

**Prebiotic reduction of brain histone deacetylase (HDAC) activity and olanzapine-mediated weight gain in rats, are acetate independent.**

Amy Chia-Ching Kao<sup>1</sup>, Ka Wai Chan<sup>2</sup>, Daniel C Anthony<sup>2</sup>, Belinda Lennox<sup>1</sup>, Philip WJ Burnet<sup>1\*</sup>

<sup>1</sup>Department of Psychiatry, University of Oxford, Oxford, OX3 7JX, United Kingdom

<sup>2</sup>Department of Pharmacology, University of Oxford, OX1 3QT, United Kingdom.

\*Address correspondence to: [phil.burnet@psych.ox.ac.uk](mailto:phil.burnet@psych.ox.ac.uk)

Philip Burnet, PhD

Department of Psychiatry

University of Oxford

Warneford Lane

Oxford, OX3 7JX

Running Title: **Epigenetic effects of B-GOS and sodium acetate**

Keywords: **Acetylation, prebiotics, B-GOS, olanzapine**

## Abbreviations

Bimuno galacto-oligosaccharide (B-GOS®)

short-chain fatty acids (SCFAs)

histone deacetylase (HDAC)

histone acetyltransferase (HAT)

N-methyl-D-aspartate receptor (NMDAR)

glyceryl triacetate (GTA)

standard error of the mean (SEM)

## **Abstract**

The intestinal microbiome is emerging as a novel therapeutic target owing to the wide range of potential health benefits that could result by manipulating the microbiota composition through relatively simple interventions. Ingestion of the prebiotic Bimuno™ galactooligosaccharide (B-GOS®) is one such intervention that has been shown to attenuate olanzapine-induced weight gain and improve cognitive flexibility in rats, potentially through mechanisms involving acetate, the major short-chain fatty acid (SCFA) that is produced by B-GOS® fermentation. The present study investigated the individual influences of B-GOS® and sodium acetate intake on brain histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, cortical and hippocampal expression of HDAC1-4 and N-methyl-D-aspartate receptor subunits in saline or olanzapine injected female rats. The effect of sodium acetate on olanzapine-induced weight gain was also investigated. Daily ingestion of B-GOS® for 21 days, reduced HDAC activity and hippocampal HDAC-4, and elevated levels of cortical HDAC-1 and HDAC-3 mRNAs. Sodium acetate supplementation significantly decreased HAT, but not HDAC, activity and increased hippocampal HDAC-3 and HDAC-4 mRNAs. Olanzapine-induced weight gain and fourteen genera of intestinal bacteria, were not influenced by sodium acetate intake. Together these data suggests the effects of B-GOS® in rats cannot be replicated by acetate ingestion, and that mechanisms beyond the production of this SCFA are likely to underlie the psychotropic and metabolic actions of this prebiotic.

## 1. Introduction

We have recently demonstrated in rats that daily supplementation with the Bimuno™ galactooligosaccharide (B-GOS®) prebiotic prevented olanzapine-mediated weight gain (Kao et al., 2018) and improved cognitive flexibility (Gronier et al., 2018). Prebiotics are substrates that are selectively utilized by host microorganisms and confers health benefits (Gibson et al., 2017). The fermentation of B-GOS® yields significant amounts of the short chain fatty acids (SCFAs) acetate, propionate and butyrate (Grimaldi et al., 2017), and we have demonstrated that B-GOS®-derived acetate enters the circulation and influences central gene expression (Gronier et al., 2018). Increasing acetate availability to the brain has been shown to impart epigenetic changes which involve the addition or removal of acetyl groups on histone molecules bound to genomic DNA (Gao et al., 2016). Histone modifications are modulated by the activities of histone deacetylase (HDAC) and histone acetyltransferase (HAT). Increased HDAC activity removes acetyl groups on histones, which decreases DNA unwinding thereby limiting gene expression. Conversely, HAT adds acetyl groups on to histones, which promotes DNA uncoiling and elevates gene transcription (Gorisch et al., 2005). The changes in central gene expression that we have previously reported in response to prebiotic feeding (Gronier et al., 2018; Savignac et al., 2013; Williams et al., 2016), may have been the downstream result of bacteria-derived acetate on epigenetic mechanisms.

The therapeutic potential of acetate has been advocated by research on neurodegenerative disorders such as Canavan's disease (Arun et al., 2010; Mathew et al., 2005), where oral acetate improved motor function in a genetic model of the disorder, and in studies of behavioural phenotypes associated with glutamate receptor N-methyl-D-aspartate receptor (NMDAR) blockade, showing that acetate attenuated cognitive deficits (Singh et al., 2016). The latter study has particular clinical relevance given that NMDAR hypofunction has been implicated in the pathophysiology of schizophrenia (Krzystanek et al., 2015; Krzystanek et al., 2016; Tarazi et al., 2003). Region specific increase in NMDAR subunits have been observed following B-GOS® feeding and acetate supplementation in rats (Gronier et al., 2018; Kao et

al., 2018; Savignac et al., 2013), further illustrating that prebiotics, and possibly prebiotic-derived acetate, may mediate the central effects of these carbohydrates.

The attenuation of olanzapine-induced weight gain by B-GOS® may be mediated by acetate, as direct administration of the latter suppresses food intake (Frost et al., 2014), which conceivably counter-balances olanzapine-induced augmentation of energy consumption (Deng et al., 2012; Skrede et al., 2012). A proposed mechanism for the satiating effect of acetate, is that it increases colonic secretion of anorectic gut hormones (Christiansen et al., 2018), which might also explain why a prebiotic that grows bacteria has the same effect on olanzapine-mediated weight gain as antibiotic administration (Davey et al., 2012) or germ-free status (Morgan et al., 2014). That is, elevated secretion of anorectic gut hormones have been reported in germ-free mice (Khosravi et al., 2015), and with the administration of broad spectrum antibiotics (Rajpal et al., 2015), which suggests that increased gut hormone secretion is an effect shared by acetate administration and the reduction or elimination of gut bacteria. Therefore, it is reasonable to investigate whether the intake of acetate alone would have the same metabolic effects as B-GOS®.

The aim of this study was first, to determine the individual influences of B-GOS® and sodium acetate intake on HDAC and HAT activities in the rat brain, and the expression of HDAC encoding genes. The specific quantification of HDAC1-4 mRNAs was chosen as their central expression are influenced by acetate intake, and/or they modulate NMDA receptor expression (Nghia et al., 2015; Soliman et al., 2012). Second, we further examined whether sodium acetate ingestion would prevent olanzapine-induced weight gain as we have reported for B-GOS® (Kao et al., 2018). Third, we examined the effect of sodium acetate supplementation on the abundance of selected gut bacterial genera which are altered by B-GOS®. This was to verify whether acetate production in the gut, through prebiotic fermentation, contributes to changes in the levels of gut bacteria.

## 2. Materials & Methods

Custom DNA oligomers were purchased from Eurofins Genomics, UK. Olanzapine was purchased from Oxford Pharmacy Stores UK, and dissolved at 10mg/ml in sterile saline solution (0.9% NaCl) adjusted to pH 6.5 with concentrated hydrochloric acid. Sodium acetate was purchased from Sigma-Aldrich. Nuclear extraction and HDAC activity kit was obtained from EpiQuik and HAT activity kit was purchased from Abcam, UK. Colorimetric acetate assay kit was purchased from Abcam, UK.

### 2.1 Animals

Female adult Sprague-Dawley rats (n = 48), 220-250g (6-8wks), were housed three per cage with rodent chow and water *ad libitum* on a 12hr light-dark cycle (21±1°C, humidity 50±5%). Female rats were used as olanzapine-induced weight gain is more robustly conferred compared to male rats (Davey et al., 2012; van der Zwaal et al., 2014). All procedures were performed in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and associated Home Office guidelines. The local Animal Welfare and Ethical Review Body at the University of Oxford approved the procedures specific to this study.

