The Effects of Dietary Probiotic Administration on Anxiety and Cognition.

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'Above all, don't fear the difficult moments – the best comes from them'

– Rita Levi-Montalcini

Thesis Summary

This thesis explores the effects of dietary probiotic administration in anxiety-like behaviour and cognitive functioning. Desbonnet *et al* (2008) reported degradation of serotonin in the frontal cortex following dietary probiotic administration implicating cognition and anxiety-like behaviour. Therefore, the effects of direct serotonin manipulation on behaviour were examined in parallel to probiotic treatment.

Identification of a task reliant on both frontal lobe and serotonergic functioning lead to the use of the probabilistic reversal learning task. Probiotic treatment did not produce effects on this task. Exploration of the effect of probiotic treatment on a watermaze reversal learning paradigm, known to be reliant on frontal lobe and hippocampal functioning indicated improvements with probiotic treatment. Further examination of this improvement in memory was explored in a novel object discrimination task and found to be significantly improved following probiotic treatment. These results produce clear indication the probiotic treatment improves aspects of learning and memory.

The effects of dietary probiotics on anxiety-like behaviour were also explored. The results from the behavioural data indicated that these tests did not assess the same facet of emotional behaviour therefore conclusions could not be drawn on the effects of probiotic on anxiety-like behaviour.

Examinations of the effects of probiotics at a cellular level were conducted through the use of H^1 nuclear magnetic resonance spectroscopy. Results from this illustrate distinct alterations in the metabolite profile following probiotic treatment. The finding from this study provide a new rational for the further exploration of the effects of probiotic treatment on behaviour and cognition.

Contents

General Introduction

1.1. Summary

The introduction of this thesis discusses the bacterial composition of the human gastrointestinal tract and correlations between microbiotal perturbations and psychiatric illness. The correlations are discussed in the context of the brain-gut axis and current evidence on the potential routes of action between the two presented. The effects of manipulations to the bacterial profile of the gut using probiotics and the consequences in central nervous system are explored, thus explaining the rationale for the current thesis.

1.2. The Human Gastrointestinal Tract

The human intestinal microflora consists of a diverse range of microbial species (Howarth & Wang, 2013). The colonisation of the intestine begins postnatally when ~~vaginal~~ delivery exposes the infant to a diverse range of bacteria giving the initial microbial profile a maternal signature (Cryan & Dinan, 2012; Palmer *et al.,* 2007). Resident or commensual bacteria colonise the gut and a relatively stable composition is established at about 2 years of age (Palmer *et al*, 2007). A fully developed adult human intestine contains nearly 100 trillion bacteria (Gill *et al,* 2006) with over 1000 bacterial species and approximately 7000 strains (Ley *et al,* 2006). This number of bacterial cells is equal to 10-100 times more eukaryotic cells than in the human body (Gill *et al,* 2006). This diverse ecosystem has been referred to as the forgotten organ (O'Hara & Shanahan, 2006) as it is fundamental in the development of adaptive immune system responses (Hooper & Macpherson, 2010; Bäckhed *et al,* 2005), endocrine system function (Sudo *et al,* 2004), intestinal barrier homeostasis (Husebye *et al,* 2001), as well as for the appropriate structural development of the smooth muscle layers required for gastrointestinal tract motility (Berg, 1996; Stappenbeck *et al,* 2002).

Although established in early life the composition of the gut microbiota can be influenced over the course of a lifetime, causes of perturbations of the microflora include diet, infection, stress and exposure to medications, such as antibiotics (Koenigsknecht & Young, 2013; O'Mahony *et al,* 2009; Scribano & Prantera, 2013; Wu *et al,* 2011). Perturbation in the natural balance of the gut microbiota has been associated with numerous illnesses including irritable bowel syndrome (Dai *et al,* 2013), inflammatory bowel disease and obesity (Mondot *et al,* 2013). Despite exposure to environmental factors over the course of a lifetime, the intestinal microbiome tends to defer to its initial composition, which was established in infancy (Forsythe *et al,* 2010). This indicates that while environmental factors influence the indigenous populations of gut microbiota during a lifetime, the preferential state is that which was established in infancy. Furthermore, the composition of the human microbiota can be categorised into one of 3 main categories, partially determined by genetics (Gulati *et al,* 2012) but strongly associated with long-term diets (Wu *et al,* 2011). Three main bacterial enterotypes have been identified, with each characterized by the predominance of a single microbial genus: *Bacteriodes, Prevotella* and *Ruminococcus*. *Bacteroides* are strongly associated with a diet high in animal fats and proteins as common in western culture. *Prevotella* is predominantly associated with carbohydrate-based diets common in agrarian societies (Wu *et al,* 2011) while *Ruminococcus* are largely found in the intestinal tract of cows and sheep where their diet is primarily plant based (Devillard *et al,* 2004). Acute changes in the diet of healthy volunteers from high in carbohydrates to high in fats and vice versa, has not been shown to produce any distinct changes in bacterial enterotypes (Wu *et al,* 2011). However, the complexity and stability of other microbial genus predominant in the human gastrointestinal tract (which include *bifidobacterium* and *lactobacilis)* are considered 'transient' (Bercik *et al,* 2012). Increases in their populations are associated with the ingestion of certain foods and have been shown to be sensitive to environmental factors, including exposure to antibiotics (Bercik *et al,* 2012). The presence of these bacterial strains has been associated with inhibition of listerial infections *in vivo*. This is largely due to competition between these indigenous bacteria with pathogens for nutrients and receptors

and the production of antimicrobial proteins (Kopp-Hoolihan 2001; Sekirov *et al,* 2010). The prevention of pathogenic microbial colonisation is only one of the beneficial functions of *lactobacilli* and *bifidobacterium*. Other functions include the protection of the intestinal barrier defence system, metabolism of carcinogenic substances and lowering of potentially neurotoxic components (Holzapfel *et al,* 1998). It is the beneficial effects of these bacteria on the host when ingested in adequate amounts, that classifies them as *'probiotics'* (Dinan & Quigley, 2011).

Studies examining the prevalence of gastrointestinal dysfunction, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), have found a high comorbidity with psychiatric disorders. Indeed, approximately 30% of patients with major depressive disorder (MDD) are reported to have IBS (Logan & Katzman, 2005). The incidence of depression in sufferers of Crohn's disease, a type of IBD, both preceding and following diagnosis, is shown to be greater than chance, with relapse correlated with depressive episodes (Logan & Katzman, 2005). Reduced populations of *lactobacilli* are evident in suffers of Crohn's disease and other forms of IBD (Ott *et al,* 2004). Of course, the incidence of depression in these patients may not be surprising given the nature of the symptoms. However, emotional disturbances can have physical effects on microbial compositions. For example, stress has been shown to reduce *lactobacillis* populations (Lutgendorff *et al,* 2008). It is the existence of this comorbidity of depressive symptoms and gastrointestinal disorders that have lead to the proposition that probiotic treatment may ameliorate depressive symptoms. Empirical evidence from clinical trials is limited in this area - as the first hypothesis to suggest that probiotics may be an adjuvant therapy to depression was suggested by Logan & Katzman in 2004. Much of the empirical evidence to date has focused on the preclinical arena.

A double-blind placebo controlled trial using one hundred and twenty-four healthy volunteers found that after 3 weeks of consuming a *lactobacillus* and *bifidobacterium* containing yogurt , the participants who originally scored in the bottom third on mood and cognition tests were significantly improved compared with controls (Benton *et al,* 2007). A more recent clinical study

again using strains of *lactobacillus* and *bifidobacterium*in a double-blind placebo-controlled trial, and found that 30 days of consumption had beneficial psychological effects on healthy volunteers as measured by the Hopkins Symptom Checklist, the Hospital Anxiety and Depression Scale and Coping Checklist (Messaoudi *et al,* 2011). Chronic fatigue syndrome is frequently associated with anxiety and gastrointestinal irregularity, including lower levels of *bifidobacterium* and small intestine bacterial overgrowth (Logan *et al,* 2003). Treatment with a strain of *lactobacillus* was shown to increase intestinal *bifidobacterium* and *lactobacillus* populations after 2 months in chronic fatigue syndrome sufferers, as well as reducing anxiety as measured by the Beck Depression and Anxiety Inventories (Rao *et al,* 2009).

The evidence for the mutual interaction of the gut and brain in psychiatric disorders at a clinical level is limited. However, recent years have seen a vast increase in the number of studies examining this interaction at a pre-clinical level, these studies will be considered in the next section.

1.3. The Brain-Gut Axis

Much of the evidence for the existence of a gut-brain axis stems from studies on stress and microbiota. One of the initial studies focused on the development of hypothalamic-pituitary-adrenal (HPA) axis reactivity in mice raised in a germ-free (GF) environment. This original study by Sudo *et al* (2004) demonstrated that postnatal exposure to microbes at an early developmental stage is required for the development of a functional HPA axis response to stress. The exaggerated HPA response in GF mice, as indicated by higher concentrations of plasma adrenocorticotrophic hormone (ACTH) and corticosterone and lower levels of brain derived neurotrophic factor (BDNF), a key neurotrophin in neuronal growth and survival, in the cortex and hippocampus, was shown to be partly corrected following reconstitution of faeces of control animals at an early stage (Sudo *et al,* 2004). However, later stage reconstitution did produce these results. Decreased NMDA (2A) receptor expression was also found in the hippocampus and cortex in GF mice compared with

controls (Sudo *et al,* 2004). This landmark study prompted further research into the effects of microbiota on CNS function.

Several studies have used maternal separation as a model of stress (Gareau *et al,* 2007; O'Mahony 2009). These have confirmed that alteration to HPA activity in response to stress directly affects microbiota of the gut. Furthermore this effect is more pronounced during neonatal development. A study by O'Mahoney (2009) demonstrated that maternal separation for three hours per day from postnatal day (PND) two, to PND twelve, caused perturbations in the gut microbiota and elevated HPA-axis function in adulthood. This study further revealed that maternal stress also increased systemic immune responses, increased anxiety and visceral hypersensitivity. Rhesus monkeys have been shown to have a substantial decrease in l*actobacilli* populations following three days of maternal separation; this then returned to normal levels by day seven (Bailey & Coe, 1999). Probiotic treatment was found to normalise the perturbation in corticosterone following maternal separation in rats providing evidence for the bidirectional nature of the relationship between the HPA axis and gut microflora (Gareau *et al,* 2007). Further to this, results from a study by Desbonnet *et al* (2010) indicated that the behavioural, neurochemical and immune system alterations caused by maternal stress could be normalised by the probiotic *Bifidobacterium Infanis.* Maternal stress has also been shown to negatively affect intestinal permeability (Garcia-Rodenas *et al*, 2006). The primary impact of increased intestinal permeability is directly on the enteric nervous system (ENS), as this allows for greater intestinal microbiotal translocation, which in turn, may affect immune and neuronal cells of the ENS (Soderholm & Perdue, 2001). It has also been shown that this 'leakiness' results in increased circulating levels of immunomodulatory bacterial cell wall components such as lipopolysaccharide (Soderholm & Perdue, 2001).The effects of stress on the composition of gut microbiota are not limited to the postnatal period. Chronic stress in adulthood has been shown to alter the relative abundance of Clostridium in the caecum of adult mice following chronic psychosocial stress (Bailey *et al,* 2011). Furthermore, alterations were also found in immune system function. Chronic stress increased levels of interleukin-6 (IL-6) and chemokine CCL2, indicative of

immune system activation (Bailey *et al,* 2011). These studies indicate that effects of stress have consequences beyond the HPA axis, affecting microbiotal composition of the gut, integrity of intestinal barrier allowing this altered microbiota of the gut to impact upon the ENS. Furthermore, correlations have been found between alterations in gut microbiota and immune system activation (Ashraf & Shah, 2014). Given the results from the studies considered previously, it is evident that a complex and mutually interactive relationship exists between the gut microflora and the HPA axis.

A developing area of research has focused on the effects of microbiotal pathogens on stress circuitry (Goehler *et al,* 2005; Lyte *et al,* 2006). Evidence from this field has shown that changes to the gut microbiota via infection with food-born pathogens, have direct effects on peripheral and central nervous system activation (Goehler *et al,* 2005; Lyte *et al,* 2006). Examination of *c-fos* levels in the vagal sensory nerve following infection with *C. rodentium* and *Campylobacter jejuni* indicated that there was significant activation of vagal sensory nerves, independent of immune activation (Goehler *et al,* 2005; Lyte *et al,* 2006). *C-fos* levels were also found to be significantly higher in several brain regions including the central amygdala (CEA), hypothalamic paraventricular nucleus (PVN) and the medial prefrontal cortex (mPFC) (Gaykema *et al*, 2004; Goehler *et al,* 2005). These areas are typically associated with vagal nerve stimulation (Naritoku *et al,* 1995). Infection with *E. Coli.* has been shown to produce similar activation of the PVN in GF mice (Sudo *et al,* 2004). Vagotomy studies have confirmed that the vagus nerve is a mediator in CNS activation found following infection with *Salmonella Typhimurium* in the rat (Wang *et al,* 2002). However, contrasting evidence has shown that vagotomy prior to infection with *Trichuris muris* did not prevent the increase in anxiety levels associated with infection in control animals (Bercik *et al,* 2010), indicating that other pathways, independent of the vagus nerve enable the CNS response to microbiotal infection and this may dependent upon the specific microbiotal agent. This research provides clear evidence for bottom-up signalling where microbiota agents have direct impact on peripheral and central nervous system function. Considered with the evidence from maternal separation studies, it is clear that interactions between the gut microflora and CNS are bidirectional and utilise multiple pathways

including the HPA axis, vagus nerve and immune system. The behavioural effects of infection with food-born pathogens have indicated that anxiety and depressive symptoms are impacted by gut microbiota. Subclinical infection with *C. Jejuni* was found to increase anxiety-like behaviour in the elevated plus maze (EPM) in the absence of immune system activation (Lyte *et al,* 1998). Similar results have been reported showing that infection with the same pathogen increases anxiety-like behaviour in the hole-board test with activation of the brain regions associated with anxiety-like behaviour: the paraventricular (PVN), basolateral amygdala (BLA), anterior cingulate, medial prefrontal cortex (mPFC) and bed nucleus of the striaterminalis (BST). Furthermore, *c-fos* expression in the BST predicted the degree of anxiety-like behaviour (Goehler *et al,* 2008). *C.Rodentium* also increased anxiety-like behaviour in this test in the absence of immune system activation, indicating that behavioural changes are unlikely to be a result of inflammation-related stress. Further to this, analysis of *c-fos* expression in vagal sensory neurons indicated that the behavioural impact of infection is mediated by vagal sensory neurons (Lyte *et al,* 2006).

Exposure to antibiotics has been shown to increase exploratory behaviour and reduce anxiety-like behaviour in step-down and light-dark preference tests when given in combination with an antifungal agent over a seven day period (Bercik *et al,* 2010). Furthermore, after a 2 week washout period, normalisation of intestinal microbiota was accompanied with normalisation of behaviour. Treatment with the antibiotic and antifungal agent did not have any behavioural impact in GF mice, thus providing evidence for the conclusion that the anxiolytic properties of antibiotics are mediated by alterations in gut microflora (Bercik *et al,* 2010).

Irrespective of the route of signalling between the gut to the brain, more and more evidence is indicating a distinct alteration in CNS functioning following alteration of the gut microbiota. Hippocampal serotonergic concentrations, and its metabolite 5-hydroxyindolacetic acid (5-HIAA), were found to be elevated in mice raised in a germ-free environment in comparison with conventional colonised control mice (Clarke *et al,* 2013). Striatal 5-HT metabolism was found to be

elevated in GF mice (Diaz-Heijtz *et al,* 2011). Furthermore, plasma tryptophan levels in male GF mice were found to be significantly higher than that of control mice (Diaz-Heijtz *et al,* 2011). Post-weaning colonisation of GF mice attenuated the behavioural aberrations in GF mice, but neurochemical function remained altered despite the fact that plasma tryptophan levels returned to baseline (Clarke *et al,* 2012). This provides further support for the theory that there is a critical window in postnatal development, during which, gut microbiotia composition plays in important role in the development of other systems such as central serotonergic signalling, BDNF expression and HPA axis activity. Another study that examined the effects of commensual bacteria compositions on neurochemistry found increased hippocampal BDNF mRNA levels and hippocampal 5-HT1a receptor expression, and decreased NMDA 2b receptor mRNA expression in the amygdala (Neufeld *et al,* 2011a). The effects on BNDF levels found in this study is in contrast to reports by Sudo *et al,* 2004. In the study by Neufeld *et al,* 2011a, the alteration to hippocampal BDNF levels was only found in male GF mice where female mice demonstrated quantitative but not a significant increase, indicating that sex may play an important role in BDNF expression. From a behavioural perspective, several studies have reported significantly lower levels of anxiety-like behaviour in a range of tests, namely EPM, open field test (OFT), and a light/dark test where anxious behaviour is thought to result in reduced exploration in illuminated area of the maze (Diaz-Heijtz *et al,* 2011; Neufeld *et al,* 2011; Clarke *et al,* 2012). Reconstitution of the gut microbiota in early life was shown to normalise behaviour in the EPM and some aspects of behaviour in the light/dark test (Diaz-Heijtz *et al,* 2011; Clarke *et al,* 2012). However, reconstitution in later life did not attenuate the aberrant behaviour in the EPM (Neufeld *et al,* 2011). Again this is further evidence that interactions in gut-brain signalling during postnatal development are key in CNS development.

Given the abundance of evidence for a gut-brain axis involving several possible signalling systems, including the immune, neurochemical, endocrine and vagal systems, current research has focused on the effects of probiotic administration on these faculties and CNS function.

1.4. Probiotics and the Brain-Gut Axis

Probiotic administration can ameliorate some of the physiological effects of stress. Several strains of *Lactobacillus* improve the integrity of the intestinal mucosal barrier preventing translocation of bacteria in stressed animals (Zareie *et al,* 2006). It is important to note that this study also showed treatment with probiotics in control animals did not significantly alter intestinal barrier integrity (Zareie *et al,* 2006). The effects of probiotic treatment on the HPA axis and immune function were the focus of a recent study by Ait-Belgnaoui *et al* (2012). *Lactobacillus farciminis* prevented hyper-permeability of the intestinal mucosal barrier as indicated by lower lipopolysaccharide translocation. Other findings from this study demonstrated that probiotic treatment prevents the HPA axis response to acute stress in rats. Plasma ACTH and corticotesterone were lower in probiotic treated stressed rats. Hypothalamic CRF expression was also lower. Neuroinflammation was also lower in these rats with reduced proinflammatory cytokine mRNA levels in the PVN compared with stressed vehicle treated rats (Ait-Belgnaoui *et al* 2012). Treatment with *Bifidobacterium infantis* produced similar results in GF mice, where restraint-stress induced increased plasma levels of ACTH and corticotesterone in control animals and no significant increase was found in probiotic treated control animals (Sudo *et al,* 2004). *C-fos* activation in the PVN was also induced 6 hours after inoculation with *B. Infantis,* before any changes in peripheral cytokine had been established. This result indicates that signalling pathways from the gut to the brain following treatment with *B. Infantis* are independent of immune system modulation. Conversely, other studies have shown significant suppression of proinflammatory cytokine release following *B. Infantis* administration (Desbonnet *et al,* 2008). As previously discussed, alterations in HPA axis functioning caused by maternal stress in mice, evidenced by increased corticotesterone levels, have been attenuated with the administration of *Lactobacillis* strains (Gareau *et al,* 2007). The use of probiotics in the maternal separation model

of stress has demonstrated the normalisation effects of *B. Infantis* on peripheral anti-inflammatory cytokine (IL-10) levels (Desbonnet *et al,* 2010).

1.5. Probiotic and Behaviour

Given the volume of evidence presented for an effect of probiotic administration on central and peripheral immune system activation, HPA axis functioning and intestinal wall integrity (Ait-Belgnaoui *et al,* 2012; Desbonnet *et al,* 2008; Gareau *et al,* 2007; Sudo *et al,* 2004; Zareie *et al,* 2006) combined with the results from GF, stress and infection studies, there has been a shift in focus on the potential effects of probiotics on neurochemical levels. A study by Desbonnet *et al* (2008) demonstrated that *B. Infantis* administration over a 14 day period significantly reduced the concentration of 5-HIAA, a metabolite of serotonin, in the frontal lobe and reduced dihydroxyphenylacetic acid (DOPAC) levels in the amygdaloid cortex. Peripheral neurochemical concentrations were also significantly affected, with higher plasma tryptophan levels found in the probiotic treated group (Desbonnet *et al,* 2008). The forced swim test (FST) was used to examine the behavioural implications of these changes in neurotransmitter level, a test routinely used to assess the efficacy of antidepressant agents (Cryan *et al,* 2005). Results indicated that the changes in frontal lobe serotonergic functioning or amygdaloid cortex dopaminergic functioning did not translate to behavioural changes in this test. Similar results were reported in maternal separation stress studies with *B. Infantis* shown to normalise basal noradrenaline concentrations (Desbonnet *et al,* 2010). However, this study did not report the alterations in serotonergic or dopaminergic functioning previously found. Equally peripheral tryptophan levels were also unaffected by treatment. Results from the forced swim test in this study indicated that chronic treatment with *B. Infantis* normalised the behaviour of the maternally separated animals. Combined, these results indicate that induced stress may diminish the potential for probiotic treatment to affect specific neurochemical functioning i.e. serotonergic and dopaminergic functioning, but may optimise the potential for probiotics to affect noradrenergic functioning. Other effects of probiotics on CNS

functioning include alterations in GABA receptor expression (B 1b and Aα2) (Bravo *et al,* 2011). $GABA_A$ receptors are the pharmacological targets for anti-anxiety medication, benzodiazepines. mRNA levels of $GABA_{B\ 1b,}$ following ingestion of *Lactobacillus,* were found to be lower in the cingulate and prelimbic cortices and elevated in the amygdala, locus coeruleus and dentate gyrus. $GABA_{A\ \alpha 2}$ were also lower in the cingluate and prelimbic cortices as well as the infralimbic cortex and amygdala whilst elevated in the dentate gyrus (Bravo *et al,* 2011). Behaviours relevant to GABAergic activity were assessed to establish whether these alterations could significantly alter behaviour. Tests relevant to an anxiety and depression were assessed as GABAergic activity is associated with psychiatric disturbances. Results from the EPM and FST showed that changes induced in GABA receptor expression by *Lactobacillus* can be detected at a behavioural level with healthy animals showing less anxiety and depression-like behaviour in these tests (Bravo *et al,* 2011). It is evident that the neurochemical effects of probiotic administration is not only region dependent but that baseline functioning determined by stress levels influences these effects.

It is clear that probiotics can alter concentrations of various neurochemicals throughout the brain. However, studies have indicated that probiotics can directly influence neurotransmitter activity. Strains of *Lactobacillus* and *Bifidobacterium,* derived from human intestines, produce GABA from monosodium glutamate under *in vivo* conditions (Barrett *et al,* 2012). Various microbial species have been shown to secrete neurochemicals given the optimum conditions (Lyte, 2011). Serotonin can be produced from candida, streptococcus, escherichia, enterococcus, dopamine from bacillus and serratia and norepinephrine from esherichia, bacillis and saccharomyces (Lyte, 2011).

Further examination into the potential for probiotics to produce neuroactive compounds has highlighted an alternative route of action that may account for their action on the brain. Described by Lyte (2011) as a microbial endocrinology- based theory, it is hypothesised that the mechanism by which probiotics exert their effects on the brain is due the activation of receptors on immune and neuronal cells both intestinally and extra-intestinally by neurochemicals secreted by probiotic

bacteria. Furthermore, it is the common recognition of these neurochemical products by both prokaryotic and eukaryotic cells that provides the foundation for gut-brain axis communication.

1.6. Thesis rationale

The purpose of the current thesis is two-fold. Firstly, given the evidence presented by Desbonnet *et al* (2008) it is clear the probiotic treatment causes alterations in frontal lobe serotonergic functioning, but failed to identify a behavioural manifestation of the changes. Therefore, Chapter 2 aims to identify a task that is reliant on frontal lobe functioning. Further examination of the effects of direct serotonergic manipulation on this task is explored in Chapter 3, before examining the impact of probiotic administration in Chapter 4. The effects of probiotics in a task known to be dependent on the frontal cortex and hippocampus, namely a watermaze reversal learning paradigm, will be explored in Chapter 4. Identification of brain areas that show improvements following probiotic treatment are explored through object recognition tasks that rely differentially on contributions of the hippocampus and subregions of the frontal cortex (Chapter 5). Secondly, several studies have implied that the behaviour effects of probiotic treatment are indicative of an anxiolytic property and proposed the use of probiotics as an adjuvant therapy for comorbid anxiety and depression (Logan & Katzman 2004). Therefore, this thesis also aims to examine the effects of probiotic treatment on anxiety-like behaviour. As previously mentioned alterations in frontal lobe serotonergic functioning have been found following probiotic treatment (Desbonnet *et al,* 2008). As this neurochemical effect is similar to that produced by serotonin selective reuptake inhibitors (SSRI), citalopram treatment on anxiety-like behaviour was also examined in Chapter 3. The impact of probiotics on anxiety-like behaviour was then explored in Chapter 4. In order to establish where potential affects could be attributed to improved serotonergic transmission this task was also conducted with citalopram.

Nuclear magnetic resonance is a powerful assessment tool that detects fluctuations in the metabolite profile. This profile is indicative of downstream genomic, transcriptomic and proteomic functioning and provides insight into cellular processes in the context of health and disease (Trushina and Mielke, 2013). Therefore, in Chapter 6 NMR was used to examine the effects of probiotic treatment on intracellular metabolism to determine whether any behavioural changes can be attributed to alterations in intracellular metabolism pathways.

The results from each task using the various manipulations from each chapter are discussed collectively in the general discussion.

Medial prefrontal cortex lesions impair performance in probabilistic reversal learning.

2.1. Summary

The studies presented in the current chapter used a probabilistic reversal learning (PRL) paradigm designed by Bari *et al* (2010). The aim of the initial study was to establish the pattern of performance in this task. The PRL task was conducted using two different stimulus reward contingencies to assess performance at two different levels of difficulty. Results from this study indicated that task difficulty affects the number of reversals achieved. Performance in the easier version of the task displayed a distinct learning pattern however, performance in the more difficult of the two tasks declined across sessions. This task was also conducted with medial prefrontal (mPFC) lesions. Results showed that the mPFC is implicated in probabilistic reversal learning, with the lesioned group completing fewer reversals, exhibiting reduced sensitivity to positive reinforcement across the sessions and reduced sensitivity to positive reinforcement in the reversal phase of the first session. Sensitivity to negative reinforcement was unaffected by the lesion therefore mPFC is integral to processing of positive feedback dissociated from negative feedback processing.

2.2. Introduction

Reversal learning is one example of cognitive flexibility in which subjects are required to adjust behaviour to changes in the stimulus-reward contingency. This requires the ability to desist in current responding to a stimulus and switch responding to a previously non-rewarded stimulus. Deficits in reversal learning and the inability to moderate responding based on reward outcome is often seen clinically in patients with Parkinson's disease, schizophrenia and bipolar disorder, as well as in developmental disorders such as autism and Tourette's syndrome (Verte *et al,* 2005).

Lesion studies have highlighted a fundamental role for the orbitofrontal cortex (OFC) in reversal learning, with lesioned rats demonstrating perseverative errors once the stimulus-reward contingency has been reversed (Chudasama *et al,* 2001; Kim & Ragozzion, 2005; McAlonan & Brown, 2003; Rygula *et al,* 2010). Interestingly, monkeys with lateral prefrontal cortex (lPFC) lesions (Rygula *et al,* 2010), or rats with medial PFC (mPFC) lesions (Chudasama *et al,* 2001; Ragozzino & Kim, 2003) do not show reversal learning deficits. In contrast, lesions to striatal regions impair reversal but only following by impaired responding to negative feedback whilst unaffecting sensitivity to positive reinforcement (Clarke *et al,* 2008). This suggests a role for striatal input to the OFC in reinforcement learning. Studies have shown differential contributions of the ventrolateral PFC and OFC in reversal performance, with lesions to the former resulting in the ability to maintain reversal abilities with familiar but not novel stimuli, while lesions to the latter impair reversal abilities regardless of whether the stimuli are familiar or novel (McAlonan & Brown, 2003). Reversal learning tasks are therefore useful indicators of OFC damage, dissociating medial, ventrolateral and dorsolateral prefrontal damage (Ghods-Sharifi *et al,* 2008; McAlonan & Brown, 2003; Rygula *et al,* 2010). Reversal learning incorporates several different stages of information processing, which may further differentiate the role of specific brain regions in cognitive flexibility. Thus negative feedback indicates that the previous response is incorrect and that an unexpected violation of the rule has occurred. As a consequence an adaption in goal-directed behaviour is required in the next trial (Holroyd & Coles, 2002). Negative feedback can also be used to test a new hypothesis once behaviour has been adapted; this is referred to as *effective negative feedback* (Walton *et al,* 2004). Several studies have indicated that the medial prefrontal cortex (mPFC) plays a fundamental role in negative feedback processing (Holroyd & Coles, 2002). In humans, source identification of negative scalp potentials following negative feedback indicated localisation on or near the anterior cingulate cortex (ACC) (Holroyd & Coles, 2002; Nieuwenhuis *et al,* 2004; Yeung & Botvinick, 2004). Imaging studies using fMRI have produced similar results indicating that the mPFC/ACC are activated following negative feedback (Holroyd *et al,* 2004, Mars *et al,* 2005). Selective excitotoxic lesions and

temporary pharmacological inactivation of the ACC in rodents impairs performance in reversal learning without increasing preservative responding to previously rewarded responses (Newman & McGaughy, 2011; Ng *et al,* 2007; Ragozzino & Rozman, 2007). This suggests that the ACC has a distinct role in processing negative feedback and in error detection unrelated to inhibition of behaviour.

Lesions to ventro-lateral prefrontal areas in non-human primates only impaired reversal learning once novel stimuli were used whereas lesions to the OFC impaired reversal learning irrespective of whether the stimuli were novel or familiar (Rygula *et al,* 2010). This indicates that the outcome of the current study could rely on the choice of experimental procedure. Post-surgery performance in reversal learning in this study was dependent on pre-surgery experience most likely due to the development of learned sets or rules that can be implemented to guide responding therefore enabling response choice to be less dependent on accurate feedback processing (Browning *et al,* 2007; Wilson and Gaffan, 2008). Other studies on the ventrolateral prefrontal cortex have found that this area is more active following negative feedback compared with positive feedback (Monchi *et al,* 2001). ERP studies have indicated a role for the right superior fronal gyrus in the processing of positive feedback compared with negative feedback (Nieuwenhuis *et al,* 2005), indicating that subregions within the lateral prefrontal cortex can be sensitive to both positive and negative feedback (Zanolie *et al,* 2008). The finding from these studies suggest that the lateral PFC interacts with the mPFC/ACC in monitoring and evaluating reinforcement (Gehring & Knight, 2000). Accurate performance in reversal learning tasks has been taken to involve both the implementation of learned rules and inhibition of current responding, as well as error detection and evaluation of feedback. This reflects an interaction between the orbitofronal cortex and ACC/mPFC regions. However, activation of these regions have been found to be negatively correlated with increased activation of the ACC and decreased activation of the OFC associated with reversal when it is individual goal-driven decision making whereas an opposing activation pattern is found when the response is experimenter guided (Walton *et al,* 2004). These results suggest that the OFC and

ACC/mPFC play dissociable roles in reversal learning. When independent decision making processes are required to detect errors and evaluate feedback, mPFC/ACC functions are employed whilst the OFC is fundamental for rule-learning.

