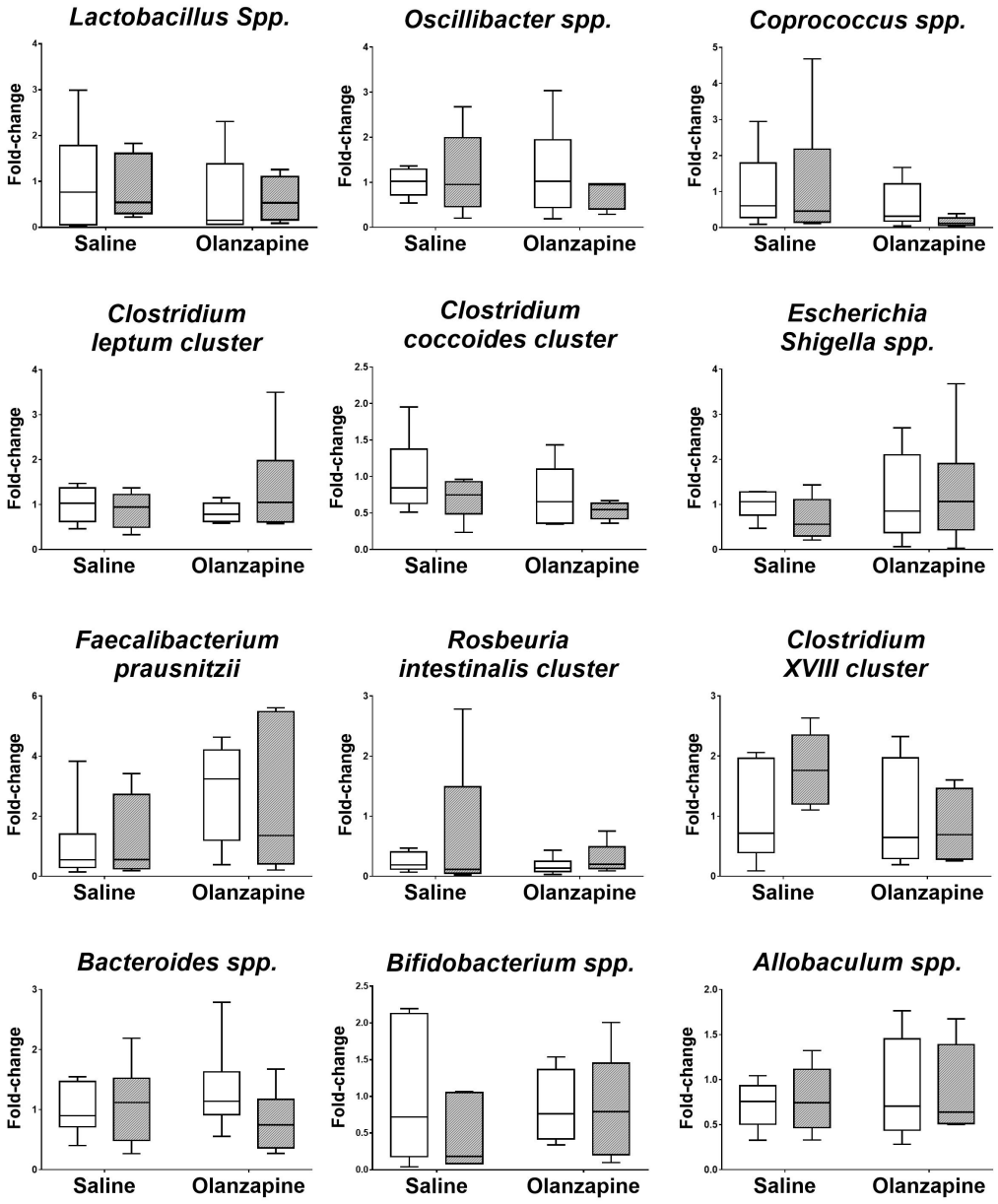


Figure 3.7: Effect of acetate supplementation (500mg/kg/day) and olanzapine administration in adult female rats. Results are expressed as fold-change relative to water/saline group and presented with medians [first to third quartile]. $*p<0.05$ compared to water/saline.

activity in the brain, and increased hippocampal levels of HDAC-3 and HDAC-4 mRNAs. Furthermore, acetate supplementation did not affect olanzapine-mediated weight gain, alter the expression of NMDAR subunits, or influence the abundance of

faecal microbial genera. The data presented in this chapter suggests that B-GOS®-associated benefits on central and peripheral physiology are not entirely, if at all, mediated by acetate produced from the microbial fermentation of this prebiotic.

3.4.1 Prebiotic Inhibition of HDAC activity & altered gene expression

The epigenetic effects of daily ingestion of the prebiotic B-GOS® is a novel finding. This further illustrates the potential of B-GOS® as a therapeutic strategy as HDAC inhibitors have been previously advocated to elicit therapeutic benefits in depression, anxiety, and schizophrenia [131, 179, 187]. A previous study demonstrated that a large single oral dose of GTA (6g/kg) inhibits brain HDAC activity by 2-fold [118]. This is consistent with previous findings where B-GOS® administration, which was shown to enchance the pool of cellular acetate [133], and was able to decrease brain HDAC activity in this study. Impaired HDAC activity pushes the HDAC/HAT enzyme equilibrium to favour transcription, possibly of neuronal genes that may ameliorate deficits in plasticity and cognition [117]. In this regard, the pro-cognitive effects of B-GOS® in rats previously reported to be associated with expression of cortical NMDARs [66], may have been preceded by HDAC inhibition.

Reduced HDAC activity was accompanied by an increase in cortical HDAC-1 and HDAC-3 mRNA in this study. An elevation in HDAC-1 mRNA was also observed after a repeated 28-day GTA (6g/kg) administration, though a reduction

in HDAC-3 expression and unaltered HDAC activity was also reported [185]. In the present study, it is possible that elevated HDAC-1 and HDAC-3 mRNAs were a homoeostatic response to HDAC inhibition, where gene transcription and/or mRNA stability was increased to replenish the inhibited HDAC pool. However, in the present study augmented HDAC-1 and HDAC-3 expression did not rescue impaired HDAC activity. It is unlikely that the reduction of HDAC function was associated with decreased hippocampal HDAC-4 mRNA, since this occurred only in the absence of olanzapine, and HDAC activity was not affected by treatment. Taken together the data suggests that B-GOS® intake for 21 days, like a single dose of GTA [118], inhibited rat brain HDAC activity, but influenced HDAC gene expression in a similar way to long-term GTA administration [185]. This suggests that B-GOS®-derived acetate may not be the sole mediator of the epigenetic actions of this prebiotic. This was confirmed by examining whether the intake of acetate could replicate the central effects of B-GOS®. Since GTA has greater brain penetration [118], and would not truly represent free acetate derived from B-GOS®, sodium acetate was used instead.

3.4.2 Sodium Acetate Intake Decreases Brain HAT Activity & Alters Hippocampal HDAC Gene Expression

Oral sodium acetate supplementation at an appetite suppressing dose (500mg/kg) [151], led to a two-fold elevation in plasma levels, which was consistent with the intake of B-GOS® [133]. The current study replicated the increased concentration of blood

acetate after repeated olanzapine injection. However, unlike B-GOS®, increased peripheral acetate reduced HAT activity without affecting HDAC functioning. This observation is in contrast to repeated GTA supplementation which, although did not impact on HDAC, increased HAT activity [185]. Hypothetically, the direction of change in HAT activity may be dependent on the uptake and steady-state concentration of acetate. Cellular acetate is converted to acetyl-CoA which is utilized for lipogenesis or enters the citric acid cycle in the mitochondria. Cytosolic acetyl-CoA can also freely diffuse into the nucleus where it would be directly available to the HAT enzyme. However, current evidence suggests that histone acetylation is dependent on cytosolic citrate (replenished by the citric acid cycle in the mitochondria) which is converted to acetyl-CoA in the nucleus, and then used by HAT [126, 188].

In this pathway, if a majority of acetyl-CoA directly enters the nucleus, excessive substrate concentrations may cause a homeostatic decrease in HAT activity via substrate inhibition. The greater brain penetrance of GTA, may have produced sufficient acetate to increase mitochondrial metabolism and the pool of citrate, and allow the acetate-citrate-HAT pathway to predominate. This of course, is speculative and can only be confirmed with acetate dose response studies of HAT activity, and measures of brain metabolism *in vivo*. Parenthetically, increased brain acetate concentrations were not detected after repeated intake of sodium

acetate, or B-GOS® or GTA feeding [66]. This may be because when free acetate enters the brain, it is readily converted to acetyl-CoA [150], conceivably at a rate that is sufficiently high to maintain fixed concentrations of this SCFA, particularly when ingested in small amount over time. Changes in brain acetate concentrations therefore, are more likely to be detected shortly after its consumption, and perhaps in high concentrations. However, the entry of acetate into the brain might also be evinced through alterations in central NMDAR gene expression.

Daily sodium acetate supplementation did not affect brain NMDAR subunit expression, unlike the administration of B-GOS® [64, 133], or GTA [66]. Since GTA increased NMDAR receptor function [119], which regulates acetate uptake into the brain [150], it is reasonable to suggest that the null effect of sodium acetate on brain NMDAR subunit expression observed here was indicative of insufficient concentrations to trigger a receptor response, which would have lowered acetate entry into the brain. Further investigations are required to corroborate this theory.

The increased expression of hippocampal HDAC-3 and HDAC-4 mRNAs following acetate supplementation and olanzapine injection paralleled the concentration profiles of blood acetate. However, the olanzapine effect on these genes was not observed in the cohort of animals used in the B-GOS® experiments, in spite of the antipsychotic causing a two-fold elevation in circulating acetate. This differential effect of olanzapine on HDAC-3 and HDAC-4 mRNAs might be related to disparities

in acetate metabolism between cohorts of rats. Inspection of plasma acetate levels revealed that basal concentrations in water/saline rats was more than half the concentration of that in the equivalent group in the B-GOS® experiment [133]. It is possible that lower pre-existing concentrations of acetate does not hinder the effect of olanzapine on HDAC-3 and HDAC-4 gene expression, whereas higher circulating levels suppresses this effect. This is evinced when the combined administration of acetate and olanzapine does not alter the expression of these genes. Overall the present data, and those from investigations of GTA, suggests that direct acetate supplementation does not fully replicate the central actions of B-GOS® intake. Whether acetate derived from the lower gut elicits a different metabolic response to that of oral acetate which is absorbed in the upper part of the gastrointestinal tract, and/or B-GOS® effects on the brain involve other SCFA derived from its fermentation, remains to be elucidated.

3.4.3 Sodium Acetate Does Not Attenuate Olanzapine Induced Weight Gain

The second part of the current study examined whether acetate intake could attenuate olanzapine-induced weight gain in female rats, given that it has been shown to be an appetite suppressant [151]. In the current model, acetate supplementation did not attenuate weight gain, and conversesly appears contribute to increase body weight, particularly over the last few days of the study. This observation is consistent

with previous work reporting acetate from a high-fat diet mediates obesity and metabolic syndrome in rats [171]. In the present study, acetate was administered continuously via drinking water; in contrast to a single, daily bolus which causes a significant, transient rise in blood acetate concentrations. It is possible that acetate intake throughout the day led to blood concentrations that favoured lipogenesis rather than suppress appetite. The suppression of olanzapine-induced weight gain by B-GOS® [133] may have been the result of the prebiotic producing more acetate or, perhaps more likely, other SCFAs derived from prebiotic fermentation such as butyrate, contributing to energy homeostasis [152].

The current data also confirmed that olanzapine increased plasma acetate [133] which is in-keeping with the notion that this SCFA induces weight gain [171]. However, given that B-GOS® also elevates circulating acetate, it can be assumed that it is unlikely to be integral to olanzapine-mediated weight gain, or to the suppression of these metabolic changes by a prebiotic. Although several investigations demonstrate altered lipid metabolism following olanzapine administration, to our knowledge, there are no other studies illustrating altered SCFAs after antipsychotics. The present findings therefore require corroboration.

3.4.4 Sodium acetate Does Not alter gut bacteria

In contrast to previous data presented in this thesis and published work [133], the current investigation did not observe changes in selected faecal bacteria, which

suggests that their alteration by B-GOS® intake was not influenced by acetate production. However, this null effect might also be the result of direct sodium acetate ingestion which did not afford enteric concentrations that were comparable to those following prebiotic fermentation. Measures of acetate in colonic tissue and faecal matter would be required to address this possibility. The current study also observed that olanzapine administration did not affect several bacterial genera, which is consistent with the findings presented in chapter 2. However, others have shown that repeated olanzapine injections to female rats increased the abundance of the phylum Firmicutes in the gut [140]. With the exception of Escherichia/Shigella spp., Bacteroidetes spp., and bifidobacteria spp., all bacterial genera analysed in the present study belong to phylum Firmicutes. It is possible that the olanzapine-mediated changes in this phylum did not involve the bacteria measured in the present study. A similar interpretation might be offered to reconcile altered Actinobacteria [41], with no changes in *Bifidobacteria spp.* after olanzapine administration. However, the earlier untargeted microbiome profiling studies used olanzapine doses and administration procedures that were different to each other, and to the current study. Clearly, a more accurate sequence-based evaluation of the gut bacteria is required to support or refute the present findings, and to identify the phyla/genera that are affected by olanzapine at the present dose used.

3.5 Conclusion

The present study provides evidence to suggest that ingestion of acetate as a way to augment cellular pools of acetyl-coA to influence epigenetic processes cannot replicate the metabolic effects of prebiotic feeding. This highlights that the gut-brain axis communication likely relies on mechanisms additional to SCFA actions. Future studies should examine the concerted effects of endocrine or immune metabolites with SCFAs.

For a research worker the unforgotten moments of life are those rare ones which come after years of plodding work, when the veil over natures secret seems suddenly to lift and when what was dark & chaotic appears in a clear & beautiful light.

The first person to show that a defect in an enzyme can cause a human disease, and together with her husband, they discovered the Cori cycle, which showed how muscle tissue uses chemical reactions to turn carbohydrates into lactic acid.

— Gerty Cori (1896 - 1957)

4

Prebiotic Supplementation Does Not Influence Serum Metabolic and Immune Markers in Medicated Psychosis Patients

A portion of the data in this chapter have been published in *Scizophrenia Research* "Pro-cognitive effect of a prebiotic in psychosis: A double blind placebo controlled cross-over study" [189]

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Abstract

Adverse cardio-metabolic side effects such as weight gain are commonly attributed with several frontline treatment options for psychosis. The oral administration of the prebiotic B-GOS® to rats, prevented olanzapine-mediated weight gain and improved cognitive flexibility have recently been demonstrated. The current study explores these findings into a clinical population receiving antipsychotic medications. A total of 39 non-affective psychosis participants aged 18-60 were randomized in a double-blind 24-week placebo-controlled crossover study. Participants received either a 12-week treatment with the prebiotic B-GOS® followed by a 12-week maltodextrin (placebo) supplement, or in the reverse order. Simple anthropometric measurements such as BMI, central adiposity (waist & hip circumference), and the concentration of serum inflammatory biomarkers (IL-6, CRP) and acetate, were measured at week 0 (baseline), 12, and 24. Using two complementary statistical models (paired *t*-tests and mixed linear regression) to confirm the findings presented. B-GOS® supplementation did not confer any significant effects on anthropometric indices. Circulating levels of IL-6, CRP and acetate were analyzed with the same

models and reveal no significant changes after prebiotics. Together the findings in this section implies that prebiotics would not be an effective strategy to manage cardio-metabolic side effects of antipsychotics in psychosis patients.

4.1 Introduction

Weight gain is a serious side effect of antipsychotics, particularly clozapine and olanzapine, and likely contributes to the increased prevalence of obesity in patients with severe mental illness, which ranges between 41-50% compared to the 20-27% in general population [190]. The propensities of antipsychotics to confer clinically significant weight gain, defined as more than 7% increase in baseline body weight, have been repeatedly demonstrated in randomized controlled trials (RCTs) [134, 191], literature reviews [7], and confirmed in some meta-analyses [71, 72]. Not only does this weight gain decrease medication tolerability, but it also presents as a significant risk factor for metabolic syndrome and cardiovascular disorders, likely owing to antipsychotic-associated impaired glucose metabolism, increased cholesterol and triglyceride levels as well as arterial hypertension [192]. Together, antipsychotic-induced weight gain and its associated cardio-metabolic effects are major contributors of morbidity and mortality in people with psychosis.

The inflammation-centred hypothesis of schizophrenia, and to some extent obesity, has received some scrutiny in the past. Elevated levels of pro-inflammatory cytokines and other markers have been documented in blood and cerebrospinal fluid

of patients with schizophrenia [87, 193]. Amongst these markers, high levels of CRP, a hepatocyte-derived pattern recognition molecule that is indicative of subclinical systemic inflammation [194], has been consistently reported in schizophrenia patients that neither antipsychotic administration nor disorder progression were able to normalize [52, 195, 196]. This implies that CRP may play a crucial role in schizophrenia pathogenesis, particularly since CRP may also contribute to cognitive impariments [197], a common clinical presentation of schizophrenia and possibly consequence of antipsychotic use [40]. Similarly, elevated levels of IL-6 have also been positively associated with illness progression [88], implying that therapeutic compounds which exhibit anti-inflammatory properties could be beneficial for patients. While antipsychotics have been shown to modulate microglia, a major sources of various inflammatory cytokines and free radicals in the brain, implying anti-inflammatory properties [198], whether this underpins their therapeutic actions remains to be investigated.