### 2.2 Sodium acetate and B-GOS® administration

The administration of B-GOS® and olanzapine to rats was previously described (Kao et al., 2018), and comprised of four different treatment groups: saline/water (n=6), B-GOS®/saline (n=6), water/olanzapine (n=6), B-GOS®/olanzapine (n=6). Similarly, for acetate supplementation with olanzapine, rodents were randomly assigned to four treatment groups: saline/water (n=6), acetate/saline (n= 6), water/olanzapine (n = 6), and acetate/olanzapine (n = 6). Sodium acetate was administered to rats via their drinking water at a dose of 500mg/kg/day (Frost et al., 2014). Animal body weight and fluid intake were monitored daily, and sodium acetate concentrations were adjusted accordingly to maintain the desired daily dose. This method of acetate delivery is consistent with our studies with B-GOS® (Kao et al., 2018), and avoids stress to animals incurred by intra-gastric gavage, a method required to administer the glyceryl triacetate (GTA) supplement (Soliman and Rosenberger, 2011).

Furthermore, the authors of the latter study state that since GTA is a lipid complex, it has greater brain penetrance than acetate itself. However, SCFAs arising from the gut are not conjugated to larger lipid molecules, and so GTA is not a true physiological representation of acetate arising from the bacterial fermentation of dietary carbohydrates.

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

### *2.3 Plasma and tissue collection*

Twenty-four hours after the final injection, animals were humanely culled by cervical dislocation and decapitated to obtain trunk blood, which was collected in heparin coated 2ml Eppendorf tubes. All blood samples were centrifuged at maximum speed on a bench-top centrifuge for 5min. The top plasma phase was pipetted into a fresh Eppendorf tube and frozen on dry ice. Whole brains were removed, bisected and the hippocampus and cortex dissected out from one hemisphere. All cortical regions and hippocampus from the other hemisphere were dissected from the mid-brain and basal ganglia as one piece of tissue. These regions were selected because prebiotic and probiotic intake significantly influences gene expression therein, and they are neuroanatomical regions that underlie the specific behaviours that are influenced by these supplements (Bravo et al., 2011; Gronier et al., 2018; Savignac et al., 2013) ). All plasma and tissue samples were stored at -80°C until analysed.

### *2.4 Plasma acetate analysis*

Levels of circulating SCFA acetate was measured using commercially available Colorimetric acetate assay kit was purchased from Abcam, UK. All samples were assayed in duplicate and protocols were carried out according to manufacturer's instructions.

## *2.5 Nuclear extraction*

Nuclear protein was extracted from the combined cortex/hippocampal tissue using the EpiQuik™ Nuclear Extraction Kit I (Epigentek, Cat. No. OP-0002), according to manufacturer's instructions. Briefly, the tissue was gently homogenised with steel beads in extraction buffer for 30min, vortexing at 3min intervals. The homogenate was centrifuged and the resulting supernatant was measured using Bradford's protein assay (Bio-rad laboratories, CA, USA). These samples were referred to as 'brain homogenate'.

### *2.5.1 HDAC activity*

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

### *2.6.2 HAT activity*

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

## *2.7 Quantitative Polymerase Chain Reaction (QPCR)*

Total RNA was extracted from isolated cortical and hippocampal tissue using the TRI-Reagent (Sigma-Aldrich, UK) according to manufacturer's instructions, and reverse-transcribed to cDNA, using a commercial kit (Life Technologies, UK). The SYBR Green methodology (Power SYBER, Life Technologies, UK) was applied to amplify mRNA encoding HDAC1-4 using



previously published primer sets. QPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). All QPCR data are presented as fold change (relative to  $\beta$ 2-microglobulin, B2M) which were calculated using the 2-Ct relative quantitation method (Livak and Schmittgen, 2001).

## 2.8 Gut microbiota analysis

The total abundance of 16S DNA encoding specific microbial genera and total bacteria in faecal pellets were analysed using QPCR and previously published primers (Morel et al., 2015). Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) after mechanical disruption. The concentrations of isolated DNA were quantified spectrophotometrically by measuring absorbance at 260nm. The SYBR Green methodology (Power SYBER, Life Technologies, UK) was used to amplify 20ng DNA on a 7900HT Fast Real-Time PCR System (Applied Biosystems, US). Duplicate samples were held at 95°C for 10 min and subjected to 40 cycles of denaturation (95°C for 15sec) and annealing/extension (60°C for 60sec). These QPCR data were also calculated using the  $2^{-\Delta\Delta C_t}$  relative quantitation method (Livak and Schmittgen, 2001) and reported as fold-changes relative to the saline/water group.

## 2.9 Statistical Analysis

Statistical analyses were performed using SPSS version 22. Normal distribution of data was confirmed using Shapiro-Wilk test. Data points greater than three standard deviations from the mean were excluded from respective analyses. All data are expressed as mean  $\pm$  standard error of the mean (SEM) unless otherwise stated. HDAC and HAT activities, mRNA abundance and plasma changes were analysed using two-way ANOVA with *Bonferroni* correction. Microbial data were analysed with non-parametric Kruskal-Wallis test, and Mann-Whitney U *post-hoc* comparisons. Body weight data were analysed with two-way ANOVA (repeated measures), comparing time (day), 'diet' (water, B-GOS® or acetate) and 'treatment' (saline, olanzapine), followed by post-hoc *Bonferroni* correction when significance was

reached. The level of significance was set at 0.05 and p-values and F-statistics are reported as exact values. Degrees of freedom were reported as  $F_{x-1, n-x}$  where  $x$  is the number of groups/comparisons, and  $n$  is the total number of animals (without  $\pm 3$ SD outliers). Thus, for the analysis of diet x treatment interaction,  $x=2$ ; and for *post hoc* comparisons between all groups,  $x=4$ .

### 3. Results

#### 3.1 Effect of B-GOS® intake on brain HDAC activity and gene expression

A significant overall effect of diet was observed in brain homogenate HDAC activity ( $F_{1,18}=5.974$ ,  $p=0.028$ ; Figure 1A), which was reduced (~20%) by B-GOS® in both saline and olanzapine-injected animals. No significant changes were observed for HAT activity in these samples (Figure 1B).

There was a significant overall effect of diet on cortical levels of HDAC-1 ( $F_{1,18}=5.396$ ,  $p=0.032$ ; Figure 1C) and HDAC-3 ( $F_{1,18}=6.745$ ,  $p=0.018$ ; Figure 1E) mRNAs. A significant 26% increase in HDAC-3 gene transcript levels was revealed between water/olanzapine and B-GOS®/olanzapine ( $F_{1,18}=10.696$ ,  $p=0.004$ ) groups. No significant effect of treatment or diet, or interactions, were observed for HDAC-2 and HDAC-4 (Figure 1D, F).

No significant overall effect of diet or treatment were observed for hippocampal HDAC-1 and HDAC-2 mRNAs (Figure 2). However, a significant diet x treatment interaction was observed for the abundance of hippocampal HDAC-3 mRNA ( $F_{1,20}=5.955$ ,  $p=0.024$ , figure 2C), where olanzapine reduced the expression of this gene relative to saline injected rats, in the absence of B-GOS® ( $F_{1,20}=11.710$ ,  $p=0.003$ ). There was also an overall effect of treatment where olanzapine reduced HDAC-3 mRNA compared to all saline injected animals ( $F_{1,20}=5.756$ ,  $p=0.026$ ). Ingestion of B-GOS® alone resulted in a 20% decreased trend in HDAC-3 mRNA ( $F_{1,20}=3.951$ ,  $p=0.061$ ). No effects of diet or diet x treatment interactions were observed for HDAC-4 mRNA, though its reduction with B-GOS® alone was significant compared to un-supplemented rats ( $p<0.05$ ).

### *3.2 Effect of B-GOS® and sodium acetate intake on plasma acetate concentrations*

Circulating concentrations of plasma acetate are presented as fold-changes relative to saline/water controls to correct for inter-cohort variation (Figure 3). A significant diet x treatment interaction was observed for plasma acetate concentrations, ( $F_{1,22} = 15.341$ ,  $p=0.001$ ). The elevation of this SCFA after either B-GOS intake or olanzapine injection returned to baseline concentrations when administered in combination (Figure 3A). Similarly, following either sodium acetate supplementation or the repeated injection of olanzapine circulating acetate concentrations were increased (Figure 3B). There were no significant overall effects of diet or treatment, though a diet x treatment interaction was observed ( $F_{1,20}=10.191$ ,  $p=0.005$ ) where the combined administration of acetate and olanzapine reduced plasma acetate concentrations.