Classical reversal-learning paradigms use stimulus-reward contingencies which present the subject with 100% rewarded correct responses. Probabilistic reversal learning requires the subject to choose between two responses both of which have a probability of reward however at contrasting contingencies. This task requires the ability to decipher the new contingency and choose responses accordingly. This is achieved by integrating feedback over a number of trials (Cools *et al,* 2002). OFC lesions impair reversal learning using this 100% stimulus –reward contingency where rule learning is paramount. However, little research has focused on the systems responsible for effective reversal learning when accurate feedback processing is required to direct subsequent response. The increased difficulty of the probabilistic reversal learning hypothetically should employ mPFC resources to direct responding as no discrete rules exist.

The current study presents two versions of the automated PRL task developed by Bari *et al* (2010). The initial study, a pilot of the this task, used a stimulus-reward contingency set to 80% reward on correct responses and 20% reward on incorrect responses (referred to as PRL80). In order to assess whether task difficulty affects sensitivity to positive and negative reinforcement in normal rats the stimulus reward contingency was subsequently changed to 60% reward on correct responses and 40% reward on incorrect responses (referred to as PRL60). This may provide another level at which to assess the effect of pharmacological interventions in future experiments. The purpose of this experiment is to determine whether the task can be performed by normal animals across a range of difficulties. The second part of the study examines the performance of animals with mPFC excitotoxic lesions. The rationale behind this is that in probabilistic reversal learning attention to positive and negative reinforcement is required to optimise performance whereas discrete rule – learning is not. Evidence from previous lesion studies has indicated that feedback processing is

reliant on mPFC functioning (Gehring & Knight, 2000). It is expected mPFC lesioned rats will show a deficit in reversal but not acquisition of the PRL task.

2.3. *Method and Procedure*

Subjects

Experiment 1: Thirty-two male adult Lister hooded rats (Harlan, UK) weighing 280-350g were used in this experiment. They were housed in pairs in a 12h:12h light dark cycle, the holding room was maintained at 55% ± 10 humidity and a temperature of 19-23 ^{o}C and were all tested during the 12 hour light cycle. The rats were 3 months old when being tested and were thoroughly habituated to handling before the study began. The rats were food restricted to 85% of free feeding body weight and had free access to water. The experiment was performed in accordance with Home Office under Animals Scientific Procedures Act 1986.

Experiment 2: Twenty-two male adult Lister hooded rats (Harlan, UK) weighing 280-380g were used in this experiment. These animals were 6 months old when testing began. They were housed under the same conditions as animals in Experiment 1.

Experiment 2: Surgical Procedure

Twenty-two rats were divided into 2 cohorts; medial prefrontal lesion (n=11) and surgical controls (n=11). The animals were anesthetized and maintained with isoflurane gas during the surgical procedure. Once anaesthetized, the rats were placed in a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA) at flat skull position (-3.3 mm). A longitudinal incision was made along the scalp and the skull exposed. The scalp was retracted using haemostats and a dental drill used to remove the skull at the point of the lesion. Ibotonic acid (63 mM) (Sigma-Aldrich, UK) was infused bilaterally using a 1µl Hamilton syringe (Reno, NV, USA) that was mounted on an injection pump (KD Scientific, Holliston, MA, USA). Ibotonic acid was administered to each injection site at an infusion rate of 0.1µl/min with the needle left in situ for 10 minutes. The coordinates and volume of ibotonic acid used at each injection site are as follows: AP +0.7 mm from bregma, ML ±0.7 mm and DV -4.5 mm

from skull surface at bregma, 0.5 µl; AP +3.7 mm from bregma, ML ±0.7 mm and DV -4.0 mm from skull surface at bregma, 0.5 µl, (Paxinos & Watson, 2008).

After surgery, the scalp was sutured and the rats were placed in a temperature controlled recovery box. They received a 5ml glucose saline subcutaneous (s.c.) injection and 1.0mg/kg s.c. injection of Meloxicam, an analgesic agent. Rats were given 2 weeks to recover before food restriction or behavioural testing.

Apparatus

The rats were trained in four five-hole operant chambers (30.5 x 24 x 21 cm, Med Associates, St Albans, VT, USA). The chambers had five apertures on one side adjacent to the food magazine. The house light, (100mA), had been removed from the operant box and placed at the top of the right wall of the external box to increase the saliency of the aperture lights. The external chamber was fitted with a fan which remained on throughout the experiment. Food reinforcement (savoury pellets, 45mg; Test Diet, Richmond, IN, USA) was delivered to a food magazine. A photo-beam was located across the mouth of each of the apertures and food magazine to detect nosepoke responses. MED-PC IV was used to custom write a program that controlled the chamber conditions. Data was recorded by a RM personal computer connected to the chambers via an interface using MEDPC-IV software (Med Associates, St Albans, VT, USA).

Training Procedure

The rats were given 3 daily sessions lasting 20 minutes to habituate to the test environment. During these sessions they received free reward pellets on a random schedule of inter-trial intervals (15, 30, 4 and 60 seconds). After the habituation sessions the rats commenced nosepoke training. Nosepoke training involved one 30 minute session per day. One side only (right or left), was trained per day. One hole per trial, immediately to the right or left of the centre hole, was illuminated for 30

seconds, during this time a nosepoke response to the illuminated aperture was rewarded with a single food pellet. The aperture lights turned off once a response was detected. No response resulted in a five second timeout during which the house-light was turned off. Nosepoke responses to a non-illuminated hole were not followed by food but had no other consequences. Retrieval of the food pellet from the magazine signalled the next trial. Once the rats had demonstrated their ability to nosepoke by completing 150 trials per session in two successive sessions of right and two sessions of left nosepoke training, task training commenced.

Task training involved random presentation of either right or left illuminated apertures for 30 seconds, again no response during this time resulted in the five second timeout. In addition, responding in a non-illuminated aperture resulted in a five second timeout. Allocation of rewards on the next trial was determined by the rewarding schedule and is uninfluenced by the preceding trial. Correct responding resulted in a single food pellet reward. Once individual performance had reached a criterion of 90% accuracy and had remained there for at least three consecutive sessions performance was deemed stable and the rats moved on to the PRL task. After 12 days of training any rats that had still not met the criteria were excluded at this stage. In Experiment 2 one rat was excluded from the surgical control group. In addition, rats displaying a side bias as identified by greater than 5% of errors on either right or left side where excluded from the study as this stage.

Test

The PRL task presented the subjects with two illuminated apertures simultaneously. The reward contingency was that a response in one aperture was rewarded on 80% of occassions and the other, ON 20% of occassions. Initial allocation of 'correct' hole was random. After 8 consecutive responses in the 'correct' aperture, as indicated by the 80% reward rate, the stimulus-reward contingency was reversed so that responding in the other (previously rewarded on 20% of occasions) aperture then became the 'correct' response. An inappropriate response in the 'correct' hole was classed as as error and thus unrewarded and was followed by a two and a half second timeout.

Again, the stimulus was presented for 30 seconds, during which if there was no response a five second timeout was issued. During the PRL task, however, responding in a non-illuminated aperture no longer warranted a timeout. Animals had one session per day and given 40 minutes to complete 200 trials. In Experiment 1, this stimulus-reward contingency of 80% reward for correct responses and 20% rewards for incorrect responses was presented for 3 sessions. This is referred to as PRL80. Following this task difficulty was increased to 60% reward on the correct response and 40% reward on incorrect response, this contingency was presented for 3 days. This is referred to as PRL60.

In Experiment 2, the rats were presented with 80% reward for correct responses and 20% reward for incorrect responses for 8 sessions.

Statistical analysis

Performance on the task was analysed by phase on the first test session, the acquisition phase comprised of the trials before the first reversal, the reversal phase comprised of any trials after the acquisition phase but prior to any further reversals should they be completed. The results from any animal that failed to complete reversals of the discrimination had their data analysed as acquisition phase and therefore did not contribute to the reversal phase data.

The data analysed from all the test session was the number of reversals completed, reward (win-stay) and negative feedback (lose-shift) sensitivity. Response to positive and negative feedback was analysed by evaluating responding on a trial after having received a reward or punishment in the previous trial. Win-stay performance was the likelihood of repeating the same behaviour having been rewarded on a pervious trial (positive feedback), lose-shift performance was the likelihood of discontinuing the behaviour following a punishment on the previous trial (negative feedback). When calculating the probabilities the number of omissions and responses in non-illuminated apertures were not included. i.e. win-stay probability is calculated by dividing total number of rewarded responses in the same hole as the previous trial by the total number of reward responses and lose-shift probability is calculated by dividing the total number of shifts in response following

punished responses divided by the total number of punished responses. Repeated measure ANOVA was carried out on all the measures. Win-stay and lose-shift probability were also analysed by phase, acquisition and reversal on the first test day. Analysis was also carried out of the perseverative errors. These were calculated at the percentage of trials where an incorrect response was recorded following reward for the same response. The number of trials to reach the criterion was also recorded, i.e. the number of trials in the acquisition phase of each session.

Where repeated measure ANOVA gave a significant interaction, analysis of the simple effects were examined by pairwise comparisons. The results are expressed as mean ± 1 standard error. The threshold for significance was $p<0.05$. Where data was non-spherical given the output from *Malchly's Test of Shericity* ,the *Greenhouse-Geisser* adjustment was used to give a corrected *F* ratio. Where interactions were significant pairwise comparisons were used to examine the simple effects of this interaction. All statistical analysis was carried out using SPSS version 20.0.

2.4. Results

2.4.1. Histology

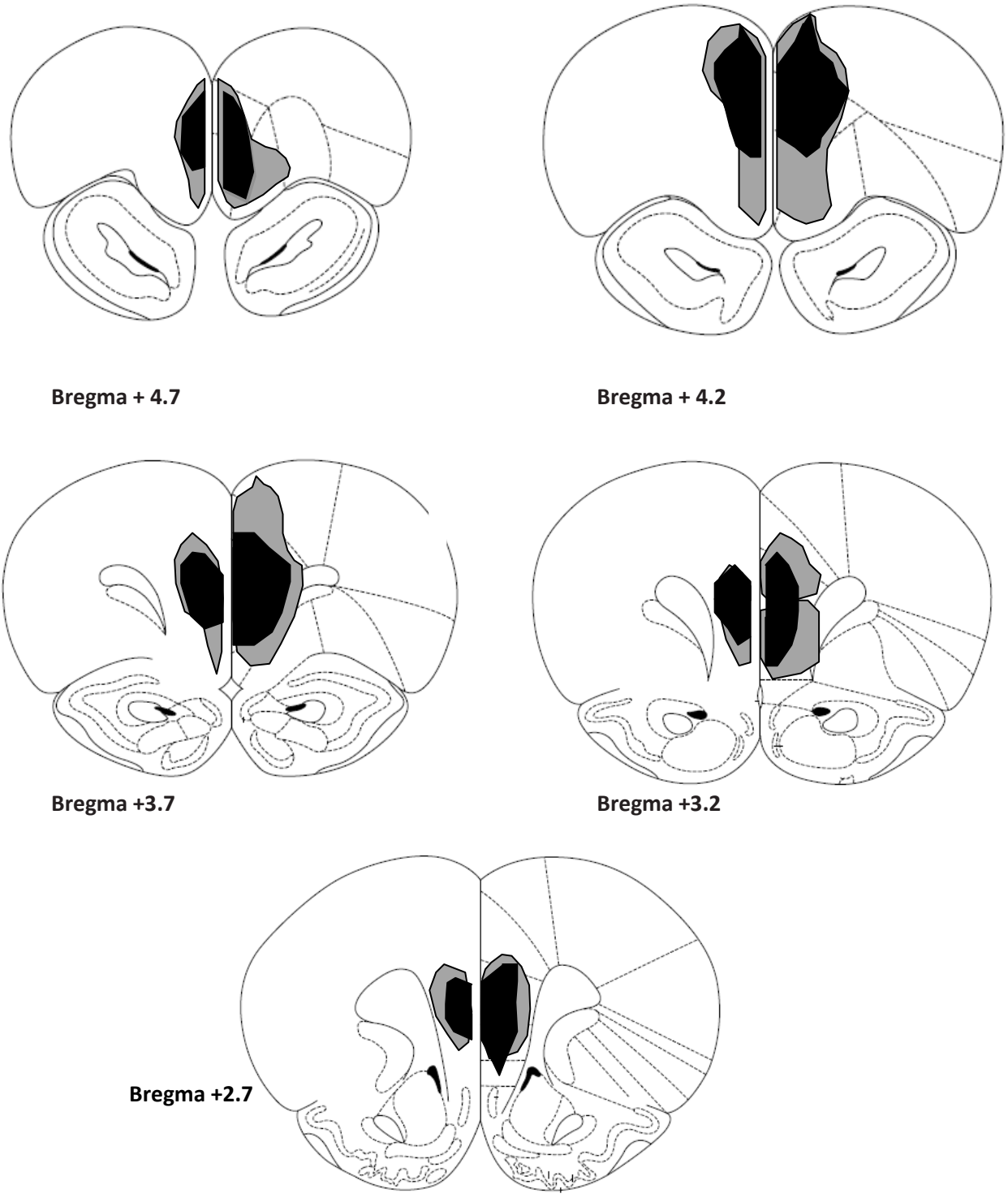

Figure 1. shows the minimum (black shading) and maximum (grey shading) prefrontal lesions in coronal sections.. Distance from Bregma given with each section (mm) (Paxinos and Watson, 1997).

Lesions to the medial prefrontal cortex resulted in substantial damage to the prelimbic (PrL) and intralimbic (IL) cortices. Moderate damage was sustained to the medial orbital frontal cortex on 2 of the 11 lesioned animals. Of these no lesions resulted in complete bilateral damage to the mOFC. 2 rats sustained damage to the cingulate cortex, which was limited area 1.

2.4.2. Experiment 1: PRL Pilot

Conditional probabilities were analysed by phase on the first day of each task (Figure 2). Repeated measures ANOVA showed that the win-stay probability did not significantly differ been the tasks ($F(1,30)=0.099$, p=NS). However, there was a significant main effect of phase with win-stay probability significantly lower during the reversal phase compared with acquisition phase across both tasks ($F(1,22)=20.44$, $p<0.001$). During the acquisition phase responses were guided by positive reinforcement, however, this was not the case during the reversal phase. There was no significant task by phase interaction ($F(1,22)=0.241$, p=NS). Lose shift probability did not significantly alter between tasks ($F(1,30)=0.054$, p=NS). However, there was a significant main effect of phase with lose-shift probability higher in the reversal phase of both tasks ($F(1,22)=23.377$, $p<0.001$). During the acquisition phase responding was completely unguided by negative reinforcement however, in the reversal phase sensitivity to negative reinforcement increased. The was no significant task by phase interaction ($F(1,22)=0.329$, p=NS).

One animal failed to acquire the task and so was excluded from the data completely. 9 animals did not complete any reversals in the first session therefore data from these animals did not contribute towards reversal phase data. Any animals which did not complete any reversals in a session had all their data from that session analysed as acquisition data, hence the variation in degrees of freedom

in the results.

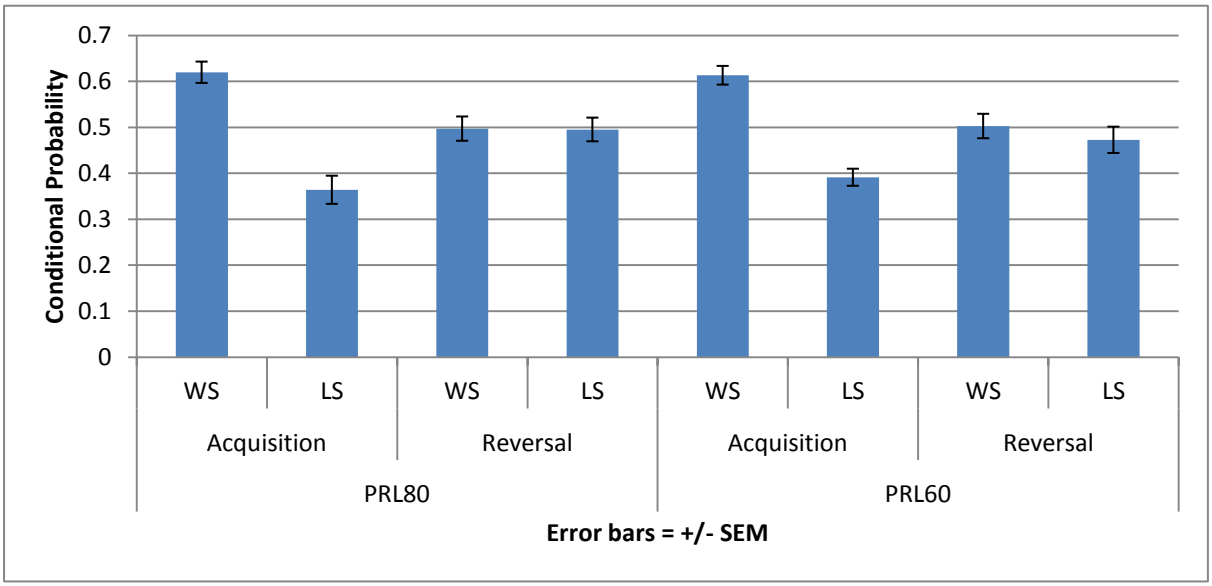

Figure 2. Conditional probability analysed by phase (acquisition and reversal) on initial session of both tasks (PRL80 and PRL60) (WS: win-stay probability; LS: lose-shift probability).

Inspection of Figure 3 suggests that the mean number of reversals completed was reduced when task difficulty was increased from PRL80 to PRL60. Repeated measures ANOVA showed that there is a significant main effect of task (PRL80 v PRL60) on number of reversals with the PRL60 task having a lower number of completed reversals ($F(1,30)=10.611$, $p<0.001$). There was no main effect of session ($F(2,60)=0.126$, p=NS), however there was a significant session by group interaction ($F(2,60)=3.673$, $p<0.05$). Analysis of the simple effects of this interaction show that session 1 (PRL80) and session 4 (PRL60) were not significantly different ($F(1,30)=0.225$, p=NS) neither were session 2 (PRL80) and session 5 (PRL60) ($F(1,30)=3.107$, p=NS). However, the third session of each task showed significant differences ($F(1,30)=11.897$, $p<0.01$).

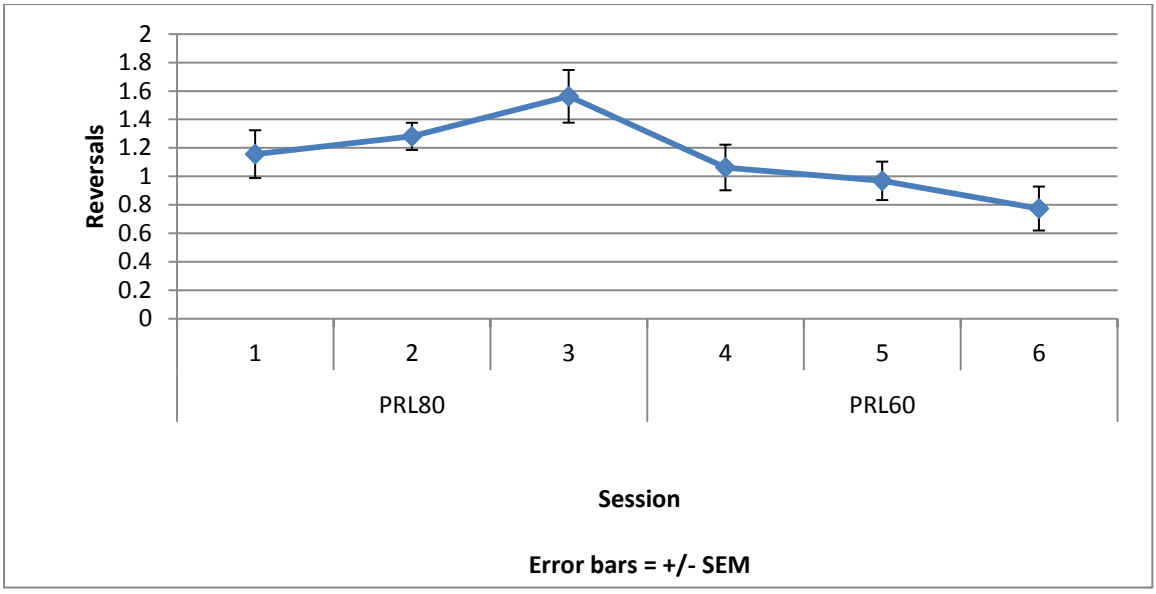

Figure 3. Mean reversal completed over 7 sessions on PRL80 task and 3 sessions on PRL60 task.

Figure 4 shows conditional probabilities over the sessions. Repeated measure ANOVA indicated that there was no significant main effect of task on win-stay probability ($F(1,30)=2.898$, p=NS). There was also no significant main effect of session ($F(2,60)=1.35$, p=NS). However, there was a significant task by session interaction ($F(2,60)=3.422$, $p<0.05$). Analysis of the simple effects of this interaction showed that there is no significant difference between the tasks on the first session of each ($F(1,30)=1.29$, p=NS) or the second session of each ($F(1,30)=1.817$, p=NS), however performance on the third session of the PRL60 task was significantly lower ($F(1,30)=7.852$, $p<0.01$).

Repeated measures ANOVA indicated that there was no significant main effect of task on lose-shift probability ($F(1,30)=0.199$, p=NS). There was also no significant main effect of session

(*F*(2,60)=2.565, *p*=NS) or task by session interaction (*F*(2,60)=0.005, *p*=NS).

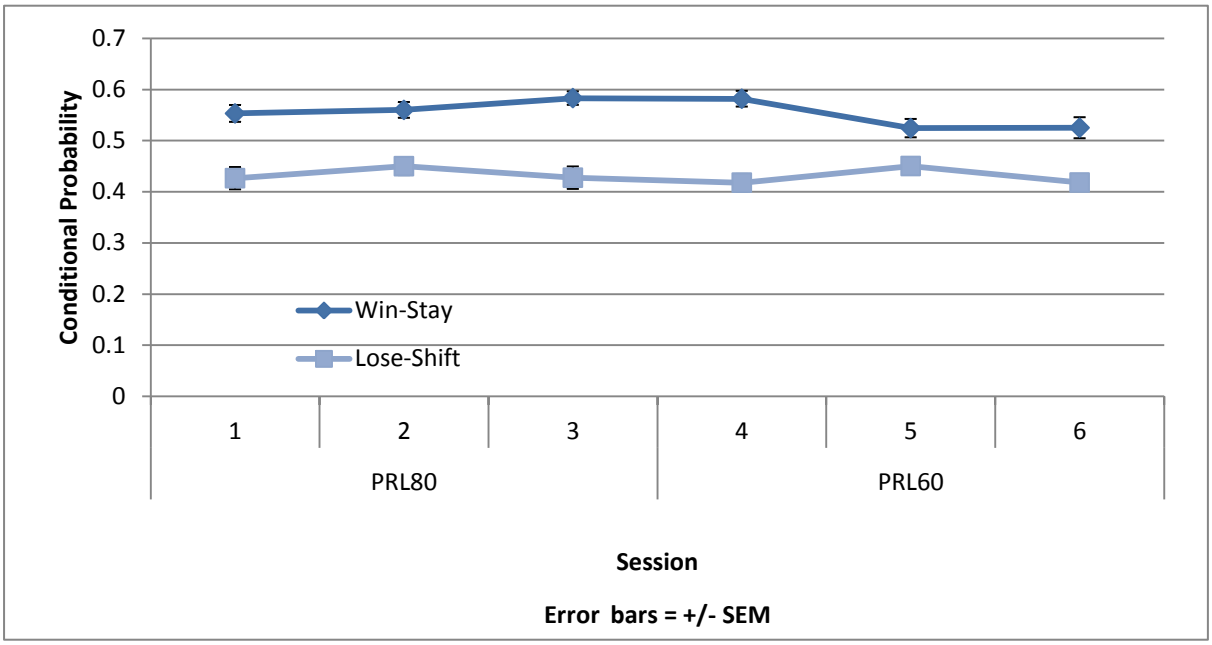

Figure 4. Mean win-stay and lose-shift probabilities of PRL80 and PRL60 tasks.

2.4.3. *Experiment 2: The effects of mPFC lesions on the PRL task*

The PRL task was conducted with mPFC lesioned rats using the PRL80 stimulus reward contingency. Figure 5 shows analysis of the conditional probability by phase (acquisition and reversal) during the initial session. Statistical analysis found no significant main effect of group on win-stay probability (*F*(1,11)=3.217, *p*=NS). However there was a significant main effect of phase (*F*(1,11)=8.065, *p*<0.05) and significant phase by group interaction (*F*(1,11)=12.001, *p*<0.01). Pairwise comparisons showed that the mPFC lesioned group were significantly impaired on win-stay probability during the reversal phase (*F*(1,11)=10.097, *p*<0.01). Analysis of lose-shift probability shows no significant main effect of group (F(1,11)=0.081, p=NS), no significant main effect of phase (*F*(1,11)=2.344, *p*=NS) or phase by group interaction (*F*(1,11)=0.0, *p*=NS).

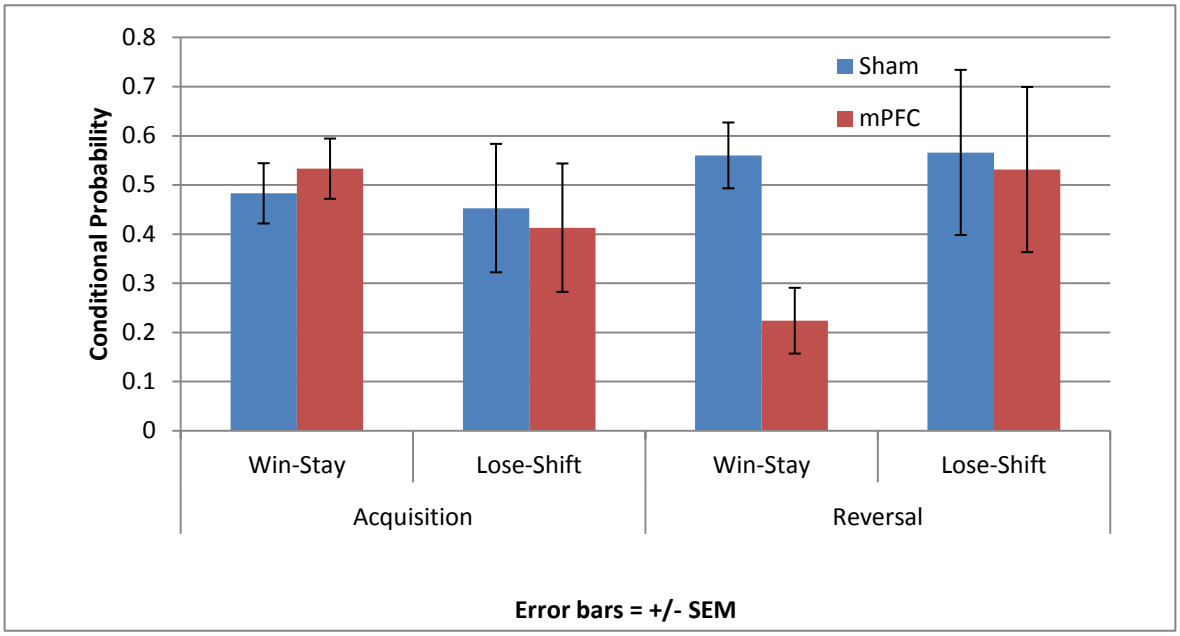

Figure 5. Conditional probabilities of sham surgery and mPFC lesioned groups during acquisition and reversal phases in reversal session 1.

Inspection of Figure 6 indicates that lesions to the mPFC impaired reversal. This is confirmed by repeated measure ANOVA, which revealed a significantly higher of number of reversals in the sham group compared with the mPFC lesion group ($F(1,15)=7.038$, $p<0.05$). There was also a significant main effect of session ($F(7,105)=2.266$, $p<0.05$) however there was no significant session by group interaction ($F(7,105)=1.904$, p=NS).

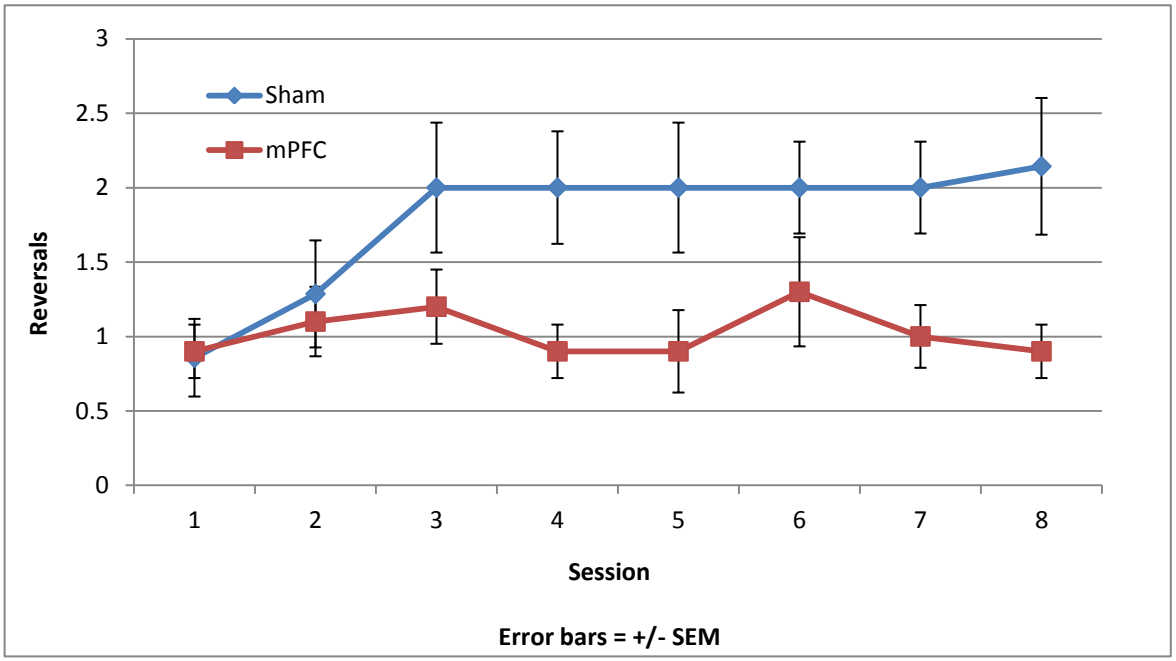

Figure 6. Mean number of reversal completed by sham surgery and mPFC lesioned groups over 8 sessions.

Inspection of Figure 7 that shows the mean win-stay probability values across training sessions were impaired across training sessions. Repeated measure ANOVA showed a significant main effect group ($F(1,15)=53524$, $p<0.05$) and a significant main effect of session ($F(7,105)=2.59$, $p<0.05$) However, there was no significant session by group interaction ($F(7,105)=0.252$, p=NS).

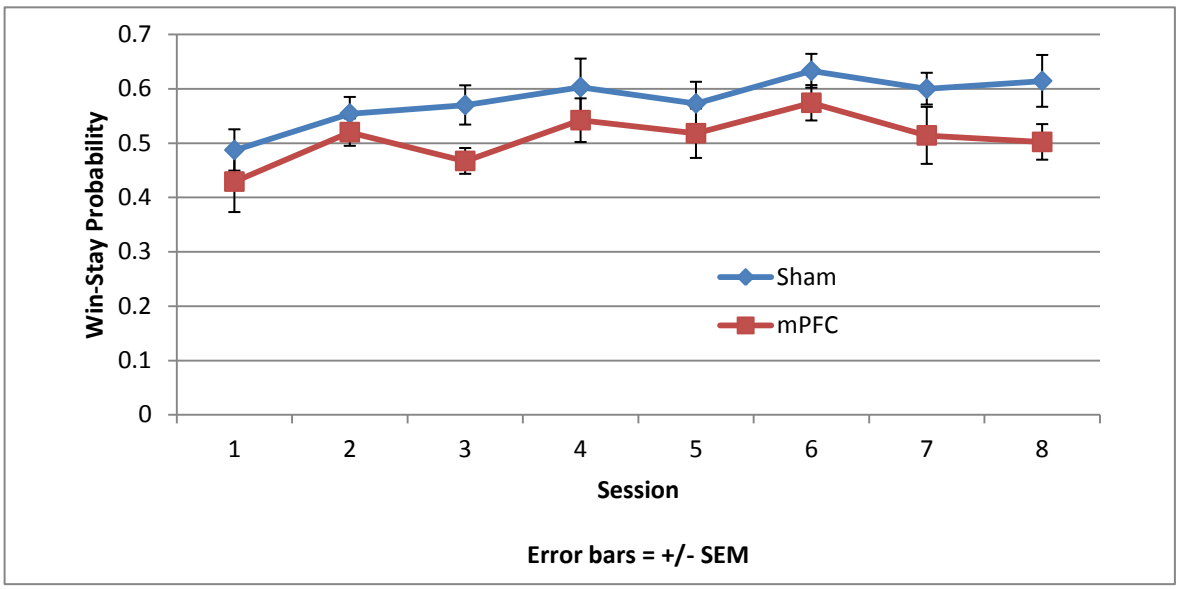

Figure 7. Win-stay probability of sham surgery and mPFC lesioned group over 8 sessions.