Augmenting the intestinal microflora with prebiotics have been shown to exhibit anti-inflammatory properies in the brain. In neurodegenerative rodent models, decreased serum CRP levels [96] and inflammatory markers such as inducible nitric oxide synthase (iNOS) and TNF-α [123] have been reported following prebiotic intake. Furthermore, antibiotics, which influence the intestinal environment by inhibiting the growth of microbes have attenuated some of the negative impacts of the antipsychotic

risperidone [199]. Since antibiotics cannot be used extensively, it nevertheless provides a precedent to explore other gut-based therapies for psychosis, such as prebiotics. Previous work in this thesis has demonstrated that supplementing rats with the prebiotic B-GOS® attenuates olanzapine-induced weight gain [133]. This anorexigenic effect has been demonstrated in overweight adults where significantly decreased markers of metabolic syndrome including CRP, insulin, total cholesterol, triglyceride and cholesterol ratio have been reported [102]. However, it may be notable that these serum observations were not accompanied with decreased total body weight (i.e., kgs) [102, 200]. It is possible that simple clinical measures such as BMI and measures of abdominal obesity (i.e., waist and hip circumference) which have traditionally been used to predict weight gain and estimate the efficacy of anti-obesity strategies were not sensitive enough. Several studies have proposed that waist circumference and waist-to-hip ratio (WHR) are apropo predictors of metabolic syndrome [201–203] and therefore provides richer insights of metabolism. Notably, in a recent a double-blind, placebo controlled, randomized trial in children, significant reductions in body weight z-score were reported, in addtion to decreased percent body fat, percent trunk fat, and serum level of IL-6 [204].

4.1.1 Specific Objectives

In the present study, the effect of administering the prebiotic B-GOS® on total body weight and immune function in medicated stable psychosis participants was

explored. This study is as a part of a larger clinical investigation, the remaining of which are detailed in Chapter 5. A proof of concept for the clinical potential of the prebiotic B-GOS® was provided by a double-blind maltodextrin-controlled randomized crossover study.

The specific aims of this study was therefore to:

- Investigate the effect of administering the prebiotic B-GOS® on anthropometric measurements
- Document the changes in serum levels of inflammatory markers (CRP and IL6) and intestinal metabolite, acetate
- Investigate correlations between anthropometric measurements and serum markers at baseline as well as before and after B-GOS®

4.2 Materials & Methods

Clasado BioSciences Ltd., UK, provided both the B-GOS® prebiotic and the maltodextrin placebo. All supplements were colourless powders packaged in 3.8g sachets for daily consumption and have been approved by the GRAS (Generally Recognised as Safe) programme, conducted by the U.S. Food and Drug Administration (FDA), to be safe and well tolerated. Detailed list of ingredients are included in appendix C.1. Blood samples were collected in gold top serum separating tubes from BD Vacutainer™ and where serum aliquots were stored at -80°C until analyses. Acetate, human CRP and human IL-6 colorimetric assays were purchased from Abcam, UK

4.2.1 Participants

Ethical approval was provided by South-Central Oxfordshire Research Ethics Committee B (reference 16/SC/0654). The study was registered on ClinicalTrials.gov, identifier number NCT03153046 (url: https://clinicaltrials.gov/ct2/show/NCT03153046).

Participants were recruited from community mental health services in Oxford Health NHS FT. Inclusion criteria were men and women, between the ages 18-60 years old with non-affective psychosis (e.g., schizophrenia, first episode psychosis), who were stable on antipsychotic medication for at least three months prior to recruitment. Considering the possibility of a ceiling effect of cognition, the main outcome in this study, participants who scored 0.5 standard deviations below the healthy average on the composite score using a cognitive test battery were invited to participate. Further inclusion criteria included subjects who were not current inpatients in a psychiatric hospital, did not present with major depressive disorders or any additional major medical disorders (including diseases affecting the human gastrointestinal tract and blood-borne disorders), were not taking any antibiotic, or dietary probiotic and/or prebiotic supplements in at least the three previous months, did not have any intolerances to lactose, and did not recently, or plan to, alter their diet. Participants were informed that B-GOS® is a lactose-derived product and may contain small amount of lactose that results in abdominal discomfort such as bloating, flatulence or abdominal pain, thus participants who self-identified

without an intolerance to lactose were also included.

4.2.2 Study Design

Participants were randomly assigned to one of two groups: one started the trial with the placebo (maltodextrin) and the other with B-GOS®. Participants were allocated a sequential subject ID, and given a 12-week supply of sachets. They were instructed to mix the contents with water and to consume the product every day at approximately the same time. Participants undertook cognitive, metabolic and inflammatory marker testing at week 0, 12, and 24. Participants were also called every two weeks to monitor any changes or additions to medication, adverse events and to record adherence. Participants were required to bring remaining sachets to each follow up meeting, where non-compliance (<60% of sachets were ingested) resulted in removal from the study.

Power Analysis

A power analysis was estimated using the mean and standard deviation scores (40.9±16.6) in patients with schizophrenia spectrum disorders on the trail making test A [205] and to achieve a significant effect (change of 7sec; α=0.05) at a power level of 0.8,a minimum of 35 participants per group was required.

4.2.3 Collection & Preparation of Whole Blood

Whole blood (10ml) was collected by peripheral venipuncture from patients at the end of each follow up session in a gold-top serum vacutainer tube, which are spray-

coated with silica and a polymer gel for serum separation. Tubes were inverted a few times to ensure thorough mixing with coated gel, before setting aside for 10-30min at room temperature to allow blood to clot. Tubes were centrifuged at 1500g for 10min at room temperature before transferring serum into fresh microcentrifuge tubes. All samples were stored at -80°C until analysed.

4.2.4 Measurement of Serum Biomarkers

Levels of circulating inflammatory markers and acetate in plasma were measured using commercially available assay kits and assayed according to manufacturers' instructions. The limit of detection were calculated as 8.64pg/ml for CRP, 6.95pg/ml for IL-6 and 0.02nmol/ml for acetate. Inter-assay coefficient of variation for all assays was 5%.

Acetate

Commercially available colorimetric kits converts free acetate into an intermediate in the presence of Acetate Enzyme Mix and Acetate Substrate Mix. This intermediate can be detected at 450nm. All samples were assayed in duplicate and protocols were carried out according to manufacturers' instructions.

C-Reactive Protein High Sensitivity

Serum was diluted 1:10,000 then assayed on pre-coated 96-well plates (ab181416). Free floating CRP in samples are bound to the immobilized antibody and detected

using HRP-conjugated streptavidin and TMB substrate solution for colour development.

Interleukin-6

Human IL-6 was measured using SimpleStep ELISA kit (ab178013) with no serum dilution. Free floating IL-6 in samples are bound to capture antibody on pre-coated 96-well plates and detected using HRP-conjugated streptavidin and TMB substrate solution for colour development.

4.2.5 Statistical Analyses

The SPSS ver. 22 program was used to perform all statistical analyses. All data are presented as mean±SEM, and were assessed for normality using the Shapiro-Wilk test. Paired *t*-tests were used to examine the overall effects of B-GOS® and placebo, on pooled data before and after the administration of supplements. A linear mixed model repeated measures analysis was also used to explore the influence of the individual components of the trial by comparing group (placebo vs B-GOS®), time (0 [baseline], 12 and 24 weeks), and order (placebo-B-GOS® vs B-GOS®-placebo). Additional analysis of each parameter(s) was only performed when initial tests and estimates of fixed effects reached significance ($p<0.05$). Pearson correlations with cognitive outcome measures and serum biomarkers were examined and reported as r^2 values.

4.3 Results

4.3.1 Population & Baseline Measures

A total of 52 volunteers were screened using the tablet-based BACs test (Figure 4.1), 10 of which did not meet the inclusion criteria (scored above 0.5 standard deviations below the healthy mean) and 3 reconsidered the study due to trypanophobia. Therefore, thirty-nine participants with psychosis were randomized at baseline. Over the course of the study, a total of 16 participants withdrew, (six were receiving maltodextrin; ten were receiving prebiotics), 12 of which withdrew in the first 12-week arm, and four in the second arm. Reasons for withdrawal included loss to follow-up, psychosis relapse, as well as side effects such as dizziness, vomiting and general discomfort. This resulted in 23 participants that completed the study. Detailed demographics of medication and ethnicity were included in the appendix (table C.1).

4.3.2 Anthropometric Measures

Measurements of weight, central adiposity (i.e., hip and waist circumference, WHR) and BMI (Table 4.1) remained unaltered following the ingestion of the prebiotic for 12 weeks. These findings were further confirmed using a mixed linear regression model that revealed no significant changes when accounting for the order of supplementation (appendix C.2).

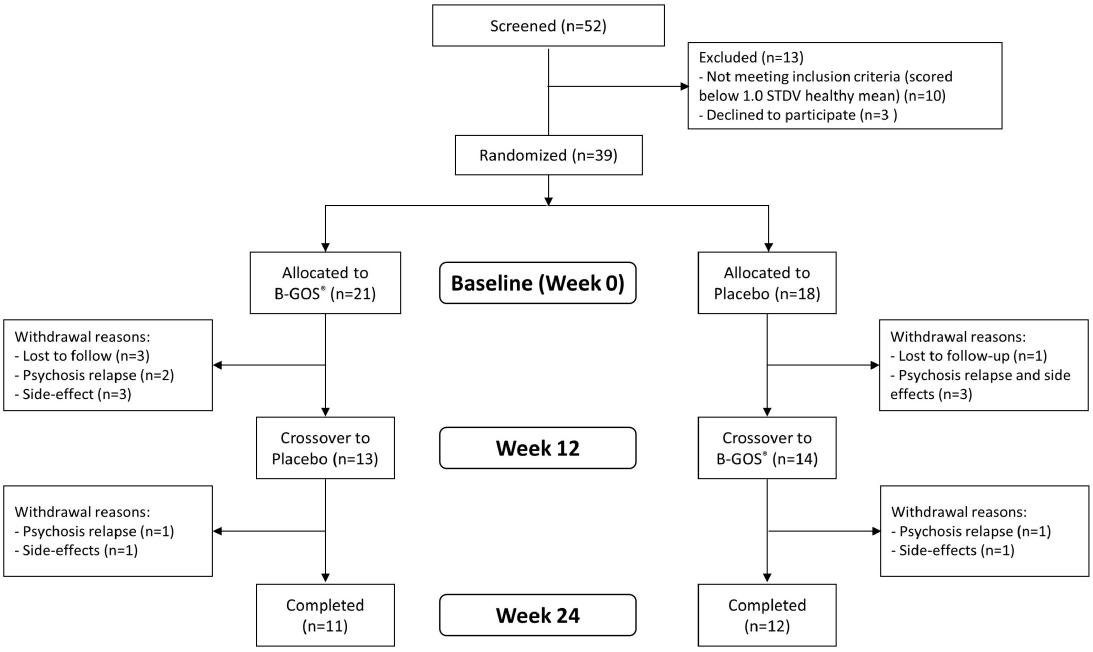


Figure 4.1: Clinical Study Flowchart.

4.3.3 Circulating SCFA & Inflammatory Cytokines

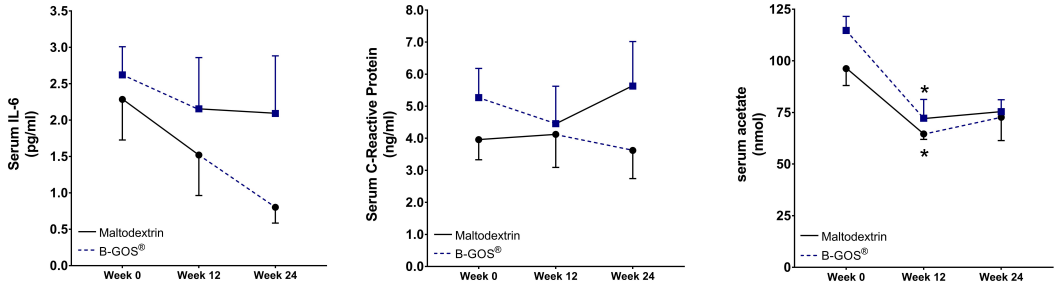
Paired *t*-tests did not reveal significant differences after B-GOS® ingestion on serum levels of acetate, CRP and IL-6 (Table 4.1).

Using the mixed linear repeated measures model (Figure 4.2), a significant effect of time on IL-6 (F=3.361, p=0.040) and acetate (F=19.512, p<0.05) were observed. Estimates of fixed effects revealed that decreases only reached significance between 0-12 weeks for both IL-6 (t=2.707, p=0.013) and acetate (t=2.859, p=0.012). The decrease in IL-6 over this period occurred in participants ingesting placebo (p=0.016), whereas acetate reduction was significant in participants supplemented with B-GOS® (p=0.03). Concentrations of serum CRP did not change over the 24-week trial following the intake of placebo or B-GOS®.

Table 4.1: Effect of B-GOS® on anthropometric measures and serum biomarkers. Paired *t*-test; *p<0.05

	Pre-B-GOS® (n=24) mean (SEM)	Post-B-GOS® (n=24) mean (SEM)	*t (p)*
Central Adiposity			
BMI (kg/m^2)	32.32 (1.569)	32.63 (1.561)	-1.27 (0.217)
WHR	0.93 (0.011)	0.93 (0.011)	-0.59 (0.559)
Hip Circumference (cm)	112.83 (6.237)	121.46 (4.84)	-1.28 (0.214)
Waist Circumference (cm)	105.2 (6.069)	113.92 (5.177)	-1.36 (0.189)
Serum Markers			
Acetate (nmol/ml)	1881.47 (118.722)	1448.29 (140.946)	1.70 (0.102)
CRP (ng/ml)	4.54 (0.825)	3.89 (0.755)	1.51 (0.150)
IL-6 (pg/ml)	2.12 (0.451)	1.61 (0.448)	1.02 (0.320)

In an exploratory analysis, the effect of medication on serum biomarkers were examined. Patient data were stratified into three groups: "clozapine", "olanzapine" and "others" and analysed using mixed linear model analyses. The concentration of circulating acetate was significantly reduced in those taking clozapine (t=3.238, p=0.011, n=10) and "others" (t=3.305, p=0.006, n=16) between week 0 and week 12. Medication did not affect any other of the parameters measured.

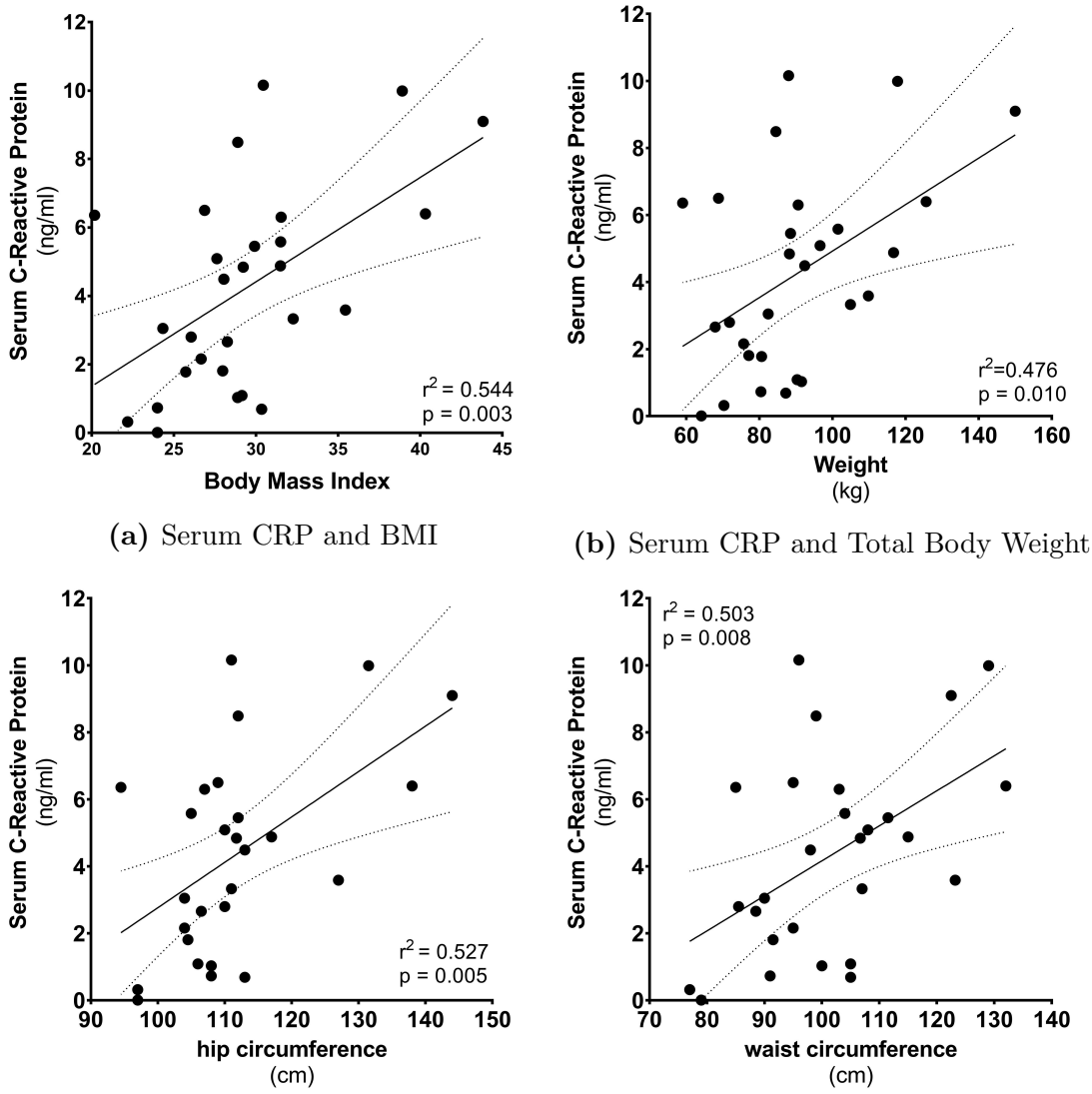


(a) IL-6 **(b)** CRP **(c)** Acetate

Figure 4.2: Changes in serum (a) IL-6, (b) CRP and (c) acetate in stable patients with schizophrenia who received firstly maltodextrin or prebiotics for 12 weeks, following by prebiotics or maltodextrin. Results are expressed as mean±SEM. General Mixed Model *p<0.05.