### *3.3 Effect of sodium acetate intake on brain HDAC activity and gene expression*

There were no significant effects of diet, treatment or a diet x treatment interaction on the activity of HDAC in the brain homogenate following acetate supplementation (Figure 4A). However, the activity of HAT was significantly decreased by acetate supplementation (overall effect of diet,  $F_{1,19} = 6.501$ ,  $p=0.019$ ; Figure 4B). A treatment x diet interaction was observed in hippocampal HDAC-3 ( $F_{1,19} = 5.076$ ,  $p=0.038$ , Figure 5C) and HDAC-4 ( $F_{1,19} = 7.336$ ,  $p=0.015$ , Figure 5D) mRNA expression. Hippocampal HDAC-3 mRNA abundance was significantly elevated by acetate ( $F_{3,17} = 5.438$ ,  $p=0.032$ ) or olanzapine ( $F_{3,17}=7.757$ ,  $p=0.013$ ) compared to water or saline respectively, but when co-administered this increase returned to baseline. This effect was paralleled by HDAC-4 gene expression (figure 5D) where a significant increase was observed when acetate ( $F_{3,17}=5.236$ ,  $p=0.035$ ) and olanzapine ( $F_{3,17}=5.766$ ,  $p=0.028$ ) were administered alone, but when administered together, HDAC-4 expression returned to control levels.

### 3.4 Sodium acetate and olanzapine-induced weight gain

The effect of acetate supplementation and olanzapine injection on the body weight of female rats is shown in Figure 6. Within-subject repeated measures analysis showed a significant time x treatment interaction ( $F_{5,100}=4.289$ ,  $p=0.004$ ), where olanzapine increased body weight over the two weeks of repeated injection compared to the water/olanzapine group. There was also a time x diet interaction ( $F_{14,280}=3.658$ ,  $p=0.031$ ). However, no overall interaction between treatment x diet was observed suggesting that both olanzapine and acetate influenced body weight independently throughout the two week period. A time x diet x treatment interaction was not observed. There was an overall effect of treatment ( $F_{14,280}=7.027$ ,  $p=0.015$ , figure 6) compared to saline injected rats, particularly from day 7 ( $p<0.05$ ).

Area under the curve analyses further confirmed these findings where an overall effect of treatment ( $F_{14,280}=6.923$ ,  $p=0.016$ ; figure 4D) was observed, driven by the difference between water/saline and water/olanzapine groups ( $F_{14,280}=9.654$ ,  $p=0.006$ ).

### 3.5 Sodium acetate and NMDAR subunit expression in the cortex and hippocampus

Sodium acetate supplementation did not significantly alter mRNA expression of NMDAR subunits GluN1, GluN2A, GluN2B or GluN2C in the hippocampus or frontal cortex (Table 1).

### 3.6 Sodium acetate and faecal bacteria

The intake of sodium acetate did not significantly alter bacterial populations (Figure 7) including *Escherichia/Shigella spp.*, *Coprococcus Spp.*, *Oscillibacter Spp.*, *C. Coccoides Spp.*, *Rosbeuria Intestinalis Cluster*, and *clostridium XVIII cluster*. The administration of olanzapine did not affect faecal microbiota. The total bacterial load in each sample (16S DNA abundance) also remained constant between groups (data not shown).

## 4. Discussion

In the present study, we compared the central epigenetic effect of daily, oral administration of either the prebiotic, B-GOS®, or sodium acetate in rats, and investigated whether the intake of acetate alone, like B-GOS® (Kao et al., 2018), attenuated olanzapine-induced weight gain. The administration of B-GOS® inhibited central HDAC activity and increased cortical HDAC-1 and HDAC-3 expression. However, the ingestion of sodium acetate inhibited HAT, not HDAC, activity in the brain, and increased hippocampal levels of HDAC-3 and HDAC-4 mRNAs. Furthermore, acetate administration did not affect olanzapine-mediated weight gain, alter the expression of NMDAR subunits, or influence the abundance of faecal microbial genera. Together, these data suggest that B-GOS®-mediated benefits on central and peripheral physiology are not entirely, if at all, mediated by acetate produced from the microbial fermentation of this prebiotic.

### *4.1 B-GOS inhibits brain HDAC activity and alters HDAC gene expression*

The central effects of HDAC inhibitors have been evaluated in neurodegenerative and neurodevelopmental cognitive disorders (Qiu et al., 2017); and in psychiatric disorders, the SCFAs butyrate (Sun et al., 2016) and valproate (Guidotti et al., 2009) have been shown to elicit therapeutic benefits in depression, anxiety, and schizophrenia. A previous study has demonstrated that a large single oral dose of GTA (6g/kg) inhibited brain HDAC activity (Soliman and Rosenberger, 2011). This is consistent with our finding that B-GOS® administration, which increased the level of circulating acetate (Kao et al., 2018), decreased the activity of brain HDAC. Impaired HDAC activity pushes HDAC/HAT enzyme equilibrium to favour transcription of neuronal genes that may ameliorate deficits in plasticity and cognition (Abel and Zukin, 2008). In this regard, the pro-cognitive effects of B-GOS® in rats which is associated with increased expression of cortical NMDARs (Gronier et al., 2018), may have been preceded by HDAC inhibition.

The reduced HDAC activity was accompanied by increased cortical HDAC-1 and HDAC-3 mRNAs. An elevation in HDAC-1 mRNA was also observed after a repeated (28 day) GTA administration, though a reduction in HDAC-3 expression and unaltered HDAC activity was also reported (Soliman et al., 2012). In our study, it is possible that elevated HDAC-1 and HDAC-3 mRNAs was a homeostatic response to HDAC inhibition where gene transcription and/or mRNA stability was increased to replenish the inhibited HDAC pool. However, in this instance augmented HDAC-1 and HDAC-3 expression did not rescue impaired HDAC activity. It is unlikely that the reduction of HDAC function was associated with decreased hippocampal HDAC-4 mRNA (Figure 2D), since this occurred only in the absence of olanzapine, and HDAC activity was not affected by treatment. Taken together our data show that B-GOS® intake for 21 days, like a single dose of GTA (Soliman and Rosenberger, 2011), inhibited rat brain HDAC activity, but influenced HDAC gene expression in similar ways to long-term GTA administration (Soliman et al., 2012). This suggests that B-GOS®-derived acetate may not be the sole mediator of the epigenetic actions of this prebiotic. We confirmed this by examining whether the intake of acetate could replicate the central effects of B-GOS®. Since GTA has greater brain penetrance (Soliman and Rosenberger, 2011), and would not truly represent free acetate derived from B-GOS®, we used sodium acetate instead.

#### *4.2 Sodium acetate intake decreases brain HAT activity and alters hippocampal HDAC gene expression*

Oral sodium acetate supplementation at an appetite suppressing dose (500mg/kg) (Frost et al., 2014), led to a two-fold elevation in plasma levels, which is consistent with the intake of B-GOS® (Kao et al., 2018). The current study replicated the increased concentration of blood acetate after repeated olanzapine injection. However, unlike B-GOS®, increased peripheral acetate reduced HAT activity without affecting HDAC functioning. This observation is also in contrast to repeated GTA supplementation which, although did not impact on HDAC, increased HAT activity (Soliman et al., 2012). Hypothetically, the direction of change in HAT activity may be dependent on the uptake and steady-state concentration of acetate. Cellular

acetate is converted to acetyl-CoA which is utilized for lipogenesis or enters the citric acid cycle in the mitochondria. Cytosolic acetyl-CoA can also freely diffuse into the nucleus where it will be directly available to the HAT enzyme. However, current evidence suggests that histone acetylation is dependent on cytosolic citrate (replenished by the citric acid cycle in the mitochondria) which is converted to acetyl-CoA in the nucleus, and then used by HAT (Peleg et al., 2016; Wellen et al., 2009).

In this pathway, if a majority of acetyl-CoA directly enters the nucleus, excessive substrate concentrations may cause a homeostatic decrease in HAT activity via substrate inhibition. The greater brain penetrance of GTA (Soliman et al., 2012), may have produced sufficient acetate to increase mitochondrial metabolism and the pool of citrate, and allow the acetate-citrate-HAT pathway to predominate. This of course, is speculative and can only be confirmed with acetate dose response studies of HAT activity, and measures of brain metabolism *in vivo*. Parenthetically, increased brain acetate concentrations were not detected after repeated intake of sodium acetate (data not shown), or B-GOS® or GTA feeding (Gronier et al., 2018). This may be because free acetate entering the brain, is readily converted to acetyl-CoA (Hirose et al., 2009), conceivably at a rate that is sufficiently high to maintain fixed concentrations of this SCFA, particularly when ingested in small amounts over time. Changes in brain acetate concentrations therefore, are more likely to be detected shortly after its consumption, and perhaps in high concentrations. However, the entry of acetate into the brain might also be evinced through alterations in central NMDAR gene expression.