Lose-shift probability was also examined across the sessions (Figure 8) however repeated measures ANOVA indicated no significant difference between the groups ($F(1,15)=0.026$. p=NS). There was also no significant main effect of session ($F(7,105)=105$, p=NS) or session by group interaction ($F(7,105)=0.678$, p=NS).

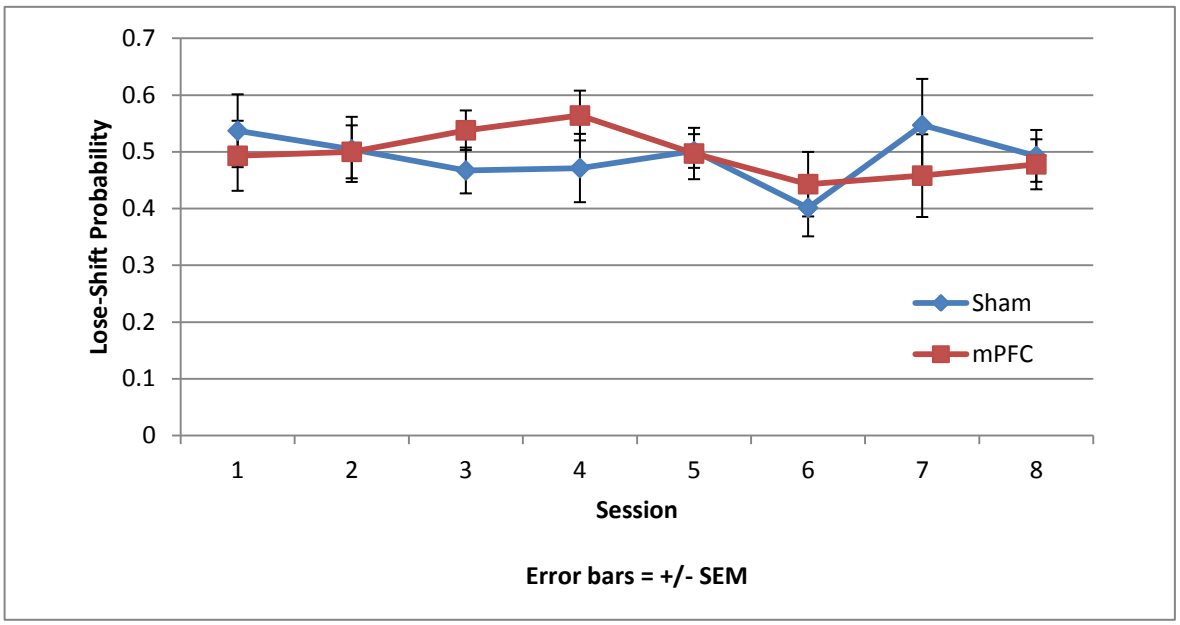

Figure 8. Lose-shift probability of sham surgery and mPFC lesioned group over 8 sessions.

Analysis of the perseverative errors indicated that there was a significant main effect of session ($F(1,13)=27.138$, $p<0.001$). However, there was no main effect of group ($F(1,13)=0.057$, p=NS) or session by group interaction ($F(1,13)=0.33$, p=NS) (figure 9).

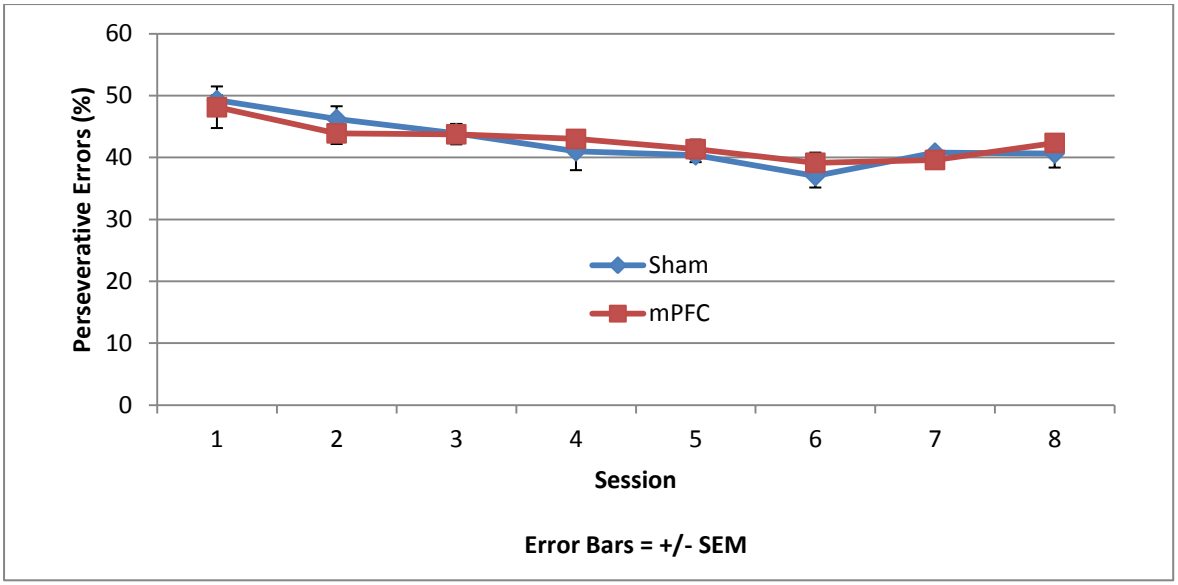

Figure 9. Perseverative errors of lesion and sham groups.

Repeated measures ANOVA of trials to criterion (figure 10) indicated that were was no significant main effect of group ($F(1,11)=3.773$, p=NS), session ($F(1,11)=2.296$, p=NS) or session by group interaction ($F(1,11)=2.749$, p=NS).

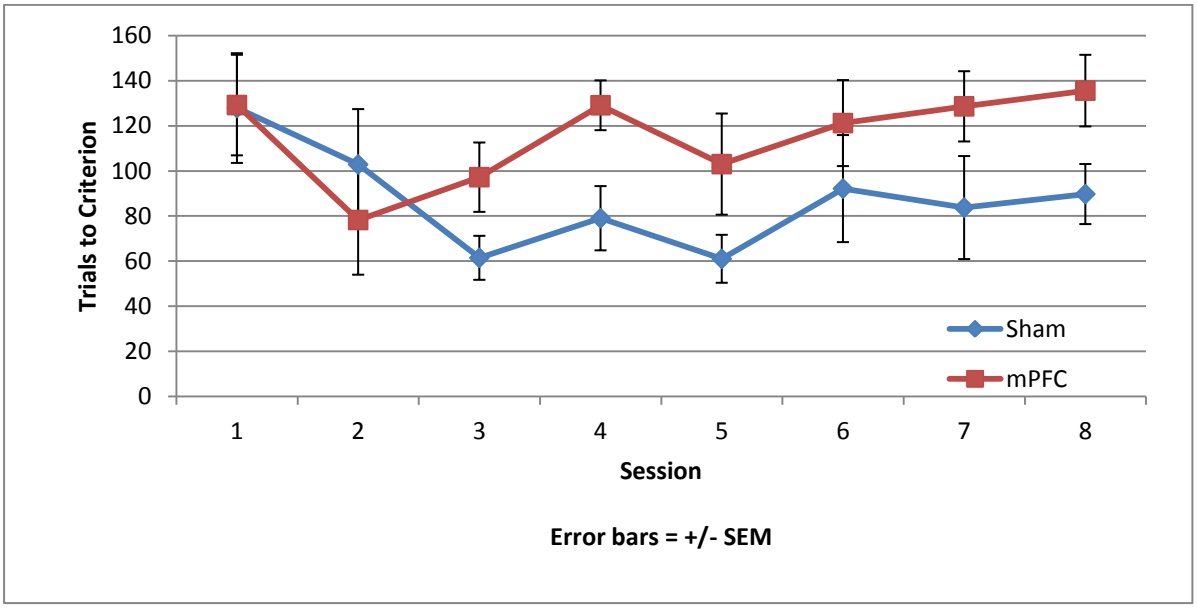

Figure 10. Trials to criterion of sham surgery and mPFC lesioned groups.

2.5. *Discussion*

The purpose of the first experiment was to pilot an automated version of the probabilistic reversal learning task to establish the pattern of performance on this task in rats. As predicted performance was influenced by task difficulty. Furthermore, performance in the tasks was found to be differentially affected by session. The number of reversals increased in the PRL80 task over the sessions while performance in the PRL60 task decreased. As the PRL80 task was run first, improved performance over the session was expected. The opposing pattern found in the PRL60 task implies that performance is not stable at this task difficulty. Analysis of conditional probabilities by phase showed that both win-stay and lose-shift probabilities was significantly affected. During the acquisition phase win-stay probability was higher than in the reversal phase in both tasks, the opposing pattern was found in lose-shift probability. This suggests that during initial rule learning there is greater sensitivity to positive reinforcement while during reversal of this rule there is greater sensitivity to errors, irrespective of task difficulty. Sensitivity to positive reinforcement was found to be significantly reduced in the more difficult task. This was illustrated by lower win-stay probability in the PRL60 task. This was not found with negative feedback sensitivity (lose-shift), which suggests that task difficulty reduces the animals' sensitivity to positive reinforcement while sensitivity to negative reinforcement remains stable.

Analysis of the perseverative errors in experiment indicated a learning effect across the session where there was a significant reduction in perseveration however this was not differentially affected by lesion. Results from this experiment showed that lesions of the mPFC significantly reduced the number of reversals completed relative to sham surgery animals. Previous studies using reversal learning with a 100% reward rate for correct responses found that lesions of the orbiofrontal cortex but not the mPFC impaired performance (Chudasama *et al,* 2001; Ragozzino & Kim, 2003). This suggested that it is the probabilistic nature of this task which engages the mPFC during reversal learning.

As attention is shifting between right or left nosepoke to 'correct' and 'incorrect' responding (not always the same in this probabilistic task), the task information could be processed as an extra-dimensional shift. Spatial discrimination between right and left is then replaced with a choice of response based on reward likelihood and could therefore be considered motivational. Extra-dimensional shifts have been found to be selectively sensitive to lesions of the IL and PL cortex (Birrell and Brown, 2000; Ng *et al,* 2007). The rats with mPFC lesions were less sensitive to positive reinforcement during the reversal phase however lose-shift probability was unaffected. This was found in the conditional probabilities across the sessions. The anterior cingulate cortex (ACC) has been associated with the processing of negative feedback (Holroyd & Coles, 2002; Nieuwenhuis *et al,* 2004; Yeung & Botvinick, 2004). The dissociable effect of mPFC lesions on negative and positive feedback sensitivity imply that these are neurally dissociable. There was substantial sparing of the ACC in mPFC lesions which may explain why negative feedback sensitivity was unaffected by the lesion.

Human studies have found greater activation in areas of the frontal cortex following positive feedback compared with negative feedback (Nieuwenhuis *et al,* 2004). It has been reported that prelimbic (Prl) and infralimbic (IL) regions of the mPFC are activated by positive reinforcement with 25% of neurons exhibiting prominent excitation during rewarded, but not unrewarded lever presses for food (Burgos-Robles *et al,* 2013). Lesions to the PrL cortex have been shown to impair goal-directed behaviour by impairing sensitivity to goal value after training (Killcross & Coutureau, 2003). This study also found that lesions to the IL cortex caused habitual responding after devaluation implying that sensitivity to reward is impaired following lesions to this area. This result is consistent with the current study that showed reduced sensitivity to reward.

This chapter provides evidence that efficient reversal learning on the PRL task requires a functionally intact medial prefrontal cortex. Furthermore, the results from the current study indicate that the impact of positive reinforcement on reversal learning is modulated by the mPFC.

Effects of citalopram administration on anxiety-like behaviour and probabilistic reversal learning.

3.1. Summary

The aim of the current set of experiments aims to test the acute effects of a serotonin selective reuptake inhibitor (SSRI) on anxiety-like behaviour, and the cognitive aspects examined by probabilistic reversal learning. The rational for this was to determine whether direct manipulation of serotonin would produce a similar pattern of behaviour as indirect manipulation though the administration of probiotics in Chapter 4.

3.2. Introduction

Serotonin selective reuptake inhibitors (SSRIs) are one of the most common family of drugs used in the treatment of depression and anxiety-related disorders, including generalised anxiety , social anxiety, panic disorder and obsessive-compulsive disorder (Bespalov *et al,* 2010; Golden, 2004; Zohar & Westenbery, 2000). SSRIs work by blocking the serotonin (5-hydroxytryptamine; 5-HT) transporter protein (5-HTT) located on the presynaptic membrane, this increases extracellular levels of 5-HT in the synaptic cleft by preventing reuptake into the presynaptic nerve terminal. Studies using microdialysis and high performance liquid chromatography (HPLC) indicate that SSRI treatment affects extracellular 5-HT levels in several brain regions (Fuller, 1994). Chronic exposure to SSRIs increase baseline 5-HT levels in comparison with acute treatment, shown only to transiently increase extra cellular 5-HT (Kreiss & Lucki, 1995). The differential effects of treatment type suggest that 5-HT reuptake inhibition *per se* is not responsible for the therapeutic effects of SSRIs administration, but rather adaptive neuronal responses over prolonged and repeated exposure are required before maximised therapeutic effects can be seen. Various studies investigating the adaptive change in neurochemical function have found that chronic treatment results in desensitisation of the 5-HT1a receptor (Ceglia *et al*, 2004; Chaput *et al*, 1986; Goodwin *et al,* 1985; Invernizzi *et al,* 1994, 1996;

Kreiss & Lucki, 1995). This receptor is found presynapically in the raphe nuclei and autoregulates serotonin release, stimulating serotonergic projections to other brain areas such as the stiatum and prefrontal cortex via a negative feedback mechanism (Kreiss & Lucki, 1994, 1995). Desensitisation of this receptor through increased extracelluar serotonin results in increased raphe firing rates to other brain areas. Acute treatment with SSRIs activate the auto-inhibitory properties of 5-HT1a autoreceptors reducing raphe nuclei firing (Blier, 2001). Hence anxiolytic effects of SSRI administration are not seen in acute treatment (in some behavioural measures of anxiety, discussed later), often anxiety symptoms in clinical populations are more pronounced during initial treatment (Birkett *et al,* 2011; Gorman *et al*, 1987; Ravinder *et al,* 2011). This has been supported in studies showing that co-administration of SSRIs and 5-HT1a receptor antagonist enhances the behavioural effects of SSRIs in both humans and animals (Muraki *et al,* 2008; Portella *et al*¸ 2011; Romero *et al*, 1996). However, administration of 5-HT1a receptor antagonists alone produce no anxiolytic effect (Muraki *et al,* 2008). As the effects of probiotic treatment on serotonin function in the raphe nuclei has not been examined, theoretically the increase in 5-HT function in the frontal lobe reported by Desbonnet *et al* (2010) may inhibit 5-HT transmission in the raphe nuclei, via negative feedback, similar to acute SSRI administration.

The initial behavioural test used in the preclinical study of anxiety was the open field test (OFT) designed by Calvin Hall (1934). Since then other tests have been developed to assess the therapeutic value of anxiolytic agents including the elevated plus maze (EPM) which is now the first choice for screening anxiolytic drugs (Ramos, 2008). Both of these tests rely on an unconditioned avoidance of threatening situations, an approach avoidance conflict is established where the animal is conflicted between exploring new areas whilst having an innate tendency to avoid potentially dangerous areas (Ramos, 2008). These tests have been used to assess the anxiolytic properties of benzodiazepines, SSRIs and tricyclic antidepressants (Oh *et al,* 2009; Prut & Belzung, 2003; Vendruscolo *et al,* 2003; Vorhees *et al,* 2011). Pharmacological studies have shown incoherency between these two tests, the benzodiazepine, chlordiazepoxide produced anxiolytic effects on the

EPM but not that OFT (Vendruscolo *et al,* 2003). Similarly, in genetic studies, in spontaneously hyperactive rats (SHRs), an animal model of anxiety, this same drug produced anxiolytic effects in the OFT but not the EPM (Vendruscolo *et al,* 2003). This demonstrates that anxiety, as with other emotional disorders, is a multi-faceted condition and that any one test may only be sensitive to one aspect of this complex behaviour. In a study using Flinders Sensitive Line (FSL) rats, a genetic model of depression, chronic dosing over 14 days of 10 mg/kg of citalopram reduced anxiety in the OFT and EPM without affecting locomotor activity (Kokras *et al,* 2011). Acute dosing with SSRIs has routinely been shown to produce anxiogenic effects in animals. The SSRI fluoxetine in acute doses increased anxiety in the EPM (Ravinder *et al,* 2011). Conversely, studies have found dose dependant effects of SSRI administration with acute and chronic doses producing an anxiolytic effect whilst sub-chronic doses having no anxiolytic effect in an elevated T-maze (Pinheiro *et al,* 2008). High acute doses of citalopram (10, 30 & 100mg/kg) have also been found to reduce conditioned fear-stress induced behaviour (Muraki *et al,* 2008). These findings suggest that behavioural measures of anxiety, potency of SSRI (citalopram v escitalopram) and dose may produce contrasting results.

In clinical populations, anxiety disorders and depression are commonly accompanied by cognitive inflexibility (Dickstein *et al,* 2009). The functional implications of this are an inability to alter behaviour in response to changes in stimulus-reward contingencies. Several studies have shown that depressed patients demonstrate an attention bias towards negative stimuli (Gotlib *et al,* 2004; Siegle *et al,* 2001), have increased sensitivity to negative feedback (Murphy *et al,* 2003) and have reduced sensitivity to positive stimuli (Roiser *et al,* 2009). An accurate way of measuring responses to positive and negative stimuli is to use the probabilistic reversal learning paradigm. In this task, mice with a 5-HTT knockout mutation demonstrated reduced negative feedback sensitivity (NFS) compared with wild types. Furthermore, administration of escitalopram (a clinical prescribed antidepressant derived from the potent s-enaniomer of citalopram) resulted in reduced NFS, increased reward sensitivity and increased number of reversals (Ineichen *et al,* 2012). Similar results were found in normal rats with a high (10mg/kg) acute dose of citalopram; increasing the number of

reversals and decreasing NFS. However, a low (1 mg/kg) acute dose produced the opposite effect on both measures (Bari *et al,* 2010). In the latter study, chronic dosing increased reversals and responsiveness to positive reinforcement.

Selective 5-HT depletion in the frontal cortex has also been shown to result in an inability to shift responding (Clarke *et al,* 2005, 2007). Global 5-HT depletion resulted in reduced reversals and reward sensitivity (Bari *et al,* 2010). In human studies, depletion of tryptophan, the biochemical precursor to 5-HT increased NFS. However, it did not affect reward sensitivity (Robinson *et al,* 2012). This supports previous findings that 5-HT is important in processing affective stimuli (Gotlib *et al,* 2004*;* Roiser *et al,* 2010; Siegle *et al,* 2001). The neurochemical mechanisms which underpin cognitive flexibility have been the focus of studies examining the role of 5-HT transmission in inhibitory processes. A study by Brown *et al* (2012) examined the contribution of inhibitory processes and anxiety in flexibility of responding. Given previous findings that SSRI administration facilitates learning in tasks which contain an anxiety producing component (Hashimoto *et al,* 2007; Montezhino *et al,* 2010) it was proposed that SSRI administration at doses which reduce anxiety improve cognitive flexibility by reducing anxiety when a previously rewarded response is no longer reinforced; allowing for more rapid switching in response patterns. Using the EPM as the standard test for anxiety, the results indicated that administration of escitalopram did not reduce anxiety at doses which also improved flexibility in responding. A second theory on the mechanism by which 5-HT manipulation affects response switching was based on findings that administration of SSRIs facilitate inhibitory processes. The theory proposed that SSRIs affect cognitive flexibility by inhibiting learned or naturally biased prepotent reponses, i.e. SSRIs facilitate inhibition of a response which previously was rewarded (learned prepotent response) or SSRIs facilitate inhibition of a naturally biased response, e.g. entering closed arms of EPM more than open arms. Results from this study indicated that escitalopram facilitates inhibition of naturally biased prepotent responses (tested using an elevated conflict test) and inhibition of learned prepotent responses (tested using serial reversal).

The purpose of the current study was to produce a behavioural profile of acute citalopram administration on anxiety-like behaviour and reversal learning. The combination of these experiments aims to provide insight into how these behaviours interact with each other. Acute dosing of SSRIs have previous shown to increase anxiety in normal animals and animal models of anxiety (FSL & SHR) and so it is expected that anxiety-like behaviour will be increased in these tests. As the reversal learning paradigm used here is based on that by Bari *et al* (2010) it is expected that acute citalopram dosing will facilitate performance on this task, by increasing NFS. This behavioural profile can then be used to compare the effects of probiotic treatment on reversal learning and anxiety-like behaviour.

3.3. *Methods and Procedure*

Subjects

Experiment 1 and 2; Open field test and elevated plus maze: Thirty-two male adult Lister hooded rats (Harlan, UK) weighing 400-550g were used in this experiment. There were housed in pairs in a 12h:12h light dark cycle, the holding room was maintained at 55% ±10 humidity and a temperature of 19-23^{o}C. There were all tested during the 12 hour light cycle. These animals were 7 months old when being tested. All animals were thoroughly habituated to handling before the study began. The experiment was performed in accordance with Home Office under Animals Scientific Procedures Act (1986).

The same animals were used for the OFT and EPM. There was one week given between finishing one study and the beginning of the next. For these tests the rats were divided into 3 groups, 1mg/kg citalopram, 10mg/kg citalopram and vehicle. The EPM was the first of the two tests to be run. The groups were then rotated so that the group which received 1mg/kg citalopram i.p. in the EPM (n=11) then received vehicle I.p. injection in the OFT, the group which received vehicle i.p. injection in the EPM (n=10) received 10mg/kg citalopram i.p. in the OFT and the group which received 10mg/kg citalopram i.p in the EPM (n=11) then received 1mg/kg citalopram i.p. in the OFT.

Experiment 3; PRL 5 mg/kg citalopram: Thirty-two male adult Lister hooded rats (Harlan, UK) weighing 270-350g were used in this experiment. These animals were 4 months old when testing began.

Experiment 4; PRL 10mg/kg citalopram: Twenty-four male adult Lister hooded rats (Harlan, UK)weighing 250-320g were used in this experiment. These animals were 4 months old when tested.

All animals were thoroughly habituated to handling before the study began. The animals were food restricted to 85% of free feeding body weight and had free access to water. The experiment was performed in accordance with Home Office under Animals Scientific Procedures Act 1986.

Citalopram Administration

The Citalopram treated groups were injected i.p with 1mg/kg, 5mg/kg or 10mg/kg of Citalopram Hydrobromide (Tocris, Bristol, UK). Citalopram was dissolved in 0.9% injectable saline to a concentration of 1mg/ml and administered 30mins prior to testing. Vehicle treated animals were injected with 0.9% injectable saline at a volume of 1ml/kg administered 30mins prior to testing. The animals were injected in a separate room and were returned to their holding room for 30 minutes before testing.

Apparatus and Procedure

Experiment 1: Open Field Test

The open field test arena was a 1m x 1m box with walls 40cm high. The activity of the animals was recorded using wide lens camera attached to a RM PC and analysed using EthoVision, an automated behavioural tracking system (Noldus Information Technology, Netherlands). All animals were naive to the room prior to testing. The room was illuminated using 60 watt lighting. All items in the room remained in place over the two days of testing. Animals were carried in groups of four in a blacked out carrying box into the room. The first animal was placed into the centre of the area whilst the experimenter remained out of sight. After the 10 minute testing periods the animal was removed from the arena and placed back into the carrying box. The arena was thoroughly cleaned before the next animal was introduced. The data was analysed according to zones, the edge of the arena was 20 cm from the wall, where these intersected were the corners, the 60cm x 60cm area in the centre was the middle. Measure taken during the test session the length of time spent in each zone, the velocity of movement, maximum distance moved and the frequency of entrances to each area. The

data given for the corners consists of all the data recorded for each of the four corners; this is the same with the data given for the edges. Each animal had two ten minute sessions in the OFT, this was run on two consecutive days.

Experiment 2: Elevated Plus Maze

The elevated plus maze consists of two intersecting platforms in the shape of a plus sign measuring 110cm x 10cm raised 70cm off the floor. Two opposing arms of the maze have 40cm black Perspex walls whilst the other two opposing arms are open. This allows for a 10cm x 10cm intersection to allow the animal to move freely between the arms of the maze. The activity of the animals was recorded using a camera attached to a RM PC and analysed using EthoVision, an automated behavioural tracking system (Noldus Information Technology, Netherlands). All animals were naive to the room prior to testing. Animals were carried in groups of four in a blacked out carrying box into the room. The first animal was placed into intersection facing an open arm. The animal was left to explore for 5 minutes before being returned to the carrying box. Once the animal had completed the test the maze was wiped down with alcohol wipes before the next animal was introduced. The data recorded during the session were the length of time spent in each zone, the velocity of movement, latency to enter each zone, maximum distance moved and the frequency of entrances to each area. The data recorded for both open arms was complied to give one data point, this was the same for the closed arms.

Experiment 3 and 4: PRL with 5 mg/kg and 10 mg/kg Citalopram

The apparatus and procedure used in this experiment ere identical to that described in Chapter section 2.3. The rats had one session per day and were given 3 days to acquire the task. They were counterbalanced on average number of reversals over days completed on the first three days and assigned to one of two group, drug or vehicle. These groups then had a further 3 days of the test phase. Injections were administered 30 minutes before behavioural testing.

Statistical analysis

For the EPM and the OFT the duration, total distance moved and the frequency of entrances into each area were all analysed as a percentage of duration, total distance moved and frequency of entrances into all areas. Maximum distance moved at any one time was calculated and analysed as a percentage of maximum distance moved in all areas. For the EPM, a one-way ANOVA was carried out on each of the following measures; duration, frequency of entrances, total distance moved, maximum distance moved (maximum distance move without stopping in at any one time), and velocity. As the OFT was conducted over two days a repeated measures ANOVA was carried out on each of the measures. The between subjects factor was dose (1mg/kg, 10mg/kg and vehicle). The results are expressed as mean ± 1 standard error. The threshold for significance was $p<0.05$. Where data was non-spherical given the output from *Malchly's Test of Shericity* ,the *Greenhouse-Geisser* adjustment was used to give a corrected *F* ratio. Where interactions were significant pairwise comparisons were used to examine the simple effects of this interaction. Tukey's post hoc analysis was carried out to examine the differences between groups. All statistical analysis was carried out using SPSS version 20.0.

Performance in the PRL task was analysed by phase, the acquisition phase was comprised of the trials before the first reversal, the reversal phase comprised of any trials after the acquisition phase but prior to any further reversals should they be completed. The results of any animals that failed to complete any reversals had their data analysed as acquisition phase and therefore did not contribute to the reversal phase data.

The data analysed from the test phase was the number of reversals completed, reward (win-stay) and negative feedback (lose-shift) sensitivity. Response to positive and negative feedback was analysed by evaluating responding on a trial after having received a reward or punishment in the previous trial. Win-stay performance was the likelihood of repeating the same behaviour having been rewarded on a pervious trial, (positive feedback), lose-shift performance was the likelihood of

discontinuing the behaviour following a punishment on the previous trial, (negative feedback). Analysis was also carried out of the perseverative errors. These were calculated at the percentage of trials where an incorrect response was recorded following reward for the same response. The number of trials to reach the criterion was also recorded, i.e. the number of trials in the acquisition phase of each session.

When calculating the probabilities, omissions and responses in non-illuminated apertures were not included. That is win-stay probability is calculated by dividing total number of rewarded responses in the same hole as the previous trial by the total number of reward responses and lose-shift probability is calculated by dividing the total number of shifts in response following punished responses divided by the total number of punished responses.

Repeated measure ANOVA was carried out on all the measures. Win-stay and lose-shift probability were also analysed by phase, acquisition and reversal on the first test day. Where repeated measure ANOVA gave a significant difference pairwise comparisons were carried out. All statistical analysis was carried out using SPSS version 20.0.

3.4. Results

3.4.1. Open Field test

Repeated measure ANOVA were carried out for each of the measures examining each area of the maze separately. *Post hoc* analysis was also conducted to examine the difference between the groups in each area.

Frequency

Frequency of entrances into all area of the arena are displayed in Figure 1. Frequency of entrances at the corners of the areas showed that there was no main effect of dose ($F(2,29)=0.377$, p=NS). *Post hoc* analysis showed there was no significant difference between any of the groups (Table 1). There was a significant main effect of day ($F(1,29)=144.666$, $p<0.001$). However, there was no significant day by dose interaction ($F(2,29)=2.989$, p=NS). A table of the raw scores in provided (Table 2). Statistical analysis of the raw scores are also showed a significant main effect of day ($F(2,29)=20.717$, $p<0.001$), no significant main effect of group ($F(2,29)=0.645$, p=NS) or day by dose interaction ($F(1,29)=1.369$, p=NS).

There was also no main effect of dose on frequency at the edges of the maze ($F(2,29)=1.563$, p=NS) or main effect of day ($F(1,29)=0.08$, p=NS). This was confirmed by *Post hoc* analysis (Table 1). There was also no day by dose interaction ($F(2,29)=0.836$, p=NS). Analysis of the raw scores also indicated no significant main effect of day ($F(1,29)=146.72$, p=NS), no significant main effect of dose ($F(2,29)=1.414$, p=NS) or day by dose interaction($F(2,29)=1.998$, p=NS)

Results from the middle portion of the arena showed that there was no main effect of dose ($F(2,29)=0.166$, p=NS). *Post hoc* analysis of this measure is displayed in Table 1. There was a significant main effect of day ($F(1,29)=158.693$, $p<0.001$) and day by dose interaction ($F(2,29)=4.229$, $p<0.05$). Analysis of the simple effects of this interaction showed that each group, 10 mg/kg ($F(1,29)=28.373$, $p<0.001$), 1 mg/kg ($F(1,29)=53.47$, $p<0.001$) and vehicle ($F(1,29)=82.755$, $p<0.001$)

differed significantly across the two sessions. Pairwise comparisons of the groups on each day are displayed in table 3. A similar pattern of results was observed when raw scores were analysed. There was no significant main effect of dose ($F(2,29)=2.983$, p=NS). However there was a significant main effects of day ($F(1,29)=313.847$, $p<0.001$) and day by dose interaction ($F(2,29)=3.994$, $p<0.05$).