4.3.4 Pearson Correlations with Serum Markers

The correlation between serum levels of acetate, CRP or IL-6 and cognitive and metabolic outcome measures were examined at baseline (Figure 4.3) which includes all participants enrolled in the study (n=39). Serum CRP was positively correlated with BMI, weight and waist/hip circumference.



(a) Serum CRP and BMI

(b) Serum CRP and Total Body Weight

(c) Serum CRP and Hip Circumference

(d) Serum CRP and Waist Circumference

Figure 4.3: Significant Pearson correlations between CRP and Anthropometric measurements at baseline. Doted lines represent the 95% confidence intervals.

Similar correlations were performed before and after B-GOS® ingestion to determine whether prebiotics intake influenced serum acetate levels and affected inflammatory biomarkers (Table 4.2). Pearson correlations suggests that B-GOS® intake does not influence serum levels of IL-6 as it remained significantly associated with BMI before and after prebiotics intake. Weight and IL-6 was significantly correlated, though after 12 weeks of prebiotic intake, this no longer reached significance. Although not significant, it is interesting that BMI and weight were negatively correlated with serum acetate levels before B-GOS® but were positively correlated after B-GOS®. Although no significance was reached, it is interesting that circulating levels of acetate was negatively correlated with BMI/weight prior to B-GOS® then became positively correlated following B-GOS® ingestion (Table 4.2).

Table 4.2: Effect of 12 weeks of B-GOS® supplementation on anthropometric measurements. Values reported as Pearson r^2 correlations with serum biomarkers and metabolic measures. BMI: body mass index, WHR: Waist-to-Hip Ratio, HC: hip circumference, WC: waist circumference.*$p<0.05$

	Acetate		CRP		IL-6	
	Pre-B-GOS®	Post-B-GOS®	Pre-B-GOS®	Post-B-GOS®	Pre-B-GOS®	Post-B-GOS®
BMI	-0.150	0.160	0.384	0.330	**0.499***	**0.436***
Weight (kg)	-0.046	0.230	0.347	0.417	**0.516***	0.258
WHR	0.024	0.154	-0.030	-0.057	-0.016	-0.189
HC (cm)	0.014	0.155	0.171	0.391	0.030	0.135
WC (cm)	0.008	0.173	0.130	0.292	0.012	0.064

4.4 Discussion

The present study investigated the effects of administering B-GOS® on anthropometric measurements and levels of circulating inflammatory markers (CRP and IL-6)

in psychosis patients. Following B-GOS® supplementation no significant changes in BMI and central adiposity were observed and inflammatory serum biomarkers IL-6 and CRP remained unchanged. In a mixed linear model of weeks 0-12, participants supplemented with the placebo had significantly lower levels of IL-6 whereas those on prebiotics had lower levels of acetate. Serum CRP levels remained unalterd over the course of 24 weeks regardless of supplement. Notably, there was an overall effect of medication, namely participants not treated with olanzapine were likely driving the reduction in serum acetate. Correlation analyses at baseline revealed that serum CRP was significantly correlated with various body weight measurements and that levels of circulating IL-6 was not influenced by 12 weeks of B-GOS® supplementation.

4.4.1 No Effect on Serum Acetate Levels

The finding that B-GOS® intake does not influence serum acetate levels nor attenuate weight gain in patients treated with antipsychotics is somewhat surprising. The fermentation of the prebiotic B-GOS® by gut bacteria produces high levels of this SCFA [148], and in rats, B-GOS® feeding elevated circulating acetate levels [66, 133]. Olanzapine administration to rats has also been seen to increase blood acetate [133]. The present study therefore measured serum acetate to determine an association with weight gain, given that it has favourable anti-inflammatory actions [119]. Conversely, B-GOS® did not influence circulating acetate levels though, notably, participants treated with clozapine and other antipsychotics had significantly lower

blood acetate levels when compared to those treated with olanzapine, irrespective of the supplement taken. This increased clearance of blood acetate by clozapine and other antipsychotics, may have confounded the analyses of this SCFA. Another confounder is that antipsychotic compounds have recently been reported to exhibit anti-microbial effects [44, 206]. This may offer a partial explanation to the decreased level of acetate in participants treated with clozapine as growth of acetate-producing microbes may have been suppressed.

4.4.2 Total Body Weight was Unaltered

The present study also demonstrated that B-GOS® supplementation does not affect body weight, BMI or central adiposity in participants with psychosis. This finding is in contrast to recent observations in rats where B-GOS® attenuated olanzapine-induced weight gain [133]. One explanation for this discrepancy could be that a majority of participants in the current study were treated with antipsychotics for several years, whereas in the aforementioned study rodents received B-GOS® several days before drug administration. This would imply that prebiotics supplementation is effectual once olanzapine-induced weight gain is established, and perhaps further examination in untreated first episode psychosis patients are warranted. While B-GOS® has been previously demonstrated to improve markers of metabolic syndrome including decreasing levels of inflammatory marker CRP, plasma insulin and total cholesterol levels, without affecting overall body weight [102], a systematic review

in humans reports that the evidence overall for the metabolic benefits of prebiotics remain contradictory [207].

4.4.3 Inflammatory Cytokines and Prebiotics

Anti-obesity effects of prebiotics was initially hypothesized to mediate through an anti-inflammatory mechanism. Elevated levels of circulating IL-6 have been reported to be a risk factor for schizophrenia [85, 86] as well as a potential predictor for first-episode and acute exacerbations of psychosis [87]. Serum IL-6 has also been positively associated with illness duration [88], depressive symptoms [89] and cognitive impairments [90]. Increased levels of serum IL-6 may contribute to metabolic dysfunctions since downstream signaling proteins, such as soluble glycoprotein 130 and soluble IL-6 receptor, have been significantly elevated in patients with metabolic syndrome [91]. However, in the present population, no significant changes in this inflammatory marker was observed with B-GOS® supplementation, and no association with obesity indices at baseline or at follow-up.

However, the effect of prebotics on circulating IL-6 have not been conclusive. One study using B-GOS®, significantly decreased serum IL-6 levels within five weeks of ingestion in a healthy geriatric population [156]. However, the authors were unable to replicate this finding in a crossover study a few years later in a comparable cohort with the same prebiotic compound [200]. These findings imply the fickle nature of the stability of the IL-6 protein or, more likely the

need to characterize additional biomarkers to elucidate a potential mechanism. Although significantly decreased levels of IL-6 were observed in type 2 diabetes patients using another prebiotic formulation [208], a recent meta-analysis reports that prebiotics does not exert any significant effects on serum IL-6 levels [96]. This is consistent with our current findings where circulating levels of IL-6 were not affected by B-GOS® supplementation.

4.4.4 Inflammation and Obesity

The interpretation that B-GOS® does not influence serum levels of IL-6 was derived from the observation that IL-6 remained significantly associated with BMI before and after prebiotics (Table 4.2). Alternatively, this could be a validation of the recruited population as positive correlations between IL-6 and total body weight have previously been reported. Elevated levels of IL-6 have been linked to obesity and type II diabetes mellitus [209], possibly through the activation of AMPK, a central mediator in several metabolic processes including fatty acid synthesis [210]. However, phenotypes of rodent with a double-knockout for the IL-6 gene includes obesity, hepatosteatosis, liver inflammation and insulin resistance compared to wildtype controls [211]. This observations imply that therapeutic strategies which decrease IL-6 concentrations or, at least block IL-6 receptor (IL-6R) signalling, could counter-intuitively lead to further cardio-metabolic impairments. To further complicate the role of IL-6, a recent report highlights a negative asociation between

serum levels of IL-6R and BMI in in psychosis patients experiencing weight gain [212], suggesting that patients with increased IL-6R would experience less weight gain. These findings suggest that IL-6 has lipolytic effects and anti-obesity potential, and since the IL-6 signalling pathway has been broadly characterised into two models: the classical model (i.e., IL-6 binding to the IL-6R on target cells allowing the IL-6/IL-6R complex to interact with GP130 on the cell membrane) and the trans-signaling model (i.e., soluble type of the IL-6 receptor binds to IL-6, and together they interact with gp130 that elicits different cellular responses) [91], further exploration of the IL-6 signalling mechanisms may provide richer insights into antipsychotic-induced weight gain, and the SCFA-dependent, or independent, anti-inflammatory potential of prebiotics.

4.4.5 Limitations

Limitations of the study will be discussed collectively in Chapter 5.

4.4.6 Future Directions

There are a plethora of serum biomarkers that can be profiled in psychosis patients that may further the mechanistic understanding of prebiotics. In a recent longitudinal study examining the impact of antipsychotics in pharmacologically-naive patients, 14 water-soluble metabolites in serum had consistently changed with pharmacological treatment [213]. Amongst these markers, a strong association was identified with the NMDAR agonist D-serine and L-glutamate acid. Since

schziophrenia has been associated with hypoglutamate functioning, compounds that increase NMDAR expression and function, such as B-GOS® [64], or other receptor agonists may confer therapeutic benefits. Although compounds that activate GLP-1R has shown therapeutic promise particularly against weight gain associated with olanzpine/clozapine [164, 165, 214], this mechanism of action has been implicated in acting on the serotonin system [168] which could potentially jeopardize the efficacy of antipsychotic agents. Current interventions for managing obesity in psychosis patients is very limited. Non-pharmacological strategies including behavioural, nutritional and physical interventions are compromised by low acceptability and adoption rates amongst psychosis patients [215] whereas pharmacological approaches are confounded by adverse side effects such as increasing cardiovascular risks, depression, anxiety and potentially suicide [216–218]. Therefore, gut-based strategies remain plausible for psychiatric therapy.

4.5 Conclusions

The current study demonstrates that the prebiotic B-GOS® did not increase circulating levels of acetate, and was unable to influence anthropometric measures of obesity and cursory biomarkers of the immune system. This report, and in addition to the findings presented thus far in this thesis, suggests that acetate may not be a critical mediator for the anti-obesity profile of the prebiotic B-GOS®. However,

this does not preclude acetate altogether as concerted actions with other SCFAs such as butyrate, or distal effects of acetate remain possible

Science and everyday life cannot and should not be separated as it provides a partial explanation for life. In so far as it goes, it is based on fact, experience, and experiment.

A chemist, molecular biologist, and one of the key figures behind unlocking the structure of human DNA, Franklin's skills in molecular biology were overlooked as Watson and Crick became known as the pair credited with the Nobel Prize in Physiology or Medicine in 1962.

— Rosalind Franklin (1920 - 1958)

5

Prebiotic Supplementation Improves Global Cognitive Functioning in Medicated Psychosis Patients

A portion of the data in this chapter have been published in *Scizophrenia Research* "Pro-cognitive effect of a prebiotic in psychosis: A double blind placebo controlled cross-over study" [189]

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Abstract

Neurocognitive impairments are increasingly recognized as a fundamental symptomatology in schizophrenia with more than 80% of patients exhibiting significant deficits, even at first episode of illness. Current pharmacotherapies do not alleviate cognitive symptoms of schizophrenia, and may even exacerbate impairments. Recent demonstrations in rodents using a prebiotic to attenuate olanzapine-induced weight gain and improve cognitive flexibility suggests that gut-based strategies may confer therapeutic benefits. The current study explores the clinical potential of improving cognitive impairments using the prebiotic B-GOS® in psychosis patients. A total of 39 medicated psychosis patients aged 18-60, treated with antipsychotic agents, were randomized in a double-blind 24-week maltodextrin-controlled crossover study. Neurocognitive functioning was evaluated using a tablet-based Brief Assessment of Cognition in Schizophrenia (BACS) which assesses multiple learning domains. All measurements were taken at week 0 (baseline), 12, and 24. Pearson correlations with serum inflammatory markers (CRP and IL-6) as well as circulating acetate from Chapter 4 was also examined with changes in cognitive performance. Experimental design and analyses have been described in

Chapter 4. Here, B-GOS® supplementation enhanced global cognitive performance in the first 12 weeks of the trial. Stratified analyses comparing verbal and executive cognitive domains suggests that this increase in global functioning following B-GOS® supplementation was driven by the improvement in executive functioning tasks, an NMDAR-based domain. No significant correlations were detected with serum biomarkers and cognitive/psychological change. Overall these findings suggest that the intestinal microbiome is a feasible therapeutic target to improve cognitive impairments in psychosis-related disorders.

5.1 Introduction

The most disabling aspect of psychosis is the cognitive impairments that are detectable early in illness and remain stable or further deteriorate for periods of up to ten years [6]. While anti-psychotic medications are effective strategies for reducing remission rates and symptom management [73, 219] lifetime cumulative exposure appears to negatively impact cognition, particularly verbal learning and memory performance [220]. Patients who require high doses of antipsychotics or polypharmacy score significantly lower than those on recommended doses or monotherapy in specific cognitive domains such as processing speeds [221], attention [222] as well as global cognitive functioning [40, 223]. Although discontinuation of antipsychotic medications have been attributed to beneficially impact clinical domains such as cognition [224] and metabolic measures [225], relapse is inevitable.

Therefore, along with conventional treatment programs, an opportunity for identifying adjunctive therapies which can reduce anti-psychotic-associated side effects such as weight gain would provide significant clinical value.

Early life increases in CRP have been reported as a risk factor for the develope-ment of schizophrenia in later life [226], and may have a role in cognitive decline [197, 227]. A recent meta-analysis in both first episode and chronic phase patients revealed elevated levels of serum CRP, regardless of antipsychotic use [195], despite reported anti-inflammatory properties of antipsychotics [198]. At the time of writing this thesis, studies that examined an association between CRP and symptom severity was minimal, where most investigations reported negative associations with cognitive impairments [197, 227]. Notably, a recent study highlights CRP, as well as IL-6 to be related to cognitive impairments in the context of neuropsychiatric disorders, including schizophrenia [228]. Therefore, therapeutic strategies that can normalize serum CRP levels, or inflammation at large, may be beneficial for cognition in psychosis patients.

Manipulating the intestinal microbiome to influence CRP levels can be one such strategy. The administration of the prebiotic B-GOS® has been shown to impart some anti-inflammatory effects including decreasing levels of CRP [96] as well as inflammatory biomarkers in the brain [123]. These reports advocate the intestinal microbiome as a viable target to suppress inflammation and attenuate

cognitive impairment. In further support of this one study has demonstrated that the administration of minocycline, a broad-spectrum tetracycline antibiotic with anti-inflammatory properties, improved negative symptoms in early stage psychosis patients treated with risperidone [199]. Since broad applications of antibiotics is not a feasible therapeutic strategy, the study provides a precedent to further explore microbial-based therapies for psychosis and examine whether they can attenuate symptoms that do not respond to conventional therapies.

While studies in rodents have demonstrated that prebiotics supplementation can improve cognitive flexibility [66] and prevent olanzapine-mediated weight gain [133], at the time of writing this thesis, four studies had examined central effects of prebiotics supplementation in humans. In children, increased feelings of fullness and reduced prospective food consumption following prebiotic ingestion was reported in older (11-12 year old) but not in younger children (7-10 year old) [229] suggesting that prebiotics may modulate neuronal circuits in the hypothalamus and brainstem which govern feelings of satiety. However, two other studies that examined the effects of prebiotics in pre-term infants did not report significant central changes in neurodevelopmental marker [230, 231], though in healthy adult volunteers, significantly higher attentional vigilance to positive stimuli and waking cortisol levels, broad physiological measure of stress, were reported [63]. It is interesting that these findings suggest that prebiotics have a lesser effect on developing brains (infants,

children) and may only influence central processes closer to maturation (adults). Since schizophrenia has been viewed as a neurodevelopemental disorder [232], it would be particularly interesting to contrast the prebiotics effect on cognition and mood between developing and developed brains.

The previous chapters in this thesis suggested the SCFA acetate, the main metabolite of bacterial fermentation of the prebiotic B-GOS® [148] could be mechanistically involved in the central and peripheral effects. Therefore, the aim of the present study was to examined if B-GOS® supplementation in medicated stable psychosis participants could improve cognitive function and whether circulating levels of serum CRP, IL-6 and acetate are involved in this process.

5.1.1 Specific Objectives

The specific aims of this study was therefore to:

- Examine whether cognitive improvements can be observed after B-GOS® supplementation in patients with psychosis
- Determine whether the prebiotic B-GOS® influences psychological well-being
- Characterize the correlations between cognitive tasks/domains and serum markers at baseline, before and after B-GOS® supplementation

5.2 Materials & Methods

In addition to materials used in section 4, cognitive tests were administered through an IPad Air 2.

5.2.1 Participants and Study Design

As detailed in section 4.