Daily sodium acetate supplementation did not affect brain NMDAR subunit expression (Table 1), unlike the administration of B-GOS® (Kao et al., 2018; Savignac et al., 2013), or GTA (Gronier et al., 2018). Furthermore, GTA increased NMDAR receptor function (Singh et al., 2016) which regulates acetate uptake into the brain (Hirose et al., 2009). Therefore, it is reasonable to suggest that the null effect of sodium acetate on brain NMDAR subunit expression was indicative of insufficient concentrations to trigger a receptor response, which would have lowered acetate entry into the brain. With regards to the above discussion, this

freely diffusing acetate may have accumulated in the cell nuclei. Further investigations are required to corroborate this theory.

The increased expression of hippocampal HDAC-3 and HDAC-4 mRNAs following acetate supplementation and olanzapine injection paralleled the concentration profiles of blood acetate (Figure 3). However, the olanzapine effect on these genes was not observed in the cohort of animals used in the B-GOS® experiments (Figure 1 and 2), in spite of the antipsychotic causing a two-fold elevation in circulating acetate. This differential effect of olanzapine on HDAC-3 and HDAC-4 mRNAs might be related to disparities in acetate metabolism between cohorts of rats. Inspection of plasma acetate concentration revealed that basal concentrations in water/saline rats (Figure 3) was more than half the concentration of that in the equivalent group in the B-GOS® experiment (Kao et al., 2018). It is possible that lower pre-existing concentrations of acetate does not hinder the effect of olanzapine on HDAC-3 and HDAC-4 gene expression, whereas higher circulating levels suppresses this effect. This is evinced when the combined administration of acetate and olanzapine does not alter the expression of these genes. Overall the present data, and those from investigations of GTA, suggest that direct acetate supplementation does not fully replicate the central actions of B-GOS® intake. Whether acetate derived from the lower gut elicits a different metabolic response to that of oral acetate which is absorbed in both the upper and lower gastrointestinal tract, and/or B-GOS® effects on the brain involve other SCFA derived from its fermentation, remains to be elucidated.

#### *4.3 Sodium acetate does not attenuate olanzapine-induced weight gain*

The second part of the current study examined whether acetate intake could attenuate olanzapine-induced weight gain in female rats. We report that acetate supplementation did not attenuate weight gain, rather it appeared to increase body weight, particularly over the last few days of the study (Figure 6). This observation is consistent with previous work reporting acetate from a high-fat diet mediates obesity and metabolic syndrome in rats (Perry et al.,



2016). In the present study, acetate was administered continuously via drinking water, which is in contrast to a single, daily bolus which causes a significant, transient rise in blood acetate concentration. It is possible that acetate intake throughout the day led to blood concentrations that favoured lipogenesis. The suppression of olanzapine-induced weight gain by B-GOS® (Kao et al., 2018) may have been the result of the prebiotic producing more acetate or, perhaps more likely, other SCFAs derived from prebiotic fermentation such as butyrate, contributing to energy homeostasis (Lu et al., 2016).

The current data also confirmed that olanzapine increased plasma acetate (Kao et al., 2018) which is in-keeping with the notion that this SCFA induces weight gain (Perry et al., 2016). However, given that B-GOS® also elevates circulating acetate, it can be assumed that this observation is unlikely to be integral to olanzapine-mediated weight gain, or to the suppression of these metabolic changes by a prebiotic. Although several investigations demonstrate altered lipid metabolism following olanzapine administration, to our knowledge, there are no other studies illustrating altered SCFAs after antipsychotics. Our findings therefore require corroboration.

#### *4.4 Sodium acetate does not alter gut bacteria*

In contrast to our previous prebiotic work (Kao et al., 2018), the current investigation did not observe changes in selected faecal bacteria, which suggests that their alteration by B-GOS intake was not influenced by acetate production. However, this null effect might also be because direct sodium acetate ingestion did not afford enteric concentrations that were comparable to those following prebiotic fermentation. Measures of acetate in colonic tissue and faecal matter would be required to address this possibility. The current study also observed that olanzapine administration did not affect several bacterial genera, which is consistent with our previous findings. However, others have shown that repeated olanzapine injections to female rats increased the abundance of the phylum *Firmicutes* in the gut (Davey et al., 2012). With the exception of *Escherichia/Shigella spp.*, *Bacteroidetes spp.*, and

*Bifidobacteria spp.*, all bacterial genera analysed in the present study belong to phylum *Firmicutes*. It is possible that the olanzapine-mediated changes in this phylum did not involve the bacteria measured in the present study. A similar interpretation might be offered to reconcile altered *Actinobacteria* (Morgan et al., 2014), with no changes in *Bifidobacteria spp.* after olanzapine administration (Figure 7). However, the earlier untargeted microbiome profiling studies used olanzapine doses and administration procedures that were different to each other, and to the current study. Clearly, a more accurate sequence-based evaluation of the gut bacteria is required to support or refute the present findings, and to identify the phyla/genera that are affected by olanzapine at the present dose used.

## **5. Conclusion**

The present study provides evidence to suggest that the ingestion of acetate as a way to augment cellular pools of acetyl-CoA and influence histone acetylation, does not replicate the epigenetic effects of the prebiotic B-GOS®. Furthermore, acetate feeding does not influence olanzapine-induced weight gain, an effect that is suppressed by this prebiotic. Although acetate is the main SCFA produced from microbial fermentation of most simple carbohydrates, our data suggest that mechanisms additional to acetate underlie the central and peripheral effects of B-GOS®.

## **Acknowledgements**

This study was funded by the Biotechnology, Biological Sciences Research Council (Grant Code: BB/I006311/1, awarded to PWJ Burnet). ACC Kao is a recipient of a Clarendon Graduate Scholarship (University of Oxford).

## **Conflicts of Interest**

The authors disclose no financial interest or conflict of interest. The B-GOS supplement and placebo were provided by Clasado Biosciences Ltd.

## References

- Abel, T., Zukin, R. S., 2008. Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. *Curr Opin Pharmacol* 8, 57-64.
- Arun, P., Madhavarao, C. N., Moffett, J. R., Hamilton, K., Grunberg, N. E., Ariyannur, P. S., Gahl, W. A., Anikster, Y., Mog, S., Hallows, W. C., Denu, J. M., Namboodiri, A. M., 2010. Metabolic acetate therapy improves phenotype in the tremor rat model of Canavan disease. *J Inher Metab Dis* 33, 195-210.
- Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., Cryan, J. F., 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A* 108, 16050-16055.
- Christiansen, C. B., Gabe, M. B. N., Svendsen, B., Dragsted, L. O., Rosenkilde, M. M., Holst, J. J., 2018. The impact of short-chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. *Am J Physiol Gastrointest Liver Physiol* 315, G53-G65.
- Davey, K. J., O'Mahony, S. M., Schellekens, H., O'Sullivan, O., Bienenstock, J., Cotter, P. D., Dinan, T. G., Cryan, J. F., 2012. Gender-dependent consequences of chronic olanzapine in the rat: effects on body weight, inflammatory, metabolic and microbiota parameters. *Psychopharmacology (Berl)* 221, 155-169.
- Deng, C., Lian, J., Pai, N., Huang, X. F., 2012. Reducing olanzapine-induced weight gain side effect by using betahistidine: a study in the rat model. *J Psychopharmacol* 26, 1271-1279.
- Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J. R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E., Bell, J. D., 2014. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nat Commun* 5, 3611.
- Gao, X., Lin, S. H., Ren, F., Li, J. T., Chen, J. J., Yao, C. B., Yang, H. B., Jiang, S. X., Yan, G. Q., Wang, D., Wang, Y., Liu, Y., Cai, Z., Xu, Y. Y., Chen, J., Yu, W., Yang, P. Y., Lei, Q. Y., 2016. Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat Commun* 7, 11960.
- Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., Reid, G., 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol* 14, 491-502.
- Gorisch, S. M., Wachsmuth, M., Toth, K. F., Lichter, P., Rippe, K., 2005. Histone acetylation increases chromatin accessibility. *J Cell Sci* 118, 5825-5834.
- Grimaldi, R., Cela, D., Swann, J. R., Vulevic, J., Gibson, G. R., Tzortzis, G., Costabile, A., 2017. In vitro fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. *FEMS Microbiol Ecol* 93.
- Gronier, B., Savignac, H. M., Di Meceli, M., Idriss, S., Tzortzis, G., DC, A., PWJ, B., 2018. Increased cortical neuronal responses to NMDA and improved attentional set-shifting performance in rats following prebiotic (B-GOS®) ingestion. *European Neuropsychopharmacology* 28: 211-224

Guidotti, A., Dong, E., Kundakovic, M., Satta, R., Grayson, D. R., Costa, E., 2009. Characterization of the action of antipsychotic subtypes on valproate-induced chromatin remodeling. *Trends Pharmacol Sci* 30, 55-60.