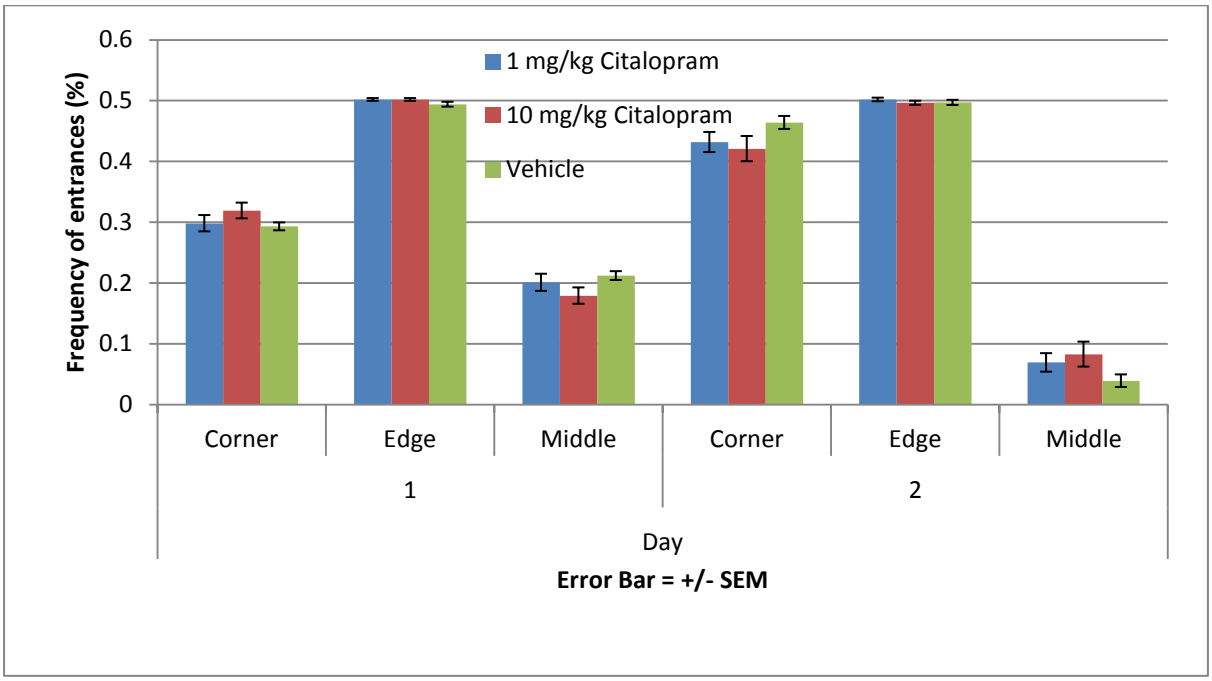

Figure 1. Frequency of entrances into the areas of the maze as a percentage of frequency of entrances into all areas of the maze.

Table 1. *Post hoc* analysis of frequency into each area of the arena.

Area	Dose (1)	Dose (2)	*F* value	*p* value
Corner	10 mg/kg	1 mg/kg	0.377	0.715
	10 mg/kg	Vehicle		0.617
	1 mg/kg	Vehicle		0.393
Edge	10 mg/kg	1 mg/kg	1.563	0.507
	10 mg/kg	Vehicle		0.28
	1 mg/kg	Vehicle		0.09
Middle	10 mg/kg	1 mg/kg	0.166	0.803
	10 mg/kg	Vehicle		0.745
	1 mg/kg	Vehicle		0.57

Table2. Raw scores of frequency of entrances

Area	Day	Dose	Frequency
Corner	1	1mg/kg	28.18
		10mg/kg	25.36
		Vehicle	26.70
	2	1mg/kg	18.10
		10mg/kg	18.75
		Vehicle	22.40
Edge	1	1mg/kg	87.67
		10mg/kg	39.64
		Vehicle	44.90
	2	1mg/kg	38.91
		10mg/kg	20.38
		Vehicle	24.40
Middle	1	1mg/kg	19.45
		10mg/kg	14
		Vehicle	19.40
	2	1mg/kg	3.10
		10mg/kg	1.88
		Vehicle	2.00

Table 3. Pairwise comparison of middle area frequency

Day	Dose (1)	Dose (2)	*P* value
1	10mg/kg	1mg/kg	0.212
		Vehicle	0.07
	1mg/kg	Vehicle	0.531
2	10mg/kg	1mg/kg	0.537
		Vehicle	0.071
	1mg/kg	Vehicle	0.216

Duration

Duration of time spent in each area of the arena is displayed in Figure 2. The duration of time spent in the corner of the arena was not found to be significantly affected by dose ($F(2,29)=0.444$, p=NS). *Post hoc* analysis showed there was no significant different between any of the groups (Table 4). There was a significant main effect of day ($F(1,29)=111.675$, $p<0.001$). However, there was no significant day by dose interaction ($F(2,29)=1.042$, p=NS). Raw scores are provided in table 5.

Analysis of the raw scores also showed a significant main effect of day ($F(1,29)=104.434$, $p<0.01$), no main effect of dose ($F(2,29)=3.281$, p=NS) or day by dose interaction ($F(2,29)=1.179$, p=NS)

There was no main effect of dose found on duration at the edges of the maze ($F(2,29)=0.32$, p=NS). *Post hoc* analysis showed no significant difference between any of the groups (Table 4). There was a significant main effect of day ($F(1,29)=57.809$, $p<0.001$) however, no significant day by dose interaction ($F(2,29)=0.061$, p=NS). Analysis of raw scores show a significant main effect of day ($F(1,29)=28.255$, p<0.001), however there was also no main effect of dose ($F(2,29)=0.66$, p=NS) or day by dose interaction ($F(2,29)=1.281$, p=NS)

Analysis of the duration in the middle of the arena showed there was no significant main effect of dose ($F(2,29)=0.098$, p=NS). *Post hoc* analysis also showed no difference between any of the groups (Table 4). There was an significant main effect of day ($F(1,29)=139.175$, $p<0.001$) and day by dose interaction ($F(2,29)=6.425$, $p<0.01$). Pairwise comparisons of this interaction showed that each group, 10 mg/kg ($F(1,29)=21.389$, $p<0.001$), 1 mg/kg ($F(1,29)=40.814$, $p<0.001$) and vehicle ($F(1,29)=86.559$, $p<0.001$) significantly differed across the two days. Pairwise comparisons of doses on each day is displayed on table 6. Analysis of the raw scores also showed a significant main effect of day ($F(1,29)=221.695$, $p<0.001$) and day by dose interaction ($F(2,29)=4.083$, $p<0.05$) and no main effect of group ($F(2,29)=1.092$, p=NS).

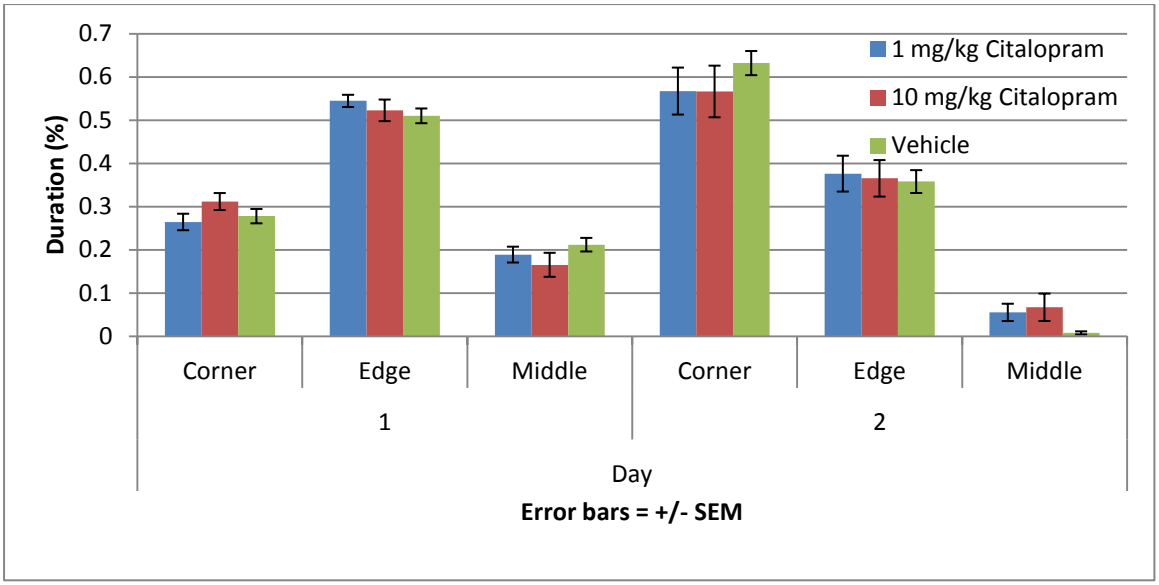

Figure 2. Percentage of time spent in each area of the maze as a percentage for total duration of the session.

Table 4. *Post hoc* analysis of duration in each area of the arena.

Area	Dose (1)	Dose (2)	*F* value	*p* value
Corner	10 mg/kg	1 mg/kg	0.444	0.831
	10 mg/kg	Vehicle		0.93
	1 mg/kg	Vehicle		0.626
Edge	10 mg/kg	1 mg/kg	0.32	0.593
	10 mg/kg	Vehicle		0.804
	1 mg/kg	Vehicle		0.443
Middle	10 mg/kg	1 mg/kg	0.098	0.839
	10 mg/kg	Vehicle		0.811
	1 mg/kg	Vehicle		0.662

Table 5. Raw scores of duration

Area	Day	Dose	Duration
Corner	1	1mg/kg	79.44
		10mg/kg	93.55
		Vehicle	83.15
	2	1mg/kg	179.48
		10mg/kg	201.9
		Vehicle	189.54
Edge	1	1mg/kg	163.73
		10mg/kg	156.69
		Vehicle	152.88
	2	1mg/kg	107.86
		10mg/kg	91.48
		Vehicle	108.04
Middle	1	1mg/kg	56.84
		10mg/kg	49.65
		Vehicle	63.84
	2	1mg/kg	12.66
		10mg/kg	6.625
		Vehicle	2.24

Table 6. Pairwise comparison of middle area duration

Day	Dose (1)	Dose (2)	*P* value
1	10mg/kg	1mg/kg	0.429
		Vehicle	0.131
	1mg/kg	Vehicle	0.448
2	10mg/kg	1mg/kg	0.672
		Vehicle	0.064
	1mg/kg	Vehicle	0.143

Total Distance

The total distance travelled in the areas of the maze is illustrated in Figure 3. Repeated measure ANOVA indicated that there was no significant main effect of dose on distance travelled in the corners ($F(2,29)=0.242$, p=NS). *Post hoc* analysis also showed no significant between any of the groups (Table 7). There was a significant main effect of day ($F(1,29)=127.203$, $p<0.001$) however, there was no significant day by dose interaction ($F(2,29)=1.272$, p=NS). Raw scores are provided in

table 8. Analysis of the raw scores also show a significant main effect of day ($F(1,29)=52.937$, $p<0.05$), no main effect of dose ($F(2,29)=0.542$, p=NS) or day by dose interaction ($F(2,29)=3.099$, p=NS).

There was also no significant main effect of dose on distance travelled at the edges of the arena ($F(2,29)=0.043$, p=NS). The *post hoc* analysis for dose is displayed in Table 7. There was a significant main effect of day ($F(1,29)=18.532$, $p<0.001$), however no significant day by does interaction ($F(2,29)=1.528$, p=NS). Again, analysis of raw scores show a significant main effect of day ($F(1,29)=66.733$, $p<0.001$), no main effect of dose ($F(2,29)=2.331$, p=NS) or day by dose interaction ($F(2,29)=0.594$, p=NS).

There was no significant main effect of dose on the total distance travelled in the middle of the arena ($F(2,29)=0.498$, p=NS). *Post hoc* analysis showed no significant difference between the groups (Table 7). There was a significant main effect of day ($F(1,29)=156.885$, $p<0.001$) and day by dose interaction ($F(2,29)=6.88$, $p<0.01$). Analysis of the simple effects of this interaction showed that each group, 10 mg/kg ($F(1,29)=30.65$, $p<0.001$), 1 mg/kg ($F(1,29)=36.64$, $p=<0.001$) and vehicle ($F(1,29)=99.538$, $p<0.001$) differed significantly across the two days. Pairwise comparisons of the doses on each day are displayed in table 9. Analysis of the raw scores also showed a significant main effect of day ($F(1,19)=195.828$, $p<0.001$) and day by dose interaction ($F(2,29)=4.824$, $p<0..05$) and no main effect of dose ($F(2,29)=0.831$, p=NS).

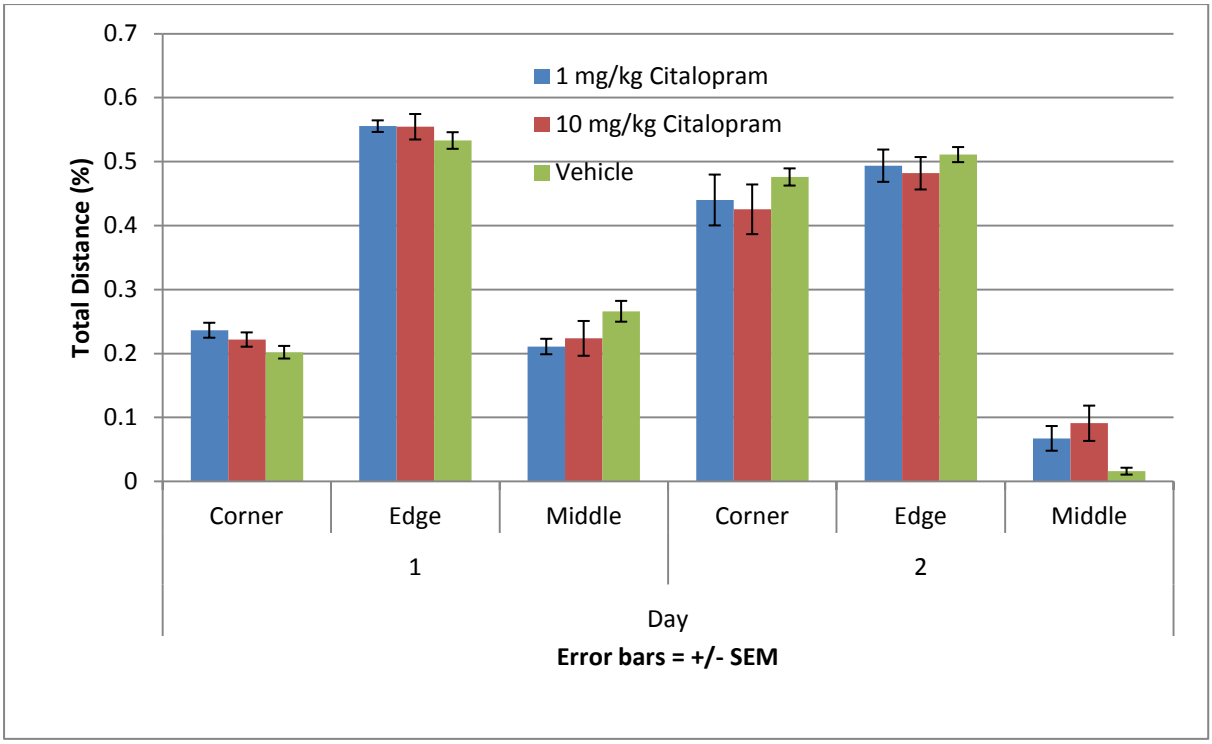

Figure 3. Total distance in each area as a percentage of total distance moved in all areas.

Table 7. *Post hoc* analysis of total distance travelled in each area of the arena.

Area	Dose (1)	Dose (2)	*F* value	*p* value
Corner	10 mg/kg	1 mg/kg	0.242	0.832
	10 mg/kg	Vehicle		0.811
	1 mg/kg	Vehicle		0.999
Edge	10 mg/kg	1 mg/kg	0.043	0.954
	10 mg/kg	Vehicle		0.983
	1 mg/kg	Vehicle		0.993
Middle	10 mg/kg	1 mg/kg	0.498	0.638
	10 mg/kg	Vehicle		0.705
	1 mg/kg	Vehicle		0.996

Table 8. Raw scores of total distance

Area	Day	Dose	Total Distance
Corner	1	1mg/kg	181.67
		10mg/kg	150.65
		Vehicle	161.42
	2	1mg/kg	228.73
		10mg/kg	242.86
		Vehicle	267.43
Edge	1	1mg/kg	430.08
		10mg/kg	381.88
		Vehicle	431.29
	2	1mg/kg	250.90
		10mg/kg	216.55
		Vehicle	298.11
Middle	1	1mg/kg	625.7
		10mg/kg	630.44
		Vehicle	872.75
	2	1mg/kg	148.98
		10mg/kg	88.13
		Vehicle	46.59

Table 9. Pairwise comparison of middle area total distance

Day	Dose (1)	Dose (2)	*P* value
1	10mg/kg	1mg/kg	0.608
		Vehicle	0.161
	1mg/kg	Vehicle	0.062
2	10mg/kg	1mg/kg	0.339
		Vehicle	*0.011**
	1mg/kg	Vehicle	0.088

(= p<0.05)*

Maximum Distance

The maximum distance travelled in each area of the arena is displayed in Figure 4. Repeated measure ANOVA showed that there was no significant main effect of dose on maximum distance travelled in the corners of the arena ($F(2,29)=0.086$, p=NS). *Post hoc* analysis also shows no significant difference between any of the groups (Table 10). There was also no significant main effect of day ($F(1,29)=1.487$, p=NS) or day by group interaction ($F(2,29)=1.028$, p=NS). Raw scores

are provided in table 11. Statistical analysis of the raw scores also showed no main effect of day (F(1,29)=1.917, p=NS) or dose (F(2,29)=0.135, p=NS) and no significant day by dose interaction (F(2,29)=0.282, p=NS).

Analysis of maximum distance at the edges of the maze also showed no significant main effect of dose (F(2,29)=0.656, p=NS). *Post hoc* analysis also showed no significant difference between the groups (Table 10). There was also no significant main effect of day (F(1,19)=0.369, p=NS), or day by dose interaction (F(2,290=0.477, p=NS). Analysis of raw scores indicated no significant main effect of day (F(1,29)=0.494, p=NS) or dose (F(2,29)=0.994, p=NS) day by dose interaction (F(2,29)=0.574, p=NS).

There was no significant main effect of dose found on maximum distance travelled in the middle of the maze (F(2,29)=2.223, p=NS). *Post hoc* analysis showed no significant difference between any of the groups (Table 10). There was also no main effect of day (F(1,29)=0.804, p=NS) or day by dose interaction (F(2,29)=1.363, p=NS). Analysis of the raw scores indicated that there was no main effect of day (F(1,29)=1.729, p=NS) or day by dose interaction (F(2,29)=2.298, p=NS), however there was a significant main effect of dose (F(2,29)=3.418, p<0.05). *Post hoc analysis* showed that there was a significant difference between 10mg/kg group and vehicle (p<0.05) but not between any other groups.

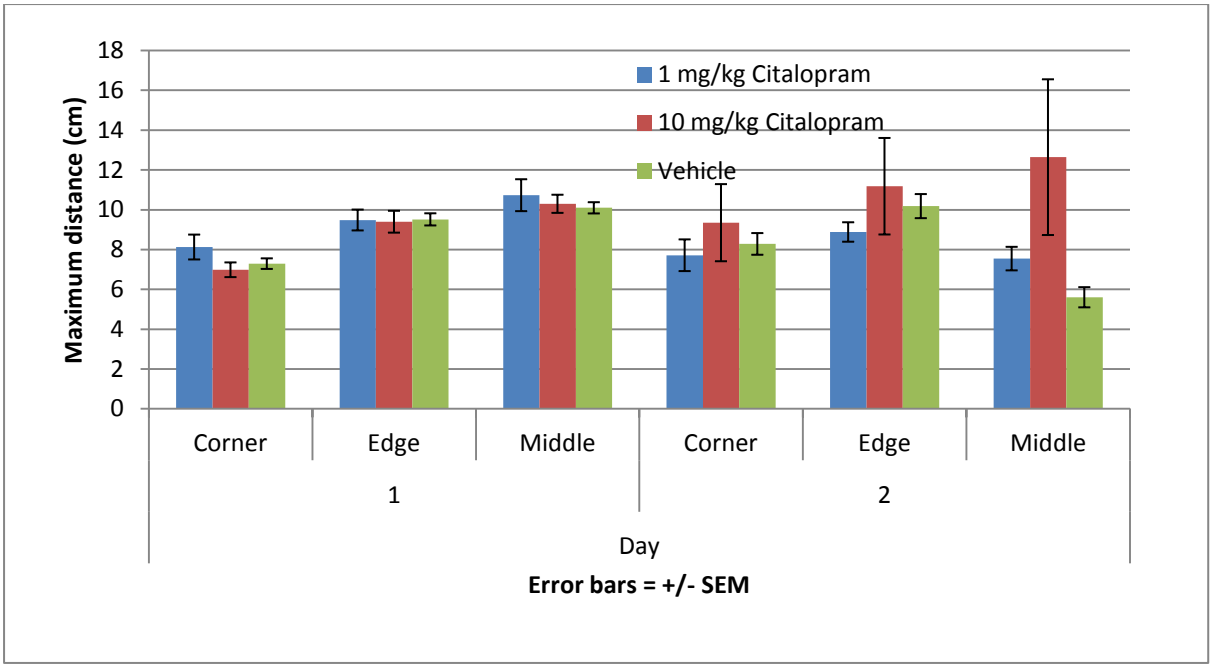

Figure 4. Maximum distance travelled in each area as a percentage of maximum distance travelled in all areas.

Table 10. *Post hoc* analysis of maximum distance travelled in each area of the arena.

Area	Dose (1)	Dose (2)	*F* value	*p* value
Corner	10 mg/kg	1 mg/kg	0.086	0.792
	10 mg/kg	Vehicle		0.692
	1 mg/kg	Vehicle		0.89
Edge	10 mg/kg	1 mg/kg	0.656	0.264
	10 mg/kg	Vehicle		0.661
	1 mg/kg	Vehicle		0.51
Middle	10 mg/kg	1 mg/kg	2.223	0.143
	10 mg/kg	Vehicle		0.059
	1 mg/kg	Vehicle		0.556

Table 11. Raw scores of maximum distance

Area	Day	Dose	Maximum Distance
Corner	1	1mg/kg	8.13
		10mg/kg	6.98
		Vehicle	7.29
	2	1mg/kg	7.83
		10mg/kg	10.46
		Vehicle	8.34
Edge	1	1mg/kg	9.48
		10mg/kg	9.40
		Vehicle	9.51
	2	1mg/kg	8.89
		10mg/kg	12.03
		Vehicle	10.23
Middle	1	1mg/kg	10.73
		10mg/kg	10.30
		Vehicle	10.10
	2	1mg/kg	7.42
		10mg/kg	13.44
		Vehicle	5.60

Velocity

The velocity moved in each area of the maze is displayed in Figure 5. Repeated measure ANOVA showed there was no significant main effect of dose on velocity in the corners of the maze ($F(2,29)=0.912$, p=NS). *Post hoc* analysis showed no significant differences between any of the groups (Table 12). The was a significant main effect of day ($F(1,29)=62.084$, $p<0.001$) and a significant day by dose interaction ($F(2,29)=4.392$, $p<0.05$). Analysis of the simple effects of this interaction showed that all groups, 10 mg/kg ($F(1,29)=5.075$, $p<0.05$), 1 mg/mg ($F(1,29)=27.53$, $p<0.001$) and vehicle ($F(1,29)=36.927$, $p<0.001$) significantly differed across the days. Pairwise comparisons of the doses on each day are displayed in figure 14. Raw scores are provided in table 13. Analysis of the raw scores shows that there was a significant main effect of day ($F(1,19)=66.74$, $p<0.001$) and day by dose interaction ($F(2,29)=2.445$, $p<0.05$) but no main effect if dose ($F(2,29)=2.553$, p=NS) .

Velocity at the edges of the arena was not significantly affected by dose ($F(2,29)=2.03$, p=NS). *Post hoc* analysis also showed no significant difference between the groups (Table 12). There was no main effect of day ($F(1,29)=0.285$, p=NS) or day by group interaction ($F(2,29)=0.43$, p=NS). Analysis of the raw scores also showed no main effect of day (F(1,29)=0.224, p=NS) or dose (F(2,29)=2.287, p=NS) or day by group interaction (F(2,29)=0.409, p=NS).

Velocity in the middle of the arena was not significantly affected by dose ($F(2,29)=0.437$, p=NS). *Post hoc* analysis showed no significant difference between any of the groups (Table 12). There was also no significant main effect of day ($F(1,29)=2.089$, p=NS) or day by dose interaction ($F(2,29)=0.037$, p=NS). Analysis of the raw scores also showed no significant main effect of day ($F(1,29)=1.211$, p=NS), or dose ($F(2,29)=2.429$, p=NS) or day by dose interaction.

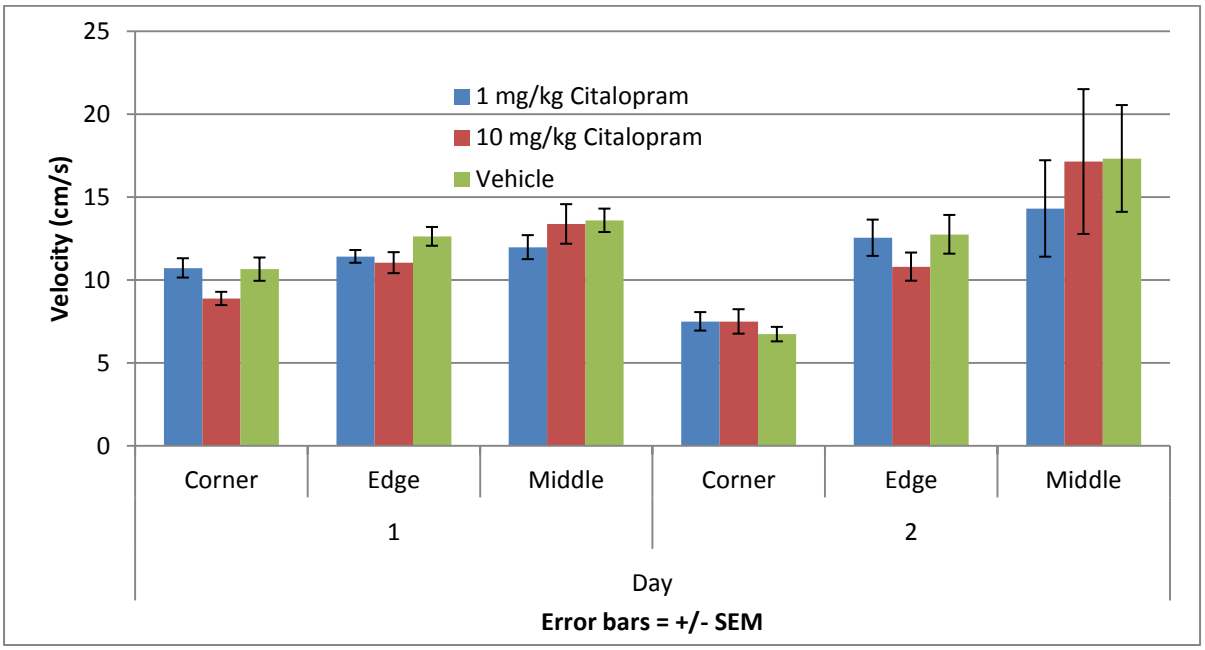

Figure 5. Average velocity in each area.

Table 12. *Post hoc* analysis of velocity in each area of the arena.

Area	Dose (1)	Dose (2)	*F* value	*p* value
Corner	10 mg/kg	1 mg/kg	0.912	0.188
	10 mg/kg	Vehicle		0.48
	1 mg/kg	Vehicle		0.553
Edge	10 mg/kg	1 mg/kg	2.03	0.23
	10 mg/kg	Vehicle		0.056
	1 mg/kg	Vehicle		0.431
Middle	10 mg/kg	1 mg/kg	0.437	0.411
	10 mg/kg	Vehicle		0.986
	1 mg/kg	Vehicle		0.455

Table 13. Raw scores of velocity

Area	Day	Dose	Velocity
Corner	1	1mg/kg	10.72
		10mg/kg	8.88
		Vehicle	10.65
	2	1mg/kg	7.23
		10mg/kg	6.84
		Vehicle	6.80
Edge	1	1mg/kg	11.42
		10mg/kg	11.04
		Vehicle	12.62
	2	1mg/kg	12.83
		10mg/kg	10.48
		Vehicle	13.01
Middle	1	1mg/kg	11.98
		10mg/kg	13.38
		Vehicle	13.59
	2	1mg/kg	14.92
		10mg/kg	24.77
		Vehicle	17.32

Table 14. Pairwise comparison of corner area velocity

Day	Dose (1)	Dose (2)	*P* value
1	10mg/kg	1mg/kg	*0.026**
		Vehicle	*0.037**
	1mg/kg	Vehicle	0.925
2	10mg/kg	1mg/kg	0.995
		Vehicle	0.379
	1mg/kg	Vehicle	0.376

(= p<0.05)*

The results from the OFT indicated that neither dose of citalopram treatment significantly affected any of the behavioural measures compared with vehicle on either day. Analysis of the raw data did not find any contrasting results.

3.4.2. Experiment 2: Elevated Plus Maze

One-way ANOVA was carried out on the measures recorded in the elevated plus maze (Figure 6). Velocity in each area of the maze is displayed in Figure 7. Statistical analysis showed no significant differences between the groups in the closed arms on frequency ($F(2,31)=1.4$, p=NS), or velocity ($F(2,31)=0.304$, p=NS) in the closed arms of the maze. However, there was a significant difference between the groups on duration ($F(2,31)=4.737$, $p<0.05$) and total distance ($F(2,31)=6.239$, $p<0.01$) and maximum distance ($F(2,31)=3.36$, $p<0.05$). *Post hoc* analysis of the differences between each group is displayed in Table 15.

Table 15. *Post hoc* analysis of all measures in the closed arms of the maze.

Measure	Dose (1)	Dose (2)	*F* value	*p* value
Frequency	10 mg/kg	1 mg/kg	1.4	0.493
	10 mg/kg	Vehicle		0.466
	Vehicle	1 mg/kg		1
Duration	10 mg/kg	1 mg/kg	4.737	*0.025 **
	10 mg/kg	Vehicle		0.063
	Vehicle	1 mg/kg		1
Total Distance	10 mg/kg	1 mg/kg	6.239	*0.009 **
	10 mg/kg	Vehicle		*0.026 **
	Vehicle	1 mg/kg		1
Maximum Distance	10 mg/kg	1 mg/kg	3.36	0.711
	10 mg/kg	Vehicle		*0.044 **
	Vehicle	1 mg/kg		0.59
Velocity	10 mg/kg	1 mg/kg	0.304	1
	10 mg/kg	Vehicle		1
	Vehicle	1 mg/kg		1

(= p<0.05)*

One-way ANOVA from the open arms of the maze showed that there was no significant differences between the groups on maximum distance ($F(2,31)=1.829$, p=NS) or velocity ($F(2,31)=1.542$, p=NS).

However, there were significant difference found in frequency ($F(2,31)=4.544$, $p<0.05$), duration ($F(2,31)=3.698$, $p<0.05$) and total distance ($F(2,31)=4.694$, $p<0.05$). *Post hoc* analysis of the differences between each group are displayed in Table 16.

