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A Microbiota-targeted strategy to attenuate antipsychotic-induced weight gain

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Timothy Cody Lipuma
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Author Contributions

During the *In vivo* study, Dr Natasha Leeuwendaal (NL), Dr Jorge Rueda (JR), Dr James Collins (JC), Dr Harriët Schellekens (HS), Tara Foley (TF), Patrick Fitzgerald (PF), and Cristina Cuesta (CC) all assisted in various research protocols.

- Partner intraperitoneal injections were carried out by the author (TL), JR, JC, HS, TF, and PF.
- Dietary and bodyweight measures were predominantly carried out by NL, with assistance from TL.
- The intraperitoneal glucose tolerance test was carried out by TL, NL, JR, JC, and PF.
- The elevated plus maze protocol was carried out by NL. Culls and sample collection were performed by TL, NL, JR, JC, and TF.
- Biomarker analysis was carried out by TL and NL.
- Gene expression benchwork was performed by TL and CC.

For the metabolomics study, Dr Kirsten Dowling calibrated the UPLC-MS and ran the samples. Dr Jane English conceptualised the metabolomics workflow, and provided supervision for data processing, statistical analysis and interpretation.

TL solely conducted all analyses and any other protocols in the methods that are not listed above.

Publications

Published Works

Boscaini, S., Leigh, S. J., Lavelle, A., García-Cabrerizo, R., **Lipuma, T**., Clarke, G., Schellekens, H., & Cryan, J. F. (2022). Microbiota and body weight control: Weight watchers within?. *Molecular metabolism*, *57*, 101427. https://doi.org/10.1016/j.molmet.2021.101427

Manuscripts in preparation

Lipuma, T., Schellekens, H., & English, J.A. Insights from different manipulations of obesity and weight loss: Perspective on ghrelin.

Prospective Journals: *Current opinion in endocrinology, diabetes, and obesity*; *Molecular metabolism*

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Prospective Journals: *Translational psychiatry*; *Psychopharmacology*

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Prospective Journals: *Translational psychiatry*, *Metabolomics*

Abbreviations

5-HT – 5-hydroxytryptamine (serotonin)

AAEC – Animal experimentation ethics committee

ACC – acetyl-CoA carboxylase

AgRP – Agouti-related peptide

APA – American psychiatric association

BBB – Blood-brain barrier

BDNF – Brain-derived neurotrophic factor

BGM – Brain gut microbiome

BW – Body weight

B-WISE – Body weight, image, and self-esteem evaluation

CART – Cocaine and amphetamine regulated transcript

CB - Cannabinoid

CCK – Cholecystokinin

CD68 – Cluster of differentiation 68

CFU – Colony forming units

CHR – Clinically high risk

CHREBP – Carbohydrate response element binding protein

CVD – Cardiovascular disease

DA – D-amino acid oxidase

DMH – dorsomedial nucleus

DPBS – Dulbecco's phosphate-buffered saline

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EPM – Elevated plus maze

FAAH – Fatty acid amide hydrolase

FAS – Fatty acid synthase

FATP5 – Fatty acid transport protein-5

FC – Fold change

FDR – False discovery rate

FFAR – Free fatty acid receptor

FMT – Faecal material transplant

FXR – Farnesoid X receptor

GABA - γ-Aminobutyric acid

GBD – Global burden of disease

GHLR - Ghrelin

GHSR – Growth hormone secretagogue receptor

GLP-1 – Glucagon-like peptide 1

GPCR – G-protein-coupled receptor

HDL – High density lipoprotein

HPA – hypothalamic adrenal pituitary

HPRA – Health products regulatory authority

IL-1β – Interleukin 1 beta

IPGTT – intraperitoneal glucose tolerance test

IRI – Insulin resistance index

ISAPP – International scientific association for probiotics and prebiotics

KEGG – Kyoto encyclopedia of genes and genomics

LC-MS – Liquid chromatography-mass spectrometry

LEPR – Leptin receptor

LHA – Lateral hypothalamic area

LPS – Lipopolysaccharide

MTOR – Mammalian target of rapamycin

NMDA – N-methyl-d-aspartate

NPY – Neuropeptide Y

PANSS – Positive and negative syndrome scale

PCR – Polymerase chain reaction

POMC – Proopiomelanocortin

PPARA – Peroxisome proliferator-activated receptor alpha

PVN – Paraventricular nucleus

RCT – Randomized controlled trial

RT-QPCR – Real-time quantitative polymerase chain reaction

SCFA – Short chain fatty acid

SGA – Second-generation antipsychotic

SREP1C – Sterol regulatory element-binding protein 1c

TG -Triglycerides

TGR5 – G-protein-coupled bile acid receptor

TNF-α – Tumor necrosis factor alpha

UPLC – Ultra-high performance liquid chromatography

VMN – Ventromedial nucleus

Abstract

Background: Atypical antipsychotics such as olanzapine are an essential treatment for psychotic-spectrum disorders, but their use is associated with significant weight gain and increased cardiometabolic disease risk. Attenuating these side effects could improve the tolerability and adherence to antipsychotic medications. Evidence suggests that the microbiome plays a role in antipsychotic-induced weight gain, thus targeting the microbiome may be a viable therapeutic strategy to attenuate the side effect profile of antipsychotics like olanzapine. Furthermore, metabolomics approaches are being increasingly employed to elucidate disease pathophysiology and potential therapeutic targets, but these strategies have not yet been applied to the problem of antipsychotic-induced obesity and hyperphagia.

Aims: The primary aims of this study are to (1) investigate if combined microbiome-targeted treatments (probiotic [APC1472], prebiotic [xanthohumol], and their combination) with olanzapine attenuate antipsychotic-induced obesity, metabolic dysfunction, and hyperphagia in female Sprague-Dawley rats, and (2) analyse blood plasma using discovery metabolomics to generate potential mechanistic and therapeutic targets related to the side effects of olanzapine.

Methods: Animals were treated with olanzapine (2 mg/kg body weight) alone (n=12), olanzapine with probiotic (n=11), olanzapine with prebiotic (n=11), olanzapine with probiotic and prebiotic (n=12) or control vehicle (n=12) twice a day via intraperitoneal injection for 31 days. Changes in body weight, adiposity, glucose metabolism, dietary intake, anxiety-like behaviour, plasma biomarkers (corticosterone, insulin, ghrelin), and hypothalamic and hepatic gene expression were examined. Ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) and subsequent metabolomic analysis using Progensis QI and Metaboanalyst were used to characterise plasma differences between the olanzapine treatment group and controls.

Results: After the study conclusion, quality control issues with the probiotic formulation were discovered, limiting the interpretability of the data from those treatment groups. However, the olanzapine treatment displayed increased weight gain, dietary intake, and hypothalamic genes

related to ghrelinergic signalling. Olanzapine did not increase adiposity, change hepatic gene expression, plasma biomarkers, or hypothalamic genes related to anorexigenic signalling. No treatments attenuated olanzapine-induced weight gain. There were no observed differences in anxiety-like behaviour between any groups. Lastly, the metabolomics investigation revealed several highly differentially expressed metabolites; two androstanoids and one endocannabinoid (oleamide).

Conclusion: These findings indicate that olanzapine-associated increases in hypothalamic ghrelinergic signalling can occur before or without the onset of peripheral changes in metabolic health. Although the attenuation of olanzapine-associated increases in hypothalamic ghrelinergic signalling could not be assessed due to the quality control issues with the probiotic, targeting ghrelinergic signalling via microbiome-targeted approaches warrants further research. Additionally, the metabolomics analyses highlight oleamide as a novel metabolite that is potentially at the intersection of the endocannabinoid system, the microbiota, and olanzapine treatment, but further research is needed to clarify if the observed increase in oleamide is due to changes in host and/or microbial metabolism.

1. General Introduction

1.1 Second-generation Antipsychotics

1.1.1 Second-generation antipsychotics and cardiometabolic disease risk

Psychosis is associated with several mental illnesses including schizophrenia, schizoaffective disorder, delusional disorder, bipolar disorder and severe depression (American Psychiatric Association [APA], 2022). Psychosis is characterised by a reduction or loss of contact with reality, which can include "positive symptoms" such as (1) delusions (firmly held but untrue beliefs), (2) sensory hallucinations (hearing or seeing things that are not real), (3) disorganized speech (ranging from loose tangential connections to complete incoherence), (4) disorganized or abnormal motor behaviour and catatonia (strange or bizarre movements and physical affect) (APA, 2022). Psychosis can also include "negative symptoms", such as (1) diminished emotional affect, (2) anhedonia (reduced ability to experience pleasure), (3) alogia (diminished speech production), (4) asociality (reduced or lack of desire to socialize), and (5) avolition (decreased motivation in purposeful activities, such as work) (APA, 2022). Global estimates indicate that psychotic disorders have a lifetime prevalence of 3% (Perälä et al., 2007). Global life expectancy for people living with psychotic disorders is 64.7 years (Hjorthøj et al., 2017), in contrast to the general population's global life expectancy of 73.5 years (Global Burden of Disease [GBD] 2019 Diseases and Injuries Collaborators, 2020).

The reasons for this widening life expectancy gap are complex, though one important factor is likely the medications used to treat psychotic disorders. Atypical antipsychotics, such as olanzapine, are efficacious first-line treatments for psychotic disorders (Huhn et al., 2020), but their use is associated with a variety of cardiometabolic disturbances, including weight gain, hyperglycaemia, dyslipidaemia and insulin resistance–all known risk factors for cardiometabolic diseases (Pillinger et al., 2020). In a one-year study of patients on antipsychotics for the management of schizophrenia, 80% experienced significant weight gain (McEvoy et al., 2007). One study of Americans diagnosed with schizophrenia found that they were 3.5 times more likely to die in a given year than the general population, with heart disease being the leading cause of death (Olfson et al., 2015). The European first-episode schizophrenia trial (EUFEST) found that the prevalence of metabolic syndrome was similar between the general population and

antipsychotic-naive patients, but antipsychotic use led to worsening glucose levels that developed into hyperglycaemia (Fleischhacker et al., 2013). This is further corroborated by a 2019 meta-analysis by Holt and colleagues, which found that there was a 2.9% prevalence of type II diabetes in antipsychotic-naive patients, in contrast to 11.3% of patients with severe mental illness treated with atypical antipsychotics. Furthermore, a Danish cohort study found that olanzapine and aripiprazole nearly doubled the risk of developing type II diabetes, and clozapine quadrupled the rate (Rajkumar et al., 2017). Taken together, although antipsychotics are essential medications, their use is a significant risk to cardiometabolic health.

Antipsychotic use is only one component of the elevated cardiometabolic risk profile of patients with psychosis. People with psychosis and schizophrenia have increased rates of detrimental health behaviours, such as alcohol use, smoking, sedentary behaviour, and disturbed sleep, in comparison to the general population (Firth et al., 2019). Despite an increased focus on addressing these issues, evidence indicates that the life expectancy gap between those with psychosis and the general population is increasing rather than narrowing (Firth et al, 2019). The reasons for this are complex. Several meta-analyses indicate that established cardiovascular risk factors, including dyslipidaemia, diabetes mellitus, obesity, and hypertension, are more common in people with psychosis (Osborn et al., 2008; Mitchell et al., 2013; Correll et al., 2022). Additionally, a recent Mendelian randomization analysis suggests that shared genetic risk exists between schizophrenia and cardiovascular disease (Veeneman et al., 2022). Therefore, research focusing on improving the cardiometabolic health of patients with psychotic-spectrum disorders is an urgent scientific priority.

1.1.2 Psychiatric stability with side effects or psychosis and hospitalisation

Although antipsychotic medications can increase cardiovascular risk, studies concerning overall mortality are inconsistent. Recent meta-analytic evidence has shown that greater improvements in psychotic symptoms are associated with worsened metabolic outcomes (Pillinger et al., 2020). In other words, the positive association between medication adherence and symptom management is inversely correlated with metabolic health (Pillinger et al., 2020). Although antipsychotics can increase weight and negatively affect metabolism, a recent study of 62,250 Finish patients found that over the course of 20 years, antipsychotic use was associated with

reduced all-cause mortality, as well as cardiovascular and suicide mortality (Taipale et al., 2020). Taipale and colleagues (2020) noted that one potential reason for this is that though there may be short to medium-term negative metabolic and weight effects of antipsychotics, long-term adherence may lead to an increased quality of life and physical activity.

Nonetheless, surveys indicate that patients find the weight gain associated with antipsychotics to be the most distressing side effect (Achtyes et al., 2018), which negatively affects medication adherence (Dayabandara et al., 2017). Globally, there are increased trends in the prescription of antipsychotics, and the ratio of atypical/typical usage is increasing toward atypical antipsychotics (Ågren, 2021), making the attenuation of antipsychotic-induced weight gain and metabolic dysregulation of high societal value, both for subjective patient wellness and long-term antipsychotic drug adherence.

1.2 Central Regulation of Appetite, Obesity and Antipsychotics

1.2.1 The hypothalamus

Eating behaviour is a complex phenotype. There are multiple physiological, neural and genetic systems at play, featuring convergent/additive and antagonistic feedback loops, rhythmicity, and adaptability: all to promote organismal survival (van der Klaauw and Farooqi, 2015; Betley et al., 2013). The brain region central to these processes is the hypothalamus. The hypothalamus is one of the most phylogenetically primitive components of the nervous system, developing from the apical nervous system of early eumetazoans as the first central regulator of physiologically important behaviours, such as rest, nutrient control and food-seeking (Cisek, 2019). Within the hypothalamus, the arcuate nucleus contains several types of neurons that regulate appetite: both pro-opiomelanocortin (POMC) neurons and cocaine and amphetamine-regulated transcript (CART) neurons are anorexigenic (suppress appetite), while Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) neurons are orexigenic (promote appetite) (Timper and Brüning, 2017). These neurons project to second-order neurons of the hypothalamus, which make up the paraventricular nucleus (PVN), ventromedial nucleus (VMN), lateral hypothalamic area (LHA), and the dorsomedial nucleus (DMH) (Yeoh and Heisler, 2012; Fetissov, 2017). These hypothalamic nuclei express receptors for a variety of peripherally produced ligands, such as insulin and amylin from the pancreas; leptin, adiponectin, and interleukins from adipose tissue;

ghrelin and cholecystokinin (CCK) from the stomach; and Peptide YY (PYY), glucagon-like peptide 1 (GLP-1), and oxyntomodulin (OXM) from the gut (Yeoh and Heisler, 2012). These systems are graphically represented in Figure 1 (extracted from Yeoh and Heisler, 2012).

1.2.2 Olanzapine pharmacology and central mechanisms of olanzapine-induced hyperphagia

Despite its known clinical efficacy and side effect profile, the mechanisms by which olanzapine increases appetite is an ongoing area of investigation (Correll et al., 2011). Both clinical and pre-clinical mechanistic evidence suggest that the obesity and metabolic side effects caused by olanzapine are attributed to its largely antagonistic affinity for neurotransmitter receptors that are expressed throughout the body, including dopamine, histamine, acetylcholine, muscarine, epinephrine, norepinephrine, and adrenergic receptors (Siafis et al., 2018). As there are receptor subtypes expressed both centrally and peripherally, interindividual variability in drug response and severity of side effects may be related to genetic differences in receptor expression (Siafis et al., 2018). This is in contrast to the cytochrome P450 system's metabolism of olanzapine (responsible for hepatic first-pass metabolism) as current meta-analytic evidence examining the effects of cytochrome P1A2 (CYP1A2) polymorphisms on olanzapine metabolism found no differences in pharmacokinetics (Takuathung et al., 2019). While the biology of olanzapine is complex, clinical evidence indicates that antipsychotic-related weight gain is due to increased caloric intake (Gothelf et al., 2002; Fountaine et al., 2010). Clinical data suggests that increased caloric intake is related to olanzapine-induced increases in appetite. In first-episode psychosis patients treated with olanzapine, patients who endorse greater changes in appetite (defined as a 10% increase from baseline appetite, measured via a Likert-scale questionnaire) had a 9.1 kg increase in body weight over 12 weeks, in comparison to 3.9 kg for patients who did not endorse an increase in appetite (Huang et al., 2020). Notably, 77.4% of participants endorsed an increased appetite, while only 22.6% did not (Huang et al., 2020). This highlights that while there is interindividual variability in olanzapine response, the majority of patients will experience weight gain.

Although this thesis focuses on hypothalamic and peripheral metabolic changes caused by olanzapine, it is worth noting that these are not the sole contributors to its side effect profile.

Olanzapine is a dopamine antagonist, and the dopamine hypothesis of schizophrenia posits that an excess of dopaminergic signalling is part of the aetiology of psychosis, thus competitive antagonistic ligands, such as olanzapine, would normalise dopaminergic signalling when consistently present at pharmacological levels of 60-80% receptor occupancy (Seeman, 2021). However, while dopamine antagonism may stabilise psychotic symptoms, it could also dampen other dopamine-related systems and behaviours. A small randomised, double-blind clinical trial employing fMRI methods to examine the effects of olanzapine on reward activation in typically developing participants found that reward-related activation of the ventral striatum, anterior cingulate, and inferior frontal cortex was reduced in the olanzapine-treated group (Abler et al., 2007). Thus, reduced dopamine signalling may impact sensitivity to food reward, which can contribute to increased dietary intake. Changes in serotonergic signalling are also implicated in olanzapine-induced obesity and hyperphagia. Olanzapine is an antagonist for serotonin receptors (5HT2a and 5HT2c), and one role of serotonergic signalling is postprandial appetite suppression (Mukherjee et al., 2021). While investigating the far-spanning pharmacologic effects of olanzapine are beyond the scope of this thesis, these mechanisms warrant continued investigation as well.

In the context of hypothalamic signalling, early clinical data indicated that olanzapine use is associated with increases in ghrelin (an appetite-promoting hormone), food craving, and binge eating (Murashita et al., 2005; Kluge et al., 2007). The majority of ghrelin production occurs in X/A-like oxyntic gland cells (P/D_1 cells in humans) (Kojima et al., 1999; Schellekens 2010), and it exerts its behavioural effects by binding to hypothalamic ghrelin receptors, also called the growth hormone secretagogue receptor (GHSR) (Yin et al., 2014; Moose et al., 2020). Of note is there are two isoforms of ghrelin, and while acyl ghrelin and deacyl ghrelin are found in the bloodstream, only acyl ghrelin has high-affinity binding to GHSR, thus acyl ghrelin is also referred to as active ghrelin (Yin et al., 2014; Moose et al., 2020).

Meta-analytic evidence examining circulating levels of ghrelin in olanzapine-medicated patients with schizophrenia found that olanzapine therapy is associated with decreased peripheral ghrelin (Goetz and Miller, 2019) which contrasts with the increased serum ghrelin seen in other studies (Murashita et al, 2005; Kluge et al., 2007). In the context of ghrelin signalling, these findings

may not be as contradictory as they first appear. Several studies subsequently discussed help clarify the time course and mechanisms of ghrelinergic signalling in the context of olanzapine, obesity, and hyperphagia.

1.2.3 Disentangling central vs peripheral effects of ghrelin

By comparing intragastric versus intracerebroventricular administration of olanzapine, Girault et al. (2012) found that the metabolic side effects of olanzapine are driven by peripheral mechanisms, rather than central, as evidenced by peripheral administration of olanzapine leading to hyperglycaemia and insulin resistance, but not intracerebroventricular administration. However, while a portion of olanzapine-related side effects may be attributable to peripheral pharmacological effects, its impact on eating behaviour (and a subsequent increase in eating) likely also contributes to peripheral side effects. In a study of chronic olanzapine administration in three cohorts of rats (8, 16, and 36 days of administration) Zhang and colleagues (2014) measured serum levels of ghrelin, weight, and hypothalamic *Ghsr*. Hypothalamic *Ghsr* and circulating ghrelin were quantified at the end of each respective cohort's treatment duration. While weight continuously increased in all three cohorts, there was only an increase in daily food intake during days 4-12, and peripheral ghrelin was only increased on day 8 of treatment, but not on days 16 and 36 (Zhang et al., 2014). Interestingly, hypothalamic *Ghsr* was increased across at all time points, and in a separate experiment within the same study, Zhang and colleagues (2014) demonstrated that intracerebroventricular injection of a ghrelin receptor antagonist reversed olanzapine-associated changes in appetite-regulating hypothalamic genes (increased in orexigenic *Npy, Agrp,* and decreased anorexigenic *Pomc*) and chow intake. Thus, while olanzapine may exert direct pharmacological effects on peripheral tissues, increases in central ghrelinergic signalling is sufficient to increase eating behaviour, and peripheral levels of ghrelin can vary with when it is measured in the time course of olanzapine treatment.

To further contextualise the complexity of ghrelinergic signalling, inferences may be drawn from clinical and preclinical models of obesity interventions, specifically by examining how ghrelinergic signalling changes pre-and post-intervention. Tamboli and colleagues (2017) found that before undergoing Roux-en-y gastric bypass surgery, intravenous administration of ghrelin to class III obese patients was associated with increased secretion of pancreatic polypeptide (a

proxy for top-down vagal activity) and decreased growth hormone (initially produced and released from the hypothalamus) in obese vs lean patients. Two weeks post-surgery, the effects of intravenous ghrelin on pancreatic polypeptide and growth hormone were similar between obese and lean patients, but blunted peripheral insulin sensitivity persisted in the 2-week follow-up period (Tamboli et al., 2017). Thus, in the context of obesity, ghrelin signalling was associated with perturbed hypothalamic and peripheral effects, but following gastric bypass surgery central effects of ghrelin are uncoupled from peripheral effects (Tamboli et al., 2017).