Hirose, S., Momosaki, S., Hosoi, R., Abe, K., Gee, A., Inoue, O., 2009. Role of NMDA receptor upon [14C]acetate uptake into intact rat brain. *Ann Nucl Med* 23, 143-147.

Kao, A. C., Spitzer, S., Anthony, D. C., Lennox, B., Burnet, P. W. J., 2018. Prebiotic attenuation of olanzapine-induced weight gain in rats: analysis of central and peripheral biomarkers and gut microbiota. *Transl Psychiatry* 8, 66.

Khosravi, Y., Seow, S. W., Amoyo, A. A., Chiow, K. H., Tan, T. L., Wong, W. Y., Poh, Q. H., Sentosa, I. M., Bunte, R. M., Pettersson, S., Loke, M. F., Vadivelu, J., 2015. *Helicobacter pylori* infection can affect energy modulating hormones and body weight in germ free mice. *Sci Rep* 5, 8731.

Krzystanek, M., Bogus, K., Palasz, A., Krzystanek, E., Worthington, J. J., Wiaderkiewicz, R., 2015. Effects of long-term treatment with the neuroleptics haloperidol, clozapine and olanzapine on immunoexpression of NMDA receptor subunits NR1, NR2A and NR2B in the rat hippocampus. *Pharmacol Rep* 67, 965-969.

Krzystanek, M., Bogus, K., Palasz, A., Wiaderkiewicz, A., Filipczyk, L., Rojczyk, E., Worthington, J., Wiaderkiewicz, R., 2016. Extended neuroleptic administration modulates NMDA-R subunit immunoexpression in the rat neocortex and diencephalon. *Pharmacol Rep* 68, 990-995.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) Method. *Methods* 25, 402-408.

Lu, Y., Fan, C., Li, P., Lu, Y., Chang, X., Qi, K., 2016. Short Chain Fatty Acids Prevent High-fat-diet-induced Obesity in Mice by Regulating G Protein-coupled Receptors and Gut Microbiota. *Sci Rep* 6, 37589.

Mathew, R., Arun, P., Madhavarao, C. N., Moffett, J. R., Namboodiri, M. A., 2005. Progress toward acetate supplementation therapy for Canavan disease: glyceryl triacetate administration increases acetate, but not N-acetylaspartate, levels in brain. *J Pharmacol Exp Ther* 315, 297-303.

Morel, F. B., Oozeer, R., Piloquet, H., Moyon, T., Pagniez, A., Knol, J., Darmaun, D., Michel, C., 2015. Prewaning modulation of intestinal microbiota by oligosaccharides or amoxicillin can contribute to programming of adult microbiota in rats. *Nutrition* 31, 515-522.

Morgan, A. P., Crowley, J. J., Nonneman, R. J., Quackenbush, C. R., Miller, C. N., Ryan, A. K., Bogue, M. A., Paredes, S. H., Yourstone, S., Carroll, I. M., Kawula, T. H., Bower, M. A., Sartor, R. B., Sullivan, P. F., 2014. The antipsychotic olanzapine interacts with the gut microbiome to cause weight gain in mouse. *PLoS One* 9, e115225.

Nghia, N. A., Hirasawa, T., Kasai, H., Obata, C., Moriishi, K., Mochizuki, K., Koizumi, S., Kubota, T., 2015. Long-term imipramine treatment increases N-methyl-d-aspartate receptor activity and expression via epigenetic mechanisms. *Eur J Pharmacol* 752, 69-77.

Peleg, S., Feller, C., Ladurner, A. G., Imhof, A., 2016. The Metabolic Impact on Histone Acetylation and Transcription in Ageing. *Trends Biochem Sci* 41, 700-711.

Perry, R. J., Peng, L., Barry, N. A., Cline, G. W., Zhang, D., Cardone, R. L., Petersen, K. F., Kibbey, R. G., Goodman, A. L., Shulman, G. I., 2016. Acetate mediates a microbiome-brain-beta-cell axis to promote metabolic syndrome. *Nature* 534, 213-217.

Qiu, X., Xiao, X., Li, N., Li, Y., 2017. Histone deacetylases inhibitors (HDACis) as novel therapeutic application in various clinical diseases. *Prog Neuropsychopharmacol Biol Psychiatry* 72, 60-72.

Rajpal, D. K., Klein, J. L., Mayhew, D., Boucheron, J., Spivak, A. T., Kumar, V., Ingraham, K., Paulik, M., Chen, L., Van Horn, S., Thomas, E., Sathe, G., Livi, G. P., Holmes, D. J., Brown, J. R., 2015. Selective Spectrum Antibiotic Modulation of the Gut Microbiome in Obesity and Diabetes Rodent Models. *PLoS One* 10, e0145499.

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

Singh, S., Choudhury, A., Gusain, P., Parvez, S., Palit, G., Shukla, S., Ganguly, S., 2016. Oral acetate supplementation attenuates N-methyl D-aspartate receptor hypofunction-induced behavioral phenotypes accompanied by restoration of acetyl-histone homeostasis. *Psychopharmacology (Berl)* 233, 1257-1268.

Skrede, S., Ferno, J., Bjorndal, B., Brede, W. R., Bohov, P., Berge, R. K., Steen, V. M., 2012. Lipid-lowering effects of tetradecylthioacetic acid in antipsychotic-exposed, female rats: challenges with long-term treatment. *PLoS One* 7, e50853.

Soliman, M. L., Rosenberger, T. A., 2011. Acetate supplementation increases brain histone acetylation and inhibits histone deacetylase activity and expression. *Mol Cell Biochem* 352, 173-180.

Soliman, M. L., Smith, M. D., Houdek, H. M., Rosenberger, T. A., 2012. Acetate supplementation modulates brain histone acetylation and decreases interleukin-1beta expression in a rat model of neuroinflammation. *J Neuroinflammation* 9, 51.

Sun, J., Wang, F., Hong, G., Pang, M., Xu, H., Li, H., Tian, F., Fang, R., Yao, Y., Liu, J., 2016. Antidepressant-like effects of sodium butyrate and its possible mechanisms of action in mice exposed to chronic unpredictable mild stress. *Neurosci Lett* 618, 159-166.

Tarazi, F. I., Baldessarini, R. J., Kula, N. S., Zhang, K., 2003. Long-term effects of olanzapine, risperidone, and quetiapine on ionotropic glutamate receptor types: implications for antipsychotic drug treatment. *J Pharmacol Exp Ther* 306, 1145-1151.

van der Zwaal, E. M., Janhunen, S. K., la Fleur, S. E., Adan, R. A., 2014. Modelling olanzapine-induced weight gain in rats. *Int J Neuropsychopharmacol* 17, 169-186.

Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., Thompson, C. B., 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* 324, 1076-1080.

Williams, S., Chen, L., Savignac, H. M., Tzortzis, G., Anthony, D. C., Burnet, P. W., 2016. Neonatal prebiotic (BGOS) supplementation increases the levels of synaptophysin, GluN2A-subunits and BDNF proteins in the adult rat hippocampus. *Synapse* 70, 121-124.