Table 16. *Post hoc* analysis of all measures in the open arms of the maze

Measure	Dose (1)	Dose (2)	*F* value	*p* value
Frequency	10 mg/kg	1 mg/kg	4.544	*0.019 **
	10 mg/kg	Vehicle		0.173
	Vehicle	1 mg/kg		0.946
Duration	10 mg/kg	1 mg/kg	3.698	*0.033 **
	10 mg/kg	Vehicle		0.632
	Vehicle	1 mg/kg		0.457
Total Distance	10 mg/kg	1 mg/kg	4.694	*0.015 **
	10 mg/kg	Vehicle		0.204
	Vehicle	1 mg/kg		0.747
Maximum Distance	10 mg/kg	1 mg/kg	1.829	0.683
	10 mg/kg	Vehicle		0.211
	Vehicle	1 mg/kg		1
Velocity	10 mg/kg	1 mg/kg	0.381	1
	10 mg/kg	Vehicle		1
	Vehicle	1 mg/kg		1

(= p<0.05)*

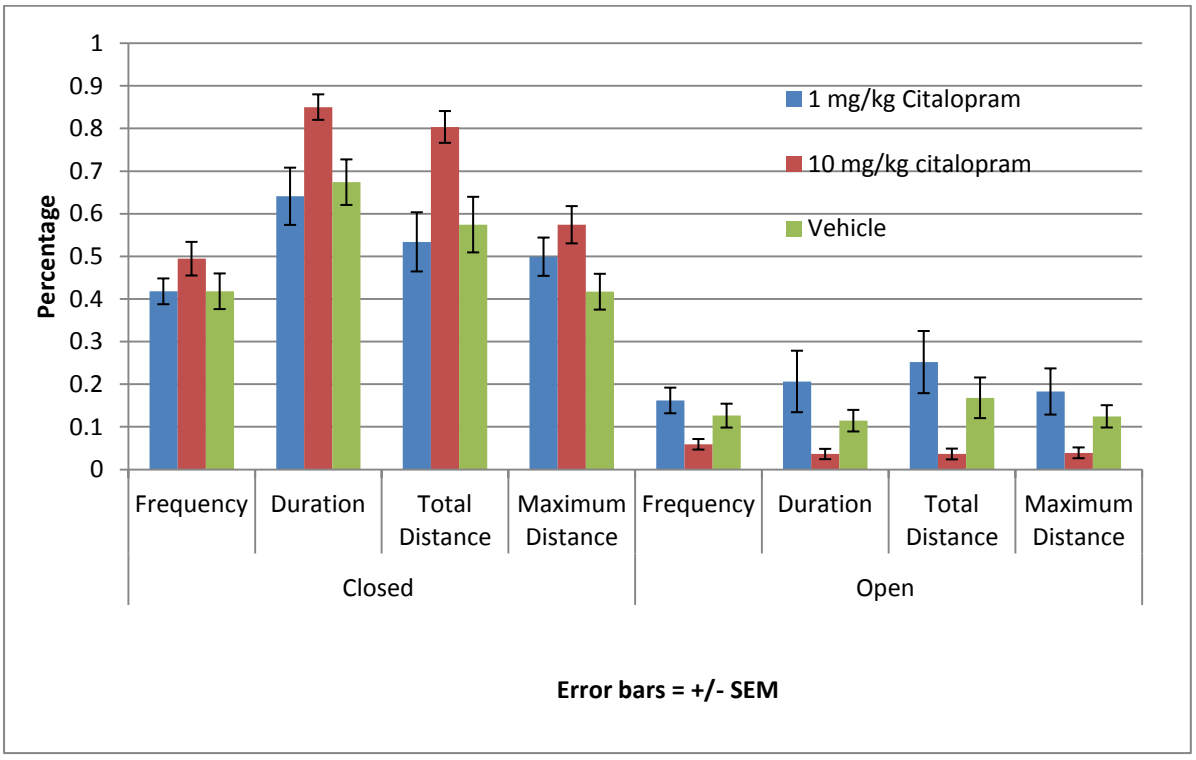

Figure 6. Frequency of entrances, maximum distance, total distance and duration in closed and open arms of the maze.

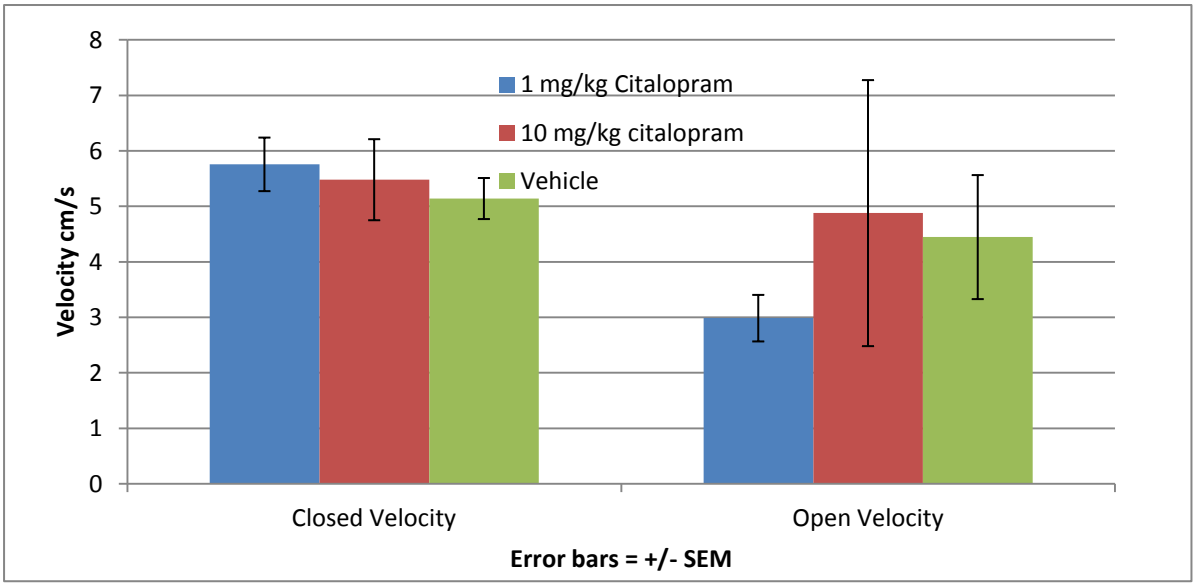

Figure 7. Mean velocity in the closed and open arms of the maze.

The results from the EPM indicated that the two doses of citalopram induced opposing effects on the duration of time spent and total distance travelled in the closed arms of the maze with the higher dose increasing both of these compared with the lower dose. The frequency, duration and total distance travelled in the open arms was also found to be significantly lower in the 10 mg/kg dose compared with 5 mg/kg. The total distance and maximum distance moved by the 10 mg/kg treated group in the closed arms was also found to be higher in the closed arms. These results indicate a distinct dissociation of behaviour in this task dependant on dose with the 10 mg/kg group displaying more anxiety-like behaviour than the 1 mg/kg group.

3.4.3. *Experiment 3: PRL with 5 mg/kg Citalopram*

Conditional probabilities were analysed by phase, acquisition and reversal on the first session of the test phase (Figure 8). Repeated measure ANOVA indicated that there was no main effect of group on win-stay probability ($F(1,23)=1.8$, p=NS) or phase by group interaction ($F(1,23)=0.041$, p=NS). There was however, as significant main effect of phase with win-stay probability found to be higher in the acquisition phase ($F(1,23)=16.183$, $p<0.05$). Analysis of lose-shift probability also shows no significant main effect of group ($F(1,23)=2.369$, p=NS) or phase by group interaction ($F(1,23)=0.001$, p=NS). There was also no main effect of phase ($F(1,233)=0.848$, p=NS).

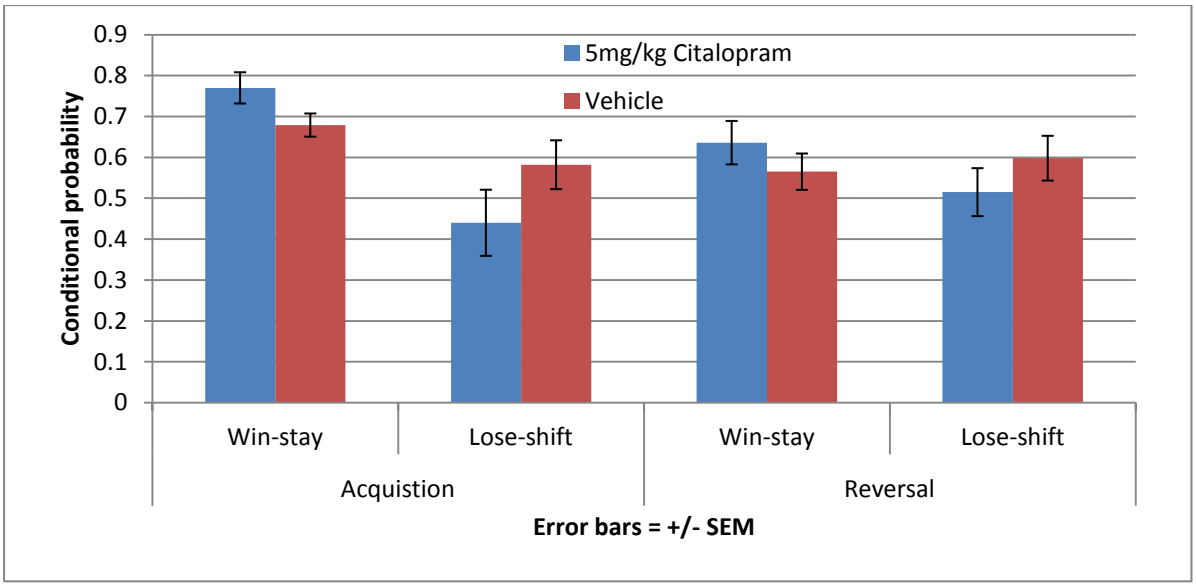

Figure 8. Conditional probability during the acquisition and reversal phase in the 5 mg/kg Citalopram and vehicle treated groups.

Repeated measures ANOVA on the number of reversals completed indicated the there was no significant main effect of group ($F(1,26)=0.745$, p=NS) (Figure 9). The was also no main effect of session ($F(2,52)=2.637$, p=NS), phase by group interaction ($F(1,26)=1.991$, p=NS) or session by phase by group interaction ($F(2,52)=1.377$, p=NS). However,there was a significant main effect of phase ($F(1,26)=21.891$, $p<0.001$) and session by group interaction ($F(2,52)=3.317$, $p<0.05$). Analysis of the simple effects of this interaction revealed that there was a significant difference between the groups

on the first session of test phase ($F(1,26)=4.446$, $p<0.05$), with no significant differences between the groups on any of the other sessions. The *F* and *p* values for these are displayed in Table 17.

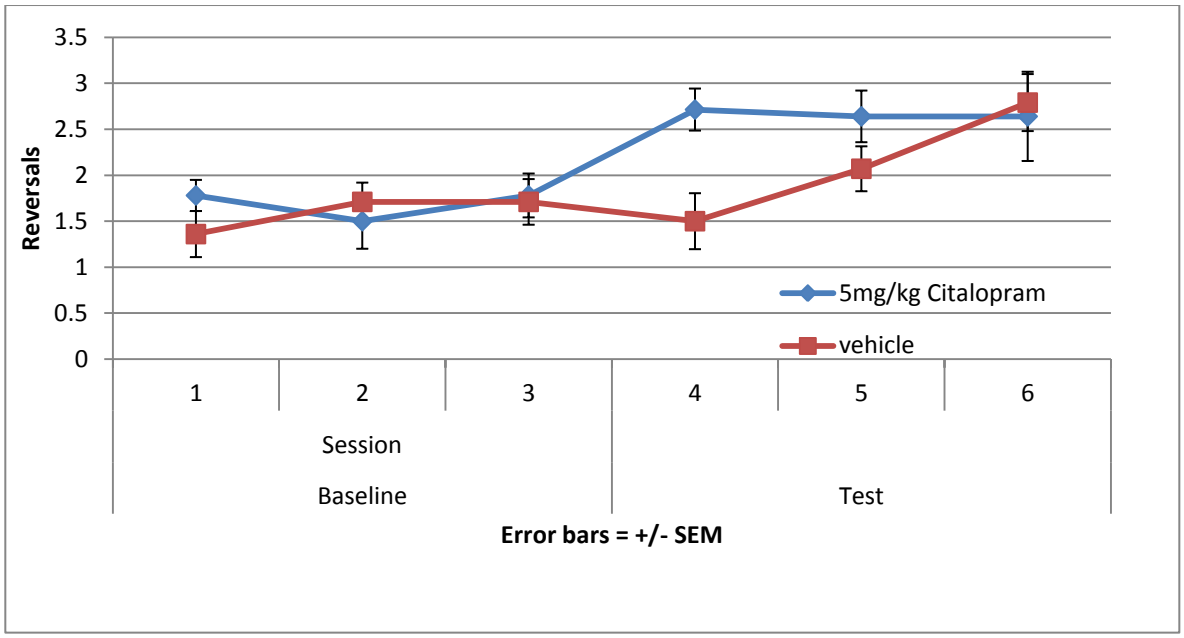

Figure 9. Mean number of reversals competed in the baseline and test phase in the vehicle and 5 mg/kg Citalopram treated groups.

Table 17. Pairwise comparisons of reversals across sessions in 5 mg/kg and vehicle treated groups.

Phase	Session	*F* value	*p* value
Baseline	1	1.55	0.224
	2	0.317	0.578
	3	0.037	0.849
Test	4	4.446	*0.045**
	5	1.098	0.304
	6	0.047	0.831

(= p<0.05)*

Repeated measure ANOVA conducted on win-stay probability indicated that there was no main effect of group ($F(1,26)=0.009$, p=NS) or session ($F(2,52)=3.059$, p=NS) (Figure 10). However, there was a main effect of phase ($F(1,26)=6.046$, $p<0.05$). There was no significant phase by group interaction ($F(1,26)=1.974$, p=NS), session by group interaction ($F(2,52)=0.323$, p=NS), phase by session interaction ($F(2,52)=1.895$, p=NS) or phase by session by group interaction ($F(2,52)=0.93$, p=NS).

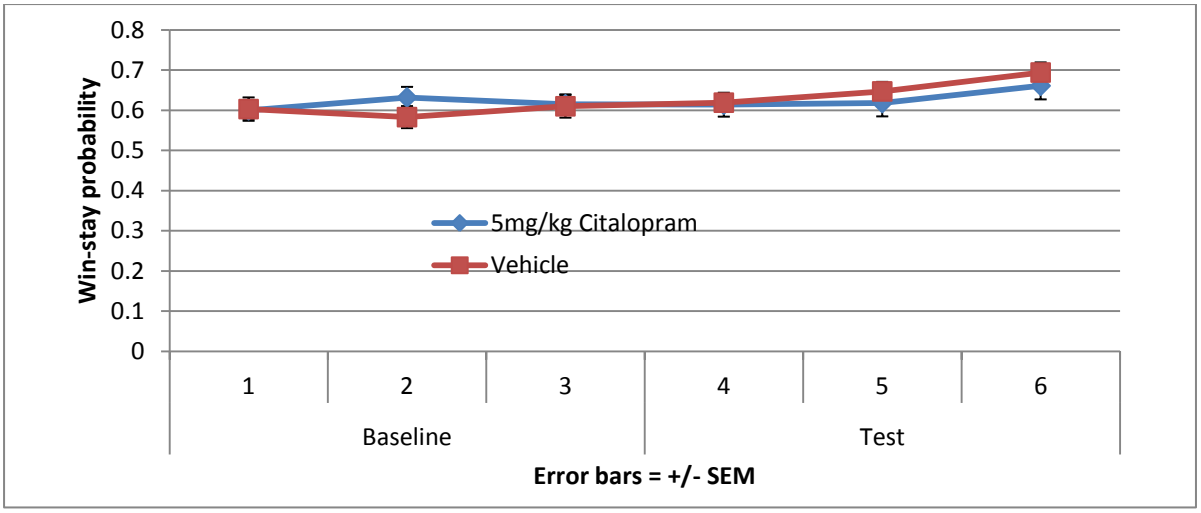

Figure 10. Win-stay probability in the baseline and test phase in 5 mg/kg Citalopram and vehicle treated groups.

Repeated measures ANOVA of the lose-shift performance indicated that there was no main effect of group ($F(1,26)=1.494$, p=NS), phase ($F(1,26)=3.187$, p=NS) or session ($F(1,26)=1.313$, p=NS) (Figure 11). There was also no significant phase by group interaction ($F(1,26)=0.817$, p=NS), session by group interaction ($F(2,52)=2.053$, p=NS) phase by session interaction ($F(2,52)=1.89$, p=NS) or phase by session by group interaction ($F(2,52)=0.273$, p=NS).

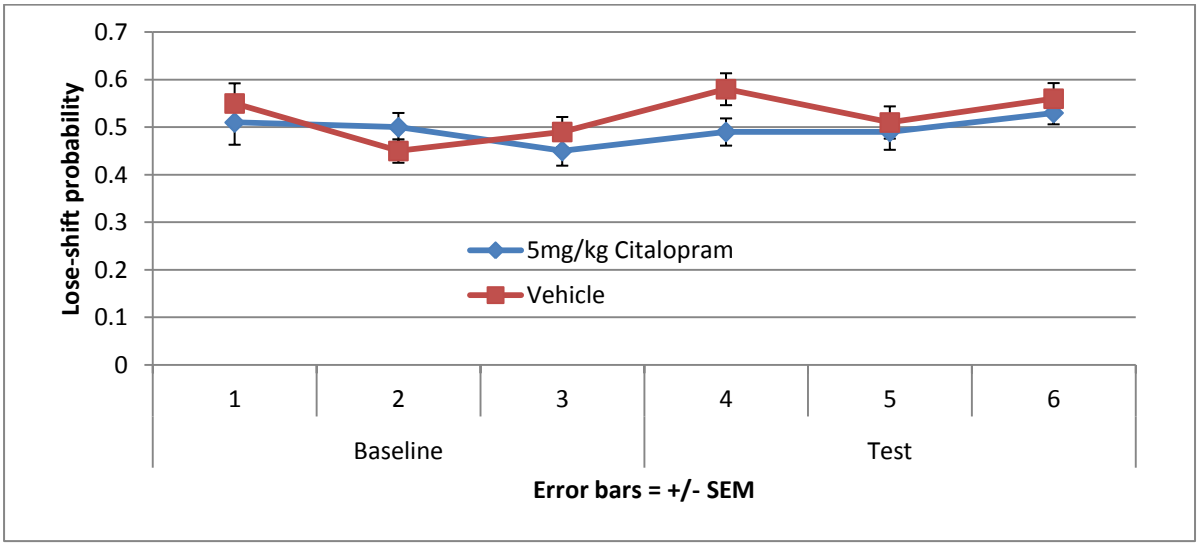

Figure 11. Lose-shift probability in baseline and test phase in 5 mg/kg Citalopram and vehicle treated groups.

Repeated measures ANOVA was also carried out on perseverative errors (Figure 12). Results showed there was no significant main effect of group ($F(1,25)=0.601$, p=NS) or session by group interaction ($F(1,25)=1.151$, p=NS). However, there was a significant reduction in perseverative errors across the sessions ($F(1,25)= 4.401$, $p<0.05$).

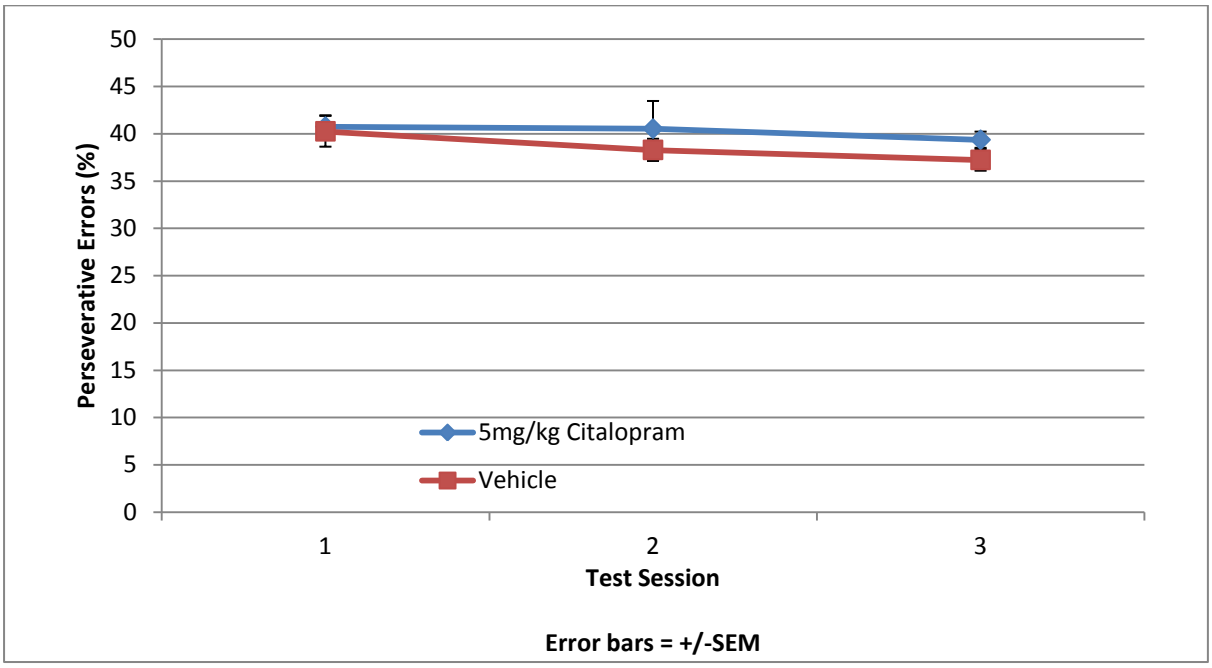

Figure 12. Perseverative errors in test phase of the 5mg/kg citalopram and vehicle treated groups.

Analysis of the number of trials to reach criterion of the first reversal indicated that there was no main effects group ($F(1,26)=1.479$, p=NS) or session ($F(1,26)=0.51$, p=NS). However, there was a significant session by group interaction ($F(1,26)=10.089$, $p<0.05$) (Figure 13). Pairwise comparison of this interaction indicated that there was significant difference between the groups on the first test session ($F(1,26)=6.331$, $p<0.05$) only.

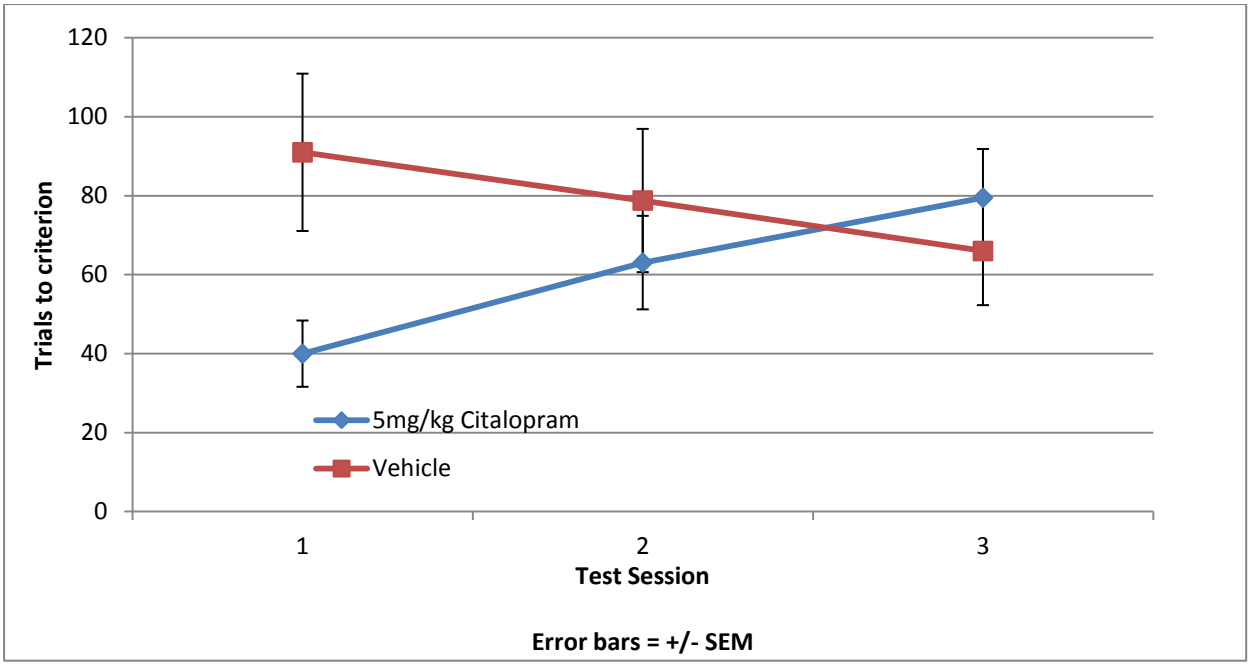

Figure 13. Trials to criterion in test phase of 5mg/kg citalopram and vehicle treated groups.

3.4.4. *Experiment 4: PRL with 10mg/kg Citalopram*

Analysis of conditional probabilities by phase on the first test session indicated that there was no significant main effect of group on win-stay probability ($F(1,18)=0.12$, p=NS) (Figure 14). There was also no main effect of phase ($F(1,18)=1.011$, p=NS) or phase by group interaction ($F(1,18)=0.787$, p=NS). Analysis of lose-shift probability also showed no significant main effect of group ($F(1,18)=0.279$, p=NS) or phase by group interaction ($F(1,18)=0.004$, p=NS). However there was a significant main effect of phase ($F(1,18)=9.994$, $p<0.01$).

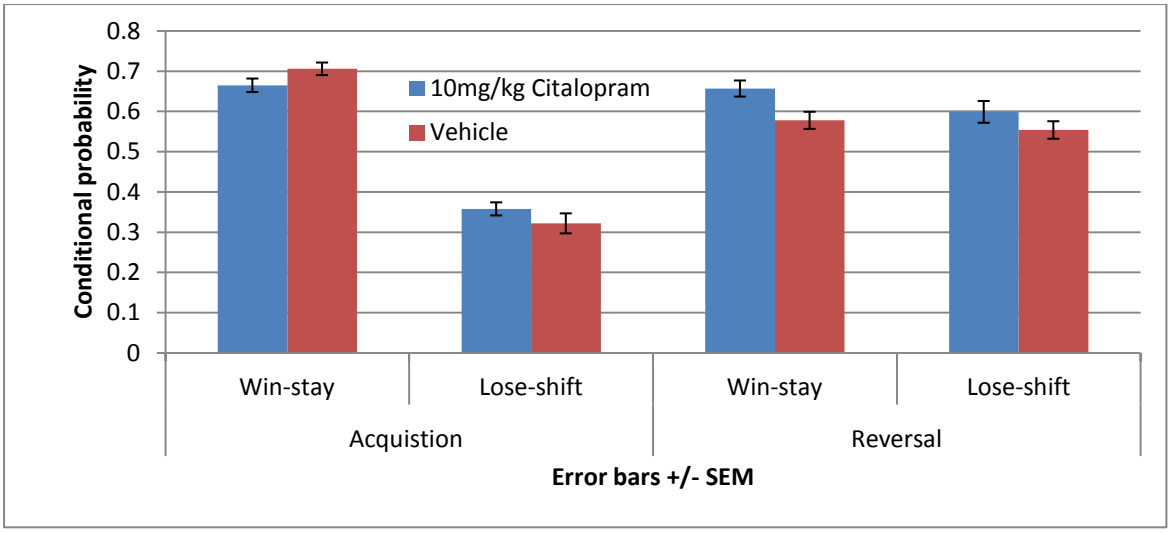

Figure 14. Conditional probability during the acquisition and reversal phase in the 10 mg/kg Citalopram and vehicle treated groups.

Figure 15 shows the mean number of reversal completed over sessions. Repeated measures ANOVA showed there was no significant main effect of group ($F(1,18)=2.57$, p=NS), phase ($F(1,18)=3.276$, p=NS) or session ($F(2,36)=0.11$, p=NS). There was also no significant main phase by group interaction ($F(1,18)=0.634$, p=NS), session by group interaction ($F(2,36)=0.525$, p=NS), phase by session interaction ($F(2,36)=0.502$, p=NS) or phase by session by group interaction ($F(2,36)=0.502$, p=NS).

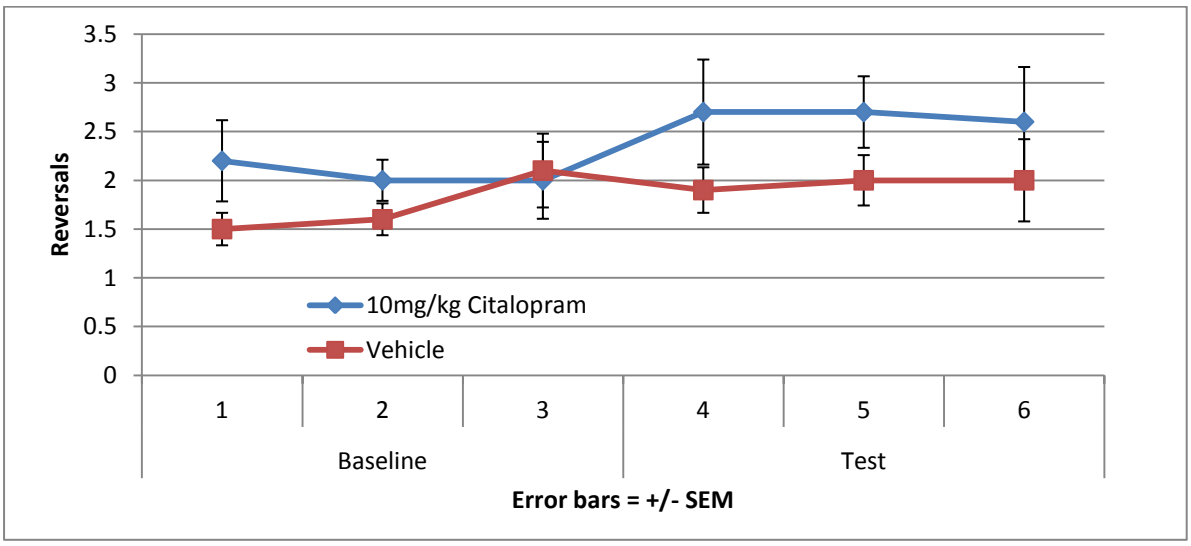

Figure 15. Mean number of reversals competed in the baseline and test phase in the vehicle and 10 mg/kg Citalopram treated groups.

Win-stay probability across the sessions is displayed in Figure 16. Repeated measures ANOVA showed that there was no significant main effect of group ($F(1,18)=2.367$, p=NS) or session ($F(2,36)=1.631$, p=NS). However, there was a significant main effect of phase ($F(1,18)=5.446$, $p<0.05$). There was no significant phase by group interaction ($F(1,18)=2.179$, p=NS), session by group interaction ($F(2,36)=1.597$, p=NS) or session by phase by group interaction ($F(2,36)=0.712$, p=NS). However, there was a significant phase by session interaction ($F(2,36)=4.184$, $p<0.05$). Analysis of the simple effects of this interaction showed that the first ($F(1,26)=0.034$, p=NS) and second ($F(1,26)=0.746$, p=NS) session of each phase were not significantly different however, the third sessions of each were significantly different ($F(1,26)=5.121$, $p<0.05$).

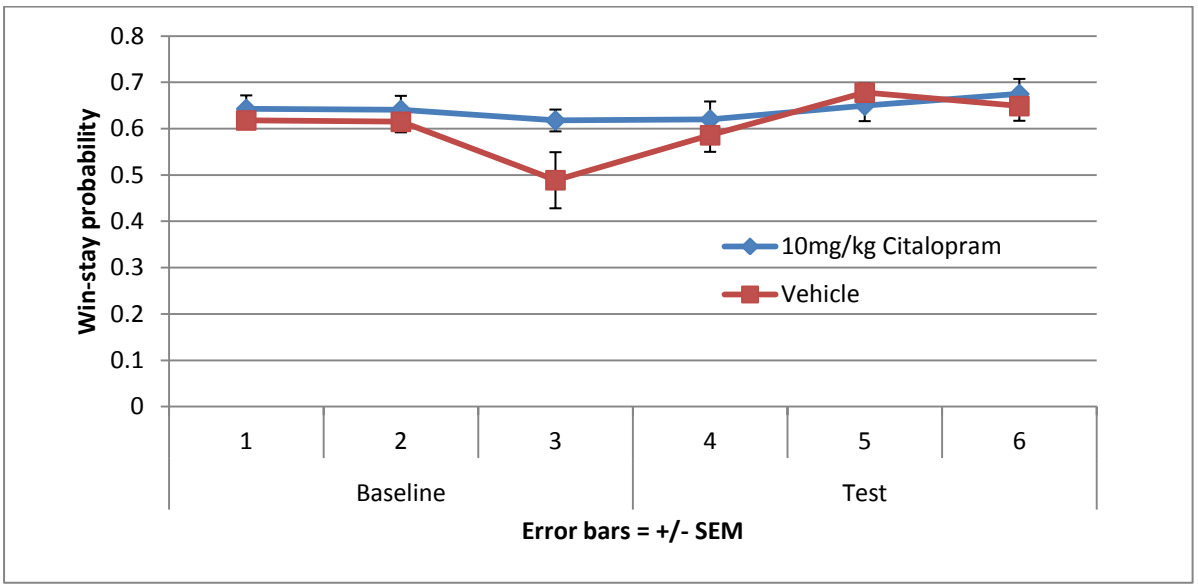

Figure 16. Win-stay probability in the baseline and test phase in 10 mg/kg Citalopram and vehicle treated groups.