A related pre-clinical study examined how sleeve gastrectomy, after induction of obesity via a high-fat diet, was associated with hypothalamic changes in gene expression of *Ghsr* (Fedonidis et al., 2014). At 90 days post-surgery, hypothalamic gene expression of *Ghsr* was significantly decreased in the sleeve gastrectomy group in comparison to sham surgery or obese controls. However, at 30 days post-surgery, hypothalamic *Ghsr* expression was increased (Fedonidis et al., 2014). Contrastingly, *Lepr* expression (the receptor for leptin, a satiety-promoting cytokine released from adipose tissue) was lower in the gastrectomy group (in comparison to obese and sham surgery) at 30- and 90-days post-surgery (Fedonidis et al., 2014). Fedonidis and colleagues (2014) also found that cerebellar *Ghsr* expression decreased at both time points following surgery, as did hypothalamic *Lepr* expression. This suggests that the expression of genes regulating appetite and energy balance is variable across brain regions. Furthermore, the associations between brain regions and gene expression are likely moderated both by time and health state (i.e., weight, stage of weight loss).

Taken together, while both clinical and preclinical animal studies data suggest that peripheral levels of ghrelin decrease as a function of olanzapine-induced obesity, preclinical studies suggest that central ghrelinergic signalling may play a causal mechanistic role in olanzapine-induced obesity through increasing food intake. Thus, targeting of ghrelinergic signalling may be a viable strategy to attenuate the hyperphagia, subsequent weight gain, and cardiovascular disease risk associated with antipsychotics.

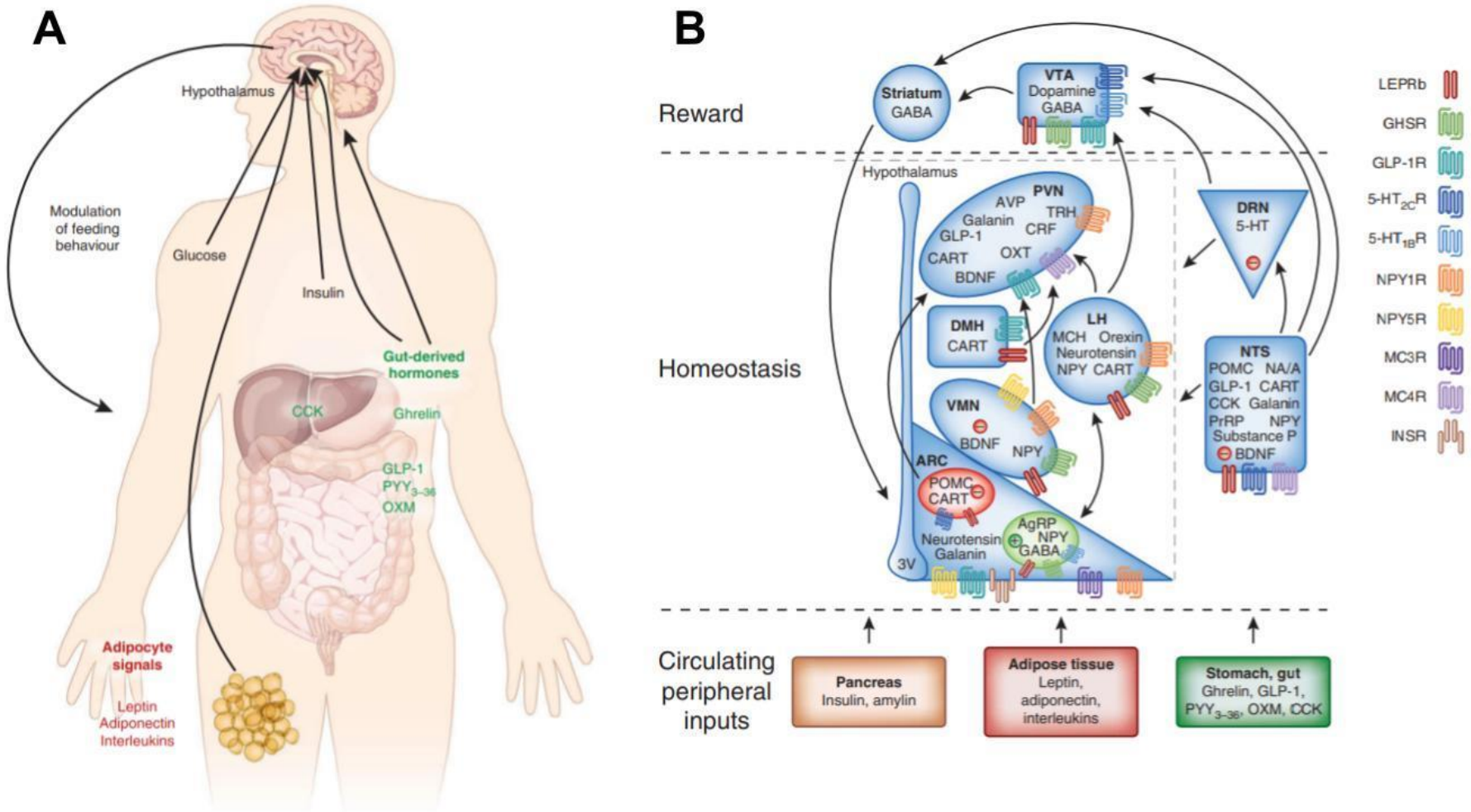


Figure 1. (A) Brain sensing of gut- and adipocyte-derived hormones regulating appetite; (B) Schematic representation of the hypothalamic nuclei and other relevant higher brain regions involved in hedonic and homeostatic regulation of appetite. Appetite is a complex behaviour with polygenic, multi-tissue regulation of anorexigenic (appetite supressing) and orexigenic (appetite promoting) pathways.

Subfigure A key: GLP-1, glucagon-like peptide 1; PYY_{3-36}, peptide YY residues 3-36; OXM, oxyntomodulin; CCK, cholestokinin. Subfigure B key: 3V; third ventricle; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; VMN, ventromedial nucleus; LH, lateral hypothalamus; ARC, arcuate nucleus; VTA, ventral tegmental area; DRN, dorsal raphne nucleus; NTS, nucleus of the solitary tract; CRF, corticotropin-releasing factor; GLP-1, glucagon-like peptide1; MCH, melanin-concentrating hormone; OXT, oxytocin; AVP, vasopressin; TRH, thyrotropin-releasing hormone; NA, noradrenaline; A, adrenaline; PrRP, prolactin-releasing peptide; PYY_{3-36}, peptide YY residues 3-36, OXM, oxyntomodulin; CCK, cholecystokinin; MC34 and MC4T, melanocortin receptors; NPY1R and NPY5R; neuropeptide Y receptors; LEPRb, long signaling isoform of leptin receptor; INSR, insulin receptor; GHSR, ghrelin receptor; GLP-1R, GLP-1 receptor. Figure extracted from Yeo and Heisler (2012).

1.3 Associations of Antipsychotics and the Gut Microbiota

1.3.1 The brain gut microbiome axis

The brain gut microbiome (BGM) axis is a prominent area of research at the intersections of physical and mental health, as they share a significant overlap of BGM axis mechanisms, including gut hormone signalling, lipid metabolism, glucose homeostasis, neuroimmune signalling, neurodevelopment, and neurotransmission (Cryan et al., 2019; Kelly et al., 2021). For example, the gut-brain axis bidirectional communication via central, enteric, autonomic, neuroendocrine and immunological systems (Carabotti et al., 2015). Within the gut, there are trillions of bacteria which outnumber the eukaryotic cells of the human body (Dinan et al., 2015). These bacteria influence host physiology vis the BGM axis through several modes, including the vagal afferents and efferents, the hypothalamic pituitary adrenal (HPA) axis, immune and inflammatory signalling, microbial metabolites (amino acids, lipids), and neuroendocrine signalling (Cryan et al., 2020). One of the seminal works in the field which highlighted targeting the microbiota to influence the brain was investigated by Sudo and colleagues (2004). Their study found that germ-free mice had elevated corticosterone responses to stress, as well as reduced levels of growth factors (brain-derived neurotrophic factor, BDNF) in hippocampal and cortical areas of the brain (Sudo et al., 2004). Their experiments also established the differential impact of gut bacteria on host health; gnotobiotic (colonized with one bacteria) *Bifidobacterium infantis* (a health-promoting bacteria) mice had a stress response closer to that of healthy conventional mise, while gnotobiotic *Escherichia coli* (a pathogenic bacteria) mice had a stress response closer to that of sickly germ-free mice (Sudo et al., 2004). Lastly, they demonstrated that the detrimental effects of being germ-free could be reversed by faecal material transplant (FMT) from healthy conventional mice—but only if this transplant was done at the early stages of the germ-free mouse's life (Sudo et al., 2004). Since their work, the past few decades have focused on influencing the composition of the microbiome to improve host brain and body health.

1.3.2 Origins and composition of the microbiome

The origins, plasticity, and developmental effects of the gut microbiome are a complex and ongoing area of research. While previous research has shown that children delivered via caesarean section, in comparison to children delivered vaginally, display higher levels and diversity of the Firmicutes phyla, and lower levels and diversity of Actinobacteria and

Bacteroidetes (Palmer et al., 2007; Domingues-Bello et al., 2010), recent investigations suggest that other sources of microbial diversity, such as breast milk, play a compensatory and more important role in colonising the infant microbiota of caesarean section babies (Bogaert et al., 2023). Furthermore, Bogaert and colleagues (2023) found that an average of 58.5% of the infant microbiome was attributable to maternal transmission.

The composition of gut microbes is highly plastic and is associated with changes in response to stress, circadian regulation, sex, immune function, age, diet, genetics, drug use, and exercise (Feng et al., 2020). Most commonly known are the effects of antibiotic drugs, which reduce bacteria from the genera *Bifidobacterium* and *Faecalibacterium*, which are thought to be beneficial to human health (Ferrer et al., 2017). An area of high research activity is the investigation of probiotics, beneficial bacteria thought to confer positive health effects, and prebiotics, a broad variety of compounds [types of dietary fibre and polyphenols] that increase good bacteria, which can then impact the BGM axis (Sanders et al., 2019).

1.3.3 The microbiota as a therapeutic target for attenuating antipsychotic side effects

Davey and colleagues (2013) provided initial evidence that the effects of antipsychotics may be contingent on the microbiota. Rats fed a high-fat diet with olanzapine rapidly gained weight, but this was attenuated by concomitant antibiotic cocktail treatment (Davey et al., 2013). A study by Morgan et al. (2014) demonstrated that olanzapine has antibacterial properties against *Escherichia coli* and *Enterococcus faecalis*, and while germ-free mice were not susceptible to olanzapine-induced weight gain, germ-free mice that underwent faecal material transplant (FMT) rapidly gained weight with olanzapine treatment, and their composition shifted toward an increased ratio of Firmicutes to Bacteroidetes. This ratio is similar to the microbiota of obese populations (Xu et al., 2022). Furthermore, the aforementioned study by Davey and colleagues (2013) found that the obesity-associated compositional shift of the Firmicutes to Bacteroidetes ratio was normalised in the antibiotic cocktail group. There is also evidence that psychotropic medications, such as fluoxetine, escitalopram, lithium, valproate, and aripiprazole all exert differential effects on the microbiota (Cussoto et al., 2019). Furthering the hypothesis that the microbiota plays a key role in the side effect profile of second-generation antipsychotics (SGA), a follow-up study by Cussoto and colleagues (2021) demonstrated that the bioavailability of olanzapine increased 1.8-fold in microbiome-depleted rats while causing no changes in hepatic

CYP450 expression, potentially disentangling the direct physiologic effects of the drug from its interactions with gut microbiota. This hypothesis is furthered by the correlation between antibiotic administration and reduced bacterial/faecal glucuronidase activity, which is the primary metabolic pathway for second-pass metabolism of olanzapine (Cussoto et al., 2021). Thus, fluctuations in the gut microbiota, and expression of glucuronidases, could play a role in olanzapine metabolism. Lastly, by blocking the effects of gut-brain communication via vagotomy, Zhu and colleagues (2022) found that the olanzapine-induced increases in white adipose tissue, decreases in brown tissue, weight gain, and increased expression of hypothalamic orexigenic *NPY* and *AgRP* were all blocked. Interestingly, the obesogenic ratio of Firmicutes and Bacteroidetes was also reversed in the vagotomy group, which suggests a top-down influence of the brain on microbiome composition (Zhu et al., 2022). However, recent clinical studies have suggested that while the increased Firmicutes to Bacteroidetes ratio may induce obesity in rodents, and it may correlate with human obesity, shifting this ratio through microbiome-targeted interventions (e.g., through probiotics or FMT) does not improve human obesity (Boscaini et al., 2022; Montenegro et al., 2023) and early positive findings in the field may have encouraged interpretive biases (Montenegro et al., 2023). Taken together, while *in vivo* research may suggest the effects of olanzapine on both central and peripheral health may be mediated via the brain-gut-microbiome axis, clinical data suggests that human obesity (and likely olanzapine-induced obesity) is more complex.

1.3.4 Mechanisms of antipsychotic and BGM axis interactions

A recent systematic review by Chen and colleagues (2023) summarises several studies demonstrating the effects of SGAs on the BGM axis. A variety of systems are implicated, including lipid metabolism (short-chain fatty acids [SCFAs] and bile acids [BAs], sphingolipids, phospholipids), intestinal neurotransmission, central brain function, and the immune system, all of which may contribute to weight gain and metabolic syndrome (Chen et al., 2023). This is graphically represented in Figure 2 (extracted from Chen et al., 2023).

Bile acids are first produced from cholesterol in the liver and are then transported for storage in the gallbladder, where they can then be released into the distal small intestine to promote digestion (Wahlström et al., 2016). Primary bile acids, such as cholic acid, can be further metabolised by gut bacteria into secondary bile acids, such as deoxycholic acid (Wahlström et

al., 2016). Although they are more popularly known for their physical properties, i.e., emulsification of dietary lipids and fat-soluble vitamins, bile acids also bind to the farnesoid X receptor (FXR), a nuclear receptor which is highly expressed throughout the intestines and liver (Staels and Fonesca, 2009; Chen et al., 2023), though FXR is also expressed in the heart, ovaries, testes, thymus, eyes, and spleen (Lefebvre et al., 2009; Teodoro et al., 2011). Within hepatic tissues, binding of BAs to FXR is associated with a variety of downstream metabolic effects, including decreased expression of *SREBP1c* (leading to decreased triglyceride production) and increased expression of *PPARα* (leading to increased lipid catabolism and production of ketone bodies) (Staels and Fonseca, 2009). BA activation of enterocyte-expressed FXR enhances glucose absorption (van Dijk et al., 2009). Another ubiquitously expressed target for BAs is a G-protein-coupled receptor (GPCR) called the Takeda G-protein-coupled BA receptor (TGR5) (Chen et al., 2023). TGR5 primarily has affinity for secondary BAs, highlighting the essential role of gut bacteria in TGR5 signalling (Wahlström et al., 2016; Chen et al., 2023). BAs may also cross the blood-brain barrier (BBB) and bind with neuronal membrane receptor TGR5, increasing expression of *AgRP* and *NPY*, which has been shown to promote appetite (Morrison and Preston, 2016). Taken together, there are several pathways by which BAs may interact with the microbiome, and subsequently impact central and peripheral signalling. Crucially, different BAs possess different affinities for TGR5 and FXR, further highlighting the modulation of BA production via the gut microbiome to impact health (Wahlström et al., 2016; Chen et al., 2023).

Another mechanism by which the microbiota impacts the host is through the generation of SCFAs. SCFAs, such as butyrate, propionate and acetate are produced through microbial metabolism of host dietary components, such as fibre and resistant starch when they reach the large intestine, where they can be absorbed into systemic circulation or used as an energy source for colonocytes (Boscaini et al., 2022; Ney et al., 2023). The biological effects of SCFAs are pleiotropic, as SCFAs can (1) regulate gene expression through contributing acetyl-CoA substrate for histone acetyltransferases and direct inhibition of histone-deacetylase complexes (2) bind to extracellular GPCRs, such as dedicated free-fatty acid receptors (FFARs) and GHSR (3) increase expression of tight junction proteins to improve BBB and intestinal barrier function (4) directly act on the brain cross the BBB where they can act as energy substrate, impact neuroimmune function, and modulate neurotransmission (5) indirectly act on the brain through binding to type-L enteroendocrine colonocyte FFARs, which then secrete anorexigenic

hormones peptide PYY and GLP-1 via vagal afferents (6) lastly, interact with peripheral organs via diffusion across cell membranes, direct transport via monocarboxylate transporters (MCTs) and direct interaction with FFARs and other GPCRs (O'Riordan et al., 2022; Ney et al., 2023). In a two-year longitudinal clinical study of schizophrenia patients, high-risk patients, and healthy controls, valeric acid was lower in patients with schizophrenia and patients who developed schizophrenia, and caproic acid was lower in high-risk patients and patients who developed schizophrenia (Peng et al., 2022). However, there were no differences in levels of acetic, butyric, or isovaleric acid among the three groups (Peng et al., 2022). A scoping review by Singh and colleagues (2022) indicates that while antipsychotic-induced shifts in gut microbiota and SCFAs are well established, the direction of associations between SCFAs and microbial composition in schizophrenia populations is mixed. Thus, clinical studies assessing the causality and impact of SCFAs in translational research are needed to clarify this system's therapeutic role.

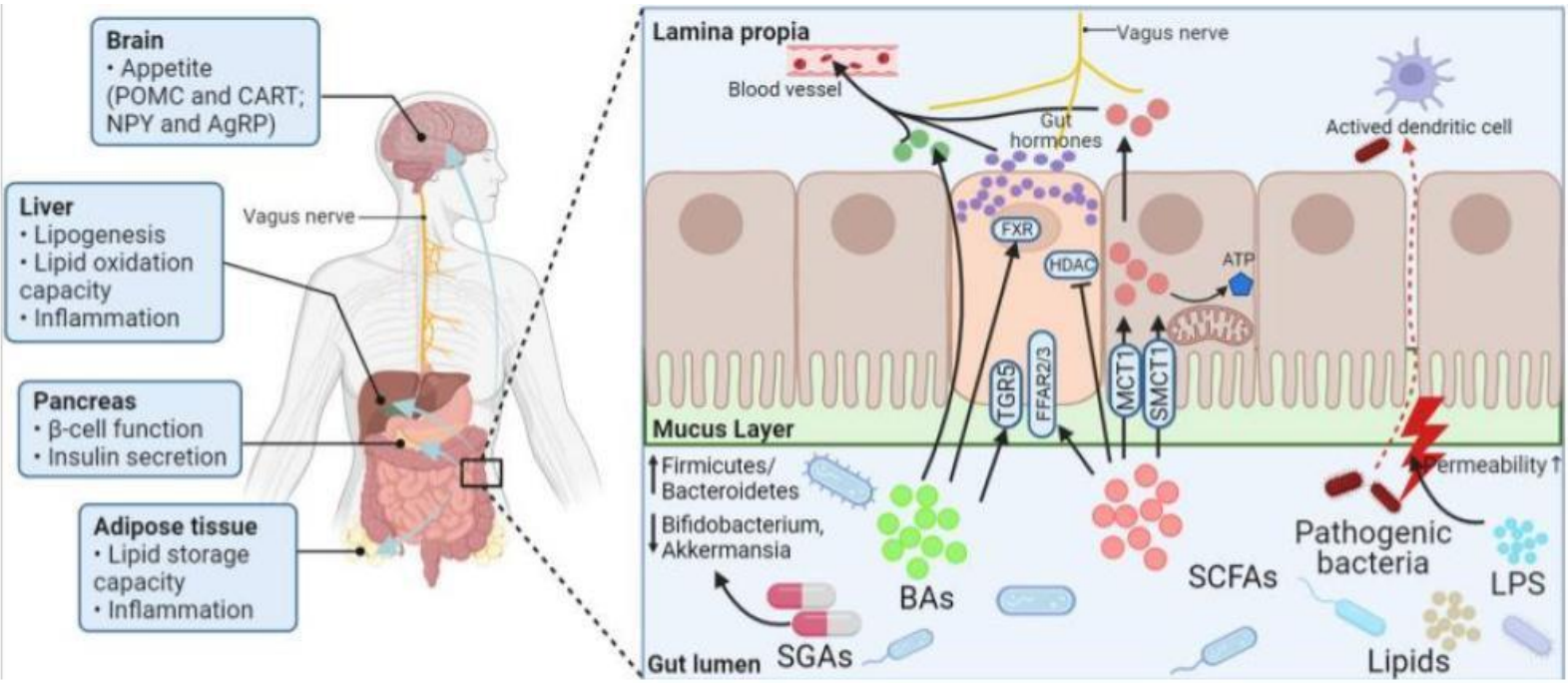


Figure 2. Schematic presentation of the potential mechanism of microbiome-mediated alterations of lipid metabolism secondary to SGA treatment. SGAs shift microbial composition toward a more obesogenic composition, which subsequently changes lipid metabolism, lipopolysaccharides (LPS), SCFAs, and BAs. These metabolites interact with a number of transmembrane and intracellular receptors of intestinal cells. Central regulation can be impacted by direct action on vagal afferents as well as circulation of gut-derived metabolites which then cross the blood brain barrier. These gut-derived metabolites also impact a variety of energy-related and immunometabolic processes in peripheral tissues, such as the liver, pancreas, and adipose. Lastly, changes in gut composition and available metabolites (SCFAs) can alter gut-barrier junction, which can then lead to increased interactions of the immune system with LPS.

Figure key: POMC, proopiomelanocortin; CART, cocaine-and-amphetamine-regulated transcript; NPY, neuropeptide Y; AgRP; agouti-related peptide; FXR, farnesoid X receptor; HDAC, histone deacetylase; ATP; adenosine triphosphate; TGR5, Takeda G protein-coupled receptor 5; FFAR2/3, free fatty acid receptors 2/3; MCT1, proton-coupled monocarboxylate transporter 1; SMCT1, sodium-coupled monocarboxylate transporters 1; BAs, bile acids; SCFAs, short-chain fatty acids; LPS, lipopolysaccharides; SGAs, second generation antipsychotics. Figure extracted from Chen et al., 2023.