## Figure legends

**Figure 1.** Administration of B-GOS and olanzapine on HDAC and HAT activities in cortex/hippocampus homogenates (**A, B**) and gene expression in the frontal cortex (**C-F**). Oral administration of B-GOS® (0.5g/kg/day) to adult female rats reduced activity of (**A**) HDAC, but not (**B**) HAT, and influenced cortical mRNA levels of HDAC1 and HDAC3, but not HDAC2 and HDAC4 (**C-F**). Animals were fed B-GOS® 7 days prior to, and throughout, the two-week administration of olanzapine. Results are expressed as mean  $\pm$  SEM for each group (n = 5/group). \*p<0.05 overall effect of diet; #p<0.05

**Figure 2.** Administration of B-GOS and olanzapine on hippocampal HDAC mRNA expression. An overall effect of olanzapine was observed on HDAC3 (**C**). There was no significant effect of B-GOS. Results are expressed as mean  $\pm$  SEM for each group (n = 6 for water/saline, water/olanzapine; n=5 for acetate/saline, acetate/olanzapine). \*overall effect of treatment, p<0.05.

**Figure 3.** Effect on plasma acetate concentrations after (**A**) B-GOS® and (**B**) sodium acetate supplementation. Sodium acetate (500mg/kg) and olanzapine (10mg/kg) were administered to female rats via the drinking water and by injection, respectively. Animals ingested acetate 7 days prior to, and throughout, the two-week administration of olanzapine. Results are expressed as mean fold-change  $\pm$  SEM for each group relative to saline/water controls. (**A**: n = 6/group; **B**: n = 6 for water/saline, water/olanzapine; n=5 for acetate/saline, acetate/olanzapine). \*p<0.05 compared to water/saline.

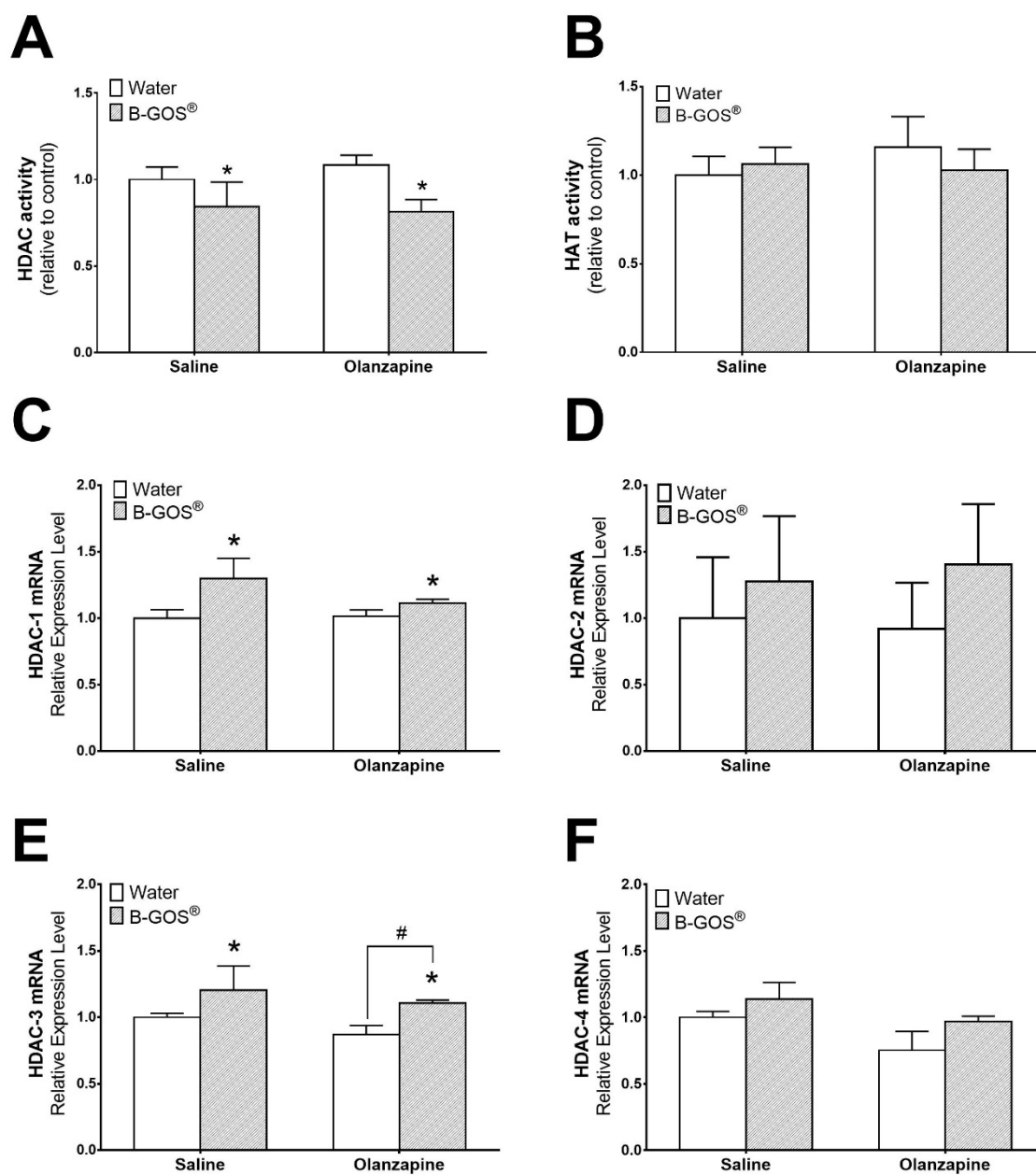
**Figure 4.** Administration of sodium acetate and olanzapine on HDAC and HAT activities in (**A, B**) cortex/hippocampus homogenates and (**C-F**) gene expression in the frontal cortex. Oral administration of sodium acetate (0.5g/kg/day) to adult female rats did not affect HDAC, but reduced HAT (**B**) activity, and did not influence cortical mRNA levels of HDAC1-4 (**C-F**). Results are expressed as mean  $\pm$  SEM for each group (n = 6 for water/saline; n=5 for water/olanzapine, acetate/olanzapine, acetate/saline). \*p<0.05.

**Figure 5.** Administration of sodium acetate olanzapine on hippocampal HDAC1-4 mRNA expression. Acetate or olanzapine alone did not affect (**A**) HDAC-1 or (**B**) HDAC-2, but increased (**C**) HDAC3 and (**D**) HDAC-4. Results are expressed as mean  $\pm$  SEM for each group (n = 6 for water/saline; n=5 for water/olanzapine, acetate/olanzapine, acetate/saline). \*p<0.05, compared to water/saline group.

**Figure 6.** Effect of Sodium Acetate on Olanzapine-Induced Weight Gain. **(A)** Percentage weight gain was calculated from the day before olanzapine administration. **(B)** Area under the Curve. Results are expressed as mean  $\pm$  SEM for each group (n=6/group). \*p<0.05 compared to water/saline.

**Figure 7.** No effect of sodium acetate on faecal bacteria composition. Results are expressed as fold-change relative to the saline/water group, and presented with medians [first to third quartile]. Open boxes = water; shaded boxes = sodium acetate. (n=6/group). \*p<0.05 compared to saline/water.