5.2.2 Cognitive Test Battery & Psychological Index

Cognitive functioning was assessed using the Brief Assessment of Cognition in Schizophrenia (BACS) test battery [233, 234]. A tablet-based application designed by NeuroCog Trials examined five cognitive domains using six subtests (Table 5.1). The scores of the six subtests are used to construct a composite score that estimates global cognitive functioning. All scores are converted into a T-scores by comparing to the mean and standard deviation from a healthy controls database.

Table 5.1: Cognitive Domains Assessed by BACS Test

Cognitive Domain	**Tablet Task**
Verbal Learning & Memory	Verbal Memory
Working Memory	Digit Sequencing
Motor Function	Token Motor
Verbal Fluency	Semantic Fluency Letter Fluency
Processing Speed	Symbol Coding
Executive Function	Tower of London

Verbal Memory

Patients are presented with 15 words and then asked to recall as many as possible. This procedure is repeated 5 times where the outcome measure was the total number of words recalled.

Digit Sequencing

Patients are presented with clusters of numbers of increasing length. They are asked to tell the experimenter the numbers in order, from lowest to highest where the outcome measure was the number of correct responses.

Motor Speed

Patients are given 100 plastic tokens and asked to place them into a container as quickly as possible for 60sec. The outcome measure was the number of tokens correctly dragged into the container.

Verbal Fluency

A summative score from two separate tests including Category Instances (Semantic Fluency) and Controlled Oral Word Association Test (Letter Fluency). Patients are given 60sec to name as many words as possible within a given semantic category (i.e., animals), and in two separate trials, patients are given 60sec to generate as many words as possible that begin with a given letter (e.g., S or T). The total number of words from the three trials is the outcome measure.

Symbol Coding

Symbol Coding examines attention and processing speed. As quickly as possible, patients write numerals 1–9 as matches to symbols on a response sheet for 90sec. A key explaining how unique symbols corresponds to the individual numbers 1-9 are provided. The outcome measure is the number of correct responses.

Tower of London

Tower of London examines reasoning and problem solving. Patients look at two pictures simultaneously. Each picture shows three different-coloured balls arranged on three pegs, with the balls in a unique arrangement in each picture. The patients are told about the rules in the task and are asked to provide the least number of times the balls in one picture would have to be moved to make the arrangement of balls identical to that of the other, opposing picture. There are two alternate forms. The outcome measure is every correct response.

Depression Index

Patients were requested to complete the Becks Depression Inventory to measure the severity of depression. This is a 21 multiple choice, self-reported inventory to monitor characteristic attitudes and symptoms of depression throughout the study [235].

5.2.3 Collection & Preparation of Whole Blood

As detailed in chapter 4.

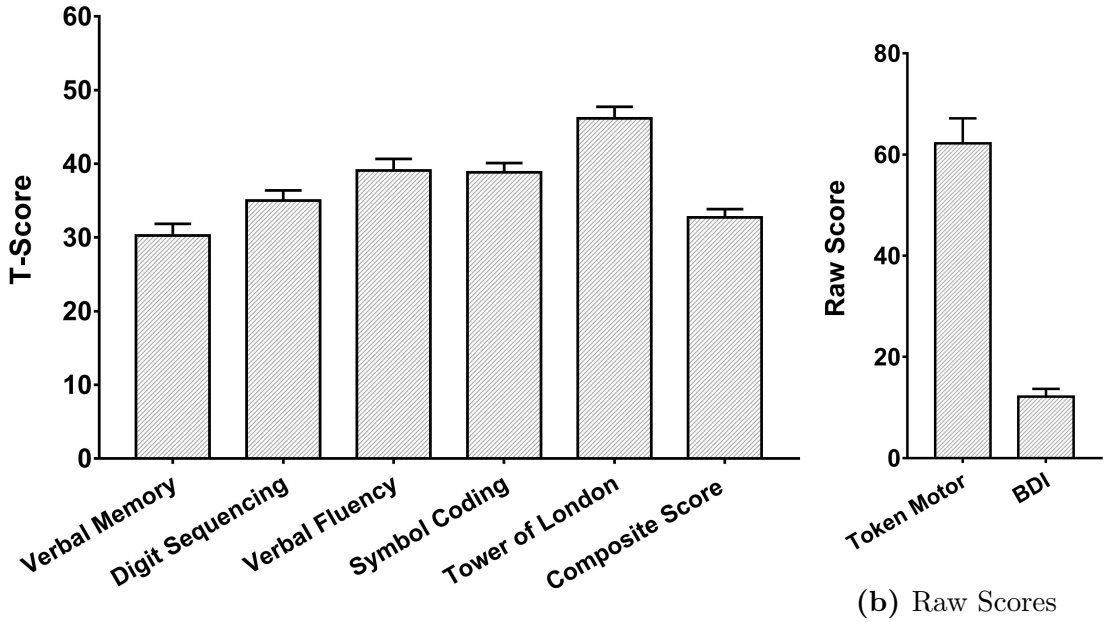
5.2.4 Statistical Analyses

As detailed in chapter 4.

5.3 Results

5.3.1 Participants & Baseline Demographics

All scores were adjusted to a standardized T-score to account for age and gender. The token motor test did not have a paper-pen equivalent and was thus presented as a raw value along with BDI (Figure 5.1).



(a) T-Scores

(b) Raw Scores

Figure 5.1: Baseline Cognitive and Psychological Functioning in Psychosis Patients. Results are expressed as mean±SEM of T-scores derived from reported healthy controls.

5.3.2 Global Cognition in Two Statistical Models

Analysis using paired *t*-tests (Table 5.2) revealed a significant increase in the composite T-score (effect size, Cohen's d=0.427) following B-GOS® intake. Although significance was not reached in the individual subtests, improvements in each domain was noted.

Table 5.2: Effect of B-GOS® on cognitive and psychological measures. All scores are reported as T-scores means (SEM) adjusted for age/gender, except BDI and Token motor which are presented as raw scores. *p<0.05 using paired *t*-tests.

	Pre-B-GOS® (n=24) mean (SEM)	Post-B-GOS® (n=24) mean (SEM)	*t (p)*
BDI	10.04 (1.81)	10.04 (2.624)	0 .00 (1.00)
Composite Score	33.83 (1.501)	36.17 (1.396)	**-2.09 (0.047)***
Verbal Memory	35.24 (2.132)	35.41 (2.215)	-0.10 (0.923)
Digit Sequencing	35.86 (1.84)	36.9 (2.038)	-0.82 (0.423)
Verbal Fluency	41.62 (2.191)	40.66 (1.657)	0.62 (0.542)
Symbol Coding	37.18 (1.599)	38.72 (1.688)	-1.59 (0.127)
Tower of London	46.45 (1.746)	49.1 (1.767)	-1.32 (0.200)
Token Motor	60.33 (5.828)	63.5 (6.245)	-0.79 (0.439)

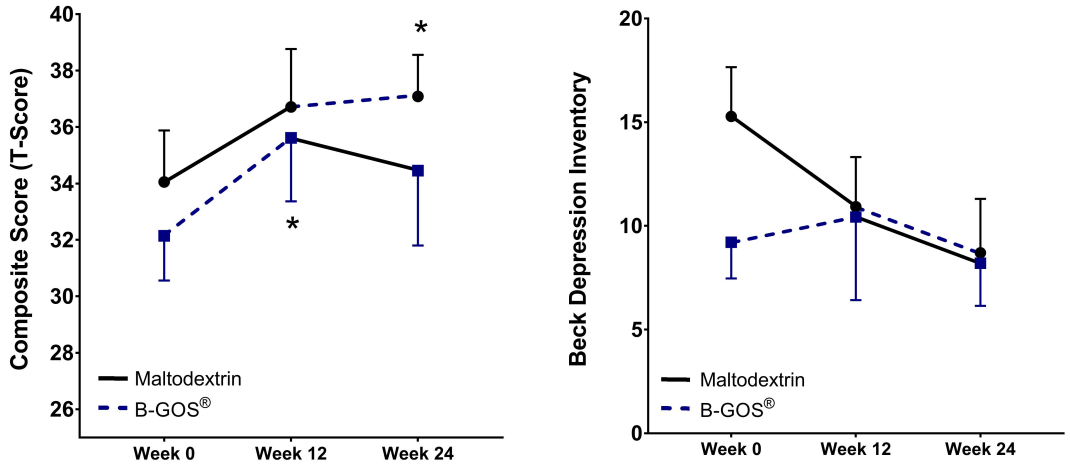
Mixed Linear Modelling

Using a mixed linear repeated measures model which included the order of supplements as a co-variable, the influence of the individual components of the trial was examined. There was an overall significant effect of time on the BACS composite T-score in all participants (Figure 5.2a; F=6.998, p<0.05), corroborating the aforementioned paired *t*-test analyses (Table 5.2). The estimates of fixed effects indicated that the increase in cognitive performance occurred at 0-12 weeks (t=-3.638, p<0.05, n=39). When T-scores were compared in this arm, significant

improvement was revealed in patients supplemented with B-GOS® (Figure 5.2a; t=-2.410, p=0.033, n=13), but not in those receiving placebo (t=-1.443, p=0.186, n=14). The calculated effect size for B-GOS® in this part of the study was 0.669. Analysis of the 12-24 week period revealed that neither the intake of B-GOS® (t=-0.466, p=0.651, n=11) nor placebo (t=-0.059, p=0.954, n=11) altered T-scores. However, the overall change in T-scores between 0-24 weeks was only significant in subjects completing the trial with B-GOS®.

5.3.3 Depression Index was Unaffected

Using paired t-test analysis, no significant effect of B-GOS® was observed (Table 5.2). In a mixed linear regression model, BDI scores decreased with time (F=5.116, p=0.013) irrespective of group, and estimates of fixed effects demonstrated that



(a) Composite T-Score **(b)** Becks Depression Inventory

Figure 5.2: Changes in composite T-score and BBDI in stable patients with schizophrenia who received firstly maltodextrin or prebiotics for 12 weeks, following by prebiotics or maltodextrin. Results are expressed as mean±SEM. *p<0.05 compared to baseline week 0 using mixed models.

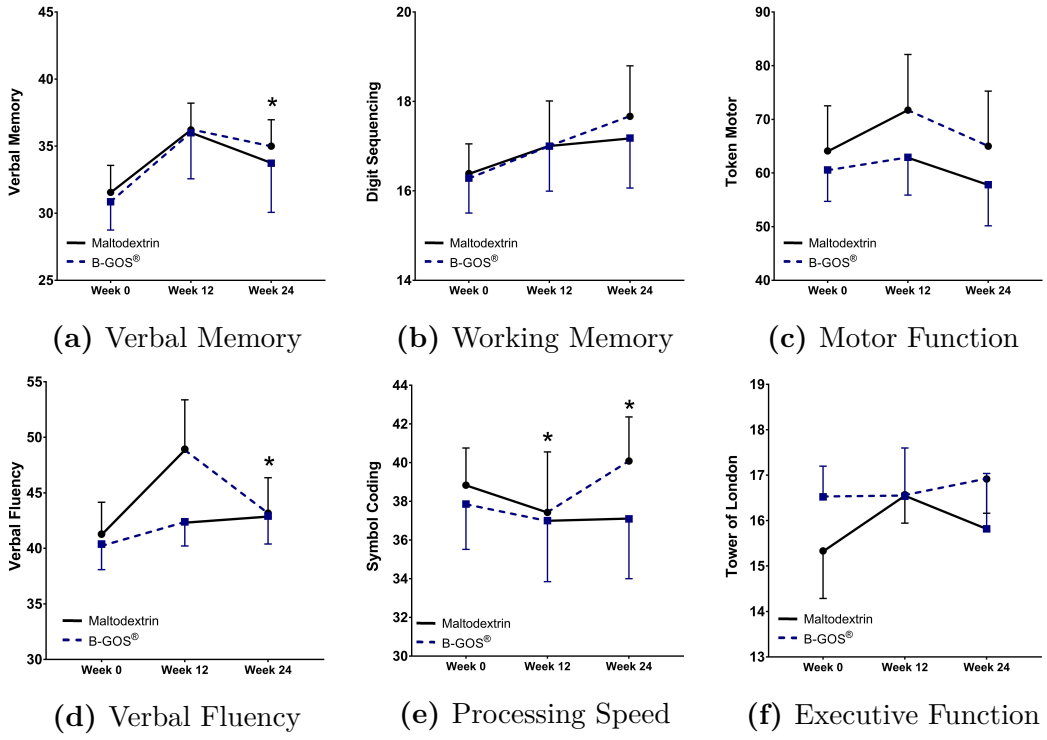
this was significant at 0-12 weeks (t=3.145, p=0.004). There was a decreased trend (F=4.467, p=0.052) at 0-12 weeks on patients supplemented with maltodextrin. No effect of B-GOS® was observed (Figure 5.2b).

5.3.4 BACs Subtests Analysis Using Mixed Linear Regression

No significant effect of B-GOS® were observed when applying the paired t-test model. Consistent with this, mixed linear regression (Figure 5.3) revealed no significant fixed effects on digit sequencing, token motor, and Tower of London over the 24 week period. However, a significant trend over time was observed in verbal fluency (F=3.015, p=0.067) as well as verbal memory (F=5.237, p=0.012). The estimates of fixed effects in this model showed a moderate trends between baseline and week 12 (p>0.073). There was a significant effect of time on the symbol coding subtest (F=3.741, p=0.038). Our fixed effects estimates suggest that this was driven by the change between week 0-12. Further analysis revealed no significant differences were observed between prebiotics and maltodextrin, implying that the significant effect of time owes to learning effects.

5.3.5 Stratified Cognitive Domain Analysis

In two complementary statistical models, there was no change in cognitive domains following B-GOS®, despite descriptive improvements in each domain and an overall global improvement (Table 5.1). The data was analysed with paired t-tests after

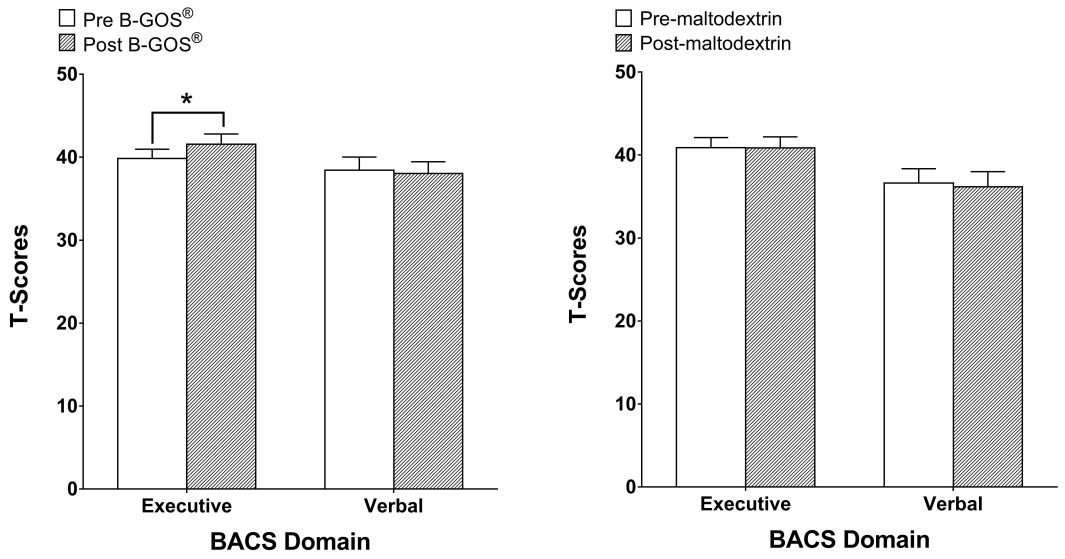


(a) Verbal Memory **(b)** Working Memory **(c)** Motor Function

(d) Verbal Fluency **(e)** Processing Speed **(f)** Executive Function

Figure 5.3: Changes in BACS Subtest in stable patients with schizophrenia who received maltodextrin for 12 weeks then B-GOS® (circles) or B-GOS® for 12 weeks then maltodextrin (squares). Results are expressed as mean±SEM of T-scores adjusted for age/gender. *p<0.05 compared to baseline week 0 using mixed models.

stratification cognitive domains into two functions: "verbal" (verbal memory, verbal fluency) and "executive" (digit sequencing [measures verbal working memory], symbol coding, Tower of London). Interestingly, there was a significant overall pre- versus post- effect of B-GOS® on the pooled executive domains (Figure 5.4; t=-0.205, p=0.043, n=72, effect size d=0.242), but not on the verbal tests (t=-0.672, p=0.533, n=48). The overall effect of B-GOS® on the composite T-scores, therefore, was likely driven by the combined effect of the digit sequencing, symbol coding and Tower of London subtests.



(a) B-GOS®

(b) Maltodextrin

Figure 5.4: Stratified analyses of cognitive subtests into two functions: "verbal" (verbal memory, verbal fluency) and "executive" (digit sequencing [measures verbal working memory], symbol coding, Tower of London) in stable patients with psychosis. Comparison pre- and post- (a) B-GOS® and (b) maltodextrin are reported for each cognitive function. Results are expressed as mean±SEM. *p<0.05 using paired *t*-test.

5.3.6 Pearson Correlations with Serum Markers

The correlation between serum levels of acetate, CRP or IL-6 and cognitive and metabolic outcome measures were examined at baseline (Figure 5.5), and after B-GOS® intake (Table 5.3). A positive correlation between performance on the Tower of London subtest (Figure 5.4b) and levels of serum acetate levels was observed at baseline. This association persisted in participants prior to B-GOS® intake (Table 5.3), but was no longer significantly associated after 12 weeks. No other significant correlations were detected between serum inflammatory markers and cognitive/psychological measurements before and after the intake of prebiotics (Table 5.3).