Lose-shift probability is displayed in Figure 17. Repeated measure ANOVA show there was no significant main effect of group ($F(1,18)=0.001$, p=NS). There was also no main effect of phase ($F(1,18)=2.953$, p=NS) or session ($F(2,36)=.334$, p=NS). There was no significant phase by group interaction ($F(1,18)=3.692$, p=NS), session by group interaction ($F(2,36)=2.03$, p=NS) or phase by session by group interaction ($F(2,36)=0.445$, p=NS). However, there was a significant phase by

session interaction ($F(2,36)=12.57$, $p<0.001$). Analysis of the simple effect s of this interaction showed that session one of each phase was not significantly different ($F(1,18)=0.071$, p=NS) however the second ($F(1,18)=7.537$, $p<0.05$) and third session ($F(1,18)=37.829$, $p<0.001$) were significantly different between phases.

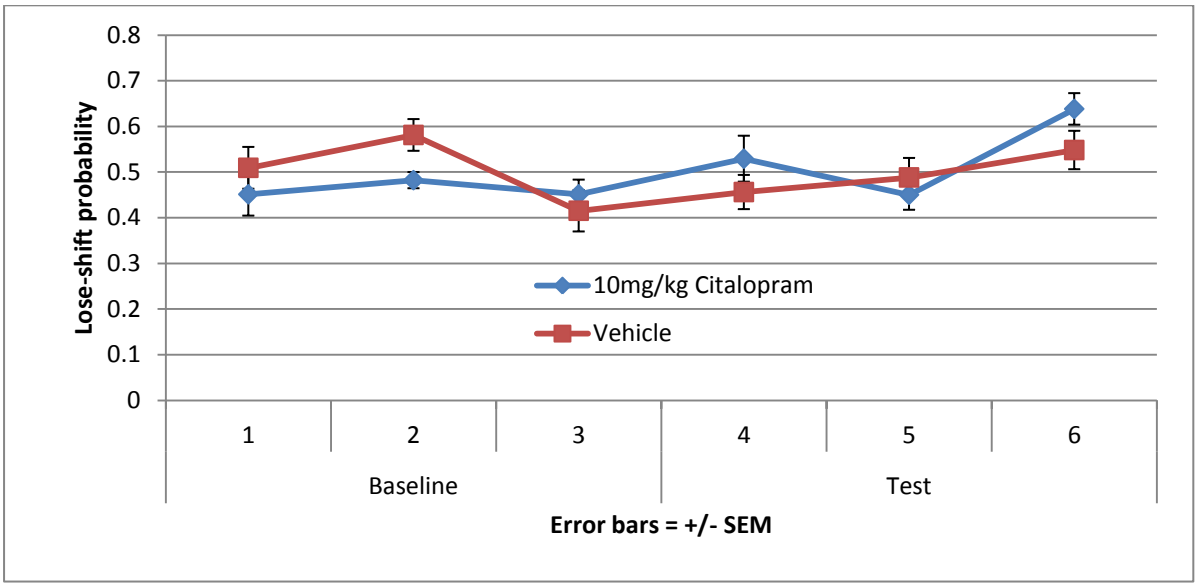

Figure 17. Lose-shift probability in baseline and test phase in 5 mg/kg Citalopram and vehicle treated groups.

Analysis of the perseverative errors indicated that there was no significant main effect of group ($F(1,18)=1.357$, p=NS) or session ($F(1,18)=0.631$, p=NS), there was also no significant session by group interaction (F(1,18)=0.038, p=NS) (Figure 18).

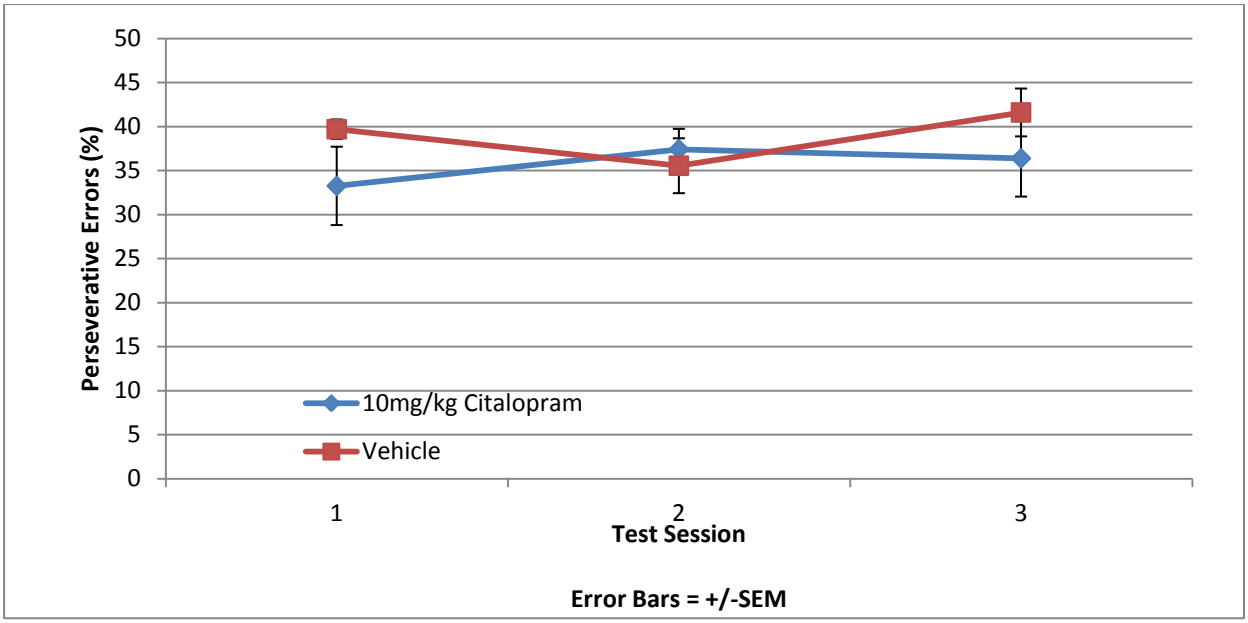

Figure 18. Perseverative errors of 10mg/kg and vehicle treated groups.

Repeated measures ANOVA of the trials to criterion indicated there was no significant main effects of group ($F(1,18)=0.687$, p=NS) or session ($F(,18)=0.006$, p=NS). There is also no significant session by group interaction ($F(1,18)=0.164$, p=NS) (Figure 19).

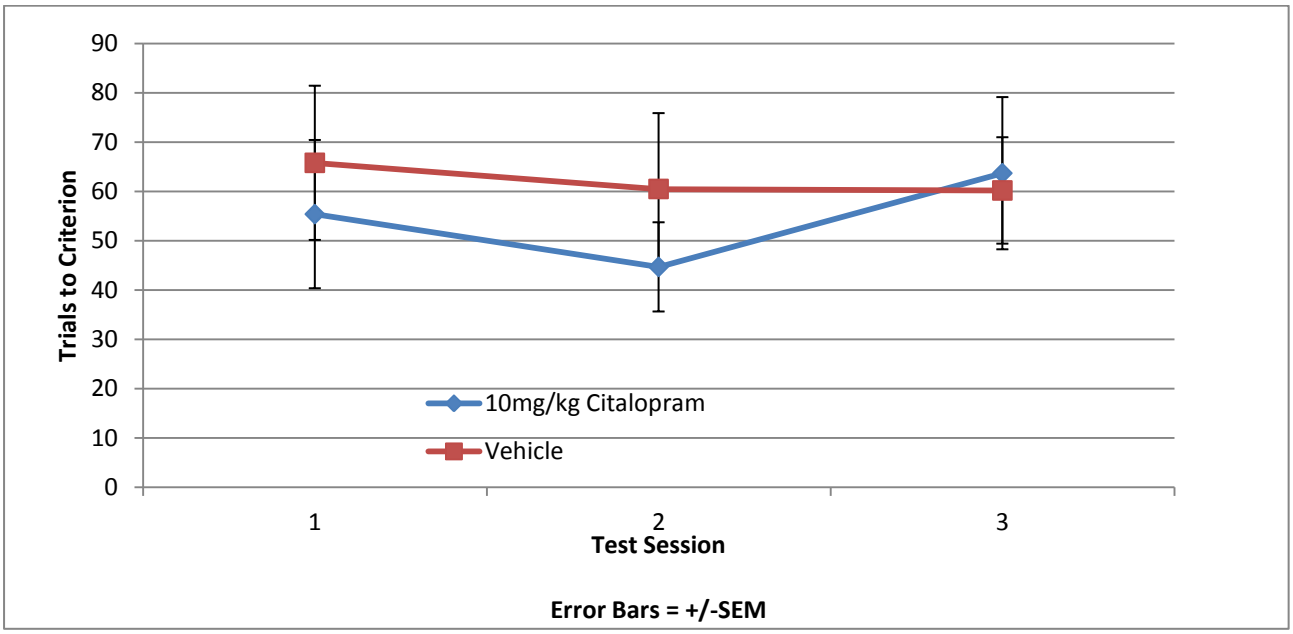

Figure 19. Trials to criterion of 10mg/kg citalopram and vehicle treated group.

The results from this experiment indicate that 5 mg/kg citalopram increased the number of reversal in the first session of the test phase. However this was not found with the higher dose of 10 mg/kg. There was no effect of either drug dose on sensitivity to either positive or negative reinforcement across the sessions. Analysis of the effect of the drug doses on feedback sensitivity during acquisition and reversal also showed that citalopram did not affect these two variables.

3.5. *Discussion*

The lower dose of 5 mg/kg Citalopram was found to increase the number of reversals on the first day of the test sessions. This result is consistent with studies that manipulate endogenous 5-HT levels through either the use of SSRI treatment or depletion of 5-HT levels (Bari *et al,* 2010; Clarke *et al,* 2005, 2007; Ineichen *et al,* 2012).

The present study followed a similar protocol to that used by Bari *et al* (2010). In the study by Bari *et al* (2010) an acute dose of 5 mg/kg citalopram was not found to significantly increase the number of reversals but 10 mg/kg citalopram produced a significant increase. In contrast, in the current study 5 mg/kg increased the number of reversals achieved on the initial test session while no effect with found with the 10 mg/kg treated group. The differential response in reversal learning between the two drug doses may be attributable to 5-HT receptor activity. The serotonergic projections from raphe nuclei extend to the frontal cortex, previous implicated in reversal learning (Chudasama & Robbins, 2003; Dias *et al,* 1996; Iversen & Miskin, 1970; Schoenbaum *et al,* 2002). Activation of the 5-HT1a autoreceptors by citalopram in the raphe nuclei reduces serotonergic signalling to the frontal cortex. However, repeated exposure desensitises the receptor. The lower dose of citalopram used in the present study may not have sufficiently activated 5-HT1a receptors in the raphe nuclei whilst still increasing extracellular 5-HT levels in the frontal cortex. Therefore the drug may not have inhibited projections to the frontal cortex. However the higher dose may have activated this receptor with desensitisation occurring over the test sessions. The differences found in this study compared with Bari *et al* (2010) may be attributed to any number of variables that can affect serotonergic receptor expression in the dorsal raphe nuclei, including rearing conditions (Gardner *et al,* 2009) and prior exposure to antidepressant drugs (Limón-Morales *et al,* 2014). Dorsal raphe nuclei 5-HT1a receptor function is affected, via a negative feedback mechanism, by 5-HT function in other areas (Almada *et al,* 2009; Kreiss & Lucki,1994). Juvenile stress attenuates dorsal hippocampal

5-HT1a receptor function in adult rats suggesting another factor that may have produced differences between these two studies (Matsuzaki *et al,* 2011).

No effect of win-stay or lose-shift probability was seen with either drug dose in the current study. This impies that responsiveness to feedback is not modulated by the serontergic system. Increased number of reversals exhibited by the 5 mg/kg treated group may be attributed to improved function in other subregions of the frontal cortex. The ability to reverse learned stimulus-response patterns though inhibition of responding and rule implementation is well established to be reliant on the orbitofrontal cortex (OFC) (Rudebeck *et al,* 2013). The administration route of citalopram in this study increases global extracellular 5-HT therefore it may have facilitated function of the OFC in inhibition of responding and enhanced rule-learning. This improvement in performance may have been due to the rats deciphering a pattern of behaviour which optimises the number of rewards. This trend was also exhibited by the 10mg/kg treated group however it did not reach statistical significance

The study by Bari *et al* (2010) indicated that SSRI administration alters sensitivity to positive reinforcement however the specific mechanism by which 5-HT affects value-based decision making is unclear (Nakamura, 2013). Given the evidence for the effect of other variables on 5-HT1a receptor function, previously discussed, it is important to consider differences in the prior experience of the animals were comparing results between studies.

In the EPM there was a significant difference between the lower dose of 1 mg/kg and the higher dose of 10 mg/kg in several measures of anxiety-like behaviour. The rats treated with the 1 mg/kg dose of citalopram had a higher number of entries, duration and total distance moved in the open arms and lower duration and total distance moved in the closed arms compared with the 10 mg/kg treated group . These results indicate that although neither dose significantly alters behaviour compared with vehicle both doses produce contrasting effects on anxiety-like behaviour with the lower dose inducing a reduction and the higher dose causing increases. This result parallels findings

by Mombereau *et al* (2010) where higher acute doses of citalopram produced a significantly higher anxiogenic effects on an elevated zero maze (EZM) (an elevated circular maze comprising of two enclosed quadrants and two open quadrants) compared with a lower dose. The SSRI fluoxetine when administered at the same dose, 10 mg/kg, was also found to produce an anxiogenic effect (Ravinder *et al,* 2011).

The results from the OFT study however, found no main effect on dose on any of the measures. This test implies a lack of coherency with the EPM. This has been previously reported in pharmacological and genetic studies (Vendruscolo *et al,* 2003) and supports opinion that construct differences mean each test assesses only one facet of an animals' emotional profile.

Another area of concern regarding the use of a battery of tests to assess anxiety in animals is that sequential testing increases familiarly with testing therefore reducing anxiety. This may in part explain why no drug effect with found in the OFT, which was the second test to be administered. Furthermore, results from the OFT, which was run for two days found a main effect of day suggesting that anxiety-like behaviour changed over the two days. On some of these measures drug dose produced an interaction by day implying that adaption to environment is differentially affected by drug dose. For example, the 1 mg/kg dose showed lower frequency of entries into the corners and edges of the maze and higher frequency of entries into the middle on the first day compared with the 10 mg/kg group, although this was not significant this pattern was reversed on the second day. This suggests that although the drug is not significantly impacting anxiety-like behaviour in this test, adaption to novel environments may be differentially affected by different doses of citalopram.

The results from the current study indicate that serotonergic transmission is implicated in reversal learning and in acute doses may induce anxiety-like behaviour. The lack of coherency between the two behavioural tests used in this study to assess anxiety levels highlight the problems in the use of behavioural testing to examine emotional disorders and indicate the need for caution when interpreting and comparing behavioural results across studies.

Effects of the dietary probiotics, bifidobacterium bifidum (CUL20), bifidobacterium lactis (CUL34) and lactobacillus acidophilus (CUL21 & CUL60) on anxiety-like behaviour, locomotor activity, reversal learning and probabilistic reversal learning.

4.1. Summary

The current study examined the effect of chronic probiotic treatment on anxiety levels using the open field test (OTF), an elevated plus maze (EPM) and a locomotor activity (LMA) test to assay the effects of the probiotics on general activity. Cognitive flexibility was assessed using a probabilistic reversal learning task and a watermaze reversal learning paradigm. The results from these experiments indicated that there was no effect of probiotics on anxiety-like behaviour in the EPM. However, in the OFT the probiotic treatment reduced exploration, suggesting a possible anxogenic effect on performance. However, results of the LMA test showed a reduction in activity levels across successive sessions which may account for the reduced exploration rates of the probiotic group in the second day of testing in the OFT. Results from the PRL task indicated that there was no difference between the groups on several measures of performance including sensitivity to reward and non-reward. However, eight of the thirty-two animals tested failed to pass the training phase of this task indicating the age of the animals may have reduced the power to detect a probiotic treatment effect on reversal learning. In contrast, probiotic treatment improved spatial discrimination learning in the watermaze.

4.2. Introduction

Depression has been cited as being the fourth leading cause of disability worldwide accounting for 4.4% of total disability adjusted life years (DALYs) and almost 12% of all total years lived with non-fatal disability world-wide (Ustun *et al,* 2004). Depression is co-morbid with anxiety disorders

(Braam *et al,* 2014). These psychiatric conditions are often accompanied by gastro-intestinal (GI) disorders with the onset of GI disturbances associated with stressful life events (Lutgendorff *et al,* 2008). Studies have found that over 50% of patients with irritable bowel syndrome (IBS) also had mood disorders (Lutgendorff *et al,* 2008). The symptomatic presentation of mood disorders was also found to be exacerbated during periods of increased gastrointestinal disturbance (Graff *et al,* 2009; Whitehead *et al,* 2002).

One of the common pharmaceutical interventions for IBS is antidepressants (Neufeld & Foster, 2009). This treatment for IBS gives a clear indication that the link between GI disturbances and mood disorders is believed to be a top-down process with CNS functioning negatively impacting intestinal constituents to cause GI disturbances. This has also been demonstrated in preclinical trials where early-life stress in rodents induced alterations of the bacterial profile of the gut (O'Mahony *et al,* 2009). Indeed, there exists an abundance of evidence to suggest a top-down component in gut-brain communication (Bailey & Coe, 1999; Garcia-Rodenas *et al*, 2006). However, the focus of recent research clearly indicates bi-directional communication between the gut and the brain with composition of the GI tract shown to impact CNS functioning (Bercik *et al,* 2010; Gaykema *et al*, 2004; Goehler *et al,* 2005; Lyte *et al,* 2006; Sudo *et al,* 2004; Wang *et al,* 2002).

Logan and Katzman (2004) were first to suggest a bidirectional pathway between the gut and the brain and that probiotics could utilize the bottom-up communication pathway and be used as an adjuvant therapy in the treatment of depression. Probiotic administration has been shown to alter functioning in numerous systems beyond the gut including the immune system, neuroendocrine system (Ait-Belgnaoui *et al* 2012; Desbonnet *et al,* 2008, 2010; Gareau *et al,* 2007; Sudo *et al,* 2004) neurotransmitter metabolism and production (Barrett *et al,* 2012; Desbonnet *et al,* 2010; Roshchina, 2010) and enterological integrity (Ait-Belgnaoui *et al* 2012; Zareie *et al,* 2006).

Two common strains of probiotics are *Lactobacillus* and *Bifidobacterium*. A probiotic formulation containing a species of each of these two bacteria had anxiolytic-like activity in rats using a

defensive-burying task (Messaoudi *et al,* 2011). Administration of *Lactobacillus rhamnosus* reduced anxiety-like behaviour in the elevated plus-maze (EPM) and forced swim test (FST) (Bravo *et al,* 2011). Early life stress in animal models has been shown to significantly alter the indigenous populations of *bifidobacterium* and *lactobacillus* (Bailey & Coe, 1999; Bailey *et al,* 2011). One such early life stressor is maternal separation. The effects of maternal separation on intestinal functioning include perturbations in the bacterial profile of the gut (Gareau *et al,* 2006, 2007) and aberrant behavioural in tests of anxiety and depression (Berman *et al,* 2014; Desbonnet *et al,* 2010; Diehl *et al,* 2014). The maternal separation preparation is therefore commonly used as a model of comorbid GI disturbances and depression. Interventions with probiotics resulted in increased preservative behaviour in the FST, characteristic of antidepressant effects (Desbonnet *et al,* 2010). Other studies have reported anxiolytic effects of probiotics in a passive avoidance step-down test (a behavioural test often used in animal models to assess anxiety levels) (Bercik *et al,* 2011). Tests examining the behavioural effects of probiotic on healthy animals have produced conflicting results, with *bifidobacterium* producing no beneficial effects in the FST despite causing immune and neurochemical changes (Desbonnet *et al,* 2008). When examining the behavioural effects of probiotics in animals following subclinical infection with pathenogenic bacteria, *Trichuris muris* anxiety-like behaviour in the light/dark preference test was ameliorated following treatment with *bifidobacterium.* In animal models of depression, where water avoidance stress is applied, probiotic pre-treatment was found to attenuate the physiological stress responses (Ait-Belgnaoui *et al,* 2013). Similarly, in rats with post-myocardial infarction depression probiotic treatment, starting at the on-set of reperfusion, reduces anxiety and depression-like behaviour in the FST, social interaction test and passive avoidance step-down test (Arseneault-Bréard *et al,* 2012; Gilbert *et al,* 2012).

In summary, there is a growing body of emerging evidence in the preclinical arena supporting the use of probiotics in the treatment of anxiety and depression. However, the demonstration of therapeutic efficacy has been confined to models of compromised gastrointestinal systems or neurochemical functioning. Furthermore, probiotic interventions have generally been acute,

typically two weeks. Thus the long-term effects of probiotic treatment on anxiety and depression-like behaviours have not been fully explored.

The aim of the present study was to examine the effect of chronic exposure to a probiotic formulation, *bifidobacterium bifidum, bifidobacterium lactis* and *lactobacillius acidophilus* on anxiety-like behaviour using the EPM and open-field test (OFT). A potential behavioural confound when examining anxiety-like behaviour following an intervention in these tasks is a change in baseline activity levels. In order to fully understand the impact of probiotic treatment on anxiety levels a locomotor activity test (LMA) was also carried out. Although the behavioural effects of chronic treatment with probiotic has never been explored, previous results suggest an anxiolytic-effect and therefore it was anticipated that probiotics would reduce anxiety measures in rats. The alterations in neurochemical functioning exhibited in animals treated with probiotics suggest that anxiety-like behaviours maybe not be the only component of behaviour affected.

Desbonnet *et al, (*2008) reported that probiotic treatment in rats reduced serotonergic degradation in the frontal cortex using high performance liquid chromatography (HPLC). In CHAPTER 2 experiment 2 (it was reported that lesions of the medial prefrontal cortex (mPFC) disrupted probabilistic reversal learning (PRL). Furthermore, probabilistic reversal learning was also sensitive to a serotonergic agonist, which enhanced performance (Bari *et al,* 2010; den Ouden *et al,* 2013; see CHAPTER 3 experiment 3). Based on these findings it was hypothesised that probiotic treatment would have a similar effect on PRL learning to a serotonergic agonist, via a putative effect on 5-HT activity in the frontal cortex.

Other areas of the frontal cortex have also been implicated in reversal learning, specifically the orbitofronal cortex (OFC) (Chudasama & Robbins, 2003; Izquierdo *et al,* 2004; Schoenbaum *et al,* 2002). Examination of the role of the OFC in reversal learning indicated that it is fundamental for rule-implementation and inhibition of responding while areas of the mPFC is responsible for error detection and evaluation of feedback (Gehring & Knight, 2000; Walton *et al,* 2004; Zanolie *et al,*

2008). As the PRL task assess flexibility of responding as well as sensitivity to reward and on-reward it may identify which subregions of the frontal cortex are affected by probiotic treatment. Serotonergic activity has also been implicated in reversal learning involving spatial discriminations (Brown *et al,* 2012; Boulougouris & Robbins, 2010). In order to examine the extent to which probiotic administration affected reversal learning involving a spatial discrimination, a watermaze reversal learning paradigm was employed (Russig *et al,* 2003). Earlier studies where SSRIs were used to alter serotonergic functioning indicated that serotonin transmission facilitates the inhibitory processes required to shift responding from a learned choice pattern in spatial reversal learning (Brown *et al,* 2012). Furthermore, specific 5-HT receptors have been implicated in spatial reversal learning. Systemically administered SB 242084, a 5-HT2C receptor agonist, has been found to improve spatial reversal learning (Boulougouris *et al,* 2008). Further examination of the neuroanatomical specificity of this 5-HT2C agonist indicated that the OFC is the locus of action for this drug. This was differentiated from the mPFC where targeted infusion of SB 242084 showed no improvement (Boulougouris & Robbins, 2010). Given the results from the study by Desbonnet *et al* (2008) it was expected that the reduction in metabolism of 5-HT in the frontal cortex would have similar effects on spatial reversal learning as that found by Brown *et al* (2012) when SSRIs were administered.

4.3. *Method and Procedure*

Subjects

The same cohort of rats were used for the OFT, EPM, LMA test, PRL task and the watermaze reversal task. Fifty were used in the OFT and EPM, forty-five were used in the LMA test, thirty-two for the PRL task and watermaze reversal task. The numbers of animals used in the latter experiments was dictated by time constraints and the resources available at the time of testing. Rats' weights ranged from 423g to 556g. All animals were given free access to water. During the OFT, EPM, LMA test and the watermaze reversal task, the rats were fed 30g of food per day. During the PRL the food was titrated to reduce body weight to 85% of free feeding body weight. The rats were housed in pairs in a holding room with a 12h light-dark cycle with lights on at 7am. Testing occurred during lights on hours. The temperature of the room was maintained at 19-23^{o}C and humidity at 55% ±10. During the OFT, EPM, LMA test and watermaze reversal task, rats were 15 months old. Testing on the PRL task was conducted when the rats were 24 months old. Ideally all four experiments would have been conducted when the rats were the same age, however, due to technical problems with the equipment for the PRL task this experiment could not be conducted earlier.

Feeding Procedure

All rats had their food placed into their cages in glass dishes. The probiotic treated group had their food dusted with the probiotic powder from capsules. The probiotic capsule (Obsidian Research, U.K., Port Talbot) contained four strains of bacteria, *Lactobacillis acidophilus* CUL60, *Lactobacillis acidophilus* CUL21, *Bifidobacterium bifidum* CUL20 and *Bifidobacterium lactis* CUL34. This probiotic formulation is a product sold by Cultech Ltd. (Wales, UK), a sponsor of the current research. *Bifidobacterium* and *Lactobacillus* strains have been previously used in studies examining the impact of probiotics on CNS functioning (Bravo *et al,* 2011; Desbonnet *et al,* 2009). A dose rate of 1 x 10^{8} cfu (colony forming unit)/capsule per rat was used. In the current study, the probiotic was directly

added to a fixed volume of food daily. The probiotic capsules were stored in a refrigerator at 7°C and food was prepared with fresh probiotic powder daily. Rats in the probiotic condition received this diet from weaning and throughout the study. Prior to weaning the mother received the same probiotic dose in her food during the gestation and post-natal period. The probiotic treated group and the control group were kept in separate rooms to avoid cross contamination.

Apparatus

The apparatus for the OFT, EPM, and PRL task were identical to those described in chapter 3.

Experiment 3: Locomotor activity test

Locomotor activity was recorded using 8 boxes (32x53cm), there had two photobeams running across the shortest edges, one 10cm from the edge and one 20 cm for the edge. Photobeams ran 2 cm from the bottom of the cages. Activity was recorded by a Noldus Information technology photobeam system and analysed with an Acorn PC. The floors of the boxes were a wire grid as was the top of the boxes. These were placed in 2 rows of 4 boxes in a holding rack. Both groups of animals were run simultaneously, with one row being used for the citalopram treated animals and the other used for the control treated animals. The room was illuminated with standard 70w florescent tube lighting. This remained on during the experiment. All of the animals were naive to the room prior to testing. All boxes were cleaned thoroughly before being used again to avoid smell of pervious animal affecting behaviour. All testing occurred between 1pm and 5 pm to avoid any diurnal variation. Each group was tested at the same time every day for 3 consecutive days. During the one hour testing the rats were allowed to freely explore the box, the activity was recorded in 6 sets of 10 minutes before being returned to their home cages. The number of overall 'breaks', number of times a beam was broken, 'runs', number of times one beam then the other was broken,

and 'consecutive breaks', number of times one beam was broken in succession, were recorded. The data was analysed across 'bins' and 'days'.

Experiment 5: Watermaze reversal learning paradigm

The watermaze was constructed from, a white circular pool with a diameter of 2m. The pool had a total depth of 62cm and was raised on a platform 75cm off the floor in the middle of the room (3.5mx3m). The pool was filled with water (23-25^{o}C) to a depth of 20cm. 0.5l of opacifer E308 (Roehm and Haas, U.K., Ltd., Dewsbury) was added to the water to make it opaque. The water was changed for every session. A circular ceiling was suspended 1m above the top of the pool. A video camera with wide-angled lens was placed in the centre of the suspended ceiling. The camera was connected to a video monitor and fed input into a RM PC running Windows XP. The data was analysed using WaterMaze software (Actimetrics, Inc., U.K., Edinburgh). The room was illuminated by 8, 45 watt, lights with a diameter of 20cm in the circular ceiling. Four 60 watt spot-lights in each of the four corners illuminated the rest of the room. These lights were 1.2m off the floor and angled upwards.

A circular platform with a diameter of 10cm was placed into the water. The height of the platform was 18cm and was located 2cm below the surface of the water. A beacon could be attached to the edge of the platform. The beacon was black and white stripped plastic rod of 2 cm in diameter which rose 10cm above the surface of the platform when attached.

On the walls of the room were three large distinct shapes made from various colours of card. These were all visible from the surface level of the water. At the edges of the circular ceiling two large objects were hung, one was a black rectangular basket (70cm x 40cm x 40 cm) and the second was a spherical white lampshade approximately 30cm in diameter. These were hung so that they came to 60cm above the top of the pool and approximately 20cm outside the pool. These were hung at east and west positions.

Procedure

The procedure for the OFT, EPM, LMA test and PRL task are identical to that described in chapter 3. In the PRL task, of the thirty-two who underwent 12 days of nose-poke training, twenty-four rats met the criteria to continue on the task, the other 8 were excluded from the study. The criteria required to progress to the PRL task was 90% accuracy in nosepoke training (where only one aperture was illumined per trial). The PRL task was conducted for 5 consecutive days.

The watermaze reversal task had three stages; pretraining, acquisition and reversal. The pretraining stage lasted one session and had 4 trials. The rats were carried into the room, 4 at a time, in a light-tight box. Each rat was carried to the watermaze and released from one of the four start locations (N, S, E, W). The order of the start locations was random and each rat was released from all of the start locations during the session, facing the pool wall. The platform was located in one of the four platform positions (NE, SE, SW, NW), the order of these was random and only changed after each rat in the group had one trial at that location. The order of platform locations was the same for each group of four rats. The platforms in each location were positioned in the middle of the quadrant 20cm from the edge of the pool. The platform had a beacon attached to allow for the rats to learn to swim and locate the submerged platform in opaque water. Each trial lasted 60 seconds and rats were left on the platform for 20 seconds. If after 60 seconds if the rat failed to locate the platform, the experimenter guided the animal to it, where they remained for 20 seconds. After this interval, the rat was removed from the maze, towel-dried and return to a light-tight box. Each of the four rats in the group completed the first trial before moving on to the second trial therefore the inter-trial interval was approximately 4 minutes.