**Figure 1**





**Figure 2**

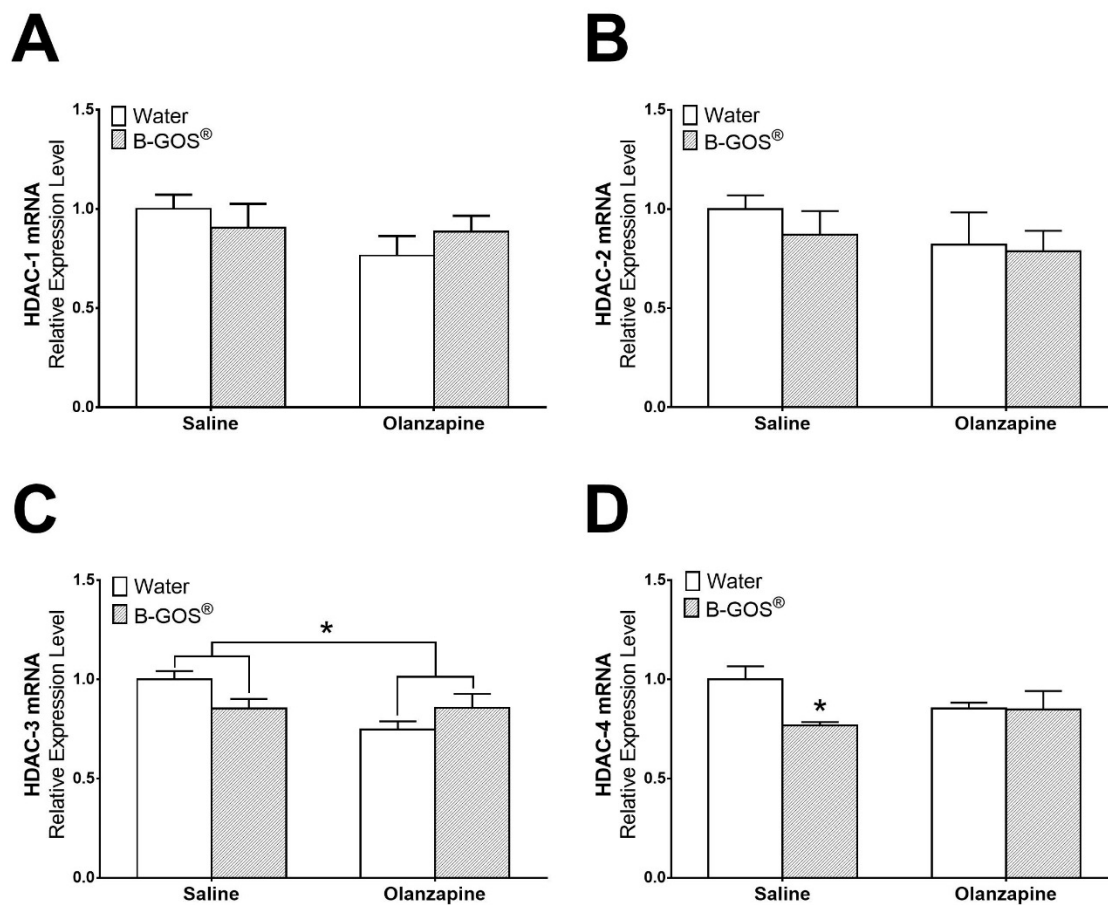
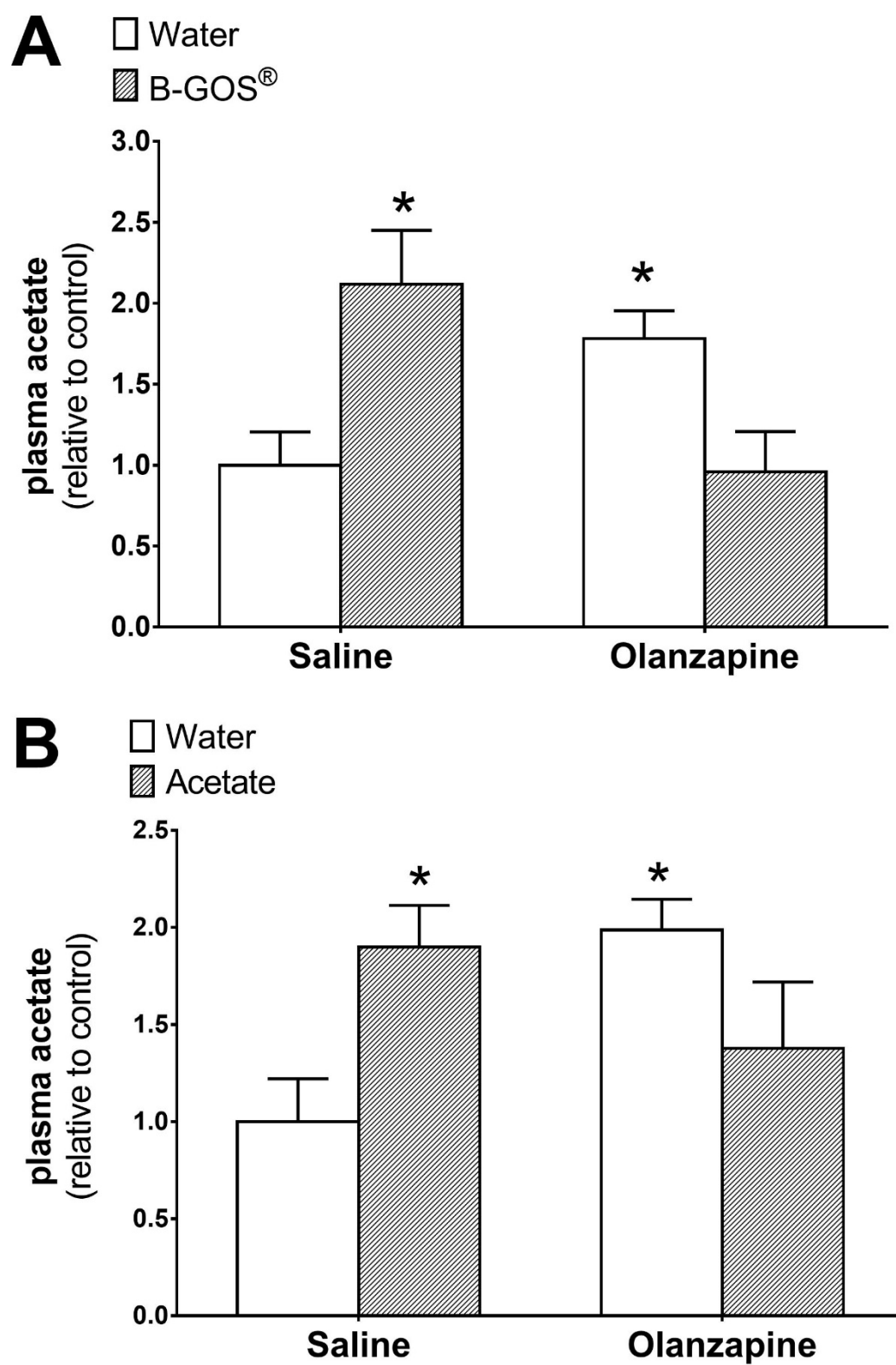
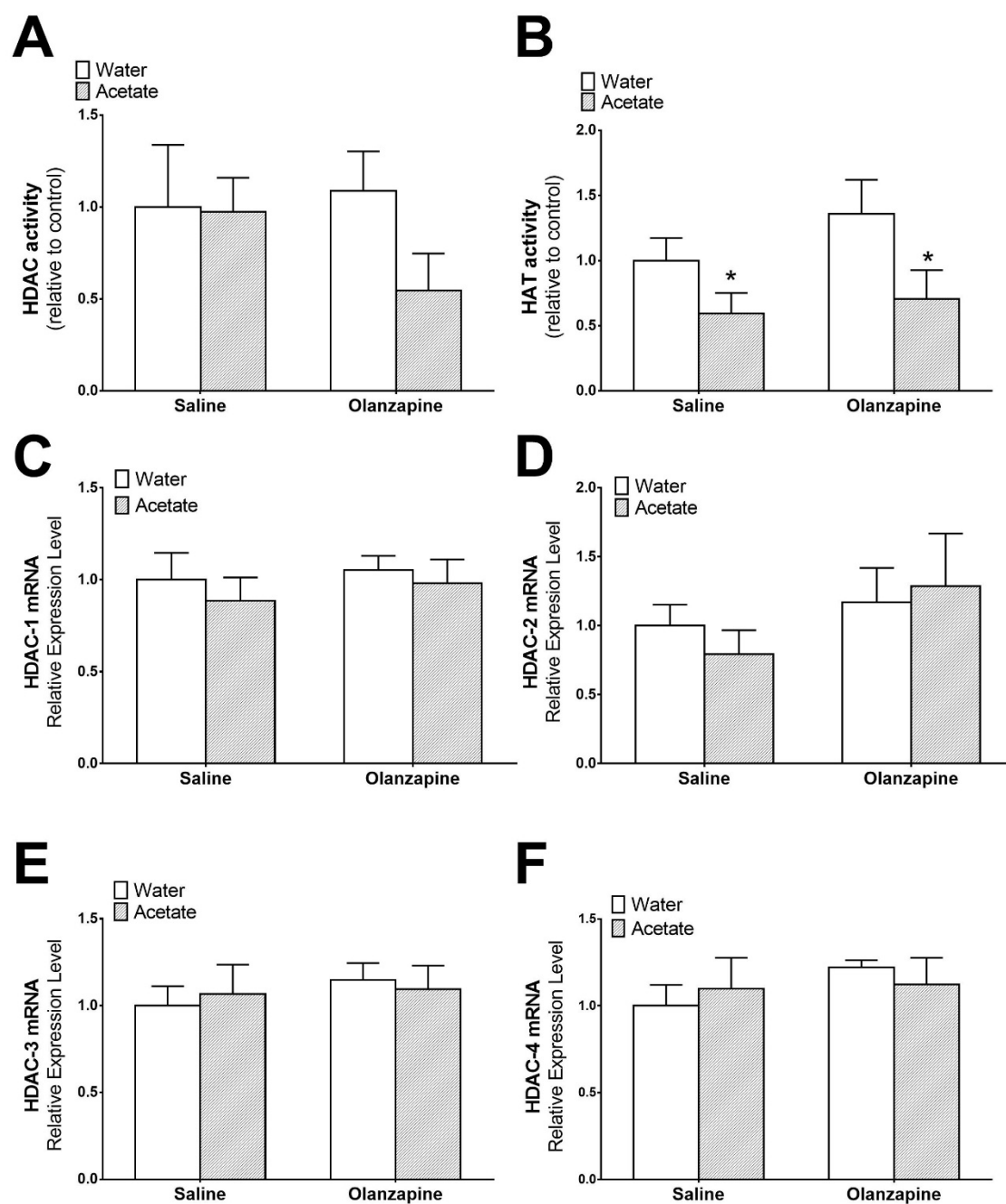