1.4 Sex Differences in the Context of Antipsychotics

Men and women have a similar lifetime prevalence of psychosis; however, men have a single age of onset peak between 21 and 25 years while women have two peak ages of onset, one between 25 and 30 years old and another after 45 years of age (Ochoa et al., 2012). Thus, the age of onset may come with different health comorbidities for men and women. There is some evidence that women are at higher risk of weight gain associated with antipsychotic use, and this weight gain is more psychologically distressing for women than men (Seeman, 2010). There may also be differences between different antipsychotics on their relative propensity for causing weight gain in men and women (Hoekstra et al., 2021), such that agents considered low risk for weight gain in one biological sex hold a different risk in the other. Lastly, a systematic review by Sankaranarayanan and colleagues (2021) concluded that while it is still unclear if disordered eating is part of psychotic-spectrum disorder psychopathology, secondary to medication, or an independent comorbidity, the association between antipsychotic use and disordered eating was greater in women.

Epidemiological studies indicate an increased association between antipsychotic use and cardiovascular disease for women, but not for men (Lai et al., 2020). This may be due to several factors. First, the overall incidence of cardiovascular disease is higher for men than it is for women (Bots et al., 2017). Second, the weight threshold before metabolic disturbances begin to set in is higher for women than it is for men, but once diabetes occurs, there is evidence that women incur more cardiovascular risk than men (de Ritter et al., 2020). Third, fat distribution patterns differ between women and men. Premenopausal women usually develop peripheral obesity with accumulation of subcutaneous fat in the gluteal area, whereas men and postmenopausal women tend toward central (abdominal) obesity (Karastergiou et al., 2012). Central obesity is associated with increased cardiovascular mortality and the development of type 2 diabetes (Frank et al., 2019). Visceral fat cells differ from peripheral fat cells in their lipolytic activity and in their response to insulin, adrenergic signalling, and density of sex hormone receptors (Frank et al., 2019).

Preclinical research also suggests a role for sex differences in antipsychotic-induced weight gain and hyperphagia. In their seminal work, Davey et al. (2012) demonstrated that olanzapine-treated female Sprague Dawley rats had increases in body weight and markers of inflammation (IL-8,

IL-1β) that was not seen in male rats, while both sexes had increases in adiposity and altered microbiota relative to controls. Gonadectomy has been shown to shift the microbiota of male mice toward a composition similar to females (Yurkovetskiy et al., 2013; Org et al., 2016). Furthermore, gonadectomized mice treated with testosterone maintained a similar gut microbiota composition to gonad-intact males (Org et al., 2016). Furthermore, a narrative review by Castellani and colleagues (2019) found that while there are several preclinical studies demonstrating that ovariectomy prevents antipsychotic-induced weight gain and hyperphagia, and peripheral administration of estradiol rescues these effects, research on the interplay of sex differences, the microbiome, and antipsychotics in humans was limited. Further research on interindividual differences among and between the sexes, and their interactions with the microbiome, may provide insight into the variability among drug response and severity of side effects.

1.5 Therapeutic translation

1.5.1 Clinical impact of adjunctive microbiome-targeted interventions in schizophrenia

While the systemic review by Chen and colleagues (2023) found that there were many animal studies examining the impact of probiotics and/or prebiotics with antipsychotics demonstrating proof of concept, they also concluded that clinical trials are necessary in order to investigate the therapeutic potential of microbiome-based interventions for improving SGA-related side effects, and that the current state of human literature does not yet indicate (1) what components of the gut microbiota and host metabolism are associated with schizophrenia (2) if microbiota changes observed in schizophrenia treated with SGAs are secondary to SGA treatment (3) if changes of the microbiota affect the clinical efficacy of SGAs. While these preclinical studies provide proof of concept, potential therapeutic targets and mechanistic investigations that are not possible in humans (i.e., vagotomy, observing transcriptional changes in brain tissue), a critical component of bench-to-bedside research is the eventual translation of research.

Nikolova and colleagues (2021) conducted a meta-analysis of 34 case-control studies examining the microbiota of psychiatric patients treated with antipsychotics (n=1519) and found there was not strong evidence for differences in the relative abundance of gut bacteria for specific disorders, but there was transdiagnostic evidence of reduced anti-inflammatory bacteria (Faecalibacterium and Coproccus) and increased pro-inflammatory bacteria (Eggerthella). A

different meta-analysis by Minichino and colleagues (2021) examined randomized double-blind clinical trials with adjunctive microbiota-targeted interventions and their impact on schizophrenia. Although there were 28 interventions, only 3 examined prebiotics or probiotics (Minichino et al., 2021). Of the 21 adjunctive antibiotic interventions (azithromycin, trimethoprim, minocycline, D-cycloserine), none were superior to placebo in terms of changes in positive (sensory hallucinations, delusions, motor tics), negative (decreases in affect, energy, sociality) or cognitive symptoms (Minichino et al., 2021).

The authors did find limited, yet positive results for the adjunctive interventions with sodium benzoate, a food preservative and antimicrobial (Lane et al., 2013; Lin et al., 2018), artemether, an antimicrobial with activity against *Toxoplasma gondii* (a protozoan that may be a risk factor for schizophrenia) (Wang et al., 2014), a probiotic with vitamin D (Ghaderi et al., 2019), and the prebiotic B-GOS (Kao et al., 2019). Both sodium benzoate studies improved positive and negative symptoms by the study completion at 6 weeks, and the study by Lane and colleagues (2013) found improvements in total symptoms and cognitive functioning, while the study by Lin and colleagues (2018) found no changes in total symptoms or cognitive functioning. The study investigating artemether by Wang and colleagues (2014) only found improvements in negative symptoms at the study end point of 8 weeks. The study by Kao and colleagues (2019) assessed cognitive and executive function and found improvements by the 24-week end point, but they did not assess positive or negative symptoms. The study by Ghaderi and colleagues (2018) included a probiotic with *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri*, and *Lactobacillus fermentum* administered once daily at a dose of 8×10^9 CFU daily and 50,000 IU vitamin D every two weeks over for a period of 12 weeks, and they observed improvements in negative symptoms, but no changes in positive symptoms.

These meta-analyses provide several insights into the current state and future directions of microbiome-targeted interventions for schizophrenia. Minichino and colleagues (2021) note that many of these antibiotics are known to deplete Bifidobacterium and butyrate-producing species, thus, one might expect a worsening of symptoms given the association of low levels of butyrate in patients with schizophrenia, but no correlations were observed. Relatedly, the antimicrobial sodium benzoate also selects against butyrate-producing and anti-inflammatory gut bacteria, but

these interventions were among the few in the study to find positive adjunctive effects (Minichino et al., 2021). The authors attribute this to the central effects of benzoate, which can cross the blood-brain barrier and inhibit D-amino acid oxidase (DA), which can then enhance the activity of N-methyl-d-aspartate (NMDA) receptors, which are thought to have decreased activity in schizophrenia (Minichino et al., 2021). The authors also attribute the positive effects of the study by Gadheri and colleagues (2018) to the effects of vitamin D, rather than microbially-mediated mechanisms (Minichino et al., 2021). The study by Kao and colleagues (2019) was the only prebiotic intervention available at the time of the meta-analysis, thus, further clinical interventions examining prebiotics may be warranted. It will be prudent to examine the other dimensions of schizophrenia, such as positive and negative symptoms, as these were not assessed in the study by Kao and colleagues (2019). Furthermore, there were few probiotic and prebiotic interventions in general, thus more studies are needed to conclusively determine if they do or do not have therapeutic potential. Lastly, the strong transdiagnostic associations of reduced anti-inflammatory and increased pro-inflammatory bacteria in the meta-analysis by Nikolova and colleagues (2021), juxtaposed against the robust null findings of adjunctive antibiotic treatment (which also shift the microbiota toward a more pro-inflammatory composition) by Minichino and colleagues (2021) suggests that the associations between schizophrenia psychopathology and pro-inflammatory microbial compositions may be correlative rather than causal.

1.5.2 Clinical impact of adjunctive microbiome-targeted interventions on SGA-associated weight gain

The relationship between the microbiome and obesity also needs further investigation to clarify directionality and causality. Although meta-analyses on the associations between obesity and metabolic disorders with microbiota observe similar trends in alteration to the shifts seen with SGAs (Xu et al., 2022), and faecal microbiome transplant (FMT) studies in animals have shown that transplanted microbiomes can cause changes in body composition and metabolism (Boscaini et al., 2022), FMT studies in humans do not lead to weight loss or changes in body composition (Boscaini et al., 2022). Thus, while preclinical studies highlight potential mechanisms and candidate therapeutic targets, well-designed clinical trials will be essential for evaluating the therapeutic potential of microbiome-targeted approaches in alleviating SGA-related side effects.

In the previously discussed meta-analysis by Minichino and colleagues (2021), no studies examined changes in appetite, though one included study did examine weight changes. Levkovitz and colleagues (2010) examined the effects of concomitant minocycline with antipsychotic over the course of six months, and at the study endpoint, participants being treated with minocycline gained significantly less weight than controls (2.08 kg vs 10.7 kg, $p< 0.05$), and only 40% in the minocycline group gained weight vs 100% of the control group. Thus, while the authors did not find meta-analytic evidence for the efficacy of minocycline in improving psychological symptoms of schizophrenia (Minichino et al., 2021), currently there is not sufficient clinical data to rule out microbiome-targeted interventions to attenuate antipsychotic-induced weight gain and hyperphagia. While antibiotics may not be a sustainable long-term strategy for managing the side effect profile of antipsychotics, manipulation of the microbiome may warrant further investigation.

Several recent clinical trials indicate a therapeutic potential for probiotic and prebiotic interventions in the attenuation of antipsychotic-induced weight gain and hyperphagia. A 2 x 2 factorial design of patients (n=113) treated with antipsychotics who had gained more than 10% of their pre-drug weight were assigned to either: (1) probiotic with prebiotic (2) probiotic alone (3) prebiotic, or (4) placebo for 12 weeks found that probiotic and prebiotic together, but not alone, attenuated weight gain (Huang et al., 2022a). Curiously, although the protocol states that positive and negative symptoms were measured, and the authors reference other studies examining changes in schizophrenia symptomatology, they do not report the baseline psychological measures or their changes in the main manuscript or supplemental figures (Huang et al., 2022a); this is also the case for measures of ghrelin, leptin, GLP-1, PYY, and adiponectin. Nevertheless, the combined probiotic and prebiotic group had a mean body mass index (BMI) decrease of 0.89 (95% CI: -1.29 to -0.48), and the placebo group had a mean BMI increase of 1.03 (95% CI: 0.36 to 1.70), and probiotic with prebiotic was superior to dietary fibre alone (p=0.044), probiotic alone (p=0.049), and placebo (p<0.001) (Huang et al., 2022a). This same research group conducted another clinical trial with two separate studies of patients with first-episode psychosis, one of 76 drug-naive patients assigned to probiotic and olanzapine or olanzapine monotherapy for 12 weeks, and the second study with 58 drug-naive patients assigned to probiotic with prebiotic and olanzapine or olanzapine monotherapy for 12 weeks (Huang et al., 2022b). In their first study, there was no weight difference between the two groups

for weight, but there was for insulin resistance index (IRI) (-0.65 [95% CI: -1.10 to -0.20]); however, there was significant attenuation when comparing weight gain (kg) (-3.45 [95% CI: -5.91 to -1.00]) and IRI (-0.95 [95% CI: -1.77 to -0.14]) in the second study (Huang et al., 2022b). Both studies used a commercially available probiotic in China (Bifico) that contained *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* at concentrations of $\geq 5.0 \times 10^7$ colony-forming units (CFU)/g, and the prebiotic used in both studies was commercially available, containing a mix of 20g traditional Chinese medicinal food plants and dietary fibres (Huang et al., 2022a; Huang et al., 2022b).

Overall, these studies highlight the therapeutic potential of synergistic probiotic and prebiotic therapies for reducing antipsychotic-induced weight gain, and prophylactic attenuation of weight if administered with antipsychotics during first-episode psychosis. Longitudinal studies should assess if successful microbiome-targeted probiotic interventions improve antipsychotic adherence, as well as long-term health outcomes (i.e., reduced onset of type II diabetes, lower incidence of severe cardiovascular events). Additionally, several meta-analyses, systematic and narrative reviews concluded that there is a lack of dose-response studies in microbiome-targeted interventions in humans, and such studies will be essential to furthering clinical investigations of the microbiome and their potential translation to standardised clinical practice (Shane et al., 2010; Reis et al., 2018; Pot and Vandenplas; 2021; Ng et al., 2023).

Lastly, it is important to address the gendered nature of the greater psychological burden of antipsychotics on women that was previously described (Seeman, 2010). There are numerous studies examining changes in body image and self-esteem in schizophrenia populations using the validated Body Weight, Image, and Self-Esteem Evaluation (B-WISE) questionnaire (Awad and Voruganti, 2004; De Hert et al., 2006; Yarborough et al., 2016). However, none of the aforementioned clinical studies investigating microbiome-targeted interventions examined changes in body image and self-esteem as an outcome. Thus, the potential impact of microbiome-targeted interventions on mental health outcomes is an important and unaddressed research question. Path analysis, an extension of mathematical regression used in research to assess causal chains in psychological (as well as biological) outcomes (Streiner, 2005; Wang et al., 2020; Perey and Koenigstorfer, 2020), will be a particularly useful tool in elucidating how microbiome-targeted interventions improve mental health in the context of antipsychotics.

1.5.3 Rationale for further preclinical microbiome-targeted interventions

The literature discussed thus far suggests that targeting the microbiome may serve as a novel and low-invasive strategy to improve the side effect profile of antipsychotics. The gut microbiome and its metabolites can affect satiety and metabolic disorders, potentially via alterations of gut peptides and/or their receptors (Leeuwendaal et al, 2021). Our group has identified the probiotic *Bifidobacterium longum* APC1472, hereafter referred to as APC1472, as a strain that can impact ghrelin receptor signalling (Torres-Fuentes et al., 2019). A preclinical investigation showed that APC1472 improved cardiometabolic outcomes and displayed anti-obesity effects in mice, and showed improvements in fasting blood glucose, ghrelin, and morning cortisol in humans (Schellekens et al., 2021). The aforementioned study by Zhang and colleagues (2014) showed that the hyperphagic, metabolic, and weight-inducing effects of olanzapine could be reversed with central administration of a ghrelin antagonist. Thus, APC1472, through its modulation of ghrelinergic signalling via the microbiome gut brain axis, could be a viable approach to reducing antipsychotic-related weight gain and metabolic dysfunction.

Prebiotics are another potential therapeutic agent for the modulation of the brain-gut-microbiota axis. One study in humans showed that women taking atypical antipsychotics, such as olanzapine, had reduced microbial diversity, and resistant starch fibre led to an increase of starch-degrading *Actinobacteria* phyla (Flowers et al, 2019). Xanthohumol, a phenolic compound found in hops, has been shown in a murine model to be protective of microbial diversity against stress (Donoso et al, 2020). Another murine model found xanthohumol to be protective against the metabolic dysfunction and weight-inducing effects of a high-fat diet (Paraiso et al., 2021). It is proposed to exert these effects through *Eubacterium* metabolising xanthohumol into a variety of estrogen-like compounds (Paraiso et al., 2019). Xanthohumol may thus serve as a therapeutic tool against the negative effects of antipsychotics on the gut microbiome, potentially protecting against the cardiometabolic and weight-inducing side effects of drugs like olanzapine.

1.6 Metabolomic Strategies for Disease Characterization and Biomarker Discovery

1.6.1 Introduction to metabolomics

Omics is an umbrella term for different methods that broadly characterise potential molecules and/or features of a biological sample. This can include transcriptomics for RNA, proteomics for proteins, metabolomics for metabolites, shotgun metagenomics for microbiota, and many other techniques (see Figure 3, Yu et al. 2022). Metabolomic approaches are considered the most "downstream" in terms of biological effects, and thus capture a wide variety of metabolites including molecules such as lipids, hormones, amino acids, small peptides, and microbial metabolites, making the metabolome the ideal phenotype to characterise gene x environment interactions (Yu et al., 2022).

In the case of metabolomics, there are two broad categories of application: discovery and validation workflows. Discovery metabolomics are used to identify candidate/putative biomarkers and are useful for hypothesis generation. Using profiling techniques, such as liquid chromatography-mass spectrometry (LC-MS/MS), thousands of metabolites are screened for differential expression changes between experimental groups. Identifications are putatively assigned to these metabolic features using a combination of software tools (such as Progensis and Metaboanalyst), human rater/identification, and molecule libraries, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2006). Targeted validation in metabolomics refers to the confirmation of the presence of certain biomarkers by targeted LC-MS using labelled standards loaded into samples, which then allows for absolute quantification and verification. While many studies examine changes in key biomarkers (i.e., ghrelin, corticosterone), there are biological pathways affected by olanzapine that cannot be captured via simple assays. Furthermore, the targeted nature of ELISAs and other bench-side approaches inherently cannot provide insight into undiscovered mechanisms. Thus, a metabolomic investigation could reveal both pathophysiology and therapeutic targets in the context of antipsychotic-induced obesity and hyperphagia.

Metabolomics, along with other omics approaches, have emerged as critical tools for advancing systems biology. Broadly, systems biology describes how multiple biological systems within an

organism interact with each other; this “deep phenotyping” has broad health and research implications, such as precision medicine (Baharum and Azizan, 2018; Kaddurah-Daouk et al., 2008, 2015; Nielsen, 2017). One potential approach is multiomics analysis, which allows for correlations between two or more omics datasets from different tissues and systems; this is particularly useful in characterising the vast crosstalk that occurs between biological systems like the brain gut microbiome axis (McHardy et al., 2013; Xu and Yang, 2021).

Several examples of multiomics approaches have demonstrated this method’s research utility. Frontier investigations on cardiometabolic disease have employed intestinal microbiome sequencing alongside blood sera and urine metabolomics, revealing a variety of potential biomarkers related to disease progress, from prodromal stages to advanced clinical stages, highlighting the use of such approaches in both diagnostics and disease monitoring (Fromentin et al., 2022). Metabolomics approaches may also be employed to assess the causal impact of microbiome-based interventions, as evidenced by a seminal work by Boehme and colleagues (2021) which identified a multitude of age-associated changes in the hippocampal metabolome, and further demonstrated that microbiome transplantation from young to old mice could partially recover the hippocampal metabolome of old mice, in addition to attenuating age-related declines in memory performance.

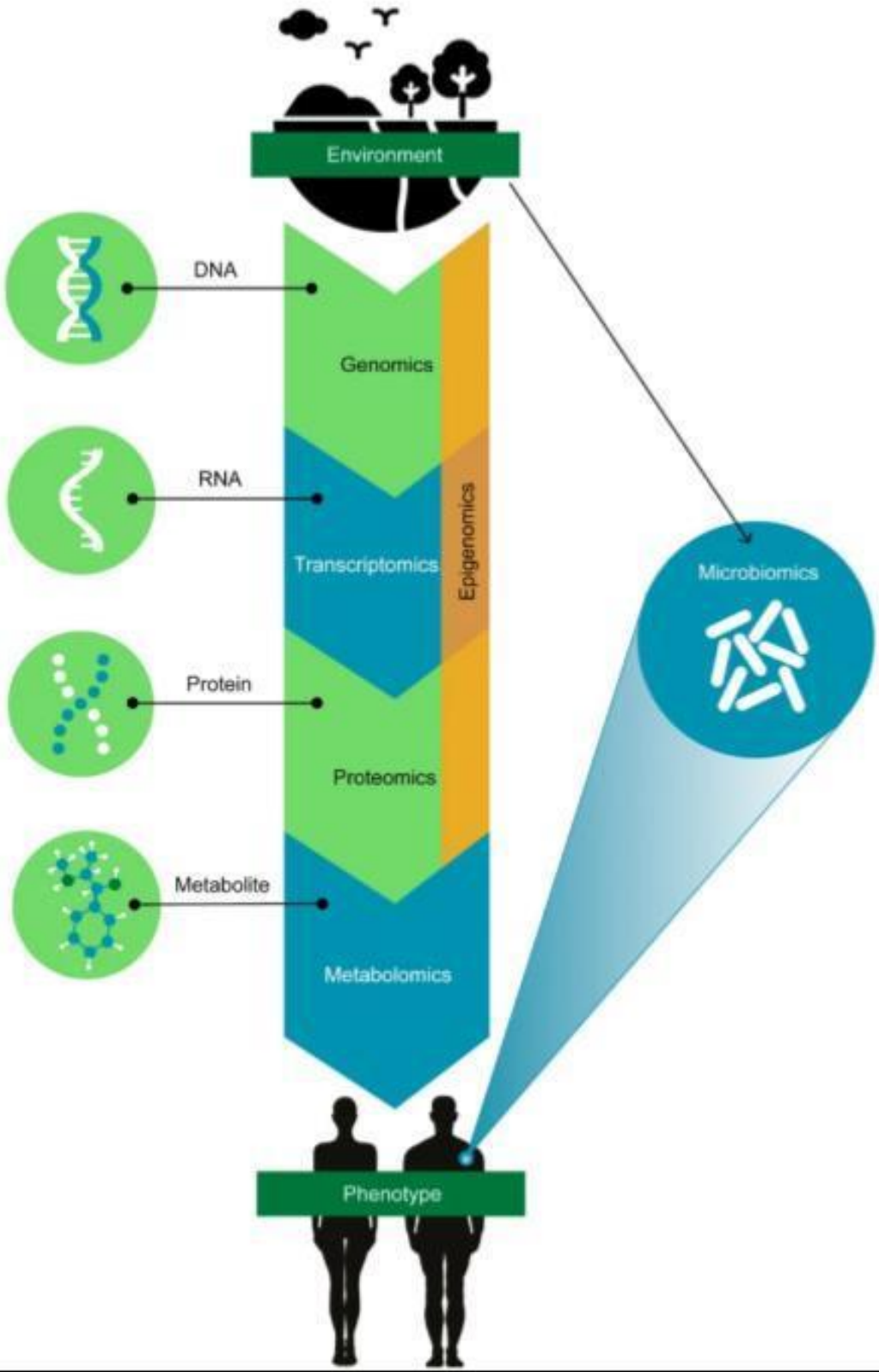


Figure 3. Connections between different types and levels of omics. Although the present study only has a metabolomics component, future investigations characterizing any disease phenotype would benefit from different -omics approaches. Figure extracted from Yu et al., 2022.