Table 5.3: Effect of 12 weeks of B-GOS® supplementation on cognitive performance and psychological index. Values reported as Pearson r^2 correlations with serum biomarkers and metabolic measures. *p<0.05

	Acetate		CRP		IL-6	
	Pre-B-GOS®	Post-B-GOS®	Pre-B-GOS®	Post-B-GOS®	Pre-B-GOS®	Post-B-GOS®
BDI	-0.178	-0.107	0.037	0.040	0.035	-0.188
Verbal Memory	-0.268	-0.045	-0.117	-0.03	0.109	-0.259
Digit Sequencing	-0.280	-0.142	0.362	-0.369	0.215	-0.036
Verbal Fluency	-0.176	0.047	-0.018	-0.369	0.038	-0.028
Symbol Coding	0.191	0.172	0.225	-0.058	-0.310	-0.345
Tower of London	0.447*	0.042	-0.07	-0.144	-0.046	-0.08
Composite Score	-0.178	-0.015	0.188	0.040	0.035	-0.188
Token Motor	-0.03	-0.146	0.233	0.074	-0.046	-0.127

5.4 Discussion

This is the first study, to the best of our knowledge, which examined the effect of a prebiotic on cognition in humans. The key finding presented here was that global cognitive performance could be improved following 12 weeks of B-GOS® supplementation in medicated patients with chronic psychosis. Furthermore, it is possible that this improvement was driven by tasks reliant on executive functioning,

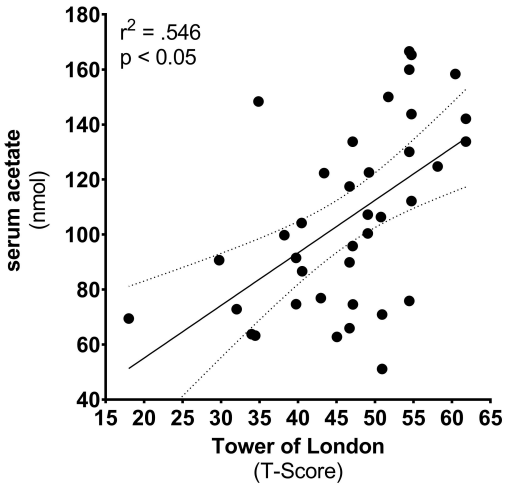


Figure 5.5: Baseline Correlation of Serum Acetate with Tower of London. Pearson correlations r^2 values are reported. Results are expressed as mean±SEM and dotted lines represent 95% confidence intervals.

rather than verbal learning and memory. This study also demonstrated that BDI scores remained stable over 24 weeks, and that B-GOS® supplementation did not change psychological well-being from maltodextrin ingestion. Serum acetate levels, though not significantly increased (Chapter 4), were significantly associated with performance on the Tower of London test, an executive functioning task.

5.4.1 Cognitive Functioning in Psychosis

The B-GOS®-mediated increase in global cognition is a novel finding. Following B-GOS® administration, cognitive flexibility in rats [66] as well as increased attentional vigilance in healthy volunteers [63] have been shown to improve. It is interesting that the observed increase in cognitive performance was not driven by a specific domain. One interpretation of this is that B-GOS® may stabilize deterioration across multiple domains, and therefore, result in improvements to global cognition when compared to placebo. Neurocognitive impairments have been hypothesized to originate from the hypofunctioning of NMDARs and other receptors at the glutamate synapse [57]. Since B-GOS® has been shown to increase cortical levels of obligatory glutamate receptor subunit GluN1 [64] as well as regulatory subunit GluN2A [133], this implies that this prebiotics may facilitate cognitive processes, perhaps with particular effectiveness to those processes that are mediated by the cortex. Long term use of antipsychotics have been shown to reduce NMDAR labelling in the hippocampus particularly with olanzapine [137, 139]. This may,

in part, contribute to the observation that antipsychotic medications exacerbate cognitive impairments in patients with schizophrenia [236, 237]. In the present study, patients were treated with antipsychotics for at least three months and were screened for presence of cognitive impairments, providing a proof of concept that augmenting beneficial bacterial populations may attenuate, or reverse the effects of antipsychotics, and improve cognitive functioning.

5.4.2 Effects on Depression and Mood

Psychological indices have also been explored following gut based therapies. Clinical studies in depressive patients have reported significant improvements in BDI scores while on probiotics [238], but not prebiotics [239]. This latter study is consistent with the findings presented in this section where BDI scores did not significantly change following 12 weeks of prebiotic ingestion. While several investigations have examined the beneficial potential of probiotics in elevating psychological indices [240, 241], recent meta-analyses appear to suggest that positive effects would be apparent in healthy volunteers [242] but not in patients with clinical depression [243]. This implies there is a psychological threshold for the central effects of probiotics, which would be consistent with the findings in prebiotics as they have been examined in patients with depression [239], or psychosis as reported in this chapter. Together this suggests that gut microbial based therapies may remain beneficial for mood, in the general population.

5.4.3 Biomarkers in Cognitive Impairments

Impairments in tasks requiring prefrontal cortical function such as self-ordered working memory and rapid information processing have been most consistently reported in schizophrenia [244–246]. Although there is an interesting narrative throughout this thesis on the role of acetate, and that at baseline, patients with psychosis with higher serum acetate levels performed better on an executive function task, the relevance of acetate remains speculative. Since serum acetate levels did not change following B-GOS®, and was no longer significantly correlated with the Tower of London task after 12 weeks, it is difficult to attribute improvements in cognition with this SCFA. Nonetheless, acetate has been implicated in epigenetic processes including HDAC, which may be modulated by BDNF regulatory mechanisms [247]. A recent review has proposed that serum BDNF in schizophrenia has accumulated the vast majority of evidence in the current literature, particularly in predicting cognitive functioning [248]. Since reduced levels of BDNF appears to be a strong biomarker for schizophrenia [246, 249] and that the administration of the prebiotic B-GOS® has been shown to increase BDNF expression in rats [64], the therapeutic potential of prebiotics cannot be completed discounted. Notably, increased cortisol metabolism has been reported in schizophrenia, possibly relating to clearance mechanisms [250, 251]. One interpretation of increased cortisol metabolism is a homeostatic response to increased cortisol levels as heightened cortisol secretion has

also been reported in clinically high risk psychosis patients [252]. Whether there is a link with the reduction of waking cortisol following B-GOS® administration [63] requires further investigation.

5.4.4 Limitations

Repeated cognitive testing in the current study resulted in a "learning effect" in all patients within the first 12 weeks where both placebo and B-GOS® groups demonstrated improved BACS scores. Although the learning effect likely confounded the interpretation of the second arm of the trial (12-24 weeks) when participants had reached a ceiling performance on the cognitive tasks. This indicates that the effect of B-GOS® could not be confirmed in patients who started with placebo because their BACS scores were already optimal and the prebiotic could not improve performance any further. Nevertheless, an overall effect of B-GOS® was demonstrated owing to the strength of the crossover study design, which increased the power for the analyses. It is also possible that there were residual or carry-over effects of the preceding intervention (B-GOS® or maltodextrin) in the absence of a wash-out period. However, the utilization of maltodextrin by beneficial bacteria such as *Bifidobacterium* has been shown to be extremely poor [253], while prebiotics with the GOS-based formulations have been shown to favour the growth of *Bifidobateria* within five weeks [200]. Since the *bifidogenic* property of B-GOS® was not seen two weeks after ingestion ceased in rats [254, 255], the influence of residual prebiotic on

cognition and metabolic parameters is very unlikely in this study. Lastly, in this pilot study direct measurements of the gut microbiome composition in participants was not performed. It is possible that the associations between microbiome and cognitive functioning could be more accurately defined thus future studies should consider measuring faecal bacterial genus.

5.5 Conclusions

This investigation provides preliminary evidence that the consumption of the prebiotics B-GOS® confers significant cognitive benefits in medicated participants with psychosis, possibly driven by improvements in digit sequencing (working memory), symbol coding (processing speed), and Tower of London (executive function) subtests. The mechanism of this effect remains unclear as the prebiotic did not have an effect on mood or metabolic and inflammatory biomarkers. These findings provide an indication that altering the gut microbiome composition has therapeutic potential for psychosis patients.

I never am really satisfied that I understand anything; because, understand it well as I may, my comprehension can only be an infinitesimal fraction of all I want to understand about the many connections and relations which occur to me, how the matter in question was first thought of or arrived at etc.

The first to explicitly articulate that a machine could manipulate symbols in accordance with rules and that number could represent entities other than quantity, pivoting perspectives from calculation to computation, the first programmer.

— Augusta Ada King, Countess of Lovelace
(1815 - 1852)

6

Conclusions and Future Implications

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6.1 Thesis Highlights

The work presented in this thesis investigated whether the prebiotic B-GOS® could be used as an adjunctive dietary strategy to improve cognitive functioning and to manage the metabolic side-effects of antipsychotic medications in psychosis.

Studies in the rodent model implicated involvement of cellular mechanisms that may underlie these prospective central and peripheral health benefits, and the proof-of-concept clinical investigation examined the therapeutic potential in medicated psychosis out-patients.

It was demonstrated in Chapter 2 that B-GOS® increased cortical expression of GluN1 mRNA, which is an obligatory subunit of the NMDAR, a receptor that is highly implicated in cognitive functioning and schizophrenia pathogenesis. Daily administration of the prebiotic also ameliorated olanzapine-mediated weight gain without compromising the pharmacological effect of the antipsychotic on the central serotonin 5-HT2AR. These effects were observed with an increase in plasma acetate levels, a major metabolite of prebiotic fermentation. However, While the administration of olanzapine and the prebiotic B-GOS® alone increased serum acetate, upon co-administration of both compounds serum levels restored to baseline. This expression profile of acetate was mirrored by ACC (enzyme that enhances the uptake of acetate into the citric acid cycle) and GPR-43 (a downstream SCFA binding receptor) following olanzapine and B-GOS® administration, highlighting the relevance of increased serum acetate. Since weight gain was observed only in olanzapine-treated animals, these finding implicate acetate as a possible mediator, thus Chapter 3 compared the effect of daily administration of sodium acetate with the prebiotic B-GOS®.

Acetate is a well-known chemical moiety that alters gene transcription when it is added to DNA histone molecules, thus it is possible that the increase in circulating and brain concentrations of acetate, as the result of prebiotic fermentation, could have epigenetic implications. Therefore the effect of the prebiotic B-GOS® on HAT/HDAC acetylation activity and expression were explored. Notably, acetyl groups are not the only moiety that can be added onto DNA molecules to modulate gene expression as methyl and phosphoryl moieties have also been reported [256]. A comparative study between B-GOS® supplementation with daily sodium acetate supplementation was carried out to gain some mechanistic insight of the prebiotic effect on neurobiology and behaviour. The finding in this thesis demonstrated for the first time that the prebiotic B-GOS® could exert an inhibitory effect on brain HDAC activity as well as increase expression of cortical HDAC-1 and HDAC-3. In contrast, sodium acetate resulted in the inhibition of HAT, not HDAC, activity in the brain and increased hippocampal levels of HDAC-3 and HDAC-4 mRNAs. Furthermore, it increased serum levels of acetate, but neither altered central NMDAR mRNA expression nor attenuate olanzpine-induced weight gain. These findings heavily suggest that the central and metabolic effects of the prebiotic B-GOS® is not entirely, if at all, mediated by the SCFA acetate.

The clinical potential of the cognitive and metabolic benefits of the prebiotic B-GOS® were evaluated in psychosis patients in Chapters 5 and 6. After 12-weeks

of B-GOS® ingestion, no changes in total body weight and other anthropometric indices were observed. Interestingly, a decreased level of serum acetate was reported furthering the narrative that acetate may not be a critical facilitator in mediating the central effects of this prebiotic. Since NMDAR hypofunctioning is hypothesized to underlie the pathophysiology of psychosis and B-GOS®-mediated increase in NMDAR subunits have been reported, the finding that B-GOS® supplementation could attenuate cognitive decline in patients was particularly interesting. In light of the study limitations discussed in Chapter 5, the findings presented in this thesis remain highly promising as the prebiotic B-GOS® addressed cognitive impairment in psychosis patients.

6.2 General Discussion

6.2.1 Acetate or Butyrate

The conclusion that acetate may not be a major mediator of the gut-brain communication was particularly interesting. The prebiotic B-GOS® has been demonstrated to favour the production of acetate and butyrate by *bifidobacterium*, albeit *in vitro* [148], suggesting that a SCFA-based mechanism may involve either or both of these compounds. In recent years, HDAC inhibitors have emerged as a novel therapeutic application in various diseases [187], including sodium butyrate which has been shown to confer some anti-depressant-like effects in mice [179], notably with potential toxicities [125]. It has been proposed that HDAC inhibitors likely signals through

nuclear factor κB (NFκB) suppression and peroxisome proliferator-activated receptor γ activation [257, 258], which are key regulators of inflammatory cytokines such as TNFα. While this mechanism is consistent with the anti-inflammatory profile of prebiotics, which has been demonstrated in rodent models of neurodegenerative disorders [122–124], the counter argument that undermines butyrate as the main mediator of gut-brain communication is implicated by coloncytes; namely gut-derived butyrate is the preferred energy source of coloncytes and chiefly functions to maintain gut integrity thereby sequestering butyrate in the intestines[113, 259]. This offers one explanation of low, to minimal, concentrations of butyrate in the periphery and further suggests that there would be minimal levels in the brain. Notably, increased clearance into tissues have also been proposed to explain low serum levels, though emperical studies will be required to confirm, or refute, this hypothesis [115]. Together these observations suggests that acetate, and perhaps in parallel to other mediators, may likely underlie the gut brain communication. However, increased serum levels of acetate have resulted in a few contradictory outcomes. High fat diet which is often fed to rodents as a model for metabolic syndrome have reported increased levels of serum acetate [171]. In contrast an anorexigenic effect has also been observed with acetate supplementation, possibly through the suppression of hypothalamic satiety signals [151]. This suppression may be the result of circulating SCFAs interacting with fatty acid receptors such as GPR-

43/41 and monocarboxylate transporter 1 (MCT-1) as shown *in vitro* [260] and *in vivo* [261, 262]. Interestingly, acetate uptake into the brain has been demonstrated to be NMDAR-dependent [150, 151] suggesting that parenchymal cells may interact with acetate. This is of course, speculative and requires further exploration.

6.2.2 Central Changes from SCFAs

The central epigenetic potential of the prebiotic B-GOS® is a novel and interesting finding. Inhibition of HDAC activity favours gene transcription that may involve neuronal plasticity and attenuate cognitive decline [117]. Using genetic knockout and overexpression mouse models, one study has reported that specifically HDAC-2 negatively regulates memory formation and synaptic plasticity, a phenotype that was rescued using HDAC inibitor vorinostat [263]. Although the findings in this thesis did not report significant effects of B-GOS® on HDAC-2, an overall decrease in HDAC activity was reported highlighting the need to further investigate the therapeutic potential. The findings presented in Chapter 3, and serum levels in Chapter 4, suggested that acetate may not be a key mediator for the epigenetic profile of the prebiotic B-GOS® further supports the value of butyrate in mediating the gut-brain communication. In a recent analysis of 79 phylogenetically diverse gut microbes isolated from healthy human donors, the three most potent HDAC inhibiting strains were all butyrate producers [264]. Nonetheless, others [148] as

well as findings present in chapter 2 of this thesis have suggested that acetate would, at least, contribute to the observed pro-cognitive effects.

6.2.3 Body Weight and Intestinal Microbiome

Antipsychotic-induced weight gain likely involve the microbiome since germ-free rodents (absent of an intestinal microbiome) do not gain weight when administered with olanzapine [41]. Furthermore, dietary SCFA supplementation has been shown to cause significant changes in reducing the proportion of Firmicutes and increasing the proportion of Bacteroidetes [152]. Whether these changes in bacterial populations are affiliated with putative weight reduction mechanisms such as enhanced triglyceride hydrolysis and free fatty acid oxidation in the adipose tissue, or increased adipogenesis/ mitochondrial biogensis remains to be examined. Furthermore, weight gain has been viewed as a chronic low grade inflammatory state, which could be mediated by SCFAs since various immune cell types including monocytes, neutrophils and macrophages express GPR-43 [112] and that the administration of SCFAs resulted in significant changes in the expressions of GPR-41/43 [152]. It has also previously been shown that re-colonization of the intestinal microbiota in germ-free mice restores their ability to respond to an acute inflammatory stimulus through an IL-1β production pathway that is partially dependent on GPR-43 [265]. Notably, administration of TNFα has been shown to suppress weight gain and influence lipid metabolism in adipocytes [161] and

macrophage infiltration of adipose tissues is associated with the development of insulin resistance [43]. These observations further complicates the role of inflammation as a mediator, consequence or homeostatic response to weight gain, and requires further investigations.

Weight Gain in Psychosis

Physiologically, serum biomarkers are also critical indicators of metabolism. The balance between the hormones leptin and ghrelin are well recognized for their roles in energy regulation, where obesity has often been the outcome of aberrant expression [266], along with increased production of inflammatory cytokines including TNF-α [267, 268]. However, recent evidence comparing several metabolic markers between psychosis patients treated with antipsychotic agents and untreated obese patients have suggested that changes in leptin/ghrelin expression are related to BMI, rather than antipsychotic exposure [84]. Notably, there has been a report showing that leptin levels are positively associated with antipsychotic use [82].