All rats were required to locate and climb on to the platform by the fourth trial before moving on to the acquisition phase. During this phase, platform locations (either SE or NW quadrants) were

counter-balanced between the groups. The beacon was removed from the platform for the duration of the experiment. Each trial lasted a maximum of 60 seconds and animals were confined to the platform for 20 seconds. If the platform was not located, the rat was guided to the platform by the experimenter and left there for 20 seconds. Rats were trained on acquisition of the platform location for 6 sessions. On the 6th day a 5th probe trial was introduced. During the probe trial the platform was removed from the pool and the rats swam for 60 seconds. The reversal phase commenced on the 7th session. During these sessions the platform location for all the trials was switched to the quadrant opposite that used for acquisition. The procedure for this phase was identical to that used in the acquisition phase, except that a 5th probe trial was completed at the end of each session to track acquisition of the new spatial bias.

Statistical analysis

Statistical analysis of the data from the OFT, EPM and PRL task were identical to that described in Chapter 3, section 3.3. In the watermaze reversal task the following measures were recorded for all of the first four trials in the acquisition and reversal phases; time taken to reach the platform, percentage of time spent in the correct quadrant, percentage of time spent within 10cm of the edge of the platform (zone time) and velocity of swimming were recorded. For the probe trials where the platform was removed the number of platform crossings was also recorded. Averages were calculated for each measure over the 4 trials of the acquisition and reversal phases to produce one data point for each phase. A repeated measures ANOVA was conducted on each of the measures.

Repeated measures ANOVA was conducted on each of the measures recorded in the LMA test, breaks, runs and consecutive breaks. Where significant interactions occurred pairwise comparisons were carried out to examine the differences between the groups.

The results are expressed as mean ± 1 standard error. The threshold for significance was p<0.05. Where data was non-spherical given the output from *Malchly's Test of Shericity* ,the *Greenhouse-Geisser* adjustment was used to give a corrected *F* ratio. Where interactions were significant pairwise comparisons were used to examine the simple effects of this interaction. All statistical analysis was carried out using SPSS version 20.0.

4.4. Results

4.4.1. Experiment 1: Open Field Test

Frequency

Figure 1 shows the frequency of entrances to the areas of the maze over two days. Statistical analysis indicated a significant main effect of group on frequency of entrances into the corners of the maze ($F(1,48)=8.871$, $p<0.05$). However, there was no significant main effect of day ($F(1,48)=0.088$, p=NS) or group by day interaction ($F(1,48)=0.518$, $p>0.05$). A table of raw scores is provided (Table 1). Analysis of the raw scores showed there was a significant main effect of group ($F(1,48)=0.839$, $p<0.05$), no significant main effect of day ($F(1,48)=0.248$, p=NS) or or day by group interaction ($F(1,48)=0.184$, p=NS).

Frequency of entrances to the edges of the maze was not significantly different between groups ($F(1,48)=0.136$, p=NS). There was also no significant main effect of day ($F(1,48)=1.910$, p=NS) or group by day interaction ($F(1,48)=0.078$, p=NS). Analysis of the raw scores showed there the was no significant main effect of group ($F(1,48)=4.135$, p=NS), day ($F(1,48)=0.006$, p=NS), day by dose interaction ($F(1,48)=0.052$, p=NS).

There was a significant main effect of group on frequency of entrances to the middle of the maze ($F(1,48)=10.268$, $p<0.01$). There was no significant main effect of day ($F(1,48)=0.702$, p=NS) nor

group by day interaction ($F(1,48)=0.795$, p=NS). Analysis of the raw scores also showed a significant main effect of group ($F(1,48)=7.731$, $p<0.05$), no main effect of day ($F(1,48)=0.511$, p=NS) or day by group interaction ($F(1,48)=0.152$, p=NS).

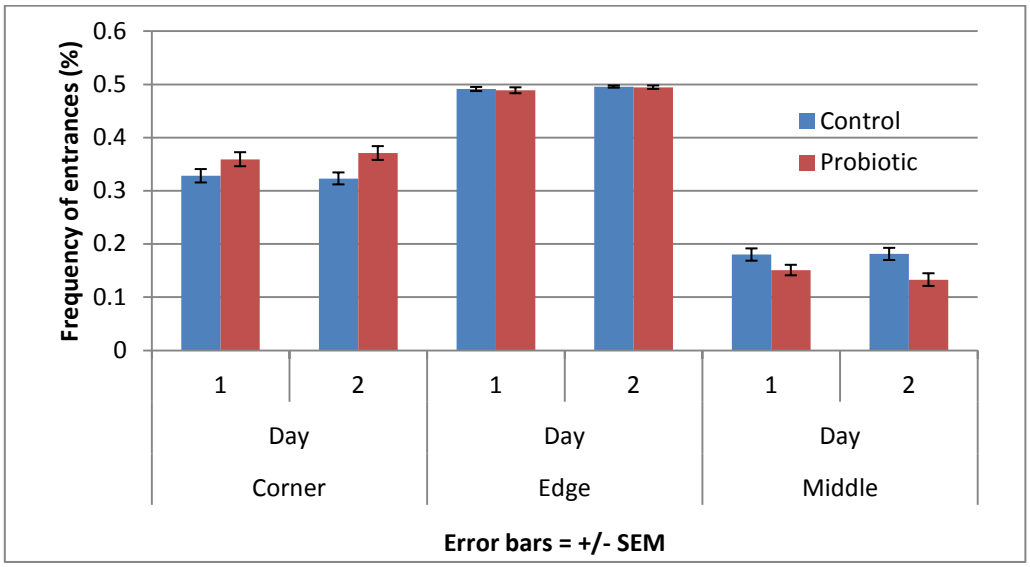

Figure 1. Frequency of entrances into the areas of the maze as a percentage of frequency of entrances into all areas of the maze.

Table 1. Raw scores of frequency.

Area	Day	Control	Probiotic
Corner	1	9.24	8.80
	2	9.76	8.92
Edge	1	14.30	12.63
	2	14.87	12.49
Middle	1	21.65	16.42
	2	21.19	14.38

Duration

Duration of time spent in each area of the maze is displayed in Figure 2. There was a significant main effect of group on duration of time spent in the corners of the maze ($F(1,48)=11.213$, $p<0.05$). There was a also a significant day by group interaction on duration of time spent in the corners of the maze ($F(1,48)=4.531$, $p<0.05$). Analysis of the simple effects of this interaction showed that there was no difference between the group on the first day ($F(1,48)=2.449$, p=NS). However, the probiotic group had a significantly higher duration on the second day ($F(1,48)=17.179$, $p<0.001$). No significant main effect of day was found ($F(1,48)=1.176$, p=NS). Raw score are provided in table 2. Analysis of raw scores also showed a significant main effect of group ($F(1,48)=5.455$, $p<0.05$) and no main effect of day ($F(1,48)=2.23$, p=NS) however there was also no day by group interaction ($F(1,48)=3.549$, p=NS).

There was also a significant main effect of group on duration of time spent at the edges of the maze ($F(1,48)=5.548$, $p<0.05$). There was no significant main effect of day ($F(1,48)=0.245$, p=NS) or group by day interaction ($F(1,48)=3.802$, p=NS). Analysis of the raw scores also showed a significant main effect of group ($F(1,48)=4.399$, $p<0.05$), no main effect of day ($F(1,48)=0.174$, p=NS) or day by dose ineraction ($F(1,48)=4.405$, p=NS).

There was a significant main effect of group on duration in the middle of the maze ($F(1,48)=18.414$, $p<0.001$). There was however, no significant main effect of day ($F(1,48)=2.518$, p=NS) or group by day interaction ($F(1,48)=1.015$, p=NS). This was also found with analysis for the raw scores. There was a significant main effect of group ($F(1,48)=17.784$, $p<0.001$), no main effect of day ($F(1,48)=2.697$, p=NS) or day by group interaction ($F(1,48)=1.002$, p=NS).

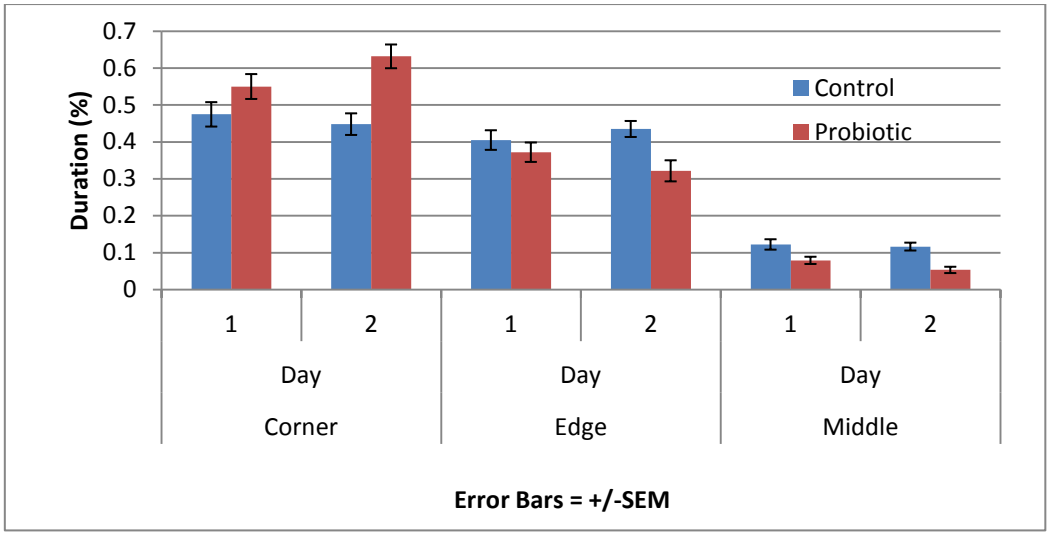

Figure 2. Percentage of time spent in each area of the maze as a percentage of the duration of the session.

Table 2. Raw scores of duration.

Area	Day	Control	Probiotic
Corner	1	71.08	82.35
	2	67.27	99
Edge	1	60.69	56.34
	2	62.5	49.83
Middle	1	72.73	47.53
	2	70.12	31.48

Total Distance

Total distance travelled in each area of the maze in displayed in Figure 3. Repeated measures ANOVA indicated a significant main effect of group on total distance moved in the corners of the maze, (F(1,48)=8.354, p<0.01). Analysis of the simple effects of this interaction showed that there was no significant difference between the groups on the first day (F(1,48)=0.108, p=NS). However, the probiotic group showed significantly higher total distance travelled on the second day (F(1,48)=8.392, p<0.01). There was also a significant main effect of day (F(1,48)=9.512, p<0.01) and a day by group interaction (F(1,48)=5.272, p<0.05). Raw scores are provided in table 3. Statistical analysis of the raw scores also showed a significant main effect of day (F(1,28)=19.081, p<0.001) however there was no main effect of group (F(1,48)=2.443, p=NS) or day by group interaction (F(1,48)=2.792, p=NS).

There was no significant main effect of group found on the total distance moved at the edges of the maze (F(1,48)=3.432, p=NS). There was also no significant main effect of day (F(1,48)=0.018, p=NS) or group by day interaction (F(1,48)=2.907, p=NS). Analysis of the raw scores also showed a similar pattern. No main effect of group (F(1,48)=0.003, p=NS) or day (F(1,18)=7.267, p=NS) was found or day by group interaction (F(1,48)=0.527, p=NS).

There was a significant main effect of group found on total distance travelled in the middle of the maze (F(1,48)=21.997, p<0.001). There was also a significant main effect of day (F(1,48)=8.625, p<0.05). However, there was no significant day by group interaction (F(1,48)=2.072, p=NS). Analysis of raw scores showed a significant main effect of group (F(1,48)-18.385, p<0.001) however there was no main effect of day (F(1,48)=0.678, p=NS) or day by dose interaction (F(1,47)=1.272, p=NS).

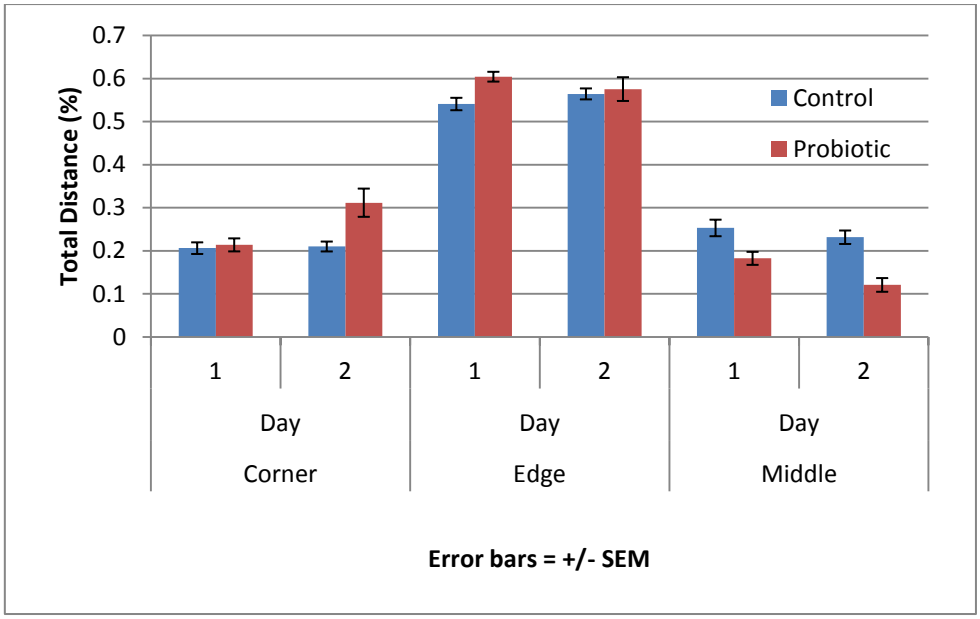

Figure 3. Total distance in each area as a percentage of total distance moved in all areas.

Table 3. Raw scores of total distance

Area	Day	Control	Probiotic
Corner	1	203.44	198.45
	2	260.23	302.71
Edge	1	554.14	594.80
	2	713.35	693.95
Middle	1	1075.56	721.38
	2	1117.11	546.71

Maximum Distance

The average maximum distance moved is the maximum distance travelled at any one time and is displayed in Figure 4. There is no significant main effect of group on the maximum distance moved in in the corners of the maze ($F(1,48)=0.235$, p=NS). There was also no significant main effect of day ($F(1,48)=3.851$, p=NS) or group by day interaction ($F(1,48)=1.578$,p=NS). Raw scores are displayed in

table 4. Analysis of the raw scores also showed no significant main effect of group ($F(1,48)=0.039$, p=NS) or day ($F(1,48)=0.835$, p=NS) or day by dose interaction ($F(1,48)=3.956$, p=NS).

A significant main effect of group was also found in the maximum distance moved at the edges of the maze ($F(1,48)=11.91$, $p<0.01$). However, there was no significant main effect of day ($F(1,48)=2.512$, p=NS) or group by day interaction ($F(1,48)=0.077$, p=NS). Analysis of the raw scores showed a significant main effect of group ($F(1,48)=0.002$, $p<0.05$) no main effect of day ($F(1,48)=3.435$, p=NS) or day by group interaction ($F(1,48)=3.656$, p=NS).

There was no significant main effect of group found in the maximum distance the middle of the maze ($F(1,48)=7.150$, $p<0.05$). There was a significant main effect of day ($F(1,48)=10.534$, $p<0.01$). There was no significant group by day interaction (1,48)=2.001, p=NS). Analysis of the raw scores showed no significant main effect of day ($F(1,48)=0.74$, p=NS) , group ($F(1,48)=1.138$, p=NS) or day by group interaction ($F(1,48)=2.425$, p=NS).

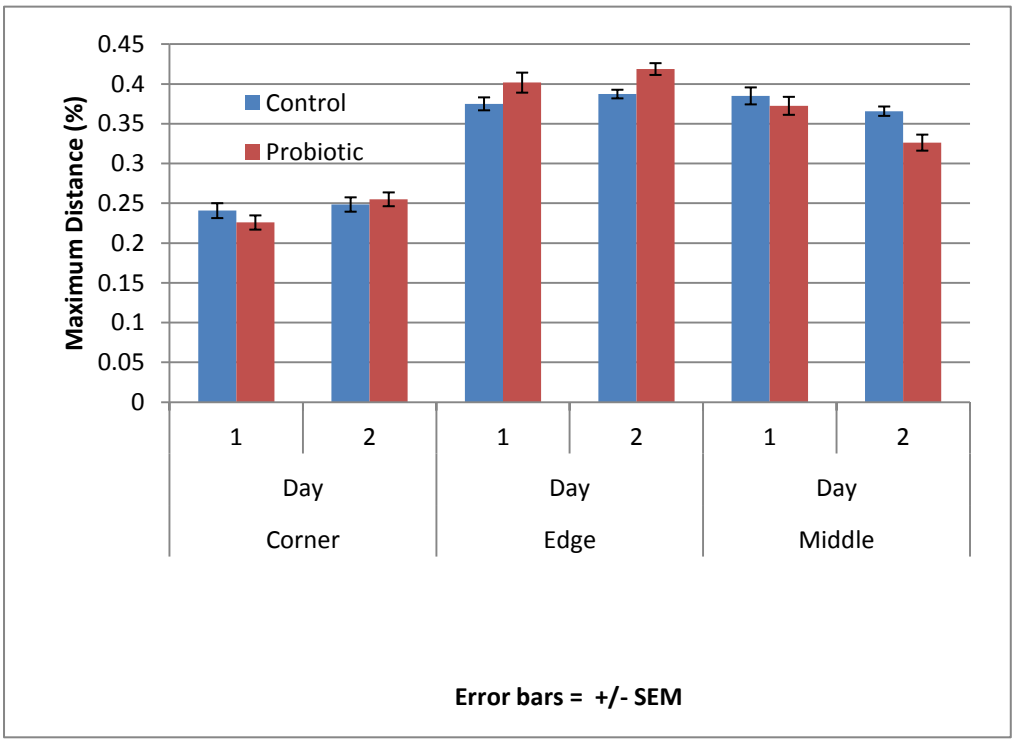

Figure 4. Maximum distance travelled in each area as a percentage of maximum distance travelled in all areas.

Table 4. Raw scores of maximum distance

Area	Day	Control	Probiotic
Corner	1	8.53	6.36
	2	7.42	9.24
Edge	1	13.65	11.48
	2	13.24	15.57
Middle	1	18.13	13.40
	2	13.72	14.62

Velocity

Velocity in each area of the maze is displayed in Figure 5. There was no significant main effect of group found on velocity in the corners of the maze ($F(1,48)=0.245$, p=NS). There was also no significant main effect of day (F(1,48)=.553, p=NS) or group by day interaction ($F(1,48)=0.172$, p=NS). Statistical analysis of the raw scores also reflected these results. There was no significant main effect of group (F(1,48)=0.009, p=NS) or day (F(1,48)=5.03, p=NS) or day by group interaction (F(1,48)=1.343, p=NS). Raw scores are provided in table 5.

There was a significant main effect of group on velocity at the edges of the maze ($F(1,48)=9.301$, $p<0.01$). However, there was no significant main effect of day (F(1,38)=1.705, p=NS) or group by day interaction ($F(1,48)=0.687$, p=NS). Analysis of the raw scores also showed a significant main effect of group ($F(1,48)=9.297$, $p<0.01$), no main effect of day ($F(1,48)=1.705$, p=NS) or day by group interaction ($F(1,48)=0.686$, p=NS).

Statistical analysis showed that there was no main effect of group on velocity in the middle of the maze ($F(1,48)=1.637$, p=NS). There was also no main effect of day ($F(1,48)=0.02$, p=NS) or group by day interaction ($F(1,48)=0.006$, p=NS). These results were also found when raw score were analysed,

no main effect of group ($F(1,48)=1.642$, p=NS), day ($F(1,48)=0.02$, p=NS) or day by group interaction ($F(1,48)=0.006$, p=NS).

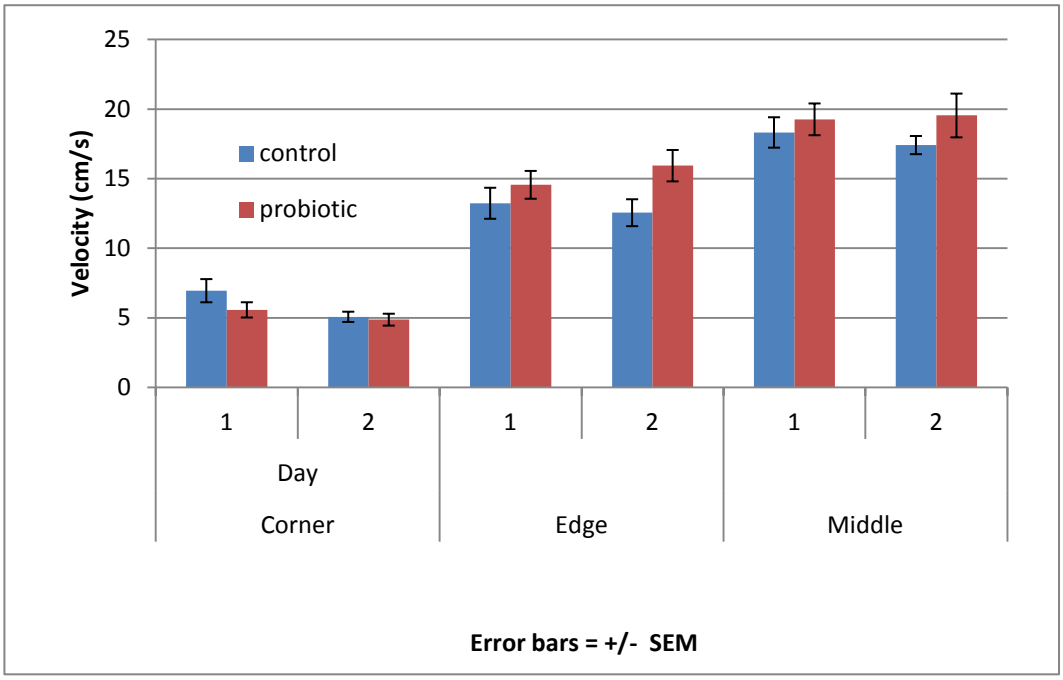

Figure 5. Average velocity in each area.

Table 5. Raw scores of velocity

Area	Day	Control	Probiotic
Corner	1	6.95	5.57
	2	5.07	4.87
Edge	1	13.23	14.56
	2	12.56	15.94
Middle	1	18.32	19.27
	2	17.42	19.55

The results of the open field test indicate that the probiotic group showed preference for the corners and edges of the maze. The control group had a significantly higher frequency of entrances, duration, total distance and maximum distance moved in the middle of the maze. This was in

contrast to the probiotic group who showed significantly greater frequency of entrances into the corners of the maze, higher duration and total distance travelled in the corners on the second day. Results from the measures taken at the edges of the maze indicated that the control group had significantly higher duration. However the probiotic group showed a higher maximum distance travelled and velocity indicating that the control group explored the edges more whilst the probiotic group used this area to move from one corner to the other.

4.4.2. Experiment 2: Elevated Plus Maze

One-way ANOVA was carried out on the measures recorded in the elevated plus maze. Statistical analysis showed no significant differences between the groups on any of the measures, closed arm frequency ($F(1,48)=0.912$, p= NS), closed arm duration ($F(1,48)=0.148$, p= NS), closed arm total distance travelled ($F(1,48)=0.649$, p=NS), closed arm maximum distance travelled ($F(1, 48)=0.122$, p=NS) (see Figure 6), closed arm velocity ($F(1, 48)=0.004$, p=NS) (see Figure 7), open arm frequency ($F(1,48)=0.912$, p= NS), open arm duration ($F(1,48)=0.062$, p = NS), open arm total distance travelled ($F(1,48)=0.649$, p=NS), open arm maximum distance travelled ($F(1,48)=0.122$, p=NS) (see Figure 6), open arm velocity ($F(1,48)=0.42$, p= NS) (see Figure 7). Raw scores from which the rations were derived are provided in table 7. Statistical analysis of the raw scores also found no significant difference between and of the groups on any of the measures.

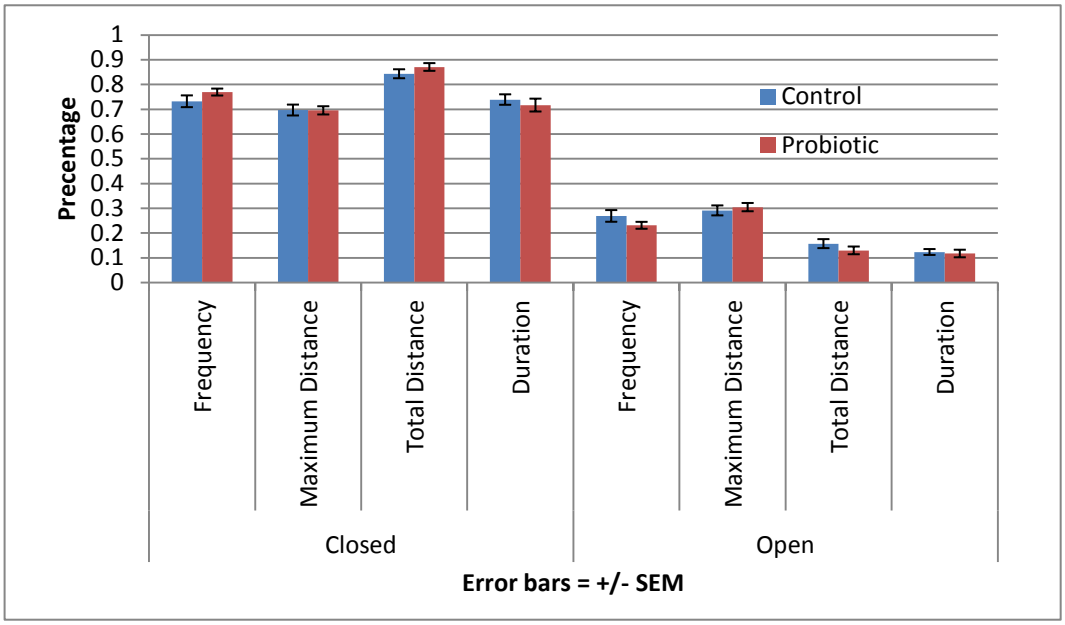

Figure 6. Frequency of entrances, maximum distance, total distance and duration in closed and open arms of the maze.

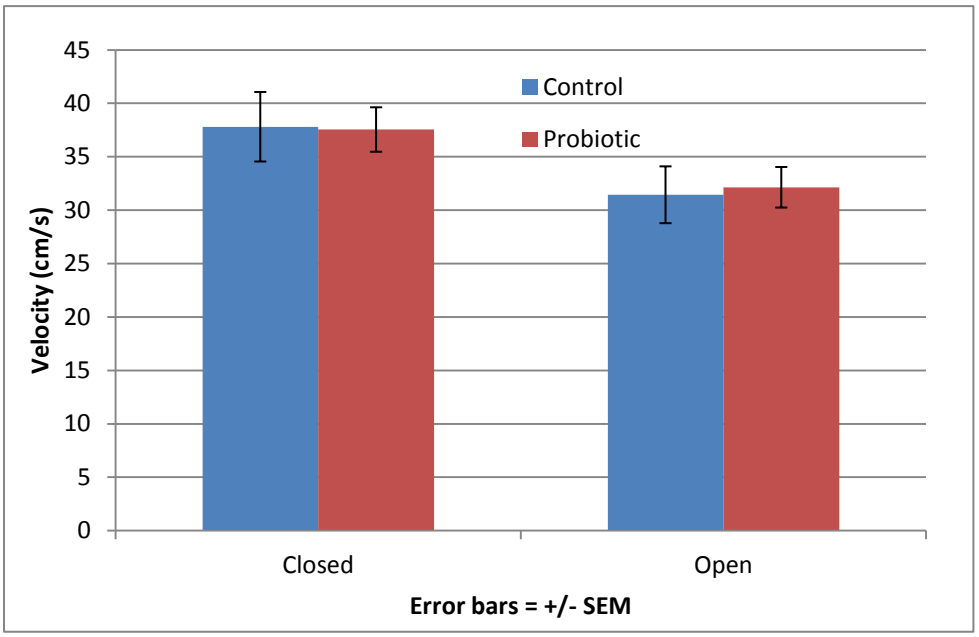

Figure 7. Mean velocity in the closed and open arms of the maze.

Table 7. Raw scores for all measures

Area	Measure	Control	Probiotic
Open	Frequency	5.08	4.70
	Duration	37.16	35.61
	Total Distance	1196.83	1175.92
	Maximum Distance	41.72	32.99
	Velocity	31.43	37.67
Closed	Frequency	14.81	15.30
	Duration	221.44	225.39
	Total Distance	7087.3	7334.26
	Maximum Distance	128.64	94.73
	Velocity	37.80	37.83

Results from the EPM indicated that treatment did not differentially affect any of the behavioural measures.

4.4.3. Experiment 3: Locomotor Activity Test

The mean number of total beam breaks is displayed in Figure 8. Statistical analysis showed there was no significant main effect of group ($F(1,43)=1.538$, p= NS) or day ($F(2,86)=1.871$, p=NS). However, there was a significant day by group interaction ($F(2,86)=14.865$, $p<0.001$). Analysis of the simple effects of this interaction showed the probiotic group had a significantly higher number of breaks on the first day ($F(1,43)=12.984$, $p<0.01$) and significantly lower on the third day ($F(1,43)=4.598$, $p<0.05$) with no significant difference found on the second day ($F(1,43)=2.616$, p=NS). There was also a significant main effect of bin ($F(5,215)=45.363$, $p<0.001$). However there was no significant group by bin interaction ($F(5,215)=0.377$, p=NS) or day by bin by group interaction ($F(10,430)=1.342$, p=NS).

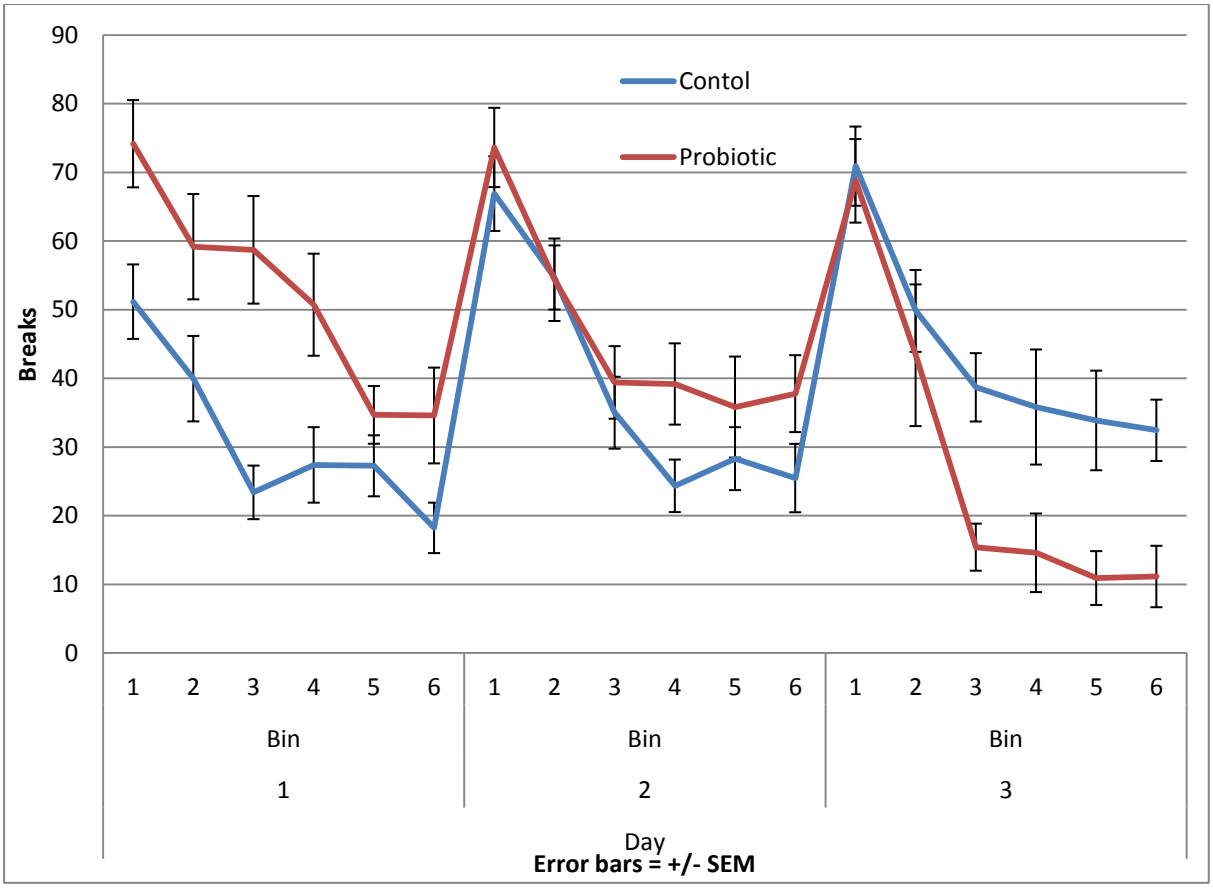

Figure 8. Total number of beam breaks achieved by the probiotic treated group and the control group.