1.6.2 Omics, psychopathology, and metabolism

Currently, psychopathology is assessed through psychological instruments and surveys, and the potential of a biological diagnostic approach continues to elude scientists. Several recent studies highlight the potential of omics approaches in characterising biological contributors and peripheral signatures of psychopathology. A multiomic study by Zhao and colleagues (2022) comparing healthy controls to patients with major depressive disorder found that the abundance of certain gut microbiota was correlated with altered metabolomes (i.e., phosphoethanolamine) and inflammatory markers (IL-1β), which were also associated with clinical survey scores for depression and reduced grey matter volume of the inferior frontal gyrus. Other studies have found a relationship between the severity of psychotic symptoms and markers of inflammation and lipid metabolism (Goldsmith et al., 2021, Dickens et al., 2021, Joaquim et al., 2020). In a 5-year longitudinal study of healthy controls and individuals with clinical high risk for psychosis (CHR), it was shown that the lipidome of CHR subjects was different from controls prior to psychosis, while controlling for age, sex, and BMI (Dickens et al., 2021). Furthermore, a machine-learning approach revealed that the development of psychosis by CHR subjects could be predicted by a decrease in ether phospholipids–which have also been implicated as a transdiagnostic biomarker of mental illness (Dickens et al., 2021). These studies demonstrate that the wide-capture approach of omics methods can highlight potential biological mechanisms underlying psychopathology, and more importantly, they can help identify patients who are at elevated risk for psychiatric disorders to facilitate early intervention.

In addition to increased diagnostic efficiency, omics approaches can help tailor physician treatment and medication choice. In a study of patients experiencing first-episode psychosis, either related to schizophrenia or bipolar disorder, better recovery outcomes (as indicated by their Positive and Negative Syndrome Scale [PANNS] scores) were associated with a particular metabolomic phenotype, and putative annotation of metabolites suggested that carnitines and phosphatidylcholines may be associated with more severe psychotic symptoms (Joaquim et al., 2020). Similarly, de Almeida and colleagues (2020) found that participants taking risperidone, olanzapine, or quetiapine had many similarly altered lipidomes, though risperidone affected certain lipids that were not impacted by quetiapine or olanzapine. They also found that particular metabolomic profiles could be used to distinguish good and poor responders to antipsychotics

(de Almeida et al., 2020). This is a potent example of the potential of omics approaches in enhancing precision medicine, which could increase patient satisfaction, health and treatment compliance–all of which are critical areas needing improvement in the area of antipsychotics.

1.6.3 Omics for cardiometabolic diseases

In addition to its utility for psychopathology, omics approaches can help progress traditional biomedical sciences as well. Lind and colleagues (2022) published a large-scale human metabolomic study (more than 11,000 participants, pooled from several datasets) employing both discovery and validation techniques, which found 15 molecules that were correlated (via multiple linear regressions) with at least one of the symptoms of metabolic disease (blood pressure, waist circumference, glucose dysfunction, HDL and TG levels). These 15 validated biomarkers all passed FDR after adjustment for BMI, and they were all related to insulin sensitivity. One molecule, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine, was associated with a risk of cardiac event, which was confirmed by longitudinal follow-up of cohort members. Importantly, patients with baseline CVD at baseline were excluded from these analyses, highlighting 1-palmitoyl-2-oleoyl-phosphatidylethanolamine as a candidate biomarker for risk of severe cardiac events in the context of metabolic syndrome. It is worth highlighting that the data in the Lind et al. (2022) study came from five cohorts of metabolic syndrome studies, yielding an N >11,000, and nearly 800 validated metabolites. Though it has limitations in that the data come solely from Swedish populations, this study highlights the statistical power gained through open science. Thus, for less common conditions like schizophrenia, collated datasets generated through open science practices may be important for the advancement of screening metabolomic profiles that indicate a high risk for cardiac events.

1.6.4 Methodological and statistical considerations for metabolomics data

Innovation in omics approaches and mass spectrometry are fast-paced and consequently are unstandardized and are not exempt from the cross-disciplinary issues of type I error, type II error, effect sizes, and sample sizes. These techniques also present unique statistical challenges, such as: (a) high dimensionality with more measures/variables relative to samples, requiring correction for False Discovery Rate (FDR) (b) by definition, discovery/untargeted metabolomic phenotyping does not lend itself well to *a-priori* estimates of effect sizes, and (c) trying to

account for dimensionality and conservative estimates of effect sizes leads to very high requirements for sample sizes (Billoir et al. 2015; Tolstikov et al., 2020).

Additionally, there is a growing school of thought, originally raised by Gelman and Carlin (2014) that errors related to effect size (magnitude of the effect, type M, and the direction of the effect/sign, type S) would more greatly aid the robustness of science than the current practice of focusing on statistical significance (alpha). The 2014 Gelman and Carlin article has been cited over 1000 times, and their argument is at the heart of many calls for a shift toward prioritising effect size over significance (Kruschke and Liddell, 2018; Gillies et al., 2020; Ellis, 2022). In the context of omics, larger effect sizes aid the identification of putative biomarkers (Kirpich et al., 2018); this is a challenge for early disease detection, as prior to disease onset or health events (i.e., stroke or heart attack), the detection of relevant biomarker signals relative to statistical noise can be unfavourable (Ning and Lo, 2010; McDermott et al., 2013).

Although an in-depth discussion regarding statistical approaches in omics is beyond the scope of this thesis, the aforementioned literature demonstrates ongoing scientific debate on best statistical approaches/practices in the context of metabolomics (Ning and Lo, 2010; McDermott et al., 2013; Billoir et al. 2015; Tolstikov et al., 2020). Thus, the metabolomic statistical approaches herein should not be seen as absolute. Instead, it should be thought of as the first steps in a sequential process of metabolomic screening and characterization of the experimental model (olanzapine-induced obesity). These results can guide follow-up validation studies and further hypotheses on both intervention targets and mechanisms of action.

1.7 Study Rationale, Aims and Objectives

Patients using antipsychotic medication report that weight gain is the most distressing treatment side effect (Achytes et al., 2018), which can lead to poor medication adherence, symptom management, or relapse of psychosis. Thus, addressing antipsychotic-induced weight gain and cardiometabolic dysfunction could lead to improved physical and mental health of patients relying on antipsychotic medications. Additionally, to our knowledge, there are no investigations characterising the metabolomic profile of olanzapine-induced obesity and hyperphagia in female rats. Thus, this thesis is useful for informing future targeted and validation metabolomics

investigations of olanzapine-induced obesity and hyperphagia. As APC1472 has displayed anti-obesity effects (Schellekens et al., 2021), possibly via ghrellenergic signalling (Torres-Fuentes et al., 2019), it may be a viable therapeutic approach in attenuating the side effects of olanzapine. Furthermore, xanthohumol is protective against the effects of a high-fat diet (Paraiso et al., 2021). Probiotics and prebiotics can be synergistically protective, as indicated by the clinical studies by Huang and colleagues (2022a, 2022b). Therefore, I hypothesized that combining olanzapine treatment with APC1472 or xanthohumol would attenuate olanzapine-induced obesity and metabolic dysfunction, and the combination of APC1472 and xanthohumol would attenuate these side effects better than either adjunctive treatment alone.

The objectives of the *In vivo* experiment are as follows:

- Investigate if co-administration of probiotic with olanzapine **attenuates antipsychotic-induced weight gain**;
- Investigate if co-administration of probiotic with olanzapine is associated with changes in **genes related to central regulation of appetite**;
- Investigate if co-administration of probiotic with olanzapine is associated with changes in **cardiometabolic biomarkers and hepatic genes**;
- Investigate if co-administration of probiotic with olanzapine is associated with changes in **anxiety-like behaviour**;
- Investigate if co-administration of olanzapine with **probiotic and prebiotic or prebiotic alone** is associated with changes in the aforementioned outcomes (weight gain, central regulation of appetite, cardiometabolic biomarkers, hepatic gene expression, anxiety-like behaviour);
- Compare the olanzapine treatment group with controls across the aforementioned outcomes to **infer potential mechanisms of olanzapine**.

The objectives of the metabolomic experiment are as follows:

- **Identify differentially expressed putative metabolites** for subsequent validation studies;
- Infer the potential **biological role of putatively identified metabolites** in the context of olanzapine-induced obesity and cardiometabolic disease.

2. Methods

2.1 *In Vivo* Methods

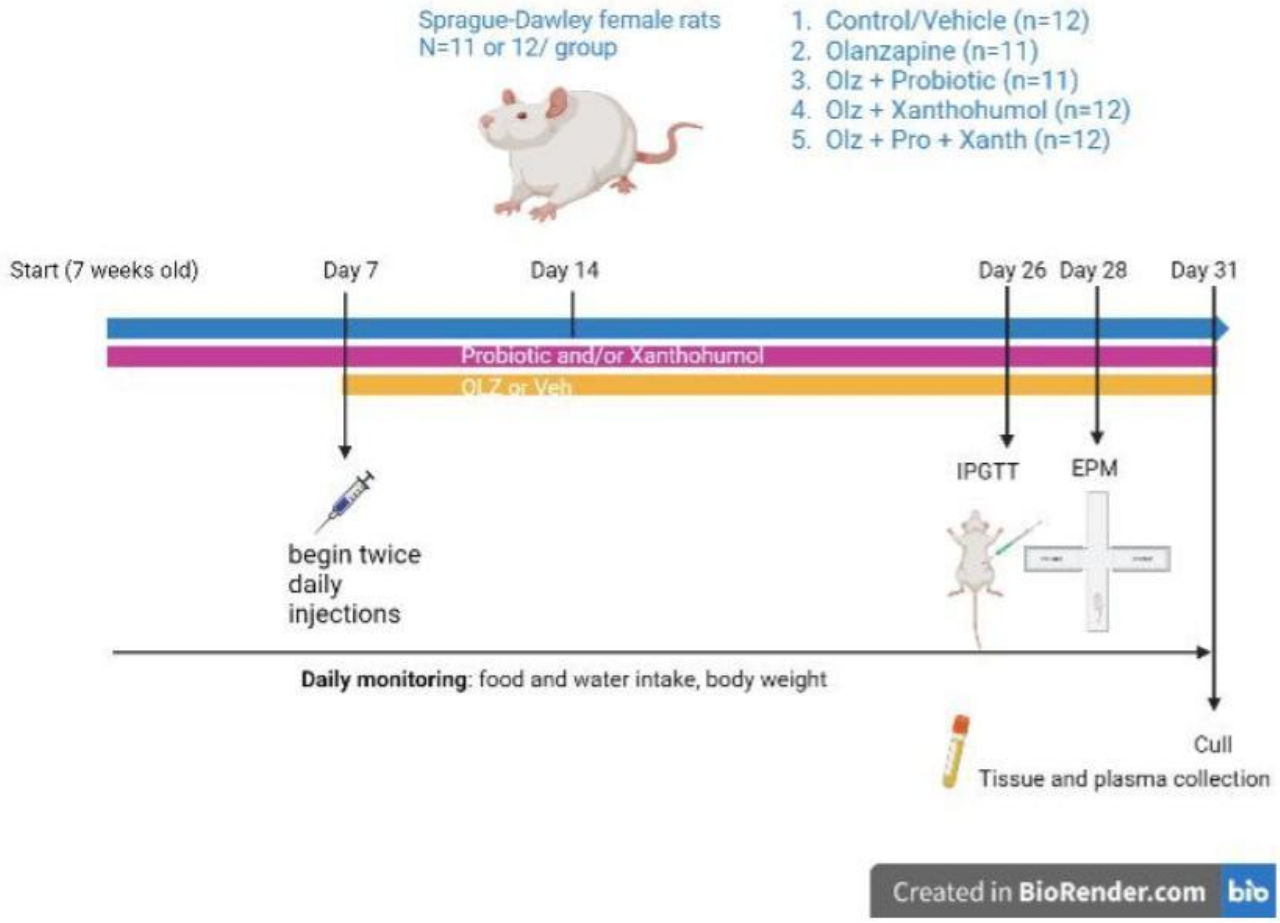


Figure 4. Descriptive figure of experimental design. 60 female Sprague-Dawley rats were split into 5 groups of 12. Xanthohumol was delivered via rat chow (0.015%) and probiotic was mixed into nightly drinking water ($7.5x10^9$ CFU/ml). Olanzapine was administered via intraperitoneal injection twice daily (2mg/kg body weight).Two rats were euthanized per animal welfare guidelines due to declining health, which were within acceptable attrition rates.

2.1.1 Conceptual figure of experiment

2.1.2 Animals

8-week-old, female Sprague-Dawley rats weighing approximately 200g were used for this study (Envigo) (n=58 with n=11-12 per group). Female rats were used as they have been shown to better reflect the elevated weight gain caused by atypical antipsychotics (Davey et al., 2012). There were five arms to the study: control (saline) (n=12), olanzapine (n=12), olanzapine + dietary xanthohumol (n=11), olanzapine + dietary probiotic (n=11) and olanzapine + dietary xanthohumol + dietary probiotic (n=12). Animals were housed four rats per cage in standard holding cages with free access to food and water in the animal care facility of University College Cork. As rats were grouped by treatment, there was no risk of coprophagia across treatments. Rats were acclimated to dietary treatment for 7 days by being given experimental chow during the evening. For subsequent days of the study, groups were fed only experimental chow. For rats receiving xanthohumol, standardised chow containing 0.015% xanthohumol was used. Animals were maintained in a standard light/ dark cycle. All cages are environmentally enriched throughout the entire duration of the project. All experiments were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) of University College Cork. All researchers had individual authorisation regulated by the Health Products Regulatory Authority (HPRA). The project authorisation number is AE19130/P145.

2.1.3 Olanzapine administration

Olanzapine injections of 2 mg/kg body weight (BW) were delivered twice daily, as per the protocol described in Davey and colleagues (2013). Drug solutions were prepared daily and administered intraperitoneally twice daily, for 21 days at approximately 9.00 AM and 4.00 PM. Control animals were injected twice daily with vehicle (saline).

2.1.4 Preparation and administration of probiotic

The original aim of the study was to examine the effects of *Bifidobacterium longum* APC1472 However, after the *in vivo* study had concluded, it was discovered that there may have been several bacterial strains used. The intent was to grow APC1472 as previously described in Torres-Fuentes and colleagues (2019) to create a probiotic solution with an average concentration of 7.5×10^9 CFU/ml. This was prepared by diluting ampules of frozen inocula (approximately 1.25×10^{10} CFU/ml) into the rat's water bottles, which were changed daily to

ensure maximum bioactivity and per HPRA regulations. Rats in the probiotic receiving groups were given free access to the probiotic solution as their sole source of water from evening until the morning (approximately 6:00 PM – 7:00 AM). Normal drinking water was given outside of these hours. However, during the course of the project, it was discovered that there may have been several bacterial strains used. The implications of this quality control deviation are addressed in the discussion section of this thesis.

2.1.5 Administration of prebiotic and diet

Standardized rat chow and experimental rat chow that was 0.015% xanthohumol by mass were purchased from ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany), the same vendor for the study by Donoso and colleagues (2020). Consumption of both normal and treatment diets were measured by comparing the mass of morning chow to the mass of chow recorded from the previous evening.

2.1.6 Weight change

Rat body weight was measured in the mornings. Changes in body weight are presented as the mean % change from baseline +/- the SEM.

2.1.7 Intraperitoneal glucose tolerance test (IPGTT)

On day 26 of the study, animals underwent an intraperitoneal glucose tolerance test (IPGTT) as described by Pilon and colleagues (2018). Animals were fasted from 6.00pm the previous night before testing (14h). A 20% glucose solution was prepared using D-glucose (Sigma-Aldrich) added to sterile saline and passed through a 0.2 μm filter into a sterile Falcon tube per the protocol established by Benedé-Ubieto and colleagues (2020). Rats were intraperitoneally injected with 20% glucose solutions dosed to 2g glucose/ Kg BW. Glucometers (LTC4) were used to collect glucose readings via tail tip bleeds at baseline, 15, 30, 60 and 120 minutes. Glucose levels are presented as the mean concentration (mmol/L) +/- the SEM.

2.1.8 Elevated plus maze (EPM)

The elevated plus maze is a commonly used test to assess levels of anxiety in rodents. On day 28 of the study, animals were evaluated for anxiety-like behaviour using the EPM. The test was performed as previously described by Cryan and colleagues (2004). Animals were habituated to the behavioural room 1 hour before testing. The maze consisted of two open arms and two closed arms extending from a common central platform. Animal behaviour was filmed, and behaviour was measured for 5 minutes from when the researcher exited the EPM area. Data are presented as the mean time spent in each arm (seconds) +/- the SEM.

2.1.9 Tissue and plasma collection

Rats were fasted overnight (16 h) before being sacrificed on the 31st day of the study via rapid decapitation. Trunk blood was collected in sterile ethylenediaminetetraacetic acid (EDTA) coated 100mL tubes. These were centrifuged at 3500 rpm for 15 minutes at 4^{o} C and were stored at – 80^{o} C for further analysis. For analysis of active ghrelin, 50 uL of plasma sample was acidified per Schellekens et al., 2021. The brain was quickly excised, dissected into brain regions, and placed in PCR-grade tubes. Tissues (liver frontal lobe, brown, gonadal, mesenteric, and subcutaneous fat) were collected following the cull, weighed to the nearest 0.0001 g and placed in aluminium foil. Both brain regions and tissues were stored in dry ice following dissection and then transported to the – 80^{o} C freezer. Faecal samples were collected from the distal colon for 16S RNA sequencing at a later time.

2.1.10 Plasma biomarker analysis via enzyme-linked immunosorbent assay (ELISA)

Insulin levels were analysed using the Rat/Mouse Insulin ELISA kit (Millipore, EZRMI-13K), corticosterone levels were assayed using the Corticosterone ELISA kit (Enzo Life Sciences, ADI-900–097), and active ghrelin levels were analysed using the Rat/Mouse Ghrelin (Active) ELISA kit (Millipore, EZRGRA-90K). Analysis and benchwork followed manufacturer instructions, and samples were run in duplicate. As the data were nonparametric, a box and whisker plot was used to visualize the data. Corticosterone and ghrelin are presented as the median concentration in pg/mL, while insulin is presented in ng/mL.

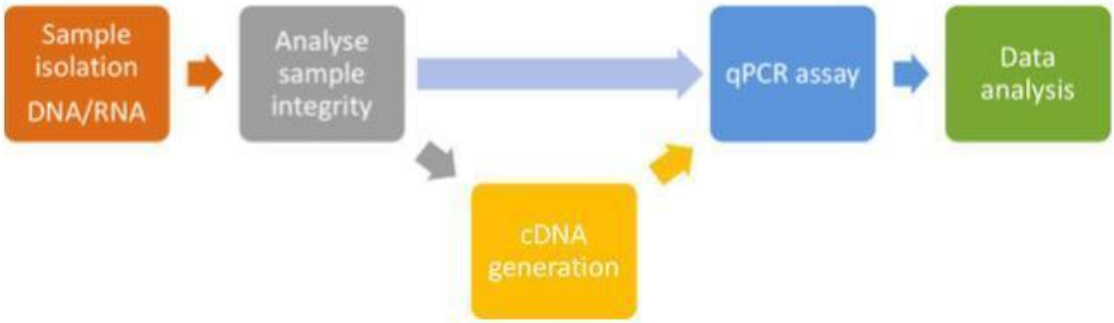


Figure 5. Concept diagram for quantitative polymerase chain reaction (qPCR) workflow. Liver (hepatic) and brain (hypothalamic) tissues were collected during culls and stored at -80°C until the start of RNA isolation and extraction. Integrity of the RNA is checked via spectrophotometer peaks. Primers for genes of interest are then added to generate cDNA from any RNA that is present. This cDNA is then amplified by PCR, and quantified via a fluorescent reaction. Note that the protocol within uses RT-qPCR, but the general workflow and concept is similar (Figure extracted from Adams, 2020).

2.1.11 Gene Expression in hypothalamus and liver

2.1.11.1 RNA extraction and cDNA synthesis

The isolation of RNA from hypothalamic and hepatic tissue was performed according to the protocol of High Pure RNA Tissue/Cell Kit (#12033674001 Roche, Basel, Switzerland), using the kits DNAse1 (Vial 2; white cap), Wash Buffer 1 (Vial 4; black cap), Wash Buffer II (Vial 5; blue cap), and DPBS.

2.1.11.2 Quantification of RNA concentration using NanoDropTM

RNA concentration and quality were quantified and analysed using the NanoDropTM ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, United States). First, RNaseZAPTM #R2020-250ML was used to clean and remove all RNase from workbench surfaces, gloves and pipettes. Then, 2 µL of elution buffer was added in the NanoDropTM spectrophotometer to be used as a blank value. Next, to measure RNA concentration and quality, 2 µL of RNA sample was added to the NanoDrop. Three replicas of each sample were measured, which generated an average concentration.
Finally, NanoDropTM was cleaned with water to switch it off.

2.1.11.3 Quality control check of RNA extraction

Several absorbance ratios are used to indicate sample purity or contaminants. RNA has a peak absorbance of A260, proteins have a peak absorbance of A280, and DNA has a peak absorbance of A230. The accepted ratio for RNA to DNA (A260/A230) are between 2.0-2.2, and the accepted ratio for RNA to protein (A260/A280) is ~2.0 (Desjardins and Conklin 2010; Matlock 2020). Samples were confirmed to be within acceptable absorbance ratios.

2.1.11.4 Reverse transcription of RNA into cDNA

The High-Capacity cDNA kit (Applied Biosystem, Warrington, UK) was used to reverse transcribe extracted hypothalamic and hepatic RNA into cDNA, per the manufacturer's instructions.