Figure 3



**Figure 4**



**Figure 5**

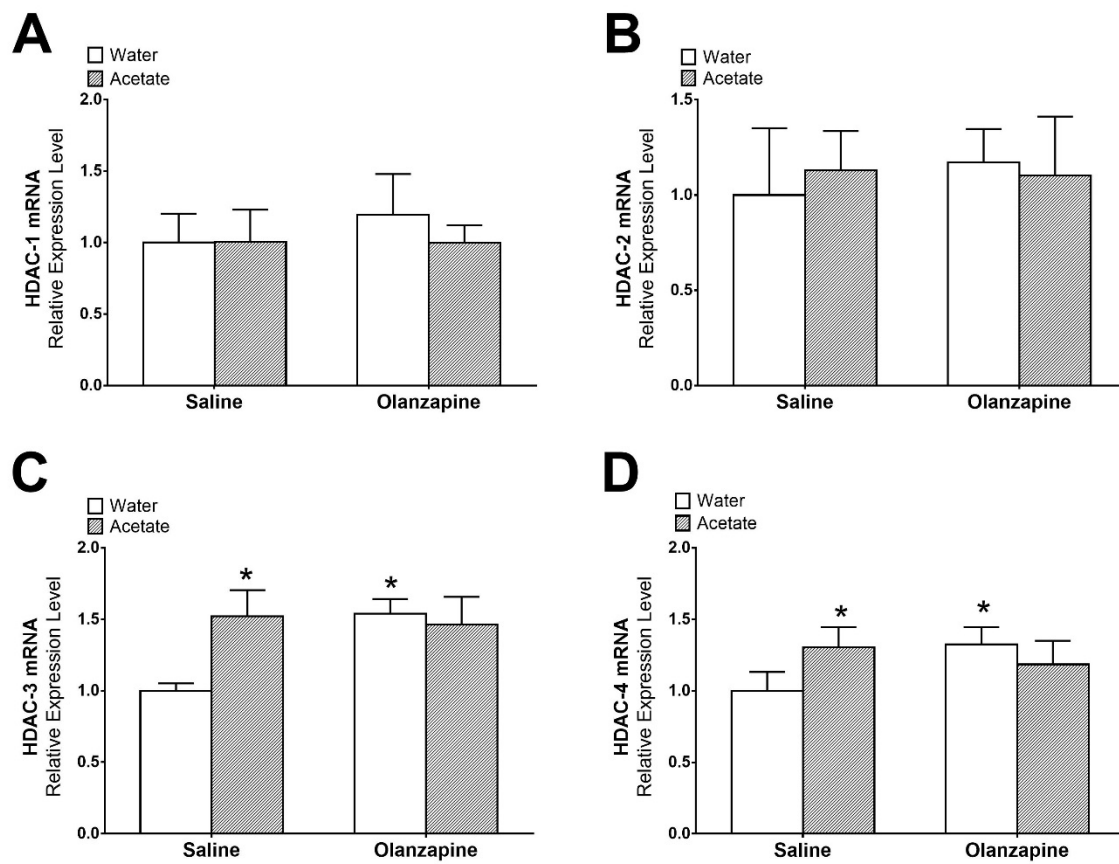
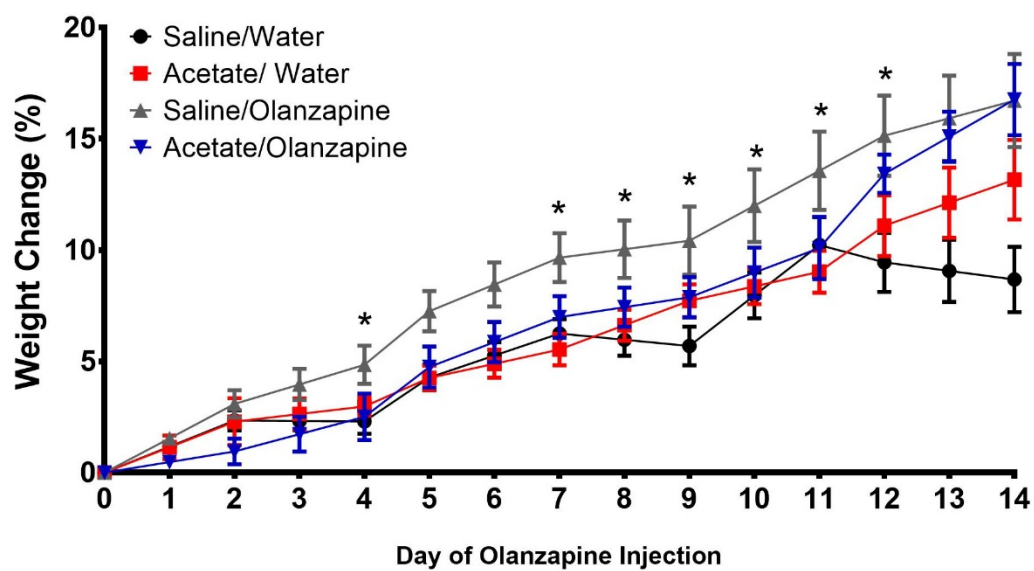
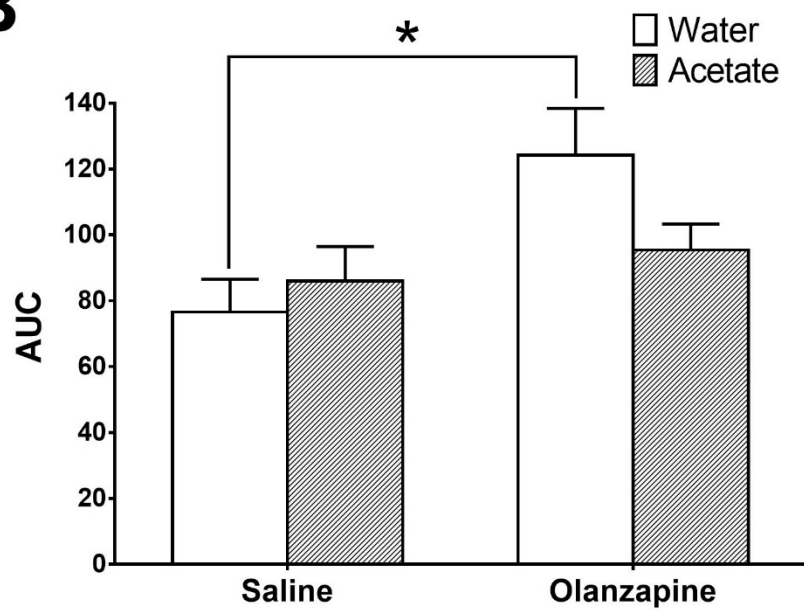


Figure 6

**A**



**B**



**Figure 7**