Differential affinity for central receptors is emerging as a favoured hypothesis. In a recent meta-analysis, antipsychotics with higher affinity for the H1R, 5-HTR2C, as well as muscarinic receptors 1 (M1) and 3 (M3) were more strongly associated with increased risk of weight gain [269]. The histaminergic systems has long been implicated in regulating feeding and energy metabolism where histamine neurotransmitters bind to the postsynaptic H1Rs thereby resulting the activation

of downstream effectors. This has been shown in chronic infusion rat models where reduce food intake was observed following neurotransmitter infusion [270] as well as in a histidine decarboxylase knockout model resulted in increased visceral adiposity and decreased glucose tolerance [271]. Similarly antipsychotics such as olanzapine and clozapine are inverse agonists of the H1R which results in increased food intake in animal models [272], and likely in patients. In addition, increased M3 receptor binding was reported to correlate with increased food intake and body weight in rats following olanzapine administation [273] and 5-HTR2C agonist lorcaserin was found to alleviated olanzapine-induced metabolic side effects [76], further implicating antipsychotic receptor pharmacology to be a major determinant in weight gain. Since the work from Chapter 2 of this thesis suggested that the effects of prebiotic supplementation occurs independent of the 5-HT2AR, yet attenuation of weight gain was achieved, it may be worth investigating the effects of B-GOS® on other neurotransmission systems.

6.2.4 Inflammation and the Microbiome

There is considerable debate on whether antipsychotic agents possess antimicrobial activity thereby affecting the intestinal microbiota and contributing to metabolic side effects. Studies on both sides have been reported demonstating potential anti-commensal [44] and anti-microbial [41] effects, as well as inability to inhibit the growth of pathogeic bacteria [45]. While antibiotics which slightly modify

the gut microbiota and exhibit similar antibacterial activity to second generation antipsychotic agents did not result in metabolic dysfunctions [43, 46], a recent systematic review has suggested that antipsychotic-related microbiome alterations may contribute to the observed weight gain and metabolic disturbances, and are likely to involve inflammatory mechanisms and suppression of resting metabolic rate [47]. Following the identification of LPS as a major mediator involved in the onset of insulin resistance and metabolic alterations associated with low-grade inflammation, both markers of weight gain and obesity [274], other microbial components (e.g., peptidoglycans or flagellin) have further been confirmed in regulating energy metabolism and contribute to glucose/lipid homoeostasis [275]. These molecules, among others including SCFAs, can lead to the production of GLP-1 and PYY (peptides that regulate energy harvesting) via activation of GPRs to favour fat production [47]. In this narrative, entry of bacterial antigens into circulation implies that the integrity of the intestinal barrier has been compromised. If antipsychotic agents indeed exhibit bactericidal activity [44], which may eliminate specific bacteria from the intestinal environment, it is possible that structure and function of the intestinal barrier, which is normally maintained by the microbiome, may be jeopardized [25, 276]. In this context, it is perhaps not surprising that antibiotics which limit the growth of all microbes in the intestine, and therefore limit release of microbial products into circulation, could attenuate olanzapine-induced

weight gain [43]. Similarly, prebiotics which induce the growth of non-pathogenic microbes, and potentially limit PAMPs from eliciting an inflammatory response, also exhibit anti-obesity effects [133].

The contradicting cardio-metabolic effects of B-GOS® presented in this thesis is also interesting. Anti-obesity and weight loss effects have been shown using prebiotics and other similar compounds such as probiotics; in a randomized, placebo-controlled trial, prebiotic supplementation led to reductions in self-reported appetite and caloric-intake as well as decreased overall weight in overweight adults [277] and, more recently in overweight and obese children [229]. Obesity can be a result of an imbalance in orexigenic and anorexigenic gut hormones such as cholecystokinin, PYY and GLP-1 and it appears that prebiotics may confer its potential anti-obesity effects by influencing satiety proteins [278]. In a systematic review of human studies examining weight gain as the primary clinical outcome [207], the possibility of floor effects was discussed since prebiotic interventions that took place between 12 to 17 weeks in obese patients [277, 279] were more likely to see reductions in body weight compared to trials that ranged from 4-8 weeks in healthy volunteers [208, 280, 281]. The findings presented in this work suggests that B-GOS® does not influence total body weight.

Central Effects

The neurobehavioural effects of antibiotics have been explored in anxiety and depression associated with diet-induced obesity where an oral course of antibiotics (i.e., metronidazole or vancomycin) decreased neuroinflammation, improved insulin signaling in the brain and reduced signs of anxiety and depression in mice [282]. Independent to the risk of cardiovascular disorders, high BMI has been associated with deficits in learning, memory and executive functioning in otherwise healthy middle aged-adults [283–285]. This association between poor cognitive performance also applies to other obesity indices such as higher waist circumference and WHR [286]. This hypothesis likely results from the view that obesity is a state of low grade chronic peripheral inflammation, which may seed inflammation in the brain. Furthermore, the expansion of visceral adipose tissues, a physical feature of weight gain, can be a potent source of inflammatory interleukins and cytokines [268]. In addition, high fat diets increase the levels of circulating free fatty acids which are able to activate the immune system by interacting with toll-like receptors (TLRs), a family of pattern recognition immune cell surface proteins that can induce the production of pro-inflammatory cytokines and macrophage infiltration [287]. In the volunteers who participated in the study presented in this thesis, higher levels of serum CRP was significantly associated with higher anthropometric metabolic measures but not cognitive performance.

6.3 Limitations and Future Studies

It is important to highlight that the findings presented in this thesis examines a single prebiotic compound, B-GOS® and extrapolating to other GOS-based compounds, and prebiotics at large, should be performed with caution. Prebiotic formulations can be structurally dissimilar (Figure 1.1) and therefore may not influence the microbiome in similar ways; it would follow that the relative production of SCFAs may also be different. The microbial properties of inulin and GOS/FOS has been demonstrated in infants where increased levels of beneficial bacteria including *lactobacilli* and *bifidobacteria* have been reported [35–37]. However, the opposite effect on microbial genera has also been reported with a different formulation of GOS [174]. A more comprehensive analysis which examines the serum metabolites in humans would likely be useful to elude to a gut-brain mechanism. Numerous metabolites are produced by the microbiota such as folate, indoles, secondary bile acids, trimethylamine-N-oxide, and also neurotransmitters (e.g., serotonin, gamma amino butyric acid) which may have prominent or auxiliary roles in maintaining host health [288]. Skewed production of serotonin in the gut (as the result of medication which influences the composition of the microbiome) may be partially responsible for weight gain in psychosis [289].

6.3.1 Abundance & Metabolic Capacity

Amongst the first reports that implicated a microbial signature in disease states [145] relative abundance of phylum level ratios were advocated to be more relevant than quantifying at the genus/species level. However, as molecular protocols are refined and the irreproducibility margins continue to decrease [290], the priority may shift from characterising the relative abundance of microbial species to determining the absolute microbial load [291]. This potentially offeres additional data on the extent or directionality of changes in taxa abundance or metabolic potential. In addition to this consideration, complimenting microbial abundance data with the metabolic capacity of microbes may offer richer insights on the ecology of the intestinal lumen [292, 293]. While the potential in this data is wide, access to state-of-the art sequencing-based approach remains to be the major limitation. The findings in this thesis reports changes in microbial genera and families that were previously demonstrated to be influenced with prebiotics, and other gut-influencing compounds [155] and is currently the best data that could be obtained.

Furthermore, there is also some criticism as to whether bacterial composition in the stool, which is more stable and is likely independent of changes in the peripheral systems of the host, may offer an accurate representation of the microbial environment in the small intestines. Changes in the microbiome of the small intestines are likely to exert greater effects on the metabolic functions of the host

[47] thus future studies should identify ways of addressing this potential discrepancy.

6.3.2 Influence of Daily Diet

Dietary differences significantly influence the composition of the intestinal architecture, implying that putative therapies may have limited application and must be personalized to the individual. It is important to note that the clinical data presented in this thesis was performed as a within-subject analysis as a way to overcome this potentially confounding parameter. While the number of subjects recruited, and therefore power of the study, remains to be the major caveat, it should be emphasized that this was the first study to examine the therapeutic potential of a prebiotic in managing metabolic side-effects and cognitive impairment of psychosis patients. While the superiority of prebiotic supplementation to adopting dietary alterations remains to be debated, in clinical populations that present with a lack of motivation, ingestion of a simple supplement is likely to be more sustainable.

6.3.3 Commercialization

Lastly, there is still significant on-going work to delineate whether disruptions in the microbiome is the cause or the effect of mental health conditions, and which disorder may benefit from microbiome-based therapeutics. These encouraging data, including the findings presented in this thesis, are tantalizing for healthcare consumers looking for alternatives to the psychotropic therapies which toxic if misued. However, utilizing potentially unconfirmed data to promise premature delivery of

personalized health is controversial and perhaps unethical. It is interesting that the gut microbiome, along with blood parameters, dietary habits, anthropometrics, physical activity, has been included as a predictor of personalized blood glucose responses to specific diets [294]. There are considerable inter-personal variables within healthy individuals that complicates the characterization of a "healthy microbiome" [295]. Due the richness of the microbiome, it is conceivable that there are multiple configurations of a healthy microbiome, and it is equally likely that the functional output of these configurations may not be unique (i.e., redundancy). Thus while sophisticated technologies can still be beneficial for data mining metagenomic biomarkers, without a full consensus from the scientists in this field, gut-based therapies should be consumed with caution. Fundamental molecular breakthroughs are still required to confirm the legitimacy of the microbiome as a valid therapeutic target for mental illness, and cardio-metabolic disorders.

Furthermore, the translational potential of the findings presented in this thesis has not been scrutinized by strict pharmacokinetic and pharmacodynamic studies. The dose of 0.5g of B-GOS® was derived from *in vivo* studies that demonstrated optimal bifidobacteria growth. In healthy adults (avg. 50-70kg) this would translate to 50g of prebiotics, which is above the natural daily intake of prebiotics.

6.4 Significance of Findings

This dissertation has provided preliminary evidence to support the bidirectional communication of the gut-brain axis. In rodents, manipulating the composition of the intestinal microbiome using a prebiotic attenuated olanzapine-induced weight gain and increased NMDAR subunit expression in the brain, likely in a epigenetic-dependent fashion, though direct central actions of the SCFA, acetate, remains equivocal. Since the findings in this thesis focused on examining direct central effect of acetate, it would be amiss to extend these findings to indirect effects of other SCFAs, such as butyrate. Although butyrate is sequestered in the intestines, it remains conceivable that central effects can emerge from microbiome interactions with other pathways, such as with the immune system. According to the findings in this thesis, cursory markers of the immune system does not appear to be involved in the prebiotic profile, however confirmation from other biomarkers are required to rule out the immune system at large. These mechanisms could underlie the improvements in global cognition, particularly executive functioning which is heavily dependent on glutamate neurotransmission. Although metabolic measures was not affected by the prebiotic B-GOS®, changes to lipid metabolism and energy-related metabolites could underpin a separate mechanism for the metabolic effects frequently observed in rodents. Lastly, the findings presented in this thesis highlights an exciting translational potential of the prebiotics B-GOS® on cognition, though

additional assessments will be required to confirm the therapeutic potential in psychosis patients, and other neurocognitive disorders at large.

Appendices

Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled.

Scottish chemist awarded the 1904 Nobel Prize in Chemistry for his discovery of the inert gaseous elements in air captures the true spirit of research.

— Sir William Ramsay (1852 - 1916)

A

Additional Data on Prebiotic Feeding

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A.1 NMDAR Expression in Male Rats

We examined the neurobiological effects of prebiotic administration in male rats. However, due to complications in replicating a male model of olanzapine-induced weight gain the following data was precluded from chapter 2.

Nonetheless, significant changes due to olanzapine administration was still observed in central brain regions demonstrating that the gender-specific effect of olanzapine in rats is prevalent. In brief, a clinically relevant dose of olanzapine

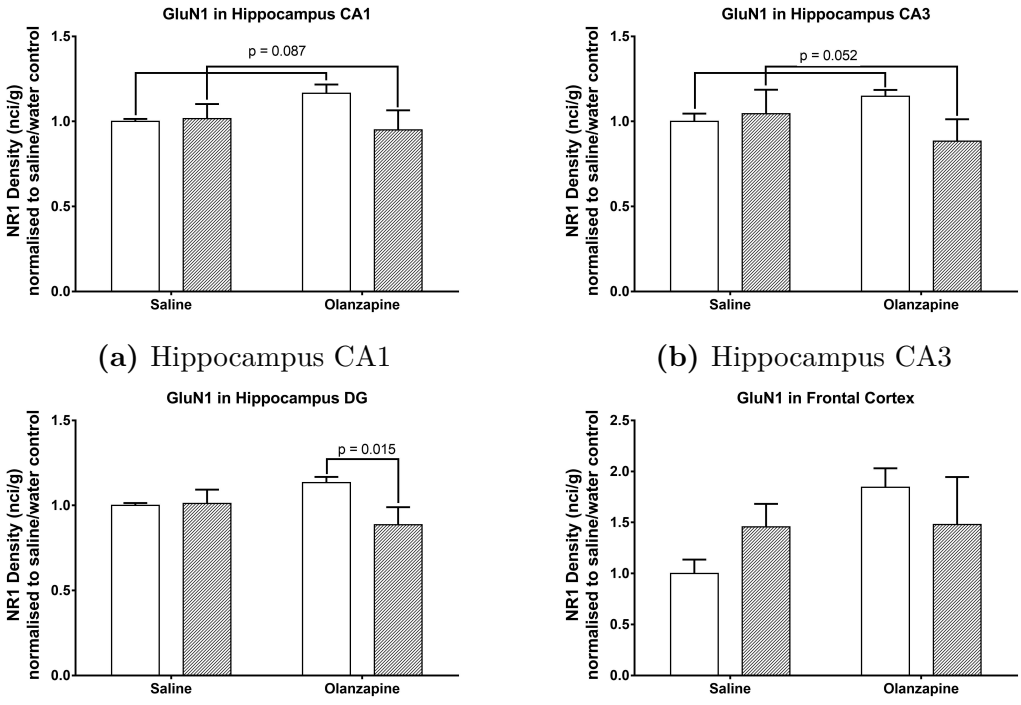
elevated GluN2B in the hippocampus, but not in rats ingesting B-GOS®, whereas cortical GluN2B levels were increased by B-GOS® feeding alone.

A.1.1 mRNA Levels

In male rats, a significant overall effect of diet on GluN1 mRNA was observed in the dentate gyrus ($F_{1,18}$=5.491; p=0.031), which reflected the B-GOS® mediated reduction GluN1 transcript ($F_{1,18}$=7.315; p=0.015). Strong to moderate trends of diet effects were observed in the CA3 ($F_{1,18}$=4.331; p=0.052) and CA1 ($F_{1,18}$=3.285; p=0.087) subfields. Significant main effects of treatment on GluN2A mRNA were observed in the CA3 region ($F_{1,18}$=4.769; p=0.042) and dentate gyrus ($F_{1,18}$=5.087; p=0.037). However, *post hoc* analysis did not reveal significant group effects in either GluN1 or GluN2A.

A.1.2 Protein Expression

A significant treatment x diet interaction was observed in the hippocampal levels of GluN2B ($F_{1,20}$=5.001; p=0.037) and GluN1 ($F_{1,20}$ = 5.740; p=0.026). The level of GluN2B in olanzapine/water rats were greater than in the saline/water group (+19%, $F_{1,20}$ = 4.545; p=0.046). A similar trend was observed following *post-hoc* analyses of GluN1 (+23%, $F_{1,20}$ = 3.738; p=0.067), with an additional decreased trend in B-GOS/olanzapine rats compared to water/olanzapine animals ($F_{1,20}$ = 4.290; p=0.051). No significant main effects of diet or treatment was detected on the expression of GluN2a in the hippocampus.



(a) Hippocampus CA1

(b) Hippocampus CA3

(c) Hippocampus Dentate Gyrus

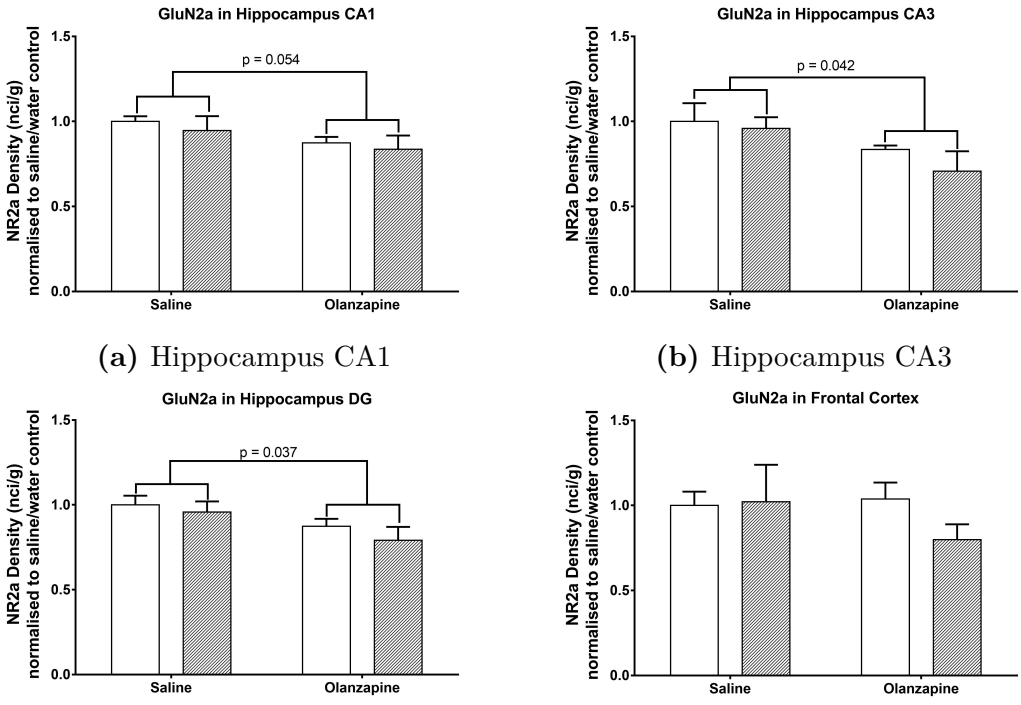
(d) Frontal Cortex

Figure A.1: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on NMDAR subunit, GluN1, in adult male rats. Animals ingested B-GOS® 7 days prior to, and throughout the two-week administration of olanzapine. Results are expressed as mean±SEM for each group (n = 6); *p<0.05, compared to the saline/olanzapine group.