2.1.11.5 Quantitative real-time polymerase chain reaction (RT-qPCR)

To measure gene expression in hypothalamic and hepatic tissue, the cDNA from the previous step was used for RT-qPCR analysis via the SensiFAST SYBR Lo-ROX Kit, Bioline (Bioline, BIO-94005, Memphis, USA), per manufacturer's instructions. Primers for genes of interest (supplied by IDT Integrated DNA Technologies, Coralville, Iowa, USA) were added to prepare master mix SensiFAST Lo-ROX. Hypothalamic primers included Npy, Agrp, Lepr, Ghrl, Ghsr, Pomc, Cart, mTOR and BDNF. Hepatic primers included Fatp5, Fas, Acc, Cd68, Srepb1c, Chrebp, Tnf-α, Ppara and mTOR. samples were loaded in triplicate in 384-well plates (Thermo ScientificTM, Waltham, United States), which were then placed in a LightCycler 480 Instrument II (Roche, 05015278001) for amplification. To calculate relative gene expression, Actβ was used for the endogenous control gene. Actβ was confirmed as a stable housekeeping gene, as indicated by null t-tests comparing threshold cycles (Ct) between Actβ in control and olanzapine-treated groups for both hypothalamic ($p = 0.2652$) and hepatic ($p = 0.9313$) tissues (Livak and Schmittgen, 2001). Relative gene expression was analysed using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method, also known as the Livak method), and expressed as a $\log_2$ fold change relative to the pooled average (Green and Sambrook 2018; Adams 2020). As the data were nonparametric, data are presented as the $\log_2$ relative fold change displayed via a box and whisker plot.

2.1.12 Statistical Analysis

IBM Statistical Package for the Social Sciences (SPPS V 28.0) was used for statistical analysis and graphs were generated using GraphPad Prism (PRISM V 9.0). Normality was tested via the Shapiro-Wilk test. For normally distributed data, a post-hoc Tukey's test was performed for comparisons containing more than three groups, while Fisher's LSD was used for groups of 3. For nonparametric tests with three groups, a Kruskal-Wallis test with a Dunn's post-hoc comparison was used. For nonparametric tests with two groups, a Mann-Whitney test was performed with a Benjamini and Hochberg false discovery rate correction. The Geisser-Greenhouse correction was applied for repeated measure ANOVAs to account for sphericity. Statistical analysis for each outcome of the experiment and results are summarised in the table below and are presented in the sequence for which the data were analysed.

Outcome	Groups	Statistical Analysis	Factor(s)	Correction	Post-hoc comparison
BW Change	All	2-way RM Anova with multiple comparisons	Treatment Group, Day	Geisser-Greenhouse	Tukey
Glucose Tolerance	All	2-way RM Anova with multiple comparisons	Treatment Group, Time (minutes)	Geisser-Greenhouse	Tukey
% Bodyfat	All	Ordinary ANOVA	Treatment Group	None (passes Brown-Forsythe test)	Tukey
Elevated Plus Maze	All	2-way Anova with multiple comparisons	Treatment Group, EPM Arm	None (only two levels of factor "EPM Arm")	Tukey
Chow Intake	Control, olanzapine, olanzapine and probiotic	Ordinary ANOVA	Treatment Group	None (passes Brown-Forsythe test)	Fisher's LSD
Plasma Biomarkers	Control, olanzapine, olanzapine and probiotic	Kruskal-Wallis	Treatment Group	None (nonparametric test)	Dunn
Hypothalamic and Hepatic Gene Expression	Control, olanzapine	Mann-Whitney	Treatment Group	None (nonparametric test)	Benjamini-Hochberg FDR

Table 1. Summary of statistical analyses and study outcomes. Corrections for sphericity were applied in repeated measure designs. Type I error corrections for post-hoc were applied for parametric tests with more than 3 groups. For parametric tests with 3 groups or less, an uncorrected Fisher's LSD post-hoc test was used. As there was significant within-group variability in measures of biomarkers and gene expression, nonparametric approaches were applied, with a Dunn post-hoc comparison and a Benjamini-Hochberg FDR correction, respectively.

2.1.13 Rationale for presented data

When RT-qPCR and Metabolomics data were being generated, it was discovered that several different strains of bacteria may have been administered to the rats due to issues with quality control of the intended monoculture (see supplemental figure 1). Thus, to reflect the chronology of our internal quality control checks, only control and olanzapine groups are reported for gene expression and the subsequent metabolomics chapter also only focuses on olanzapine versus control.

2.2 Metabolomics Methods

2.2.1 Plasma preparation

Plasma samples (n=12 controls, n=12 olanzapine-treated) were prepared in a randomised order. Samples were taken from -80˚C storage and kept on ice until thawed. Metabolite extraction was adapted from the protocol by Dunn et al (2011), as follows: cold methanol (150µl) was added to plasma (50µl), vortex mixed, and centrifuged at 15,800g for 15 minutes at 4˚C. Supernatant was transferred into an Eppendorf tube and placed in a centrifugal vacuum evaporator for 4 to 5 hours, without applying any heating. Samples were then reconstituted in a 50:50 water: acetonitrile w/ 0.1% formic acid solution (100µl), vortex mixed and centrifuged at 15,800g for 15 minutes at room temperature. Pooled quality control (QC) samples were obtained by taking 10µl from each sample. Finally, supernatants of all samples and QC were transferred into LC-MS glass vials.

2.2.2 UPLC-MS analysis

Mass spectrometry methods were similar to previous investigations (Morillon et al., 2020; 2021). Samples were maintained at 4˚C during the analysis, analysed in randomised order and in triplicate, on an ultra-high performance liquid chromatography (UPLC) Acquity system coupled to a Synapt G2-S quadrupole time-of-flight (Q-TOF) mass spectrometry system (Waters Corp, Wilmslow, UK). Data were acquired in both positive and negative electrospray ionisation modes (ESI+, also known as positive mode, and ESI-, also known as negative mode). QC samples were analysed after every ten sample injections. The chromatographic separation of the analytes was performed on a BEH C18 column (2.1x100mm, 1.7µm) maintained at 50˚C. For both ESI+ and ESI-, the elution gradient used solvent A, a mix of water and formic acid at 0.1% (v/v), and solvent B, a mix of acetonitrile and formic acid at 0.1% (v/v).In positive mode (i.e. ESI+), injection volume was 4µl, a 20-minute gradient was applied at 0.40ml/min as follows: initial condition at 0% of B for 1 minute, increased to 100% of B over 16 minutes, then maintained for 2 minutes, followed by a drop to 3% of B in 2 minutes. In negative mode (ESI-), injection volume was 4µl, and a 24-minute gradient was applied at 0.40ml/min as follows: initial condition at 0% of B for 2 minutes, increased to 100% of B over 15 minutes, then maintained for 5 minutes, followed by a return to initial conditions of 0% of B in 2 minutes.

2.2.3 *Positive mode*

In positive mode, data were acquired in “resolution” MSE mode from 50 to 1,200Da. Ion data for precursor (low energy) and fragment (high energy) were collected during the same acquisition, with a total cycle time of 0.2 seconds (0.1 second for each). Linear collision energy ramp (15- 40eV) was applied for high energy. Capillary voltage was set to 3.0kV, sampling cone at 35V, extraction cone to 5V. The source temperature was set at 120˚C, and the desolvation temperature at 650˚C. Desolvation gas flow rate was set at 950L/hr, and cone gas at 50L/hr. Mass calibration was performed using a sodium formate mix (Waters, Wexford, Ireland), as recommended by the manufacturer before each batch analysis. Real-time lock mass correction was performed using leucine enkephalin (LeuEnk, 1ng/µl) mix, injected at 10µL/min through a lock-spray probe and acquired every 30 seconds.

2.2.4 Negative mode

Data were acquired in “resolution” MSE mode from 100 to 1,500Da. Ion data for precursor (low energy) and fragment (high energy) were collected during the same acquisition, with a total cycle time of 0.2 seconds (0.1 second for each). Linear collision energy ramp (15- 40eV) was applied for high energy. Capillary voltage was set to 2.5kV, sampling cone at 35V, extraction cone to 5V. The source temperature was set at 120˚C, and the desolvation temperature at 500˚C. Desolvation gas flow rate was set at 950L/hr, and cone gas at 50L/hr. Mass calibration was performed using a sodium formate mix (Waters, Wexford, Ireland), as recommended by the manufacturer before each batch analysis. Real-time lock mass correction was performed using leucine enkephalin (LeuEnk, 1ng/µl) mix, injected at 10µL/min through a lock-spray probe and acquired every 30 seconds.

2.2.5 Data pre-processing

Data processing was performed using Progenesis QI version 2.4 (Nonlinear dynamics, Newcastle, UK), and an appropriate pooled QC was selected as the reference to chromatographically align the data and normalise to all compounds. Data were then peak picked and considered the adducts corresponding with M+H, M+H-2H2O, M+H- H2O, M+NH4, M+Na, M+K, 2M +H (ESI+), or M-H, M-H2O-H, M-Na-2H, M+K-2H, M+Cl, M+FA-H, 2M-H

(ESI-). Downstream statistical analysis was performed using the compound measurements exported from Progenesis QI.

2.2.6 Data filtering and annotation

The exact mass of metabolites was putatively annotated by database search using the Progenesis QI identification tool against the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG ID references to PubChem and the European Bioinformatics Institute were used to classify the putative metabolite's biological role. The search parameters were set for an exact mass tolerance of 5ppm for the precursor ion, and 10ppm for the fragment ion. Metabolites detected using UPLC-MS are frequently present multiple times due to fragmentation, multiple charging, chemical adduction, or dimerization. Identifications were reported for unique metabolites, after removing duplicates and metabolites of drugs or food, and after checking the retention time.

2.2.7 Data filtering and normalisation

Two datasets were generated in Progenesis QI: an annotated positive run and annotated negative run. Each dataset was then analysed for differential expression using Metaboanalyst 5.0 Features were filtered out if their relative standard deviation (RSD = SD/mean) are greater than 30% of values seen in quality control samples, which reduces the number of tests and increases power (Hackstadt and Hess, 2009). Then, data were normalised by z-score, and then $\log_{10}$ transformed. Lastly, as these data were normalised, parametric statistics (i.e., t-tests) were applied (Xia et al., 2013; Vinaixa et al., 2012).

2.2.8 Generation of volcano and principal component analysis plots visualising differential expression of metabolites

For generating a volcano plot, the Fold Change (FC) threshold was set to 1 and indicating changes from baseline/control relative to olanzapine treatment (i.e., a negative FC indicates lower levels of the metabolite in controls relative to the treatment group, a positive FC indicates higher levels of the metabolite in controls relative to the treatment group), with a post-hoc Benjamini-Hochberg FDR correction of $q < 0.05$ (Benjamini and Hochberg, 1995) applied. Lastly, 2D principal component analysis (PCA) plots were generated to visually assess group-

level differences and identify potential outliers. Shaded regions represent the 95% confidence interval.

2.2.9 Tabulation of putatively identified metabolites

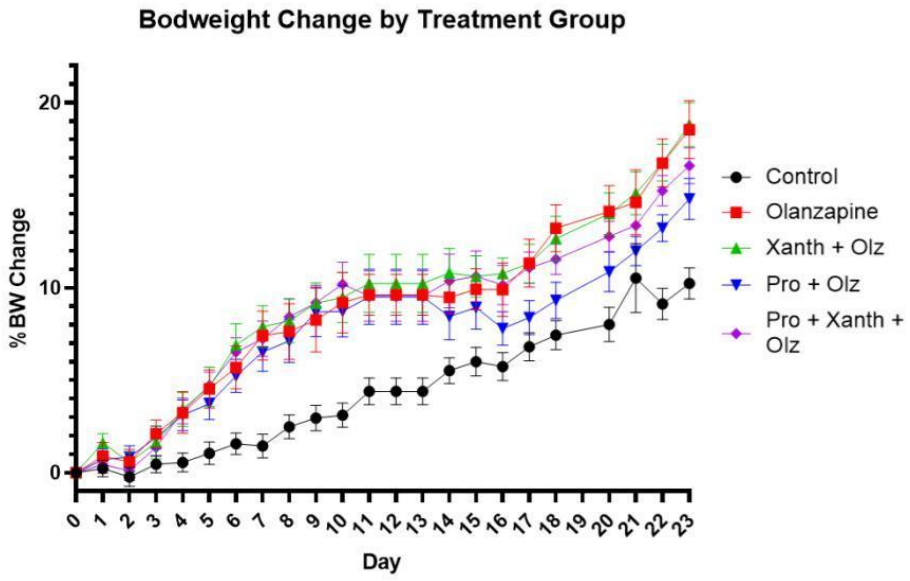
All metabolites with a significance of $p < 0.05$ were tabulated for the positive and negative run (summary table x, x2), along with their q value, KEGG ID, potential biological role/pathway, fold change (FC) comparing control relative to olanzapine, and $\log_2$(FC).

2.2.10 Simple regressions of anthropometrics and putatively identified metabolites

Concerning annotated positive and negative runs, three metabolites from the positive run passed FDR. These metabolites were then selected for simple linear regression onto anthropometric measures from the previous chapter (total body weight, % fat, and % brown fat).

3. Results

3.1 *In Vivo* Results



Significant Posthoc differences in %BW (control vs. olanzapine)	
Day	*p* value
6	0.0395
7	0.0070
8	0.0451
10	0.0262
11	0.0070
12	0.0070
13	0.0070
14	0.0176
18	0.0083
20	0.0137
22	0.0009
23	0.0019

Figure 6. Percent body weight change from baseline across treatment groups. There were overall effects of Day (F (4.624, 245.1) = 217.3, p <0.0001), Treatment Group (F (4, 53) = 5.752, p = 0.0006), and their interaction (F (88, 1166) = 2.587, p <0.0001) (2 way ANOVA with repeated measures). Posthoc Tukey's test indicated that controls had significant bodyweight differences from olanzapine treated rats for most comparisons from day 6 onward (ps < 0.05), but no intervention groups (probiotic, xanthohumol, probiotic and xanthohumol) were significantly different from the olanzapine treatment group. Data from days 19 and 24 are not included as they were IPGTT and cull days, respectively.

3.1.1 Neither probiotic, prebiotic, nor their combination attenuate antipsychotic-induced weight gain

There were main effects of Day (F (4.624, 245.1) = 217.3, p < 0.0001) and Treatment Group (F (4, 53) = 5.752, p = 0.0006), and although there was an interaction effect of Day x Treatment Group (F (88, 1166) = 2.587, p < 0.0001), post-hoc Tukey's tests indicated that there were only differences between olanzapine and control groups (ps =0.0009-0.0451). The post-hoc analysis indicates that there was a significant difference between the control and olanzapine-treated groups on most days from day six onward. No treatment groups were significantly different from the olanzapine treatment. However, in the comparison of olanzapine versus probiotic and olanzapine, the closest approach to significance was day 18 (p = 0.1454). There also appears to be a biphasic weight gain pattern in all groups treated with olanzapine. Data are presented in Figure 6.

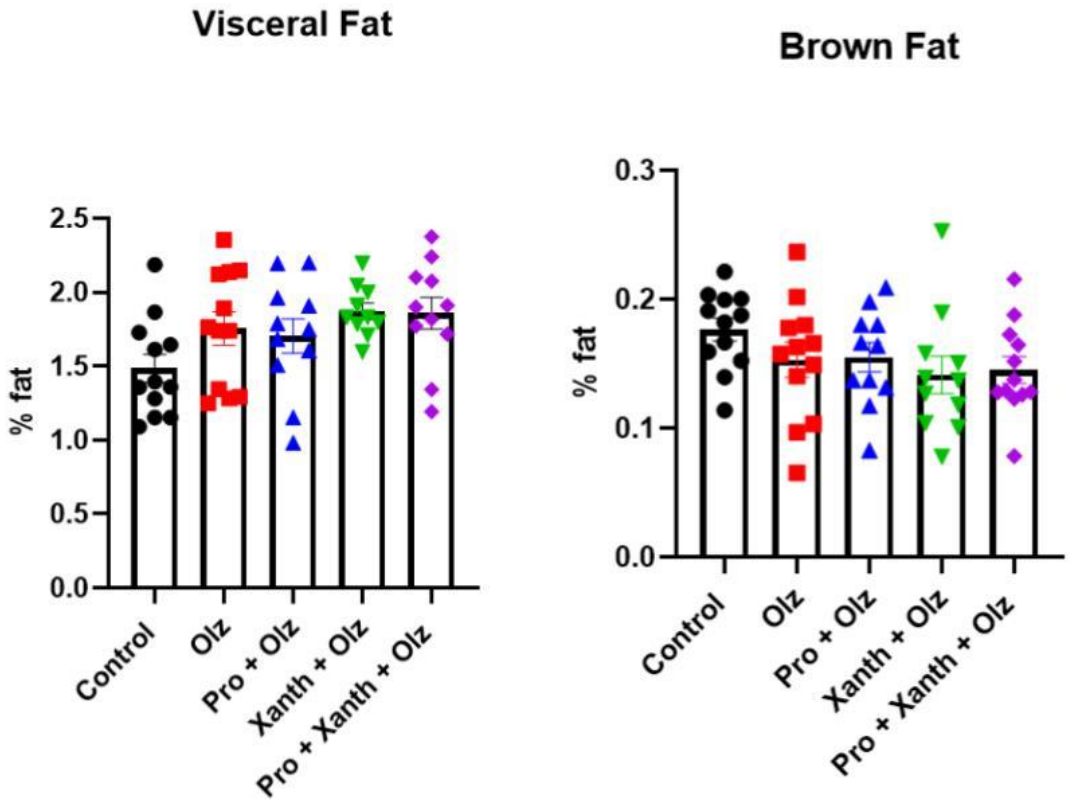


Figure 7. Visceral and brown % body fat across treatment groups. Differences at the group level approached significance for visceral fat (F (4, 51) = 2.409, p = 0.0612) but not brown fat (F (4, 53) = 1.348, p = 0.2646) (ANOVA). Posthoc tukey's test indicated no significant differences between control and olanzapine treated groups for visceral (p = 0.3056) or brown (0.6197) fat..

3.1.2 Olanzapine treatment did not increase visceral or brown fat

ANOVA indicated a near-significant difference in visceral fat (F (4, 51) = 2.409, p = 0.0612), but not for brown fat (F 4, 53) = 1.348, p = 0.2646). Importantly, the difference between the control and olanzapine-treated groups was not significant for visceral fat (p = 0.3056) or brown fat (p = 0.6197) (post-hoc Tukey's test). This indicates that the model used for olanzapine-induced obesity may have been insufficient. Data are presented in Figure 7.

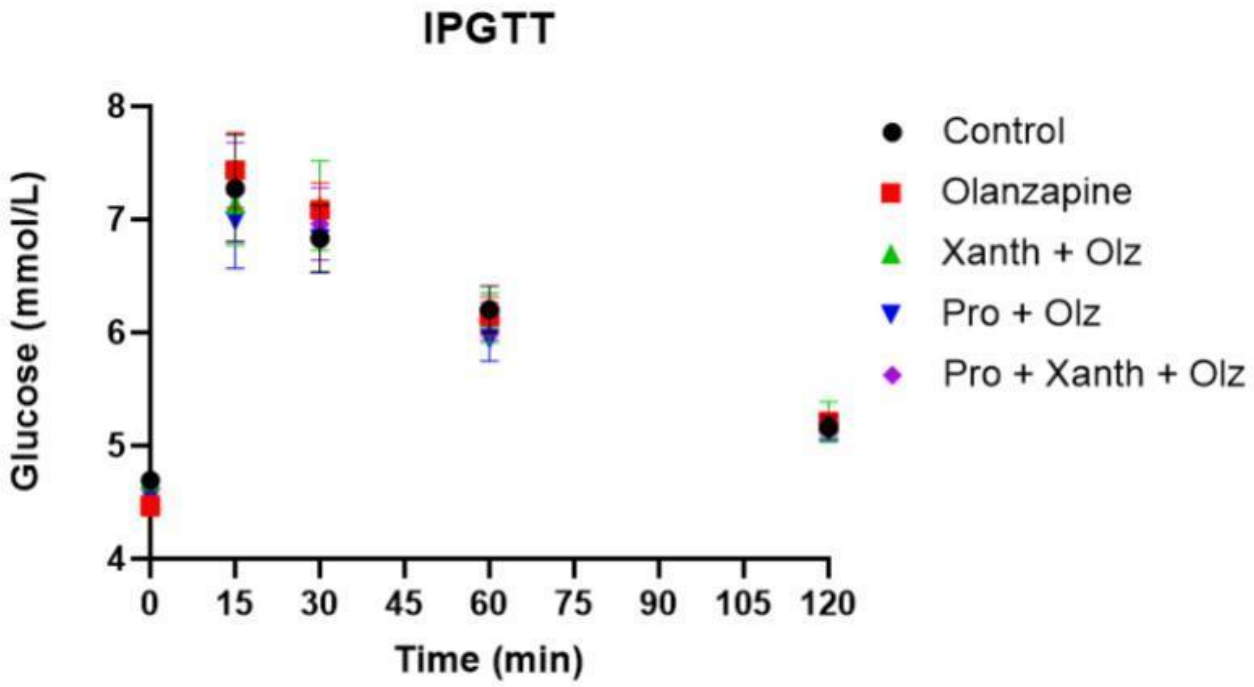


Figure 8. Intraperitoneal glucose tolerance test across treatment groups. There was a significant effect of Time (F (1.750, 94.49) = 149.6, $p < 0.0001$), but not for Treatment Group (F (4, 54) = 0.1734, $p = 0.9511$) or their interaction (F (16, 216) = 0.2429, $p = 0.9989$) (2 way repeated measure ANOVA). Posthoc Tukey's tests found no significant or near-significant differences between any treatment groups at 0, 15, 30, 60, and 120 minutes ($ps > 0.2756$).

3.1.3 Chronic olanzapine treatment did not alter glucose homeostasis

After approximately 3.5 weeks of twice-daily injections of 2mg of olanzapine/kg of body weight, rats underwent the IPGTT. There was a significant effect of Time (F (1.750, 94.49) = 149.6, $p < 0.0001$), but not for Treatment Group (F (4, 54) = 0.1734, $p = 0.9511$) or their interaction (F (16, 216) = 0.2429, $p = 0.9989$). There was no difference in levels of glucose between treatment groups at any time point (Tukey's test, $ps > 0.2756$). This indicates that the model used for olanzapine-induced was insufficient to induce glucose homeostasis dysfunction. Data are presented in Figure 8.

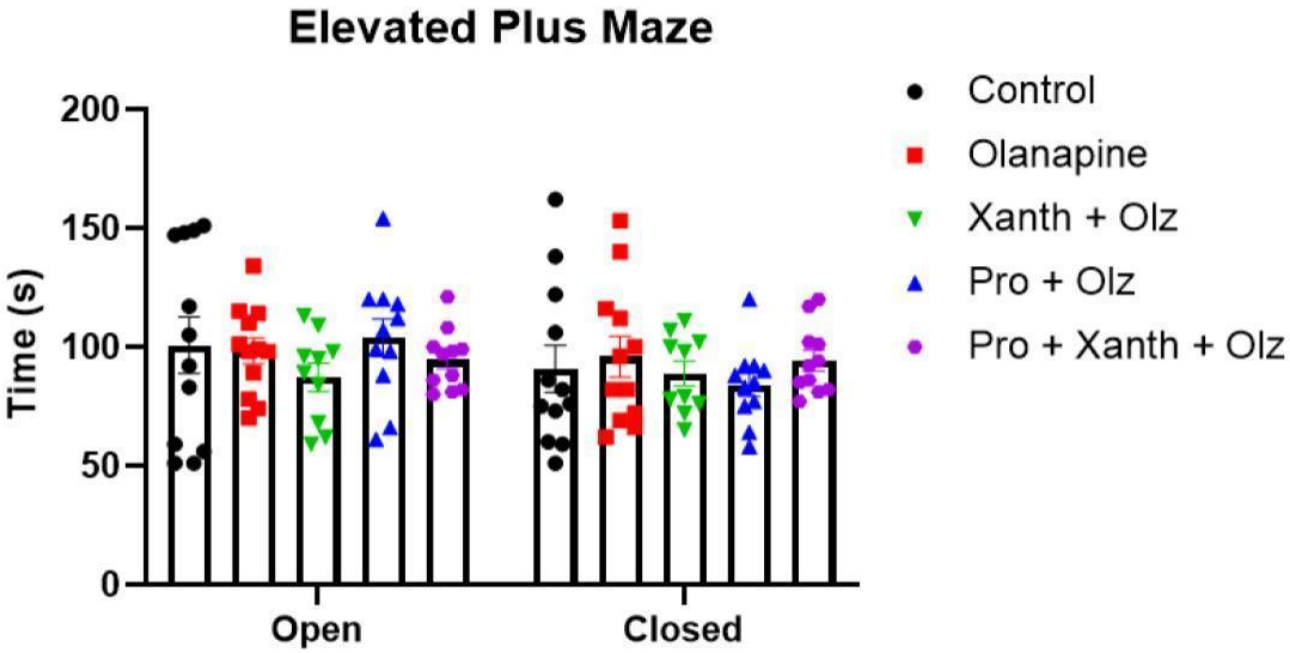


Figure 9. Elevated plus maze test. On day 26 of the study, rats underwent the elevated plus maze (EPM) protocol. There were no significant differences between groups within the open or closed arms nor were there differences between the groups across arms (2 way ANOVA with Tukey's multiple comparisons *p*s > 0.6916). There were no individual factor differences for EPM Arm (F (1, 102) = 1.722, $p = 0.1924$), Treatment Group (F (4, 102) = 0.4059, $p = 0.8040$), nor their interaction (F (4, 102) = 0.6738, $p = 0.6117$).

3.1.4 Elevated plus maze reveals no differences in anxiety-like behaviour in any group

There was no effect of Treatment Group (F (4, 102) = 0.4059, $p = 0.8040$), EPM arm (F (1, 102) = 1.722, $p = 0.1924$) or their interaction (F (4, 102) = 0.6738, $p = 0.6117$). In accordance with these null results, post-hoc Tukey's tests found no difference between treatment groups within an arm, or across arm and treatment group (*p*s $\geq$ 0.6916, 45 post-hoc tests in total). Although there was high interindividual variability within groups, no individual values were beyond +/- 2 SDs of the mean. Importantly, there was no difference between time spent in the open or closed arms of the maze, which raises concerns about the construct validity (measuring anxiety) of this procedure. This is addressed in the discussion section. Data are presented in Figure 9.

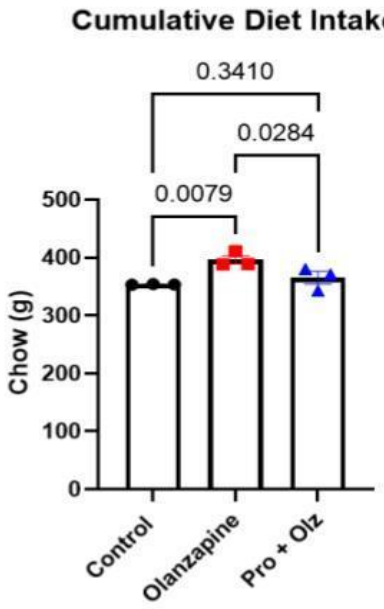


Figure 10. Dietary intake. There was a significant effect of treatment on total chow intake (F (2, 6) = 8.182, p = 0.0193) (ANOVA). Fisher's LSD posthoc comparisons indicated an increase in total chow eaten when comparing control and olanzapine treated groups (p = 0.0079), as well as olanzapine treated versus probiotic with olanzapine (p = 0.0284). Datapoints represent the total chow of a cage divided by the number of rats within the cage.

3.1.5 Olanzapine increases total chow intake, which is attenuated by concomitant probiotic supplementation

As there were no IPGTT, adipose or weight gain differences between olanzapine only and olanzapine with xanthohumol or probiotic + xanthohumol, these groups were excluded from the dietary analysis. There was a significant effect of treatment on total chow intake (F (2, 6) = 8.182, p = 0.0193), and post-hoc Fisher's LSD test indicated an increase in the olanzapine group in comparison with controls (p = 0.0079) and a reduction in chow when comparing olanzapine to concomitant olanzapine and probiotic (p = 0.0284). The difference between controls and concomitant olanzapine and probiotic was not significant (p = 0.3410). Data are presented in Figure 10.

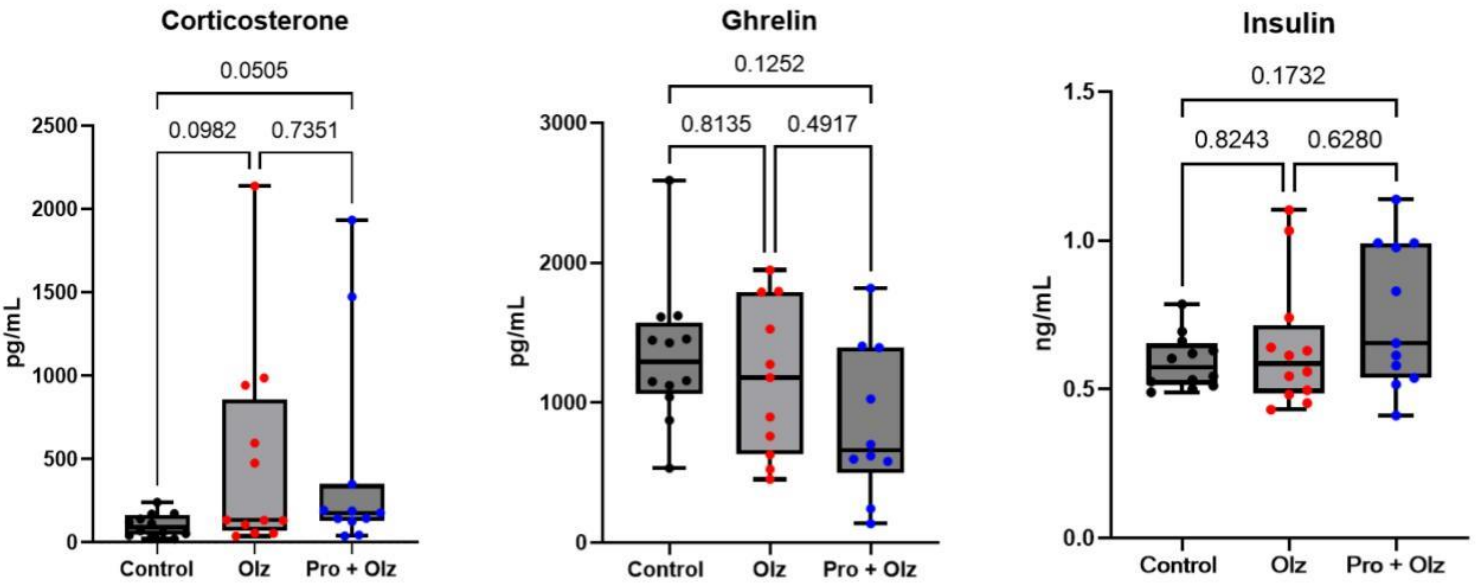


Figure 11. ELISAs of plasma biomarkers related to stress, hunger, and glucose control. Kruskal-Wallis comparisons were selected due to high within-group variability/low sphericity and low N. Differences in corticosterone approached significance between the control and olanzapine treated groups. However, there was no significant difference between the olanzapine treated and olanzapine with probiotic groups for any biomarker.

3.1.6 Olanzapine may be sufficient to increase plasma biomarkers of stress, but not appetite or glucose regulation

Due to nonnormative distributions of plasma ELISA data, nonparametric Kruskal-Wallis tests with Dunn's post-hoc comparisons were used. Levels of corticosterone approached a near-significant increase in the olanzapine-treated group ($M_{pg/mL}$ = 482.895, *SD* = 624.524) in comparison to controls ($M_{pg/mL}$ = 103.854, *SD* = 67.493) (p = 0.0982) and was significant when comparing control to olanzapine with probiotic ($M_{pg/mL}$ = 437.358, *SD* = 639.803) (p = 0.0505). This may suggest that olanzapine was sufficient to increase levels of corticosterone, but probiotic treatment did not attenuate this increase. Ghrelin was decreased by olanzapine treatment ($M_{pg/mL}$ = 1153.514, *SD* = 522.070) in comparison to controls ($M_{pg/mL}$ = 1336.882, *SD* = 506.415), but this difference was not significant (p = 0.8135). Olanzapine with probiotic also decreased ghrelin ($M_{pg/mL}$ = 828.015, *SD* = 522.287), but this was also not significant when compared to controls (p = 0.1252). Lastly, there were no statistical differences in insulin between any groups ($ps \geq 0.1911$) (control: $M_{ng/mL}$ = 0.592, *SD* = 0.091; olanzapine: $M_{ng/mL}$ = 0.644, *SD* = 0.217; olanzapine with probiotic: ($M_{pg/mL}$ = 0.749, *SD* = 0.244). Data are presented in Figure 11.

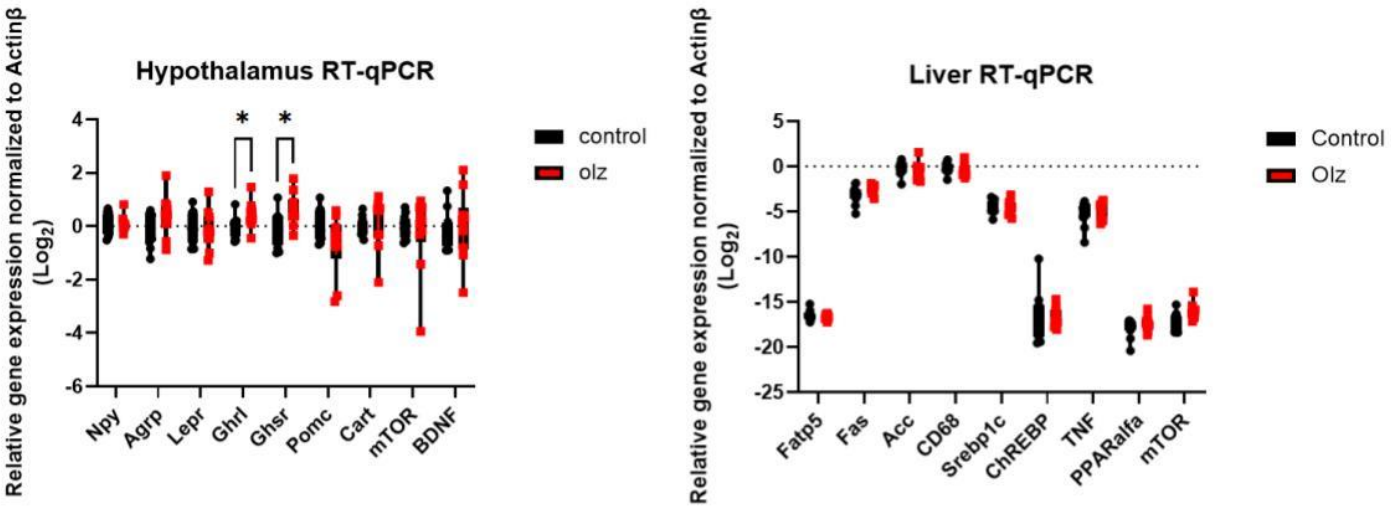


Figure 12. Relative expression of genes regulating eating behavior, energy balance, and immunological function in hypothalamic and hepatic tissue. mRNA expression for genes of interest (GOI, shown along the x axis) were normalized to the expression of the housekeeping gene *Actinβ* for both hypothalamic and hepatic tissue, and were $\log_2$ transformed to show their relative expression. Olanzapine treatment was associated with upregulated *Ghrl* and *Ghsr* in the hypothalamus (Benjamini-Hochberg FDR adjusted $p < 0.05$, Mann-Whitney test). Other genes were not differentially expressed (adjusted *ps* > 0.05).

3.1.7 Analysis of gene expression reveals an increase in ghrelinergic pathways in the hypothalamus of olanzapine-treated rats

Due to nonnormative distributions of the RT-qPCR data, nonparametric Mann-Whitney tests with a Benjamini-Hochberg FDR adjustment were used. For hepatic tissue, there was a near-significant increase in *mTOR* expression in the olanzapine-treated group (M_{FC} = -16.065, *SD* = 0.851) relative to controls (M_{FC} = -17.272, *SD* = 1.008) (adjusted $p = 0.0597$), but no other genes were approached differential expression (adjusted $ps \geq 0.4435$). For hypothalamic tissue, there was a significant increase in *Ghrl* expression in the olanzapine-treated group (MFC = .414, *SD* = 0.158) relative to controls (M_{FC} = -0.043, *SD* = 0.113) (adjusted $p = 0.0416$). There was also a significant increase in *Ghsr* expression in the olanzapine-treated group (M_{FC} = 0.631, *SD* = 0.196) relative to controls (M_{FC} = -0.155, *SD* = 0.178) (adjusted $p = 0.0416$). However, no other hypothalamic tissue genes were differentially expressed (adjusted $ps \geq 0.2766$). This suggests that Olanzapine treatment may have led to an increase in peripheral anabolic gene expression (*mTOR*) and was associated with increased expression of genes implicated in the central regulation of appetite, specifically via ghrelinergic pathways (*Ghrl, Ghsr*). Data are presented in Figure 12.

3.2 Metabolomic Results

3.2.1 Overall results

All samples (12 control, 12 olanzapine-treated) were successfully run through UPLC-MS. In our discovery UPLC-MS metabolomics analysis we identified 4173 features in positive mode, 541 of which were removed following data pre-processing via 30%QC RSD filtering. Of the 3632 remaining features, 353 were putatively identified and annotated in Progensis QI. We identified 40/353 of these putative metabolites as significantly differentially expressed ($p < 0.05$) between groups, and 3 metabolites were significant after adjustment for FDR. In negative mode, we identified 2405 features, 843 of which were removed following data pre-processing via 30%QC RSD filtering. Of the remaining 1562 features, 202 were putatively identified and annotated in progenesis QI. We identified 18/202 putative metabolites as significantly differentially expressed ($p < 0.05$) between groups, though none were significant following adjustment for FDR.

KEGG ID	Biological Role	Compound Name	FC	log2(FC)	p value	q value
C19670	Primary fatty acid amide	(9Z)-9-Octadecenamide	0.15464	-2.693	4.46E-10	1.41E-07
C15285	Androstanoid	17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol	0.59743	-0.74317	6.55E-08	1.04E-05
C14919	Androstanoid	17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one	0.76843	-0.38001	0.000398	0.042047
C15259	Androstanoid	2alpha,17-Dimethyl-19-nortestosterone	0.64288	-0.63738	0.001309	0.096264
C16522	Eicosanoid	11Z,14Z,17Z-Eicosatrienoic acid	0.72301	-0.46791	0.001518	0.096264
C15401	Androstanoid	11beta,17beta-Dihydroxy-4,17-dimethylandrost-4-en-3-one	0.65574	-0.6088	0.002648	0.11158
C05470	Corticosteroid	Tetrahydrocortisone	0.19575	-2.3529	0.002652	0.11158
C16527	FA docosanoid metabolism	Docosatetraenoic acid	0.70994	-0.49424	0.003182	0.11158
C05471	Corticosteroid	Dihydrocortisol	1.5059	0.59058	0.003267	0.11158
C08282	Monounsaturated fatty acid	Chaulmoogric Acid	0.72738	-0.45922	0.00352	0.11158
C07355	Linoleic acid metabolism	(Z)-(7S,8S)-Dihydroxyoctadeca-9-enoate	0.67723	-0.56228	0.0045	0.12844
C09484	Lactone	Isoalantolactone	0.6706	-0.57648	0.004862	0.12844
C14962	Corticosteroid	17-Hydroxy-5alpha,17alpha-pregn-1-en-3-one	0.64597	-0.63047	0.005394	0.13152
C15022	Androstanoid	11alpha,17beta-Dihydroxy-2alpha,17-dimethylandrost-4-en-3-one	0.66495	-0.58869	0.006391	0.14281
C10614	Indolizine	Securinine	0.84911	-0.23598	0.006757	0.14281
C04210	Lactococcus lactis metabolite	N(5)-(L-1-carboxyethyl)-L-ornithine	0.82293	-0.28117	0.008529	0.16897
C08317	Medium chain fatty acid	12-Hydroxylauric acid	0.73594	-0.44234	0.010393	0.19381
C13791	Medium chain fatty acid	cis-6,7-epoxystearic acid	0.78658	-0.34633	0.012652	0.21343
C15258	Estrane	17β-hydroxy-2α-methylestr-4-en-3-one	0.67856	-0.55945	0.012792	0.21343
C08367	Monounsaturated fatty acid	trans-Vaccenic acid	0.92133	-0.11821	0.015558	0.22138
C14836	Linoleic acid metabolism	1,2:4,5-Dianhydro-1-(7-carboxyheptyl)-3-deoxy-5-pentylpentitol	0.81619	-0.29303	0.016463	0.22138
C17454	Benzofuran	paeonilactone A	0.75379	-0.40777	0.016658	0.22138
C17144	Fatty alcohol	3-Octanol	0.76844	-0.38	0.016993	0.22138
C03242	Linoleic acid metabolism	Dihomo-gamma-linolenic acid	0.81564	-0.29399	0.018041	0.22138
C08380	ketone	2-Heptanone	0.68567	-0.54442	0.018615	0.22138
C02592	Bile acid	Lithocholic acid taurine conjugate	0.49683	-1.0092	0.018706	0.22138
C17644	Bile acid	ursocholic acid	0.40849	-1.2916	0.019411	0.22138
C17687	Bile acid	ALLODEOXYCHOLIC ACID	0.42102	-1.2481	0.019554	0.22138
C16320	Alpha-linoleic acid metabolism	colnelenic acid	0.61513	-0.70104	0.027835	0.30427
C04643	Bile acid	(3alpha,5beta,12alpha,17alpha,20S)-3,12-Dihydroxy-7-oxocholan-24-oic acid	0.48664	-1.0391	0.031927	0.32252
C00712	Monounsaturated fatty acid	Oleic acid	0.78259	-0.35368	0.031994	0.32252
C00695	Bile acid	cholic acid	0.43965	-1.1856	0.032558	0.32252
C06066	Lactone	5,5-Dimethyl-4-(3-oxobutyl)dihydro-2(3H)-furanone	0.84951	-0.2353	0.034423	0.33067
C01933	Amino acid	L-Norleucine	0.82997	-0.26887	0.036662	0.33602
C03240	Microbial macrolide	6-Deoxyerythronolide B	0.67884	-0.55886	0.03781	0.33602
C05660	Indole & tryptophan metabolism	5-Methoxy-3-indoleaceate	1.2459	0.31714	0.03816	0.33602
C05575	Histidine metabolism	Hercynine	0.82791	-0.27245	0.040439	0.34647
C00327	Arginine metabolism	L-Citrulline	0.92085	-0.11896	0.043166	0.35961
C14254	Androstanoid	Androstan-17-ol	1.1459	0.19654	0.044243	0.35961
C02577	Acyl-CoA	Phenoxyacetyl-CoA	0.81853	-0.2889	0.047896	0.37957

Table 2. Summary table of differentially expressed putative metabolites (positive run). A negative log2FC of 1 indicates a twofold decrease in the control group, relative to the olanzapine-treated group, whereas a positive log2FC of 1 indicates a twofold increase in control, relative to the olanzapine-treated group.

3.2.2 Identification of differentially expressed metabolites in positive run UPLC-MS

For the positive run, we identified 40 metabolites that were significantly differentially expressed between groups, and three metabolites passed FDR ($q < 0.05$): (9Z)-9-Octadecenamide 17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol, and 17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one. These metabolites were all increased in the olanzapine-treated group in comparison to the control group. A variety of metabolites from different classes of molecules were represented, including androstanoid (six compounds: C15285, C14919, C15259, C15401, C15022, C14254) inflammatory (three corticosteroid compounds: C05470, C05471, C14962), free fatty acids (nine compounds: C08282, C07355, C08317, C13791, C08367, C14836, C03242, C16320, C00712), and bile acids (five compounds: C02592, C17644, C17687, C04643, C00695). Data are presented in Table 2.