A significant treatment x diet interaction was observed in cortical GluN2B ($F_{1,18}$=5.954; p=0.025), where saline/B-GOS® rats contained greater levels of GluN2B compared to the saline/water group (+24%; $F_{1,18}$=8.882; p=0.008).

A.2 Serotonin Receptor in Male Rats

Most molecular analyses in male rats were precluded when olanzapine was unable to induce weight gain. However, in female rodents a significant treatment effect of olanzapine was observed on the levels of 5-HT2AR thus confirmation of the invalidity of the male model was carried out. No significant effect of olanzapine



(a) Hippocampus CA1

(b) Hippocampus CA3

(c) Hippocampus Dentate Gyrus

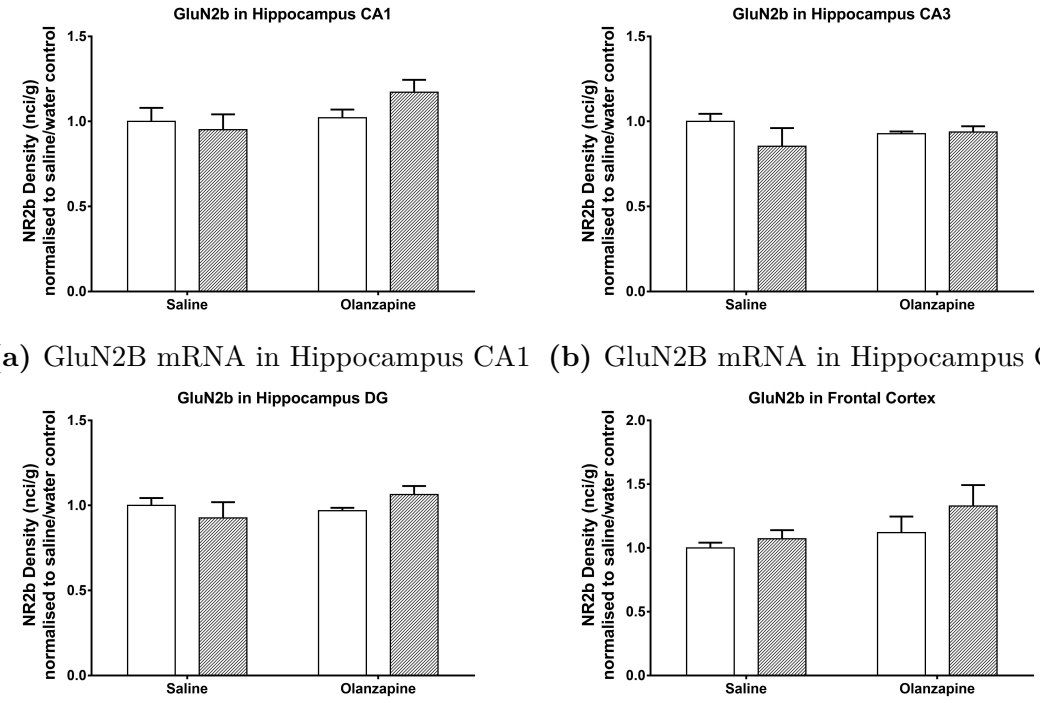
(d) Frontal Cortex

Figure A.2: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on NMDAR subunit, GluN2a, in adult male rats. Animals ingested B-GOS® 7 days prior to, and throughout the two-week administration of olanzapine. Results are expressed as mean±SEM for each group (n=6); *p<0.05, compared to the saline/olanzapine group.

was observed in the expression of 5-HT2AR implying that olanzapine was unable to exert pharmacological actions in this model. This is an interesting relationship that was beyond the scope of this thesis.

A.3 GLP-1R Expression in Male and Female Rats

We explored the potential effect of olanzapine on GLP-1R expression in the hippocampus and frontal cortex in both male and female rats, but did not observe any significant change.



(a) GluN2B mRNA in Hippocampus CA1 **(b)** GluN2B mRNA in Hippocampus CA3

(c) GluN2B mRNA in Dentate Gyrus **(d)** GluN2B mRNA in Frontal Cortex

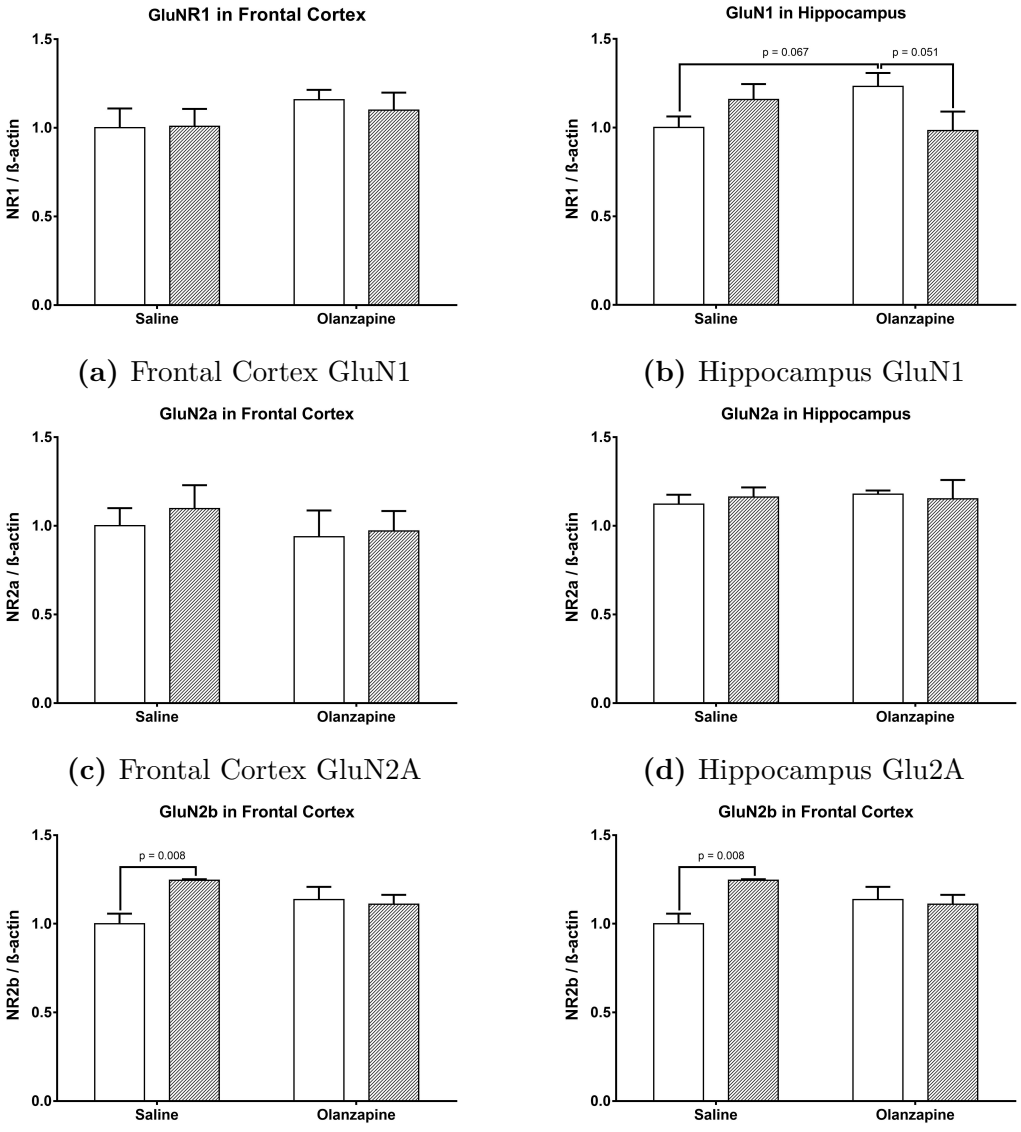
Figure A.3: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on NMDAR subunits, GluN2B, in adult male rats. Animals ingested B-GOS® 7 days prior to, and throughout the two-week administration of olanzapine. Results are expressed as mean±SEM for each group (n=6); *p<0.05, compared to the saline/olanzapine group.

Table A.1: Effect of 10mg/kg Olanzapine on Cortical and Hippocampal levels of GLP1R. Results are expressed as relative mean±SD relative to β-actin, the internal reference gene. *$p<0.05$ using two-way ANOVA.

		Saline		Olanzapine	
	Region	Water	B-GOS	Water	B-GOS®
Male Rats	Frontal Cortex	0.79 ± 0.066	0.82 ± 0.068	0.81 ± 0.064	0.84 ± 0.070
	Hippocampus	0.89 ± 0.071	0.89 ± 0.098	0.89 ± 0.066	0.85 ± 0.082
Female Rats	Frontal Cortex	0.42 ± 0.064	0.41 ± 0.059	0.39 ± 0.077	0.42 ± 0.066
	Hippocampus	0.38 ± 0.077	0.38 ± 0.045	0.36 ± 0.035	0.37 ± 0.036

A.4 Acknowledgements in Prebiotics Study

This work was funded in part by a Biotechnology, Biological Sciences Council (BBSRC) Industrial Partnership Award (Principal Investigator, PWJB; Grant Code:



(a) Frontal Cortex GluN1

(b) Hippocampus GluN1

(c) Frontal Cortex GluN2A

(d) Hippocampus Glu2A

(e) Frontal Cortex GluN2B

(f) Hippocampus GluN2B

Figure A.4: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on NMDAR subunits in adult male rats. Animals ingested B-GOS® 7 days prior to, and throughout the two-week administration of olanzapine. Results are expressed as mean±SEM for each group (n = 6); *p<0.05, compared to the saline/olanzapine group.

BB/N010035/1). B-GOS® is a registered trademark and the property of Clasado Biosciences Ltd.

Clasado Biosciences Ltd, made a 10% financial contribution towards the study, as part of the BBSRC IPA scheme.

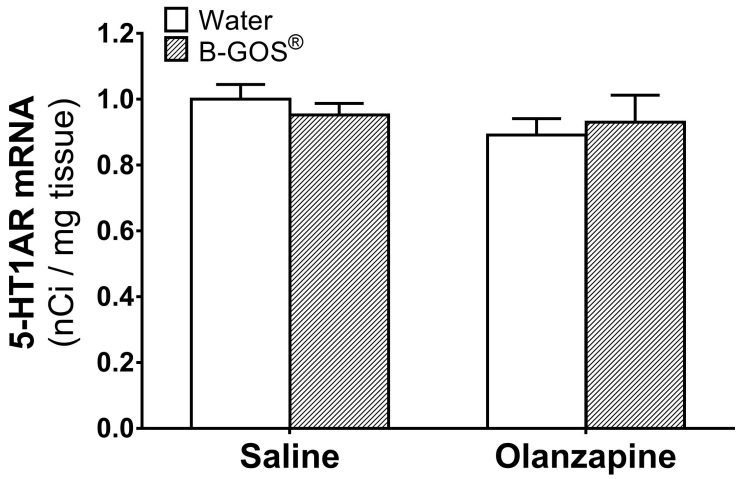


Figure A.5: Effect of B-GOS® (0.5g/kg/day) ingestion and olanzapine (10mg/kg/day) administration on serotonin receptor expression in adult male rats. Results are expressed as mean±SEM for each group (n = 6); *p<0.05.

The good thing about science is that it's true whether or not you believe in it

There is a difference between opinion and fact. Science, as a base, is always true, it is interpretation that imparts human error. Whether you believe in science or not is irrelevant.

— Neil deGrasse Tyson (1958 - present)

B

Sodium Acetate Dose Optimization

Contents

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In comparing the prebiotic effect with acetate supplementation in female rats, we found some irreproducibility in the olanzapine effect. Below details some of the peripheral data that were obtained from peripheral tissues while examining markers of acetate. They remain as interesting results but required further exploration to delineate a meaningful mechanism.

B.1 Low Dose Sodium Acetate on Weight Gain & Plasma Acetate

Prior to the data presented in Chapter 3, a dose optimization experiment was conducted to identify whether 250mg/kg/day was comparable to prebiotics intake on serum acetate concentration and total body weight. A time x treatment interaction ($F_{5,100}$=4.935; p=0.01) confirmed the rodent model where two-week intra-peritoneal olanzapine injection significantly increased body weight compared to saline-injected rats, particularly on days 6-10 ($p<0.001$; Figure B.1a). This was confirmed by the overall effect of treatment ($F_{5,100}$=11.072, p=0.003). Notably, a strong trend was observed on the overall effect of diet (acetate) suggesting that acetate supplementation may have augmented total body weight ($F_{1,20}$=TBD, p=0.053). No significant time x diet interactions or time x diet x treatment effects were observed. The area under the curve analysis confirmed these analyses where a significant overall effect of treatment was observed ($F_{1,20}$=11.521, p=0.003, figure 3.4c). *Post hoc* analyses revealed that significant olanzapine-induced weight gain in rodents supplemented with water (+86% increase between water/saline vs. water/olanzapine $F_{1,20}$=4.732, p=0.042) as well as acetate (+72% increase between acetate/saline vs. acetate/olanzapine $F_{1,20}$=6.890, p=0.016; Figure B.1b).

A significant diet x treatment interaction was observed on circulating levels of acetate ($F_{1,20}$=7.300, p=0.014, Figure B.1c) as well as an overall treatment effect

($F_{1,20}$=9.106, p=0.007). *Post hoc* analyses estimated that these overall effects were driven by the significant decrease in rodents in the acetate/olanzapine group. This decrease was significant compared with the saline/olanzapine ($F_{1,20}$=9.019, p=0.007) and acetate/saline ($F_{1,20}$=16.356, p=0.001) group. Together these observations confirm that sodium acetate administered at 250mg/kg/day was insufficient in replicating the peripheral effects of prebiotics.

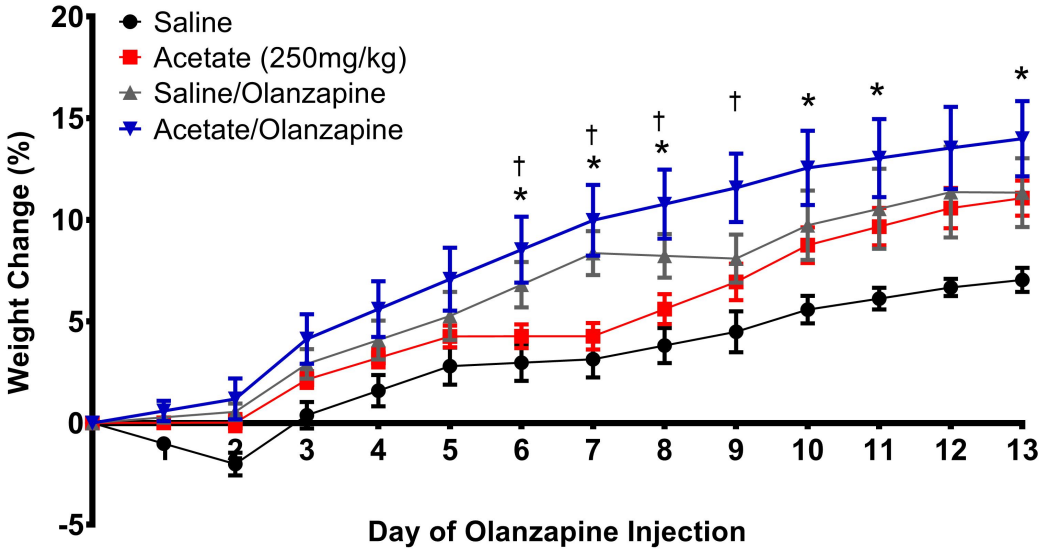
B.2 Sodium Acetate on Brain Levels of HDAC

B.2.1 Hippocampal mRNA Expression

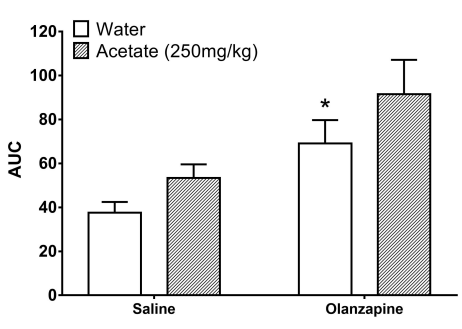
At the low dose acetate, a significant diet x treatment interaction was observed on the expression of hippocampal HDAC-1 ($F_{1,20}$=7.690, p=0.012), and a moderate diet x treatment trend in HDAC-3 mRNA expression ($F_{1,20}$=3.311, p=0.084, Figure B.2). The interaction between acetate and olanzapine on HDAC-1 was likely driven by the significant decrease due to the administration of acetate ($F_{1,20}$=6.997, p=0.016) or olanzapine ($F_{1,20}$=4.916, p=0.038). A significant overall diet effect was observed on the expression of HDAC-4 ($F_{1,20}$=4.585, p=0.045) was observed. Further analyses demonstrated that this was likely driven by olanzapine-induced decrease ($F_{1,20}$=6.442, p=0.020).