KEGG ID	Biological Role	Compound Name	FC	log2(FC)	p value	q value
C01558	Bile acid	(3alpha,5beta,7alpha,8xi,9xi,10xi,12alpha,13xi,14xi,17xi,20xi)-3,7,12-Trihydroxycholan-24-oic acid	0.39339	-1.346	0.0027305	0.33082
C17662	Bile acid	isoursodeoxycholic acid	0.42237	-1.2434	0.0034641	0.33082
C17687	Bile acid	Allodeoxycholic acid	0.45036	-1.1509	0.0093364	0.44188
C15176	Androstanoid	17-Methyl-5alpha-androst-2-ene-1alpha,17beta-diol	0.67459	-0.56793	0.013962	0.44188
C16536	Monounsaturated fatty acid	9E-heptadecenoic acid	0.50709	-0.97967	0.015195	0.44188
C00881	Pyridine Metabolism	2'-Deoxycytidine	0.70397	-0.50642	0.016838	0.44188
C05954	Arachidonic acid metabolism, prostaglandin	PGB2	0.72882	-0.45637	0.016881	0.44188
C02592	Bile acid	Lithocholic acid taurine conjugate	0.44282	-1.1752	0.023357	0.44188
C16696	Bile acid	Dolichotheline	0.24721	-2.0162	0.024454	0.44188
C08277	Dicarboxylic fatty acid	Sebacic acid	0.69476	-0.52542	0.026715	0.44188
C06321	Benzoate metabolism	(1R,6S)-1,6-dihydroxycyclohexa-2,4-dienecarboxylic acid	0.33325	-1.5853	0.028379	0.44188
C17873	Long chain fatty acid	2-hydroxy Lignoceric Acid	1.1438	0.19389	0.031562	0.44188
C19892	Carotenoid metabolism	4'-apo-beta-carotenal	0.53868	-0.8925	0.03393	0.44188
C00078	Amino acid	L-Tryptophan	1.833	0.87418	0.034294	0.44188
C06103	microbial xenobiotic metabolism	6-Hydroxycaproic acid	0.64795	-0.62604	0.037259	0.44188
C16346	Alpha-linoleic acid metabolism	17-Hydroxylinolenic acid	0.73104	-0.45198	0.038366	0.44188
C11133	Estrogen metabolism	Estrone glucuronide	0.68936	-0.53667	0.03933	0.44188
C02796	Indole metabolism	Methyleneoxindole	1.7726	0.82585	0.048488	0.46615

Table 3. Summary table of differentially expressed putative metabolites (negative run). A negative log2FC of 1 indicates a twofold decrease in the control group, relative to the olanzapine-treated group, whereas a positive log2FC of 1 indicates a twofold increase in control, relatie to the olanzapine-treated group.

3.2.3 Identification of differentially expressed metabolites in negative run UPLC-MS

For the negative run, 18 metabolites were identified as differentially expressed between experimental groups, though no metabolites passed an FDR of $q < 0.05$. However, several similar categories of signalling molecules were putatively identified and are congruent with the findings of the positive run, such as bile acids (five compounds: C01558, C17662, C17687, C02592, C16696) and free fatty acids (four compounds: C16536, C08277, C17873, C16346). Data are presented in Table 3.

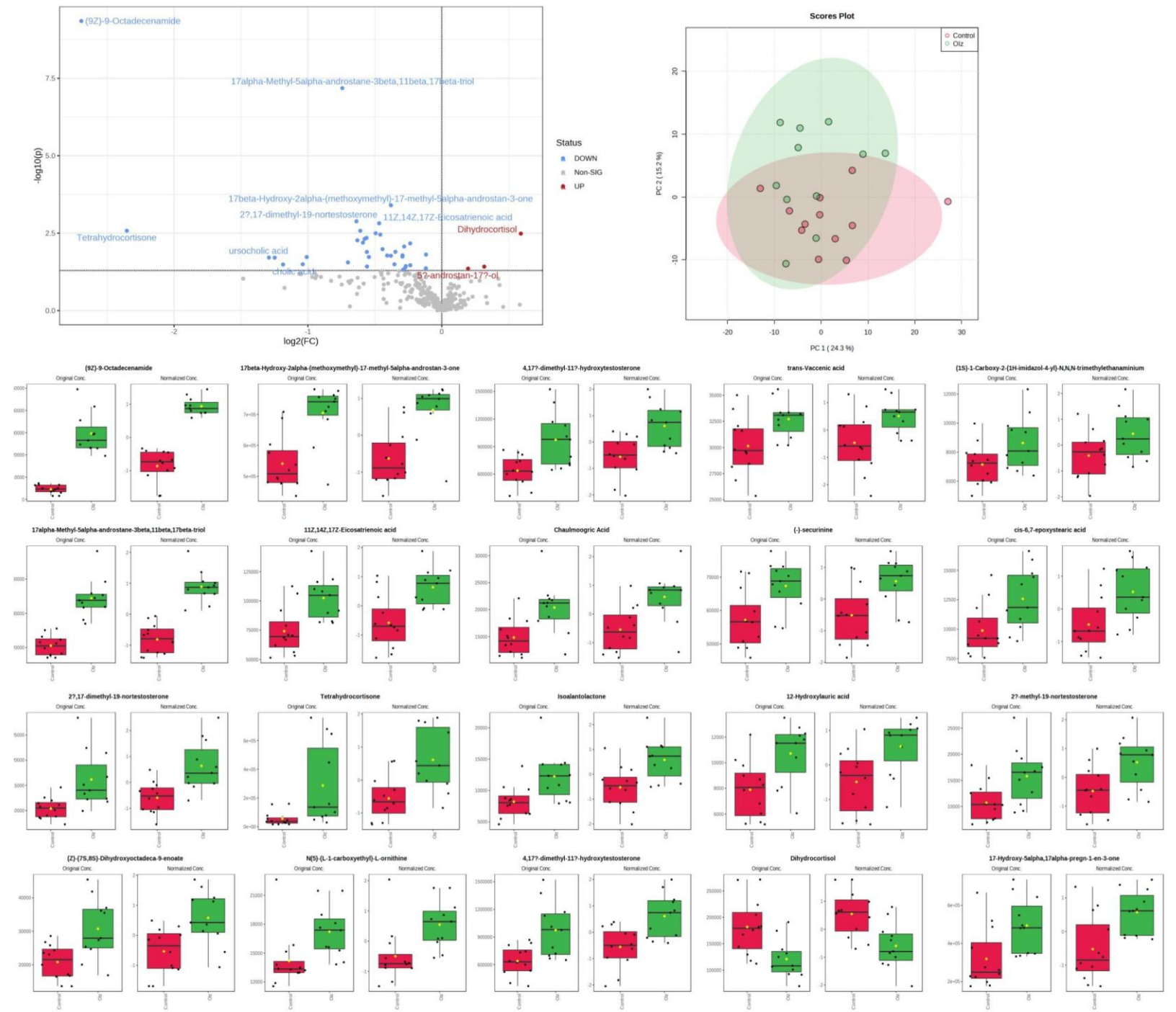


Figure 13. Metaboanalyst-generated volcano plots, individual differentially expressed putative metabolites, and principal component analysis (positive run). A negative log2FC of 1 indicates a twofold decrease in the control group, relative to the olanzapine-treated group, whereas a positive log2FC of 1 indicates a twofold increase in control, relatie to the olanzapine-treated group. The top right PCA plot features principal component 1 and 2. Individual plots of putative metabolites display the original data on the left, and the normalised (Z score and log base 10 transformed) data. (**note: for visible interpretability, only the top 20 statistically significant features are presented)**

3.2.4 Visualisation of differentially expressed metabolites in positive run UPLC-MS

The volcano plot of the positive run indicates that most of the significantly ($p < 0.05$) differentially expressed metabolites were decreased in the controls (conversely, increased in the olanzapine-treated group). The three metabolites that do pass FDR ($q < 0.05$) include (9Z)-9-Octadecenamide 17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol, and 17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one. The principal component analysis shows some differential clustering between groups and loading on the principal components. This is congruent with the greater amount of putatively identified metabolites that were significant and metabolites that passed FDR, relative to the negative run, which identified fewer putative metabolites and did not identify any metabolites which passed FDR. Data are presented in Figure 13.

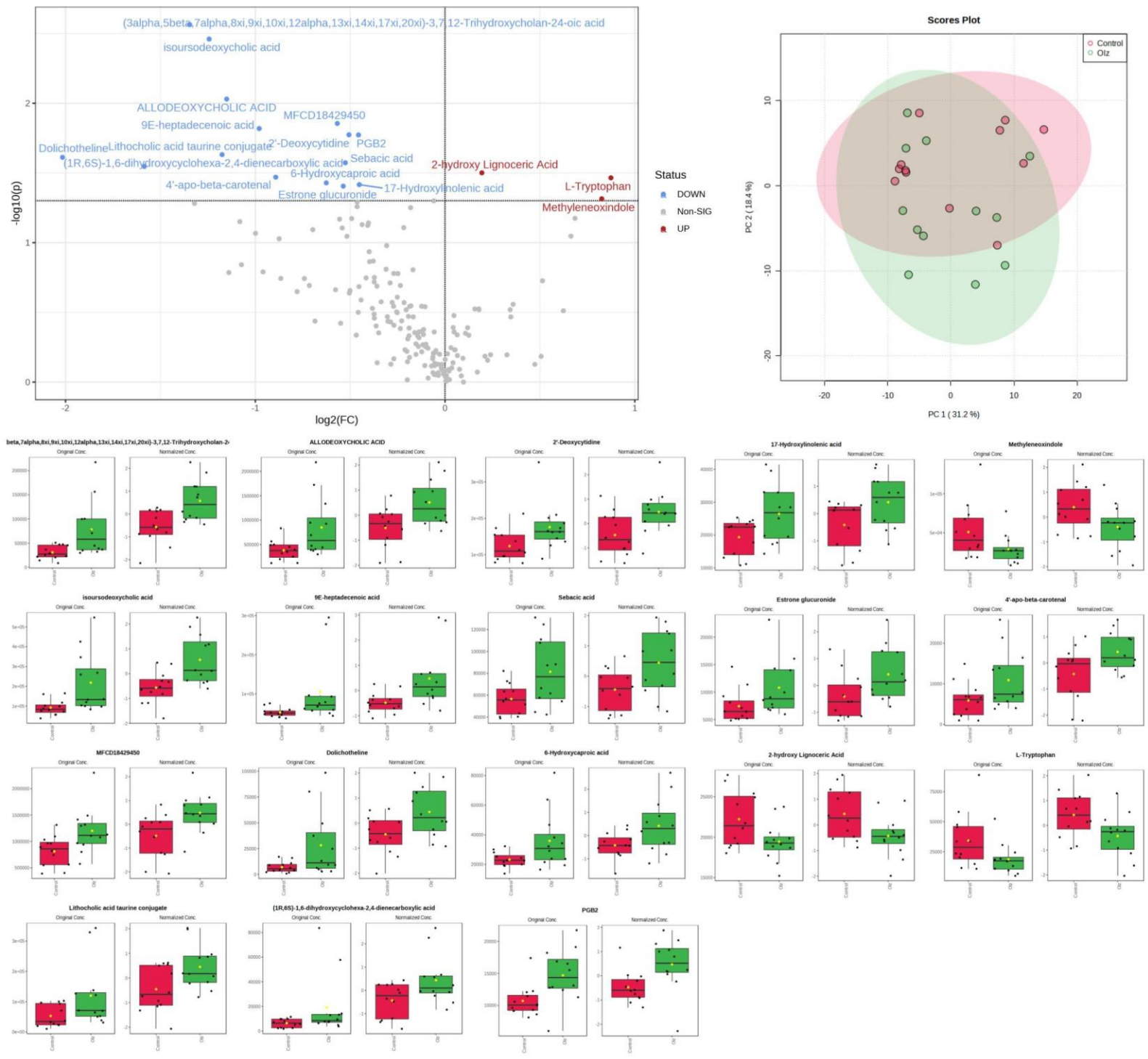


Figure 14. Metaboanalyst-generated volcano plots, individual differentially expressed putative metabolites, and principal component analysis (negative run). A negative log2FC of 1 indicates a twofold decrease in the control group, relative to the olanzapine-treated group, whereas a positive log2FC of 1 indicates a twofold increase in control, relatie to the olanzapine-treated group. The top right PCA plot features principal component 1 and 2. Individual plots of putative metabolites display the original data on the left, and the normalised (Z score and log base 10 transformed) data.

3.2.5 Visualisation of differentially expressed metabolites in negative run UPLC-MS

The volcano plot of the negative run indicates that most of the significantly ($p < 0.05$) differentially expressed metabolites were decreased in the controls (conversely, increased in the olanzapine-treated group). However, no metabolites pass FDR ($q < 0.05$). The principal component analysis shows that there was no significant group clustering or differential component loading between groups, suggesting these groups are similar/have low metabolomic variance between groups. Data are presented in Figure 14.

KEGG ID	Biological Function	Putative Metabolite (Regression DV)		Control		Olanzapine	
C19670	Primary fatty acid amide	(9Z)-9-Octadecenamide	**Anthropometric Measure (Regression IV)**	***β* (Standardized Beta)**	***p* value**	***β* (Standardized Beta)**	***p* value**
			Bodyweight	-.115	.721	**–.566**	**.07**
			% Body Fat (Mes. + Gon. + Ret.)	.001	.997	-.243	.472
			% Brown Fat	.342	.277	-.028	.934
C15285	Androstanoid	17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol					
			Bodyweight	-.093	.774	-.101	.768
			% Body Fat (Mes. + Gon. + Ret.)	.378	.225	.368	.265
			% Brown Fat	**.595**	**.041**	.328	.325
C14919	Androstanoid	17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one					
			Bodyweight	-.048	.883	-.011	.974
			% Body Fat (Mes. + Gon. + Ret.)	-.206	.522	.345	.299
			% Brown Fat	**-.570**	**.053**	.194	.567

Table 4. Summary table of results of anthropometric measures (IV) regressed onto putative metabolites (DV) using simple linear regression. To examine the associations between anthropometric measures affected by olanzapine and putative metabolites identified through metabolomics, simple linear regression was performed. Putative metabolites selected were differentially expressed between control and olanzapine (lower in controls), as indicated by a t-test FDR of $q < 0.05$ in Metaboanalyst.

3.3.6 Associations between highly differentially expressed putative metabolites and anthropometric outcomes

Simple linear regressions of the anthropometric outcomes of the previous chapter (bodyweight, % visceral fat, and % brown fat) onto putative metabolites reveal several associations. (9Z)-9-Octadecenamide decreased as a function of body weight in the olanzapine-treated group, though this relationship only approached significance (β = -.566, p = 0.070). This relationship was not seen in controls. In controls, the % of brown fat was negatively associated with 17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one, though it only approached significance (β = -.570, p = 0.053) while there was a positive association between % brown fat and 17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol, which did reach significance (β = *.595*, p = 0.410). The relationship between % brown fat and both androstanoids was not significant for olanzapine-treated groups. There were no associations between visceral fat and any putative metabolite for either controls or the olanzapine-treated group. Data are presented in Table 4.

4. Discussion

4.1 *In* Vivo Discussion

4.1.1 Caveat on analyses and quality control

As mentioned within the aims and objectives, this study was intended to evaluate APC1472 for anti-obesogenic effects when administered with olanzapine. Inocula of the bacteria were grown by collaborators and were purported to have been verified during the study, these inocula were then given to our research team. Following the completion of the animal study, quality control processes flagged potential issues with the purity and identity of the administered inocula. When this was discovered (around the time of the RT-qPCR analysis and metabolomic investigation), our research team felt the right course of action was to report the analyses that had already been completed in good faith, while also acknowledging the impact of the quality control issues on the replicability of the interventions. Thus, while these data are still valuable for understanding the mechanisms and health impacts of olanzapine-induced weight gain, the data from the probiotic intervention groups has limited interpretability. Lastly, while the probiotic group was excluded from the metabolomics analysis due to the aforementioned issues, the metabolomics workflow described in the methods was still conducted for this before this discovery. A metabolomics PCA of all three groups reveals that there was strong evidence against group differences between the olanzapine-only and probiotic with olanzapine groups, as illustrated by the near-complete overlap of these groups (Supplemental Figure 2). Thus, it would appear that the probiotic intervention had little impact on host plasma metabolomics.

4.1.2 Olanzapine alters body weight, eating behaviour, and central genetic regulation of appetite

Several of our findings are in accordance with other published data on olanzapine. Anthropometrically, olanzapine treatment led to a significant increase in weight in comparison to controls, as seen in previous studies (Davey et al., 2012; Davey et al., 2013, van der Zwaal et al., 2014). Behaviourally, there was an increase in the total dietary chow eaten in comparison to controls. Gene expression of *Ghsr*, the ghrelin receptor (also called growth hormone secretagogue receptor), was found to be significantly upregulated in the hypothalamus of olanzapine-treated rats, as was *Ghrl*, which encodes the ghrelin-obestatin preprotein. This is

congruent with a similarly designed study of olanzapine-associated increases in hypothalamic *Ghsr* expression (Zhang et al., 2014). There were also near-significant increases in serum corticosterone, a stress hormone which increases circulating glucose (previously demonstrated by Girault et al., 2012), and hepatic expression of the anabolic gene *mTOR* in olanzapine-treated rats (previously demonstrated by Liu et al., 2019) compared to controls. Taken together, these results indicate partial success in modelling the hyperphagic and weight-gain effects of olanzapine in rats.

4.1.3 Olanzapine is associated with increased levels of hypothalamic ghrelinergic signalling, but not peripheral levels of ghrelinergic signalling.

In our study, there was no difference in peripheral ghrelin between the olanzapine-treated and control groups. These findings are similar to a study by Zhang and colleagues (2014), who found that while olanzapine treatment consistently increased weight, peripheral ghrelin was increased only in days 4-12 following olanzapine treatment, but not any days thereafter. Thus, there may be different mechanisms of olanzapine-induced obesity across the course of its administration. These findings are also congruent with the non-linear, biphasic weight gain trend in our data, which was also seen in the study by Davey et al. (2012). This phenomenon may also hold in clinical populations, as a meta-analysis of olanzapine treatment in patients with schizophrenia also found decreased levels of ghrelin in blood compared to controls (Goetz and Miller, 2019).

4.1.4 Potential experimental design factors explaining null physiological findings

The significant increase in weight, total dietary chow, and upregulation of hypothalamic orexigenic genes associated with olanzapine treatment are all congruent with the rapid weight gain seen in clinical and other *in vivo* studies. Where our findings may seem incongruent are a lack of glucose dysregulation (indicated by null results for serum insulin and the IPGTT), fat metabolism (indicated by null results % body fat and *Acc*, *Fas*, *Fatp5*), inflammation and immune function (indicated null results for *Tnf-*α and *Cd68*), and other hypothalamic genes regulating appetite (orexigenic; *Agrp, Npy, mTOR,* anorexigenic; *Lepr, Cart, Pomc, BDNF*). In the interpretation of the null findings of this study, it is perhaps useful to consider study design and pathophysiology of weight gain, adiposity, and cardiometabolic dysfunction.

Several factors, such as rat strain, biological sex, and diet are relevant to preclinical investigations of weight gain. Wistar rats may have a more obesogenic response to a high-fat diet at an earlier time than Sprague-Dawley rats, potentially due to differences in the metabolic effects of their different microbial ecology (Marques et al., 2015). In terms of sex differences, males and females have different fat deposition, immunological and hormonal responses (de Moura E Dias et al., 2021; Maric et al., 2022). Researchers should also consider the differential effects of diet type, i.e., high-fat, cafeteria, standard chow, or health-promoting diets (such as the Mediterranean diet) on obesity-related outcomes (Lang et al., 2019; Bastías-Pérez et al., 2020). Follow-up studies investigating obesogenic and hyperphagic effects of olanzapine could also consider employing comparisons of different probiotics (i.e., anaerobic vs aerobic) against different diet types.

In the consideration of olanzapine-induced obesity and its side effects, meta-analytic evidence of clinical data indicates that nearly all antipsychotics are associated with weight gain (Bak et al., 2014) and that olanzapine treatment is associated with cardiometabolic side effects (Srisurapanont et al., 2021), however, there are no meta-analyses on preclinical studies of olanzapine. A narrative review by van der Zwaal and colleagues (2014) details the heterogeneous methods employed in pre-clinical models of olanzapine. Some of the relevant trends noted are (1) while most studies do not find increases in weight gain for male rats, there are increases in adiposity for males and females, even in the absence of weight gain (2) in studies where males did gain weight and adiposity, a medium-fat diet was used (3) male rats typically lost lean mass and gained fat mass, while studies on female rats were more heterogeneous, with most studies finding increases in lean mass and no changes in adiposity (with exceptions attributable to species) (4) attempts to investigate locomotor changes may be confounded by a floor-effect caused by circadian misalignment, e.g., that rats are not as active during daytime behavioural observation (5) differences in findings may also be attributable to different dosing schedules and the short half-life in olanzapine in rats relative to humans (2.5 vs ±30 h) (6) it is unclear if rodent models establish that weight gain is attributable to hyperphagia, rather than an impairment of satiety.

In summary, there are numerous sources of heterogeneity for *in vivo* study designs, which can impact replication across studies and external validity.

4.1.5 The elevated plus-maze is not compatible with the current study design

We found no changes between any of the groups in the EPM procedure. An investigation by Locchi et al. (2008) has shown that acute administration of olanzapine, but not chronic, attenuates increases in anxiety-like behaviour following restraint stress; notably, there is no statistical significance between olanzapine treated and control (saline) treated rats for time spent in the open arm (with greater time spent in the open arm being inversely proportional to anxiety). However, there were large differences in the time spent between open and closed arms. This is to be expected, as the EPM is well established in behavioural neuroscience as a measure of anxiety-like behaviour, whereby the open/exposed sections of the platform not only remove the protection of an enclosed space but also expose the rodent to a natural fear of heights (Walf and Frye, 2007; Wang et al., 2020). This contrasts with our study, which found no differences between treatment groups or across factor levels (open and closed).