The data presented here is in contrast to the high dose acetate experiment presented in chapter 3 (increased expression of hippocampal HDAC-3 and HDAC-4) and the effect of prebiotics (decreased HDAC-3 in olanzapine-treated only). One

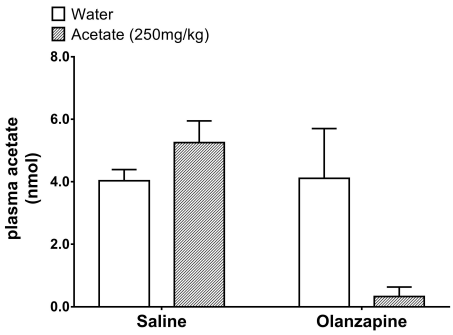
interpretation suggests that in order for sodium acetate to influence epigenetic processes, high levels of cellular acetate, acetyl-CoA, are required. Daily fermentation of prebiotics provides a consistent source of acetate to replenish levels of acetyl-coA, the necessary substrate for HAT. This pushes the enzyme equilibrium



(a) Low Dose Acetate Supplementation

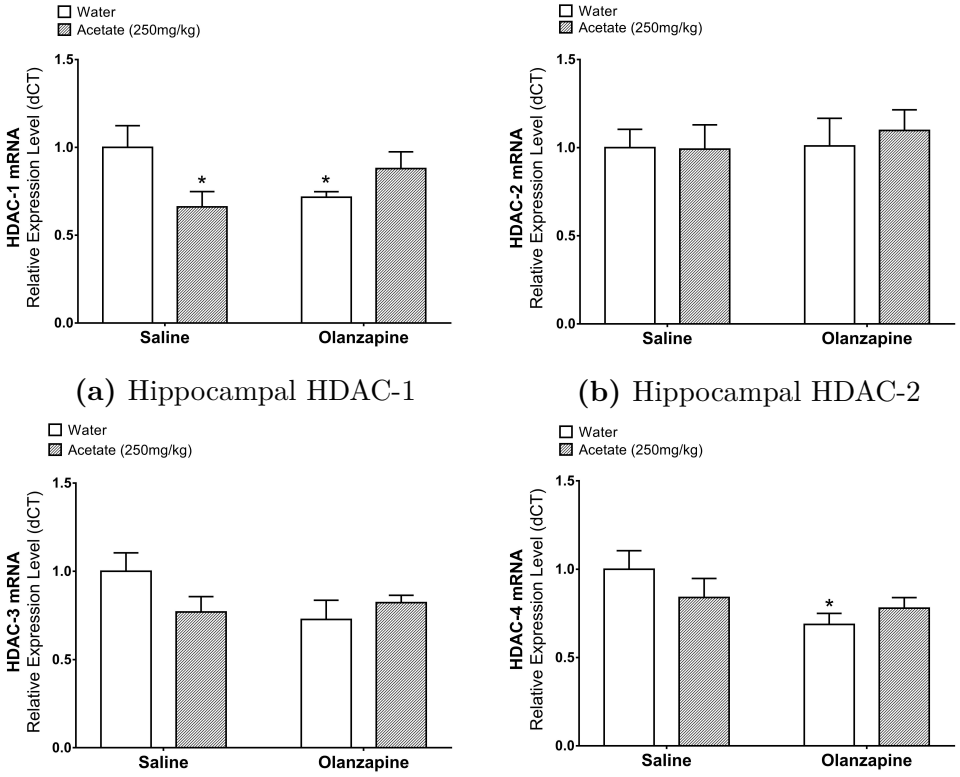


(b) Area Under the Curve



(c) Plasma acetate (250mg/kg)

Figure B.1: Effect of acetate supplementation (250mg/kg/day) and olanzapine (10mg/kg/day) administration in adult female rats. Animals ingested acetate 7 days prior to, and throughout, the two-week administration of olanzapine. (a) Percentage weight gain was calculated from the day before olanzapine administration. (b) Area under the curve analysis. (c) Circulating levels of acetate. Results are expressed as mean±SEM for each group (n=6). *p<0.05 compared to water/saline. †p<0.05 compared to acetate/saline.



(a) Hippocampal HDAC-1

(b) Hippocampal HDAC-2

(c) Hippocampal HDAC-3

(d) Hippocampal HDAC-4

Figure B.2: Effect of acetate (250mg/kg) and olanzapine (10mg/kg) administration in adult female rats on the hippocampal gene expression of HDAC. Animals ingested acetate 7 days prior to, and throughout, the two-week administration of olanzapine. Results are expressed as mean±SEM for each group (n=6). *$p<0.05$ compared to water/saline.

to favour to alter chromatin structure into open conformations which permits gene transcription. Notably, the decrease in HAT activity that we observed using a high dose of sodium acetate may be the result of a homoeostatic response since the HDAC/HAT equilibrium plays a critical role in governing gene expression.

B.2.2 Cortical HDAC mRNA Expression

At 250mg/kg, no significant effect of HDAC mRNA was observed (Table B.1). Since no significant effect of transcription was found in either the hippocampus and cortex,

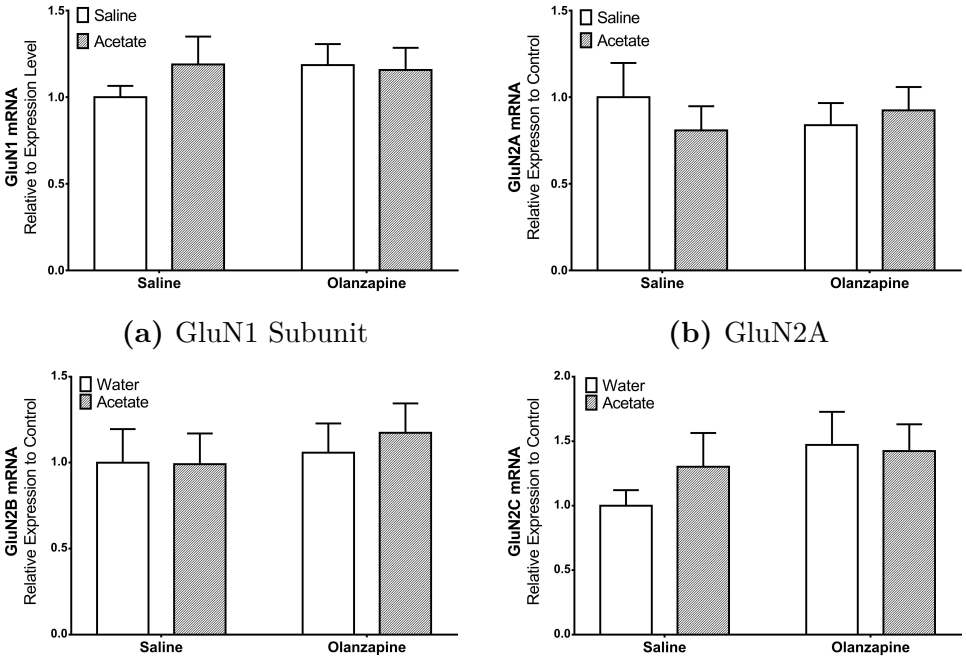
further examination of the activity of HDAC/HAT was precluded.

Table B.1: Effect of 250mg/kg Sodium Acetate on Cortical HDAC mRNA levels. Results are expressed as relative mean±SD relative to saline/water control group. *p<0.05

		Saline		Olanzapine	
		Water	Acetate	Water	Acetate
Low Dose Acetate (250mg/kg)	HDAC-1	1 ± 0.127	0.992 ± 0.193	1.079 ± 0.200	0.950 ± 0.166
	HDAC-2	1 ± 0.086	0.947 ± 0.190	0.918 ± 0.072	0.969 ± 0.234
	HDAC-3	1 ± 0.064	0.994 ± 0.091	0.983 ± 0.072	0.947 ± 0.125
	HDAC-4	1 ± 0.108	1.018 ± 0.195	0.961 ± 0.121	0.963 ± 0.173

B.2.3 Low Dose Acetate on Cortical NMDAR subunits

At 250mg/kg of sodium acetate no significant effect on the mRNA expression of cortical NMDA subunits GluN1, GluN2A-C were observed..



(a) GluN1 Subunit **(b)** GluN2A

(c) GluN2B **(d)** GluN2C

Figure B.3: Effect of acetate (250mg/kg) and olanzapine (10mg/kg) administration in adult female rats on the cortical gene expression of NMDA subunit. Animals ingested acetate 7 days prior to, and throughout, the two-week administration of olanzapine. Results are expressed as mean ± SEM for each group (n=6). *p<0.05 compared to water/saline.

B.3 Peripheral Effects of High Dose Sodium Acetate

In order to verify whether the central and peripheral systems are responding to increased levels of acetate, we documented the expression levels of ACC and SCFA receptor GPR-43 in the liver and white adipose tissue. A significant overall treatment effect ($F_{1,20}$=6.794, p=0.021, Table B.2) was observed in hepatic ACC levels where the administration of olanzapine alone decreased mRNA levels by 30% ($F_{1,14}$=5.077, p=0.041, table 3.2). Interestingly, a 2-fold increased was obsvered when B-GOS® was administered alone [133]. A significant diet x treatment interaction was also observed in hippocampal ACC ($F_{1,20}$=6.794, p=0.021). No significant changes were observed in white adipose tissues. The following table was excluded from the thesis, as well as the eventual publication due to the irreproducibility of the olanzapine effect.

Table B.2: Effect of High dose Acetate (500mg/kg) on Peripheral and Central Metabolising Enzymes. Results are expressed as relative mean ± SEM relative to saline/water control group. *p<0.05

		Saline		Olanzapine	
Region	Gene	Water	Acetate	Water	Acetate
Hippocampus	ACC	1 ± 0.327	1.05 ± 0.335	1.389 ± 0.4	0.88 ± 0.089
	GPR43	1 ± 0.622	1.129 ± 0.589	1.438 ± 0.962	1.219 ± 0.954
	MCT-1	1 ± 0.612	1.238 ± 0.221	1.444 ± 0.359	1.134 ± 0.293
Frontal Cortex	ACC	1 ± 0.445	1.14 ± 0.725	1.226 ± 0.272	1.047 ± 0.411
	GPR43	1 ± 0.335	0.911 ± 0.335	1.140 ± 0.479	1.102 ± 0.575
	MCT-1	1 ± 0.235	1.258 ± 0.305	0.958 ± 0.073	0.945 ± 0.426
Liver	ACC	1 ± 0.354	0.991 ± 0.527	0.710 ± 0.417	0.712 ± 0.413
	GPR43	1 ± 0.391	0.965 ± 0.22	0.728 ± 0.368	0.875 ± 0.539
	MCT-1	1 ± 0.297	0.959 ± 0.213	0.946 ± 0.211	1.278 ± 0.173
Adipose	ACC	1 ± 1.212	0.243 ± 0.311	0.142 ± 0.13	0.387 ± 0.363
	GPR43	1 ± 1.353	0.36 ± 0.515	0.445 ± 0.586	0.639 ± 0.378
	MCT-1	1 ± 1.251	0.714 ± 0.503	1.121 ± 0.998	1.085 ± 0.459

I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

From Memoirs of the Life, Writing, and Discoveries of Sir Isaac Newton (1885) by Sir David Brewster speaks to the depth of what we have left to discover. This world is grand and there lies an ocean of undiscovered findings that are waiting for eager and curious minds.

— Sir Isaac Newton (1642 - 1727)

C

The Prebiotic B-GOS® in Patients with Psychosis

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C.1 Prebiotic Supplement Ingredient List

The active prebiotic supplement, Bimuno™ B-GOS®, is a galacto-oliogosaccharide mixture commercially sold as B-GOS®. B-GOS® is specifically metabolized by *bifidobacteria* and contains 2.3 calories composed in the following ingredients per gram of Bimuno™: 0.96g dry matter; <01g Fat; <0.06g Protein; 0.821g GOS; 0.119g Lactose; 0.0331g Glucose; 0.0029g Galactose. B-GOS™ is a tasteless and

patent protected formulation (WO 2014155056 A1) composed of a mixture of disaccharides, trisaccharides, a tetrasaccharide and a pentasaccharide. Similarly, the maltodextrin control is a polysaccharide produced from starch and is readily absorbed as glucose. The food supplements used are safe, well tolerated and approved by the GRAS (Generally Recognised as Safe) programme, conducted by the U.S. Food and Drug Administration (FDA).

C.2 Patient Demographics

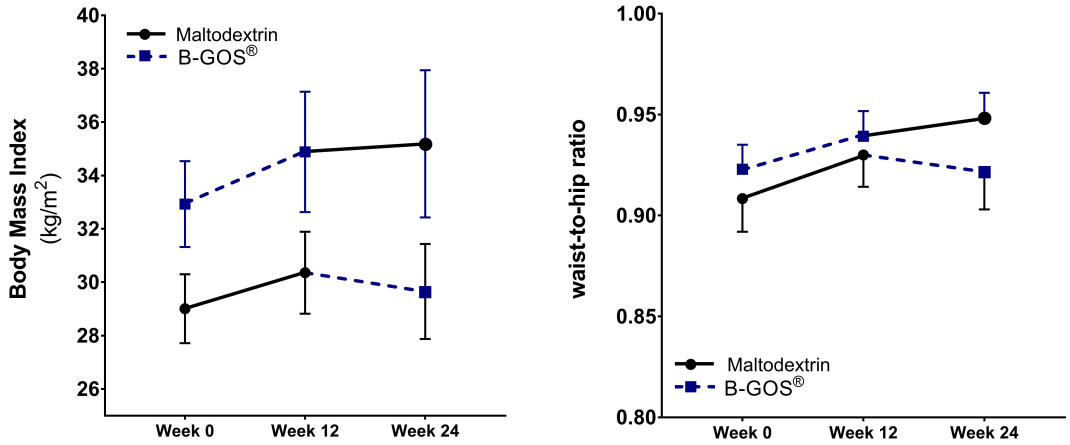
The within-subject analysis presented in Chapter 4 did not require basic demographics to be included, nonetheless the recruited population may be of interest to some.

Table C.1: Baseline demographics of participants at the start of the trials. Values are expressed as mean±SEM where applicable.

	Male (n = 24)	Female (n = 15)
Age	37.96 ± 11.235	39.80 ± 10.108
Duration of Illness (Years)	11.02 ± 9.115	6.46 ± 5.989
Antipsychotic Medication		
Olanzapine	7	4
Clozapine	9	2
Aripiprazole	4	5
Risperidone	3	2
Other	1	2
Ethnicity		
White	19	13
Caribbean	1	1
Mix	3	1
Asian	1	0

C.3 Change in BMI and WHR

In chapter 4.3.2 the *t*-test analysis of before and after the intake of the prebiotic was presented. Here we show the change in BMI and WHR in a mixed linear regression model. No significant changes were observed.



(a) Body Mass Index **(b)** Hip-to-waist Ratio

Figure C.1: Changes in (a) BMI and (b) WHR in stable patients with schizophrenia who received firstly maltodextrin or prebiotics for 12wks, followed by prebiotics or maltodextrin. Results are expressed as mean±SEM. *p<0.05.

C.4 Correlation with Cognitive Tasks

It remains an unknown as to why a negative correlation was detected at baseline between serum levels of acetate, and digit sequencing.

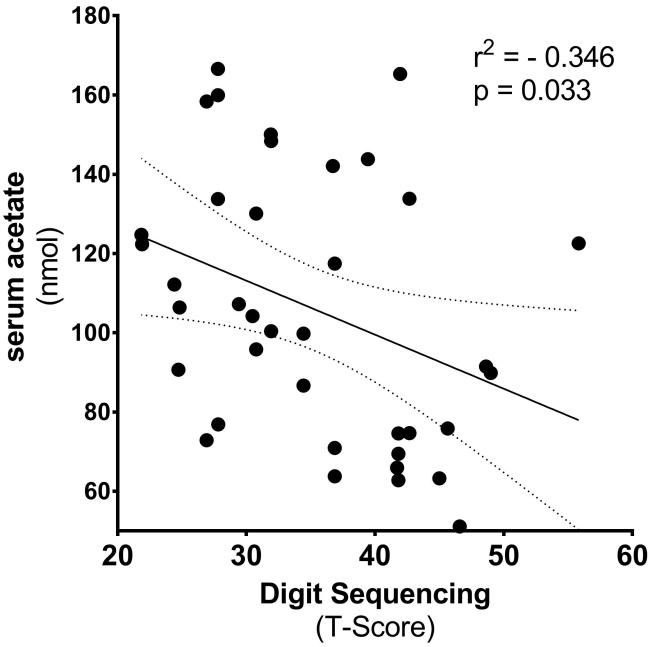


Figure C.2: Baseline Correlation of Serum Acetate with Digit Sequencing. Pearson correlations r^2 values are reported. Results are expressed as mean±SEM and dotted lines represent 95% confidence intervals. *$p<0.05$.

Alles Gescheite ist schon gedacht worden.
Man muss nur versuchen, es noch einmal zu denken.

All intelligent thoughts have already been thought;
what is necessary is only to try to think them again.

— Johann Wolfgang von Goethe

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