There are several possibilities for this seeming discrepancy. First, recent data have shown that female and male rats have differential anxiety-like responses (serum corticosterone, oxytocin, and the open field task) to male versus female human experimenters being present, and this effect is still present when the experimenter is absent, but their clothing worn is left in the experimental area (Faraji et al., 2022). Similar results have been found in mice (Sorge et al., 2014), and a recent study demonstrated that experimenter sex affects male and female mice's responses to ketamine (Georgiou et al., 2022). Across the aforementioned studies, anxiety and stress-like behaviours were augmented when the rodents were exposed to human males. Lastly, other studies have shown that handling and environmental enrichment can reduce anxiety-like behaviour in rats (Costa et al., 2012). As the rats in this experiment spent a significant amount of time being handled by several researchers, i.e., during daily weighing, and soothing before IP injections of olanzapine, our null EPM findings could reflect the reduced anxiety-like behaviour caused by chronic handling and the female sex of the EPM administrator (NL).

4.1.6 Xanthohumol does not attenuate the negative effects of olanzapine

This study was the first to investigate xanthohumol administration (as well as attempt to investigate concomitant xanthohumol and probiotic) in the context of olanzapine-induced obesity. Previous preclinical studies of xanthohumol from our research group indicate that it can buffer the effects of stress and may act via the microbiota to regulate the HPA axis (Donoso et al., 2020), and a review using Cochrane search methodologies identified xanthohumol as a potential therapeutic agent in the treatment and prevention of hyperlipidaemia, obesity, and type II diabetes (Rossi et al., 2019). We did not observe any of the positive effects of xanthohumol on weight gain seen in other studies.

There could be several reasons for this finding. Some studies indicate that xanthohumol may have antimicrobial properties against anaerobic bacteria (Cermak et al., 2017). There is also evidence that the microbiota affects the pharmacokinetics of olanzapine (Cussotto et al., 2021). Lastly, there is evidence indicating that the presence of common dietary flavonoids, such as quercetin and catechin, can affect the binding of olanzapine to the carrier protein serum albumin (Mrkalić et al., 2021). Thus, our findings highlight several areas for further investigation related to characterising the interaction between olanzapine and xanthohumol, possibly via the microbiome and/or intermolecular interactions.

4.2 Metabolomic Discussion

4.2.1 Overall findings

In this study, we employed metabolomic analysis as a follow-up to an *in vivo* study investigating the central and peripheral effects of olanzapine and subsequent weight gain and hyperphagia. This revealed several novel putatively expressed metabolites, which could then be further investigated for their potential mechanistic and/or therapeutic role in antipsychotic-induced weight gain and hyperphagia. Two androstanoids (C15285 and C14919) and oleamide, passed FDR as differentially expressed metabolites. The two androstanoids showed opposite associations with brown adipose tissue in controls, with increasing levels of C15285 being positively associated with brown adipose tissue, and C14919 being negatively associated with brown adipose tissue. In addition, we observed an increase in bile acids in the olanzapine-treated group, which is congruent with the body of literature on antipsychotic-induced obesity in both humans and rodents.

4.2.2 Observed classes of differentially expressed metabolites

Of the 40 metabolites in the positive run, five were bile acids, and of the 18 metabolites in the negative run, there were also five bile acids. Of note is that in both the positive and negative run, the expression of bile acids was increased in the olanzapine-treated group relative to controls. A targeted metabolomic investigation in humans has shown that patients with schizophrenia have decreased levels of bile acids relative to controls (Qing et al., 2022), and importantly, this sample (*n*=216, patients with schizophrenia=108) was either completely antipsychotic-naive and experiencing first-episode psychosis (*n*=62) or were hospitalised due to relapse and were unmedicated for at least one month (*n*=46). It is postulated that SGAs affect bile acid metabolism through alterations in the gut microbiome, such that bile acids (BAs) increase in response to SGA treatment, and these BAs can cross the blood-brain barrier and increase hypothalamic orexigenic gene expression (Chen et al., 2023). Meta-analytic evidence in humans suggests that BA metabolism is altered in obesity (such that BA excretion was positively correlated with obesity), and bariatric surgeries increased total fasting BAs in serum (So et al., 2020). It is worth mentioning that the study by So and colleagues (2020), by the nature of bariatric interventions, are nonrandomized and uncontrolled and are therefore prone to selection bias. Taken together, these results are consistent with literature showing that SGAs are associated with changes in BA

metabolism, but their impact and potential causality in weight gain and hyperphagia need further clarification.

In contrast to the increase in bile acids in the olanzapine-treated group, tryptophan and its microbially-derived indole metabolites were mostly increased in controls (negative run: increased L-tryptophan, methyleneoxindole; positive run: 5-methoxy-3-indoleacetate), though one indole metabolite was decreased in controls (securinine). These results should be cautiously interpreted. Although there is evidence linking the microbiota with indole-related metabolites and their anti-obesogenic effects (Li et al., 2021; Hu et al., 2022), the small number of putative metabolites identified in this study and their inability to pass FDR should, at most, suggest the inclusion of indole-related metabolites in subsequent metabolomic validation studies of olanzapine-related side effects.

4.2.3 Associations of androstanoids and adiposity

Both 17beta-Hydroxy-2alpha-(methoxymethyl)-17-methyl-5alpha-androstan-3-one (hereafter referred to by the KEGG ID C14919) and 17alpha-Methyl-5alpha-androstane-3beta,11beta,17beta-triol (hereafter referred to by the KEGG ID C15285) are androstanoids, i.e. steroids with androstane skeletons (Kim et al., 2023). To our knowledge, there is no literature describing any associations between these specific compounds and olanzapine or antipsychotics. This paucity of knowledge is not uncommon in discovery/untargeted metabolomics, as there are tens of thousands of structurally identified compounds with undescribed biological properties (Giera et al., 2022; Zhou et al., 2022). Nonetheless, regression analyses suggest a relationship between brown adipose tissue (BAT) with C15285 and C14919. Sex differences in the metabolic activation of fat have been shown in both men and women, with preclinical studies suggesting sex hormone regulation as the mechanism of this difference (Law et al., 2014; Kaikaew et al., 2021). Therefore, confirming the presence of these metabolites through a validation study followed by *in vitro* investigations of their potential mechanism of action on BAT, both with and without the presence of olanzapine, could inform further *in vivo* and clinical studies. As peripheral biomarkers of BAT in humans remains an ongoing area of study (Alito et al., 2022), the negative association of C14919 with BAT and the positive association of C15285 with BAT

indicate these androstanoids as potential peripheral biomarkers of BAT in populations not undergoing antipsychotic treatment.

4.2.4 Putative metabolite: Oleamide

9Z-9-Octadecenamide, also called oleamide, was the most significantly differentially expressed metabolite, the log 2 FC of - 2.693 indicating a more than 5x mean concentration in the olanzapine-treated group ($q < 0.0001$). Oleamide was first isolated in human plasma in 1989 (Arafet et al., 1989), and a study by Lerner and colleagues (1994) was the first to show an association between levels of oleamide in cerebrospinal fluid and sleep deprivation.
To our knowledge, no studies have studied oleamide in the context of olanzapine, nor are there any meta-analyses or systematic reviews for oleamide. Thus, if the differential expression of oleamide is confirmed in a subsequent validation study, oleamide-related signalling could prove to be a therapeutic and mechanistic target of olanzapine-induced obesity and hyperphagia.

Although further studies confirming biosynthetic mechanisms and pathways of oleamide are needed, it may be endogenously produced from oleoglycine by the neuropeptide peptidylglycine alpha-amidating monooxygenase (PAM), and/or amidation of oleic acid via oleoyl coenzyme A by cytochrome c (Mueller and Driscoll, 2009). Several lines of research suggest that oleamide can be produced through microbial dairy fermentation (Ano et al., 2015; Ano and Nakayama, 2018), and it may be gastrointestinally absorbed via the hepatic portal vein and via CD36 cells in the small intestine (Kobayashi et al., 2022). Pharmacologically, oleamide may change the affinity (e.g., allosteric modulation) of several g-protein coupled receptors, including γ-Aminobutyric acid (GABA) and serotonin (5-HT) receptors (Hiley and Hoi, 2007) and is an endogenous ligand for cannabinoid CB1 and CB2 (Pertwee, 2015). Like the endocannabinoid anandamide, oleamide is degraded by fatty acid amide hydrolase (FAAH) (Hiley and Hoi, 2007; Leung, 2013).

There are several areas of research investigating the functional role of oleamide. As previously mentioned, oleamide plays a role in sleep induction, via interactions with melanin-concentrating hormone neurons in the lateral hypothalamus (Prospéro-García et al., 2016). Another mechanism of sleep onset is a drop in body temperature, which can be mediated via vasodilation (Harding,

Franks, and Wisden, 2019) and oleamide has been found to exert vasodilatory effects on peripheral tissues (Hiley and Hoi, 2007). Oleamide may also exert diverse immunomodulatory effects, as evidenced by *in vitro* assays demonstrating that oleamide promotes maturation of naive M0 macrophages toward M1 phenotypes via upregulation of M1-associated genes and downregulating M2 genes, thereby increasing IL-1β production (Wisitpongpun, et al., 2022). Contrastingly, a study by Moon and colleagues (2018) found that oleamide suppressed carrageenan-induced inflammatory responses, including IL-1β production in macrophages. Taken together, functional outcomes of oleamide may be contingent on if it is being examined in the developmental, pre-intervention period or in response to an immunological challenge.

4.2.5 The endocannabinoid system as a therapeutic target

To date, there is a dearth of literature examining the relationship between schizophrenia and oleamide, as well as antipsychotics and oleamide. Therefore, inferences and hypotheses for further investigations may be best drawn from the intersection of schizophrenia and the endocannabinoid system, as well as obesity and the endocannabinoid system. Several meta-analyses indicate that marijuana use is a risk factor for the onset of schizophrenia and psychosis (Rault et al., 2022; Salazar de Pablo et al., 2021; Kiburi et al., 2021). Evidence from a meta-analysis found that perturbations of the endocannabinoid system are a feature of psychotic spectrum disorders, as evidenced by increased levels of anandamide and increased expression of CB1 receptors in peripheral immune cells in patients versus controls (Minichino et al., 2019), and antipsychotics are known to change expression of several appetite-related genes, including CB1 (Shams and Müller, 2014; Himmerich et al., 2015).

Furthermore, a systematic review of human and preclinical studies suggests that cannabis use has transgenerational effects on neurodevelopmental genes (Colizzi et al., 2022), and large epidemiologic studies have found that maternal and paternal use of marijuana is associated with an increased risk of psychotic spectrum disorders in offspring (Bolhuis et al., 2018). Data from systematic reviews conclude that marijuana is the most used recreational substance for patients with schizophrenia (Martinotti et al., 2022). Thus, it may be of scientific and clinical value to characterise the interplay between antipsychotics and the endocannabinoid system, and how this impacts hyperphagia, cardiometabolic outcomes, and psychotic symptoms.

Lastly, while it may seem reasonable to target the CB1 receptor as a therapeutic anti-obesity target, clinical data indicates this is a potentially dangerous approach. One pilot RCT found that obese patients with schizophrenia who received the CB1 antagonist Rimonabant had a reduction in calories eaten (medium to large effect size), but the study was terminated early as Rimonabant was withdrawn from the market (Warren et al., 2013). This was due to emerging data indicating that the use of Rimonabant was associated with increased suicidality (Warren et al., 2013). Thus, other approaches to modulating the endocannabinoid system, such as via the brain-gut-microbiome axis (Gioacchini et al., 2017; Moludi et al., 2018) should be cautious when assessing the possibility of human translation. Patients with schizophrenia are a particularly vulnerable population, and great care and scientific rigour should be exercised in preclinical drug discovery and *in vivo* testing phases. As a first step, similar to the study by Torres-Fuentes and colleagues (2019), *in vitro* probiotic screening assays should consider quantifying the effects of probiotics on the endocannabinoid system, particularly in the presence of olanzapine.

4.3 Strengths and Limitations

4.3.1 Issues with quality control

A main aim of this investigation was to assess the therapeutic potential of a probiotic, APC 1472, for its anti-obesity potential in the context of olanzapine, and the issues related to production and quality assurance are a significant limitation to the scientific knowledge gained from this study. As a nascent science, standardisation and challenges of replicability are well-known issues in the field (Kim et al., 2017; Schloss, 2018; Hornung et al., 2019). Relevant oversight bodies have been made aware of the deviations from the protocol that happened during this study (see Supplemental Figure 1). Nonetheless, even if the probiotic was correctly produced, null results remain possible. The study was designed with this possibility in mind, and the comparisons between the control and olanzapine-only groups shed further light on the mechanisms of olanzapine-induced obesity and metabolic dysfunction.

4.3.2 Limitations of exploratory metabolomics

In particular, the putatively identified metabolite, oleamide, prompts interest and further probing of the interplay between olanzapine and the cannabinoid system. However, as an exploratory metabolomic investigation, it is still necessary to undergo a metabolomic validation workflow. It could be the case that the highly differentially expressed peak identified through Progenesis is not oleamide, but a different (albeit still highly differentially expressed) metabolite. It is also worth noting that oleamide is often a component of polymers making up laboratory materials, which is known to affect bioassays through contamination (Jug et al., 2020), though the high level of differential expression between the control and olanzapine-treated groups indicates that the signal observed is likely biological in origin, rather than a contaminant. Another limitation is the approach to annotation. As the annotation workflow using KEGG did not include drugs or drug metabolites, the presence of glucuronidated olanzapine metabolites could not be assessed. As previously demonstrated by Cussotto and colleagues (2021), antibiotics can lead to a reduction in the expression of bacterial glucuronidases and an increase in olanzapine absorption. Future studies using an annotation approach including drugs and drug metabolites could investigate if microbiome-mediated glucuronidation of olanzapine explains variability in olanzapine-induced weight gain and metabolic dysfunction.

4.3.3 Improving preclinical design

There are several ways in which follow-up studies could improve the methodology used in this thesis. In modelling complex diseases like olanzapine-induced metabolic dysfunction, it is important to consider the factors of age, species, diet type, sex, and other potential variables that impact translatability and internal validity. Additionally, to assess changes in behaviour, it may be advisable to conduct a more robust behavioural battery that is also in alignment with the model organisms' circadian rhythm. Lastly, to assess if the hyperphagic and obesity-related mechanisms of olanzapine vary at different time points, collection of biological samples across a longer time course is also warranted.

Importantly, these findings challenge assumptions of concomitant probiotic and prebiotic interventions. The potential negative synergism between xanthohumol and olanzapine illustrates that positive findings from one prebiotic or probiotic study should not be assumed to hold the

same effect when combined with other agents, i.e., drugs like olanzapine, that also interact with the microbiome. This assumption is illustrated by the 2019 consensus statement made by the International Scientific Association for Probiotics and Prebiotics (ISAPP), which highlights synergistic possibilities between many probiotic and prebiotic combinations, but also raises concerns about the possibility of detrimental microbial shifts (Swanson et al., 2020).

4.4 Future Directions and Conclusion

In this study, olanzapine treatment in female rats did not lead to statistically significant changes in visceral or brown fat, glucose homeostasis, or hepatic genes involved in fat metabolism, immune activation, or inflammation. However, there were changes in central ghrelinergic signalling, total dietary chow, and weight gain. This is all indicative of hyperphagia. Metabolomic analyses revealed a general increase in bile acids, which is congruent with the observed weight gain. Furthermore, two androstanoids (C15285 and C14919) and the endocannabinoid oleamide were sufficiently differentially expressed to pass FDR. Regression analyses suggest that C15285 and C14919 could serve as peripheral biomarkers of brown adipose tissue in control, but not olanzapine-treated rats. Although oleamide just approached significance in its negative association with bodyweight in olanzapine-treated rats, its high differential expression, along with its novel associations between the microbiome, olanzapine, and the cannabinoid system, warrants further investigation and probing into its potential therapeutic and mechanistic potential.

Taken together, the increase in bile acids, dietary chow intake, body weight, and central ghrelinergic signalling, along with the null results for the IPGTT, brown and visceral adiposity, and insulin, raise the possibility that in the time course of olanzapine-induced metabolic dysfunction, increases in appetite and weight gain precede peripheral metabolic dysfunction. Although other studies have highlighted the direct metabolic effects of olanzapine on peripheral metabolism, interventions which attenuate appetite may be a more effective strategy, as increases in appetite may be the driver of weight gain and subsequent cardiometabolic disease burden. Though the impact of the probiotic intervention could not be confidently assessed due to issues with quality control, further studies that aim to modulate ghrelinergic signalling may still be warranted.

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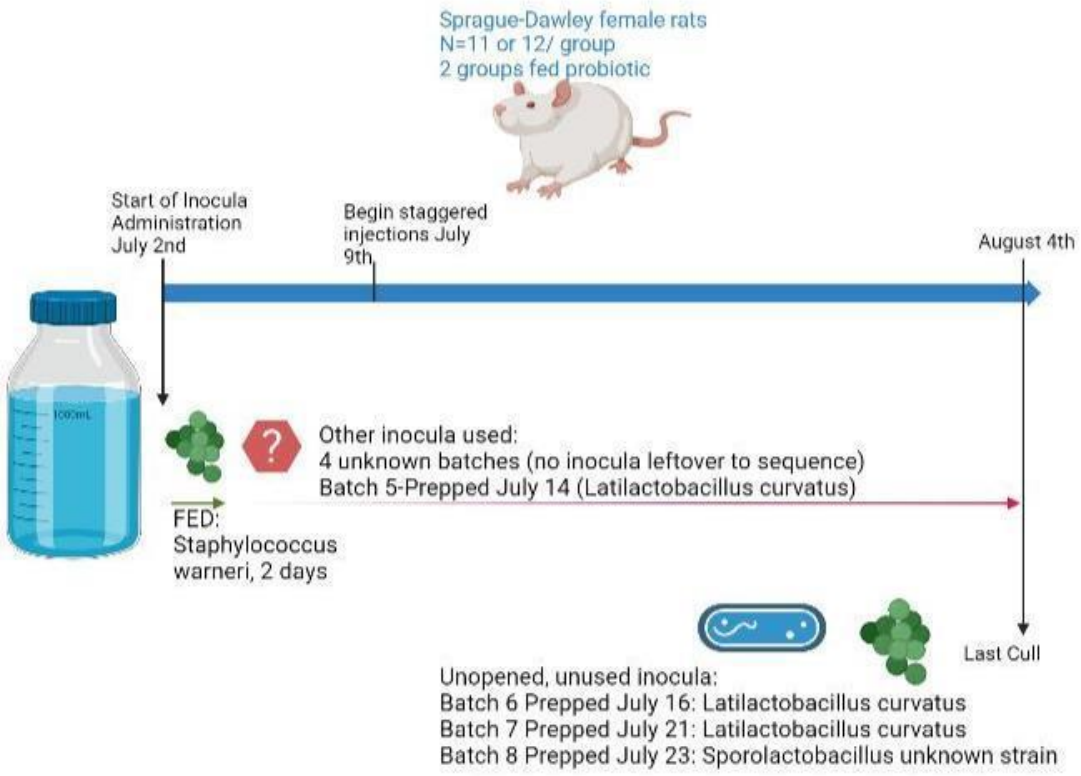
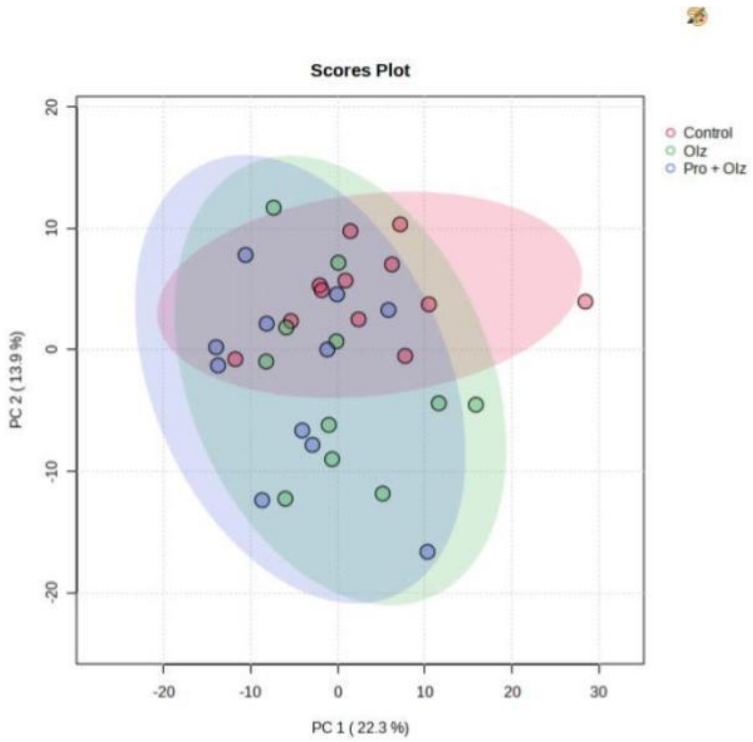
6. Supplemental Figures



Supplemental figure 1. Quality control issues with probiotic inocula. The intended strain, APC 1472, was not present in any of the leftover batches of inocula. Instead, we found several strains: *Staphylococcus warneri, Latilactobacillus curvatus*, and an unknown species of *Sporolactobacillus*. Relevant bodies (such as animal welfare) have been made aware of this unintentional deviation from the approved study protocol, and quality control measures are being taken to reduce the likelihood of this happening again. Because there were several strains administered, and no leftover inocula of several batches, it is not possible to reliably infer any effects of concurrent administration of olanzapine with probiotic.



Supplemental Figure 2. Positive run PCA of control, olanzapine, and probiotic with olanzapine groups. The substantial overlap of the control and olanzapine groups implies similar clustering of metabolites, i.e., that their plasma is not substantially different. Thus, even if there were certainty in what probiotic was administered with olanzapine, it was not sufficient to lead to a notably different metabolomic profile compared to olanzapine alone.