The number of consecutive breaks refers to the number of pairs of beam breaks which that occur at the same side of the testing chamber, displayed in Figure 9. Repeated measure ANOVA indicated that there was no significant main effect of group ($F(1,43)=1.734$, p=NS) or day ($F(2,86)=2.491$, p=NS). There was also no significant bin by group interaction ($F(5,215)=0.918$, p=NS) or bin by day interaction ($F(10,430)=1.563$, p=NS). There was however a significant main effect of bin ($F(2,215)=29.98$, $p<0.001$) and significant day by group interaction ($F(2,86)=18.417$, $p<0.001$). Analysis of the simple effects of this interaction indicated that the probiotic group had a significantly higher number of consecutive breaks on the first ($F(1,43)=14.327$, $p<0.001$) and second days ($F(1,43)=4.329$, $p<0.05$) but a significantly lower number of consecutive breaks on the third day ($F(1,43)=5.914$, $p<0.05$). There was also a significant day by bin by group interaction ($F(10,430)=2.182$, $p<0.0.05$). The results of the analysis of the simple effects are displayed in table 1.

Table1. *F* ratios and *p* values for pairwise comparison of day by bin by group interaction.

Day	Bin	*F* ratio	*p* value
1	1	7.727	0.008
	2	4.129	0.048
	3	18.141	0.000
	4	7.484	0.009
	5	1.362	0.250
	6	4.249	0.045
2	1	1.001	0.323
	2	0.213	0.647
	3	0.534	0.469
	4	4.110	0.049
	5	1.352	0.251
	6	3.583	0.065
3	1	0.123	0.728
	2	0.008	0.931
	3	13.780	0.001
	4	3.482	0.069
	5	6.286	0.016
	6	10.507	0.002

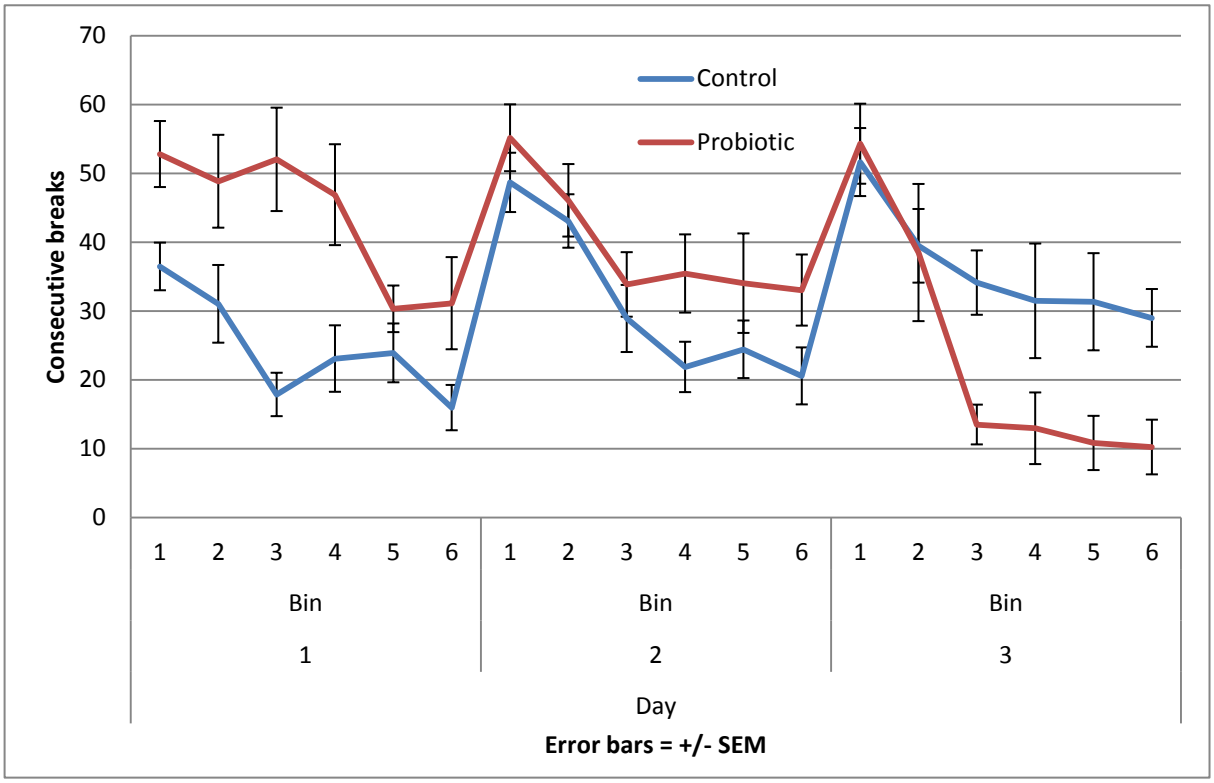

Figure 9. Mean number of consecutive breaks achieved by the probiotic treated group and control group.

The number of runs refers to the number of pairs of beam breaks which were on opposite sides of the testing chamber; this is displayed in Figure 10. Repeated measures ANOVA indicated that there was no significant main effect of group on number of runs completed ($F(1,43)=0.825$, p=NS). There was also no significant bin by group interaction ($F(5,215)=0.975$, p=NS), day by bin interaction ($F(10,430)=0.930$, p=NS) or day by bin by group interaction (F(10,430)=1.7, p=NS). However, there was a significant main effect of bin ($F(5,215)=110.286$, $p<0.001$), a significant main effect of day ($F(2,86)=10.377$, $p<0.01$) and a significant day by group interaction ($F(2,86)=10.377$, $p<0.001$). Analysis of the simple effects of this interaction showed that there was no significant difference between the groups on the first ($F(1,43)=1.837$, p=NS) and second days ($F(1,43)=0.454$, p=NS). However the control group had a significantly higher number of runs than the probiotic group on the third day ($F(1,43)=20.05$, $p<0.001$).

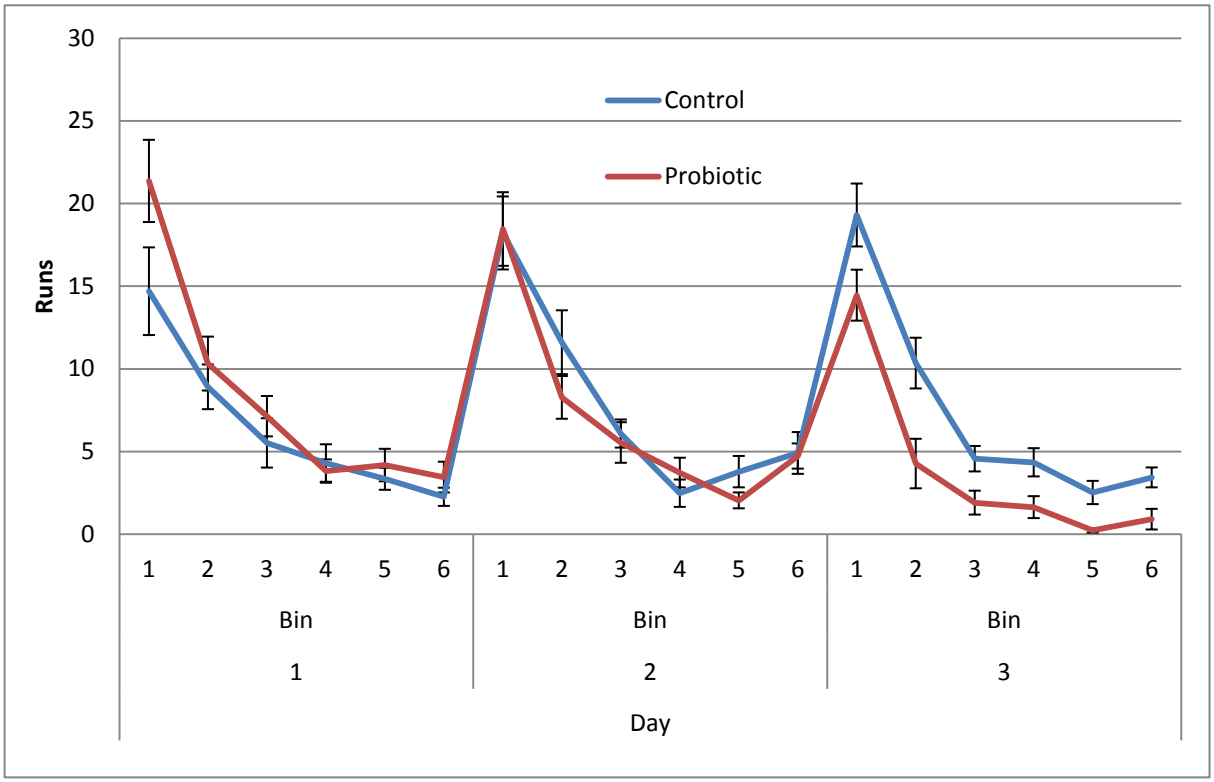

Figure 10. Total number of runs achieved by the probiotic treated group and the control group.

The probiotic group showed a significantly higher number of beam breaks on the first day and a significantly lower number on the third day. This pattern was also reflected in the number of consecutive breaks, with the probiotic having a higher number than the control group on the first day and significantly lower number on the third day. Analysis of consecutive breaks by bin showed that, on the first day, the probiotic group had a higher number of breaks on all bins except bin 5. However on the third day they had significantly lower number of breaks in the third, fifth and sixth bins. The number of runs completed also paralleled the results from the number of breaks and consecutive breaks. There was a significantly lower number of breaks completed by the probiotic group on the third day.

4.4.4. *Experiment 4: Probabilistic Reversal Learning*

Results were analysed by phase on the first day of the PRL task. All trials up to the first reversal were analysed and included in the 'acquisition phase' data. Subsequent trials until a further reversal had been reached were analysed and included in 'reversal phase' data.

Acquisition and reversal phase of session 1

Conditional probabilities during session 1 are presented in Figure 11. Statistical analysis showed no significant main effect of group on win-stay probability ($F(1,18)=0.862$, p=NS), there was also no significant group by phase interaction ($F(1,18)=0.665$, p=NS). However there was a significant main effect of phase ($F(1,18)=21.808$, $p<0.001$). Analysis of lose-shift probabilities indicated that there was no significant main effect of group ($F(1,18)=0.162$, p=NS) or group by phase interaction (F(1,18)=1.048, p=NS). However there was a significant main effect of phase ($F(1,18)=10.071$, $p<0.05$).

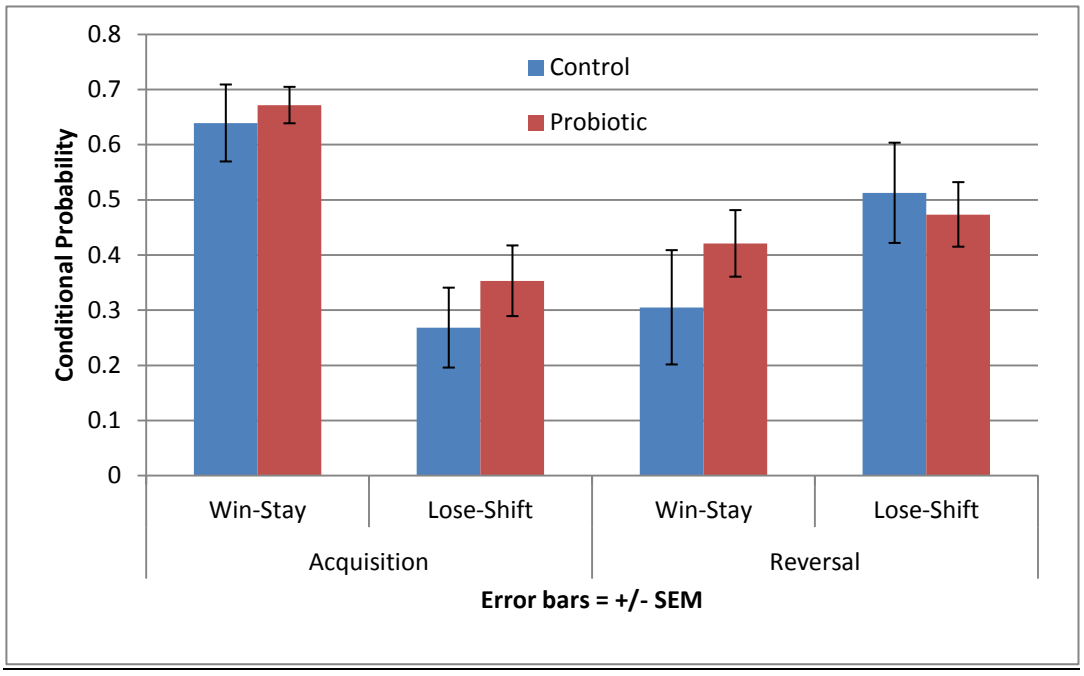

Figure 11. Win-stay and lose-shift probability analysed by phase, acquisition and reversal, on the first test session.

Reversals

Figure 12 shows the mean number of reversals over the sessions. Repeated measures ANOVA showed no main effect of group on number of reversals ($F(1,21)=0.014$, p=NS). There was also no group by session interaction ($F(4,84)=0.531$, p=NS) or main effect of session ($F(4,84)=0.887$, p=NS).

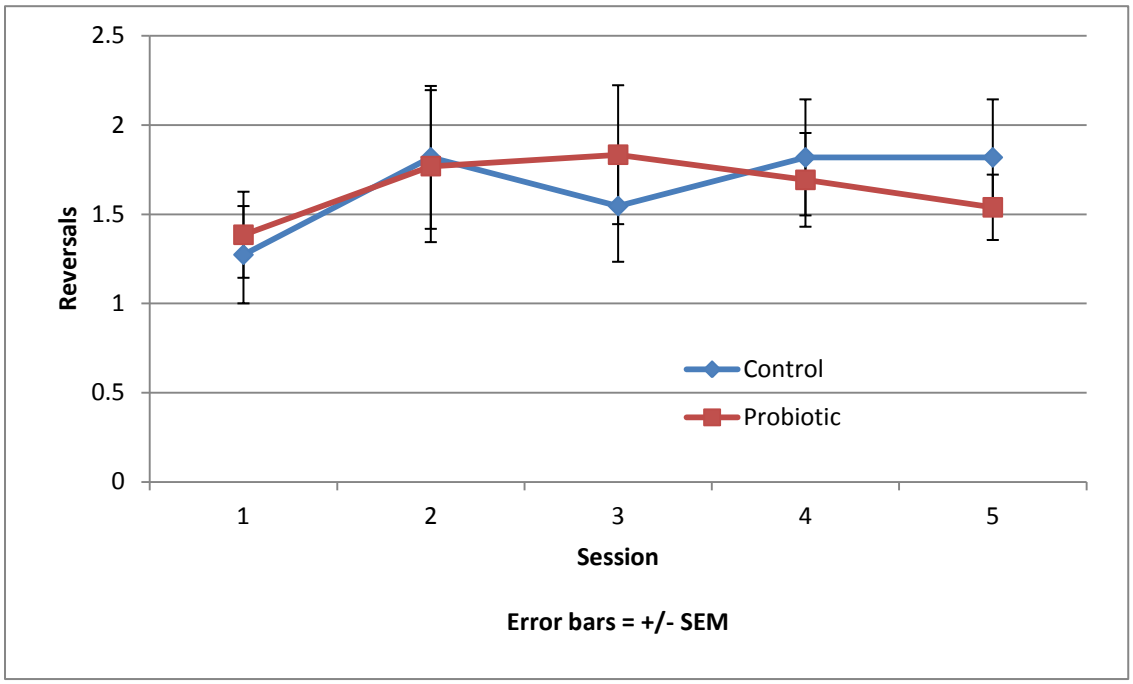

Figure 12. Mean number of reversals completed by probiotic and control group over the test sessions.

Win-stay

Win-stay probability over the test sessions is displayed in Figure 13. Statistical analysis showed no significant main effect of group on win-stay probability ($F(1,21)=0.831$, p=NS), no significant group by session interaction ($F(4,84)=0.903$, p=NS) or main effect of session ($F(4,84)=0.501$, p=NS).

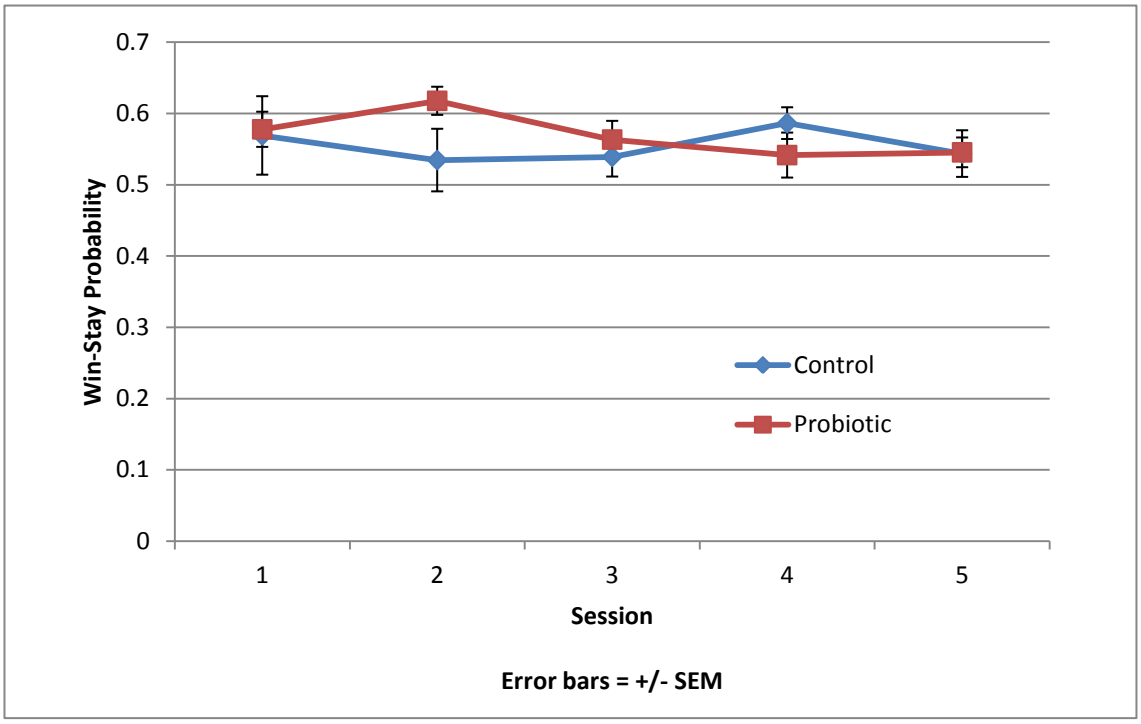

Figure 13. Win-stay probability of probiotic and control group over the test sessions.

Lose-shift

Lose-shift probability is displayed in Figure 14. Repeated measures ANOVA showed no significant main effect of group on lose-shift probability ($F(1,21)=0.015$, p=NS), no significant group by session interaction ($F(4,84)=0.621$, p=NS) nor main effect of session ($F(4,84)=1.128$, p=NS).

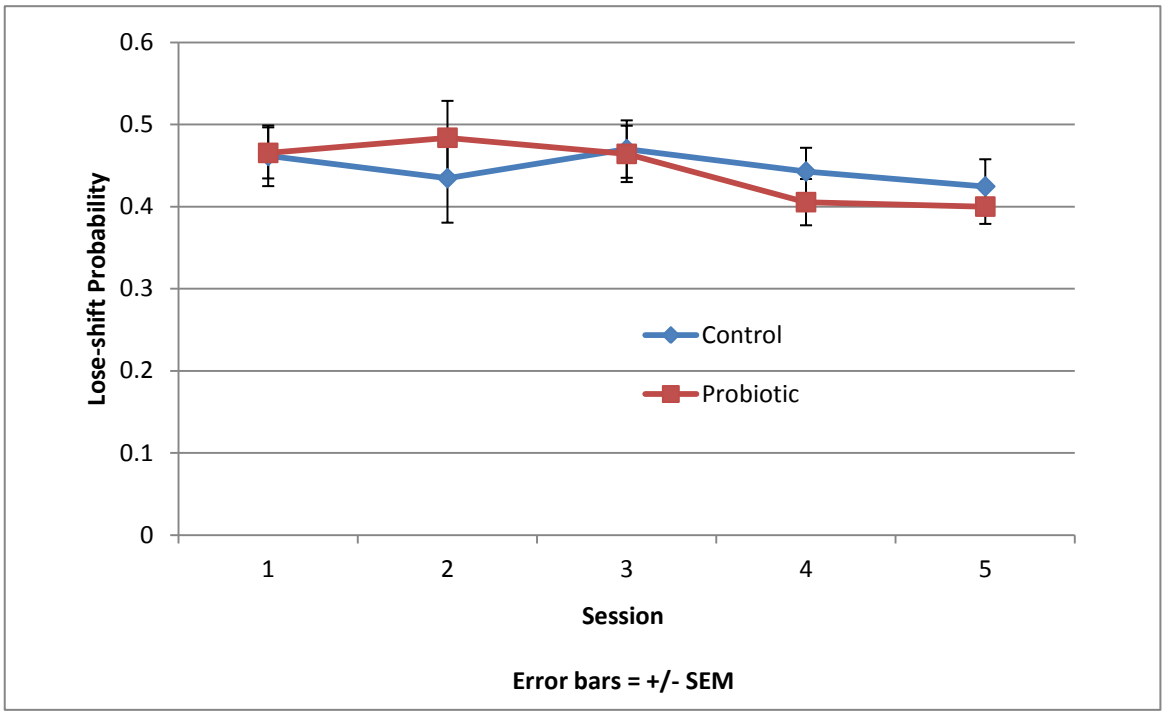

Figure 14. Lose-shift probability of the probiotic and control groups over the test sessions.

In summary, the results from the probabilistic reversal learning task indicated that probiotic administration did not significantly alter sensitivity to positive (win-stay) or negative (lose-shift) reinforcement in either acquisition or reversal in the initial day of the task. Probiotic treatment also did not significantly affect the number of reversals completed or sensitivity to positive or negative reinforcement over any of the test sessions.

Repeated measures ANOVA of the perseverative errors indicated that there was no significant main effect of group ($F(1,21)=1.155$, p=NS) or session ($F(1,21)=1.004$, p=NS). There was also no significant session by group interaction ($F(1,21)=1.163$, p=NS) (figure 15).

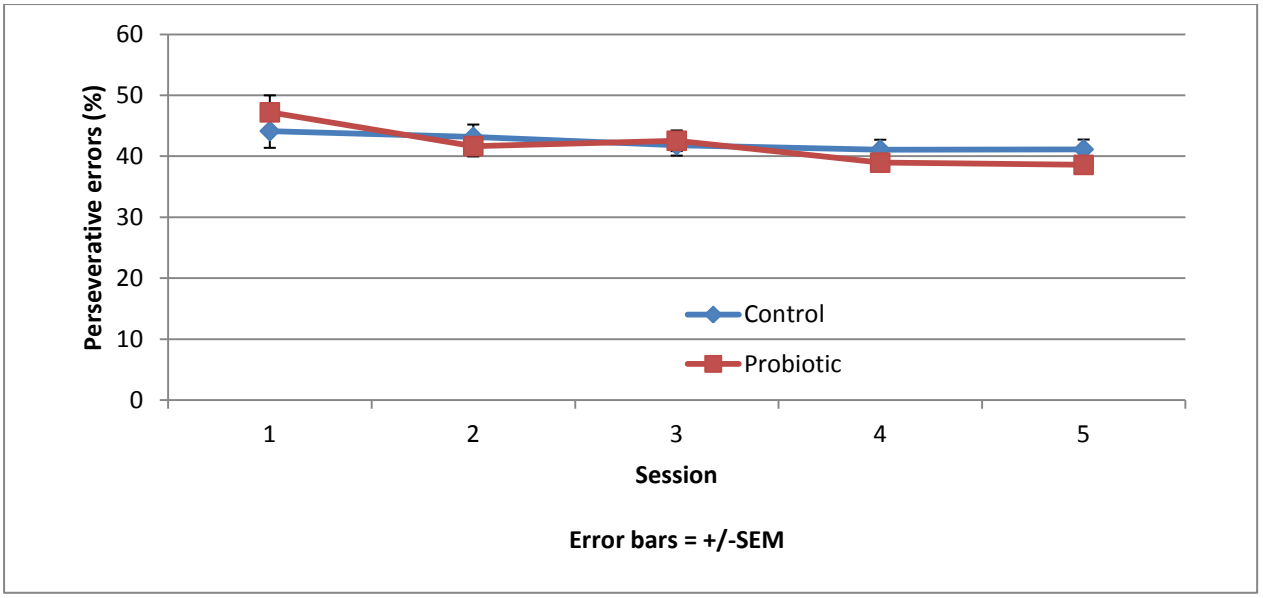

Figure 15. Perseverative errors of control and probiotic treated groups.

Analysis of the trials to criterion showed that there was no significant main effect of group ($F(1,20)=1.052$, p=NS) or session ($F(1,20)=0.059$, p=NS). There was also no significant session by group interaction ($F(1,20)=0.223$, p=NS) (figure16).

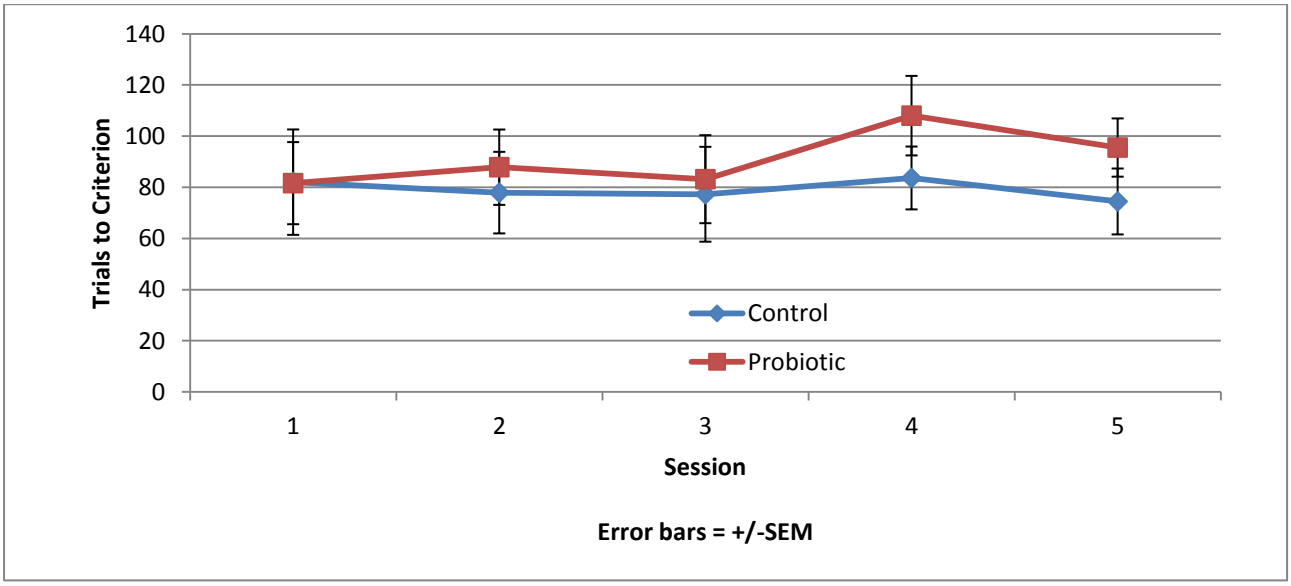

Figure 16. Trials to criterion of control and probiotic treated groups.

4.4.5. *Experiment 5: Watermaze Reversal Task*

Acquisition Phase

Time spent in the correct quadrant of the watermaze during acquisition sessions is displayed in Figure 15. A repeated measures ANOVA of the time spent in the correct quadrant indicated a significant session by group interaction during acquisition ($F(5,150)=2.491$, $p<0.05$). There was also a significant main effect of session ($F(5,150)=6.81$, $p<0.001$), however, there was no significant main effect of group ($F(1,30)=0.695$, p=NS). Pairwise comparisons revealed no significant difference between the groups on any sessions. Session 1 ($F(1,30)=1.053$, p=NS), session 2 ($F(1,30)=1.183$, p=NS), session 3 ($F(1,30)=0.014$, p=NS), session 4 ($F(1,30)=1.274$, p=NS), session 5 ($F(1,30)=3.819$, p=NS), session 6 ($F(1,30)=1.906$, p=NS).

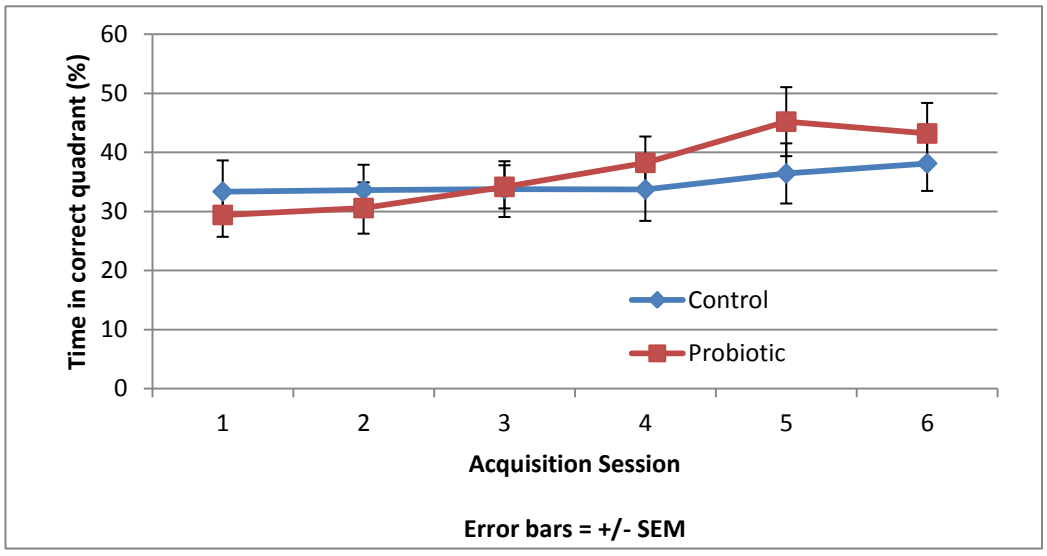

Figure 15. Mean time spent in correct quadrant during acquisition sessions.

Figure 16 displays the time spent in the correct zone during the acquisition session. Repeated measure ANOVA indicated that there no significant main effect of group on time spent in the correct zone during the sessions ($F(1,30)=1.509$, p=NS). There was also no significant interaction over the sessions ($F(5,150)=0.349$, p=NS). However there was a main effect of session ($F(5,150)=6.342$, $p<0.001$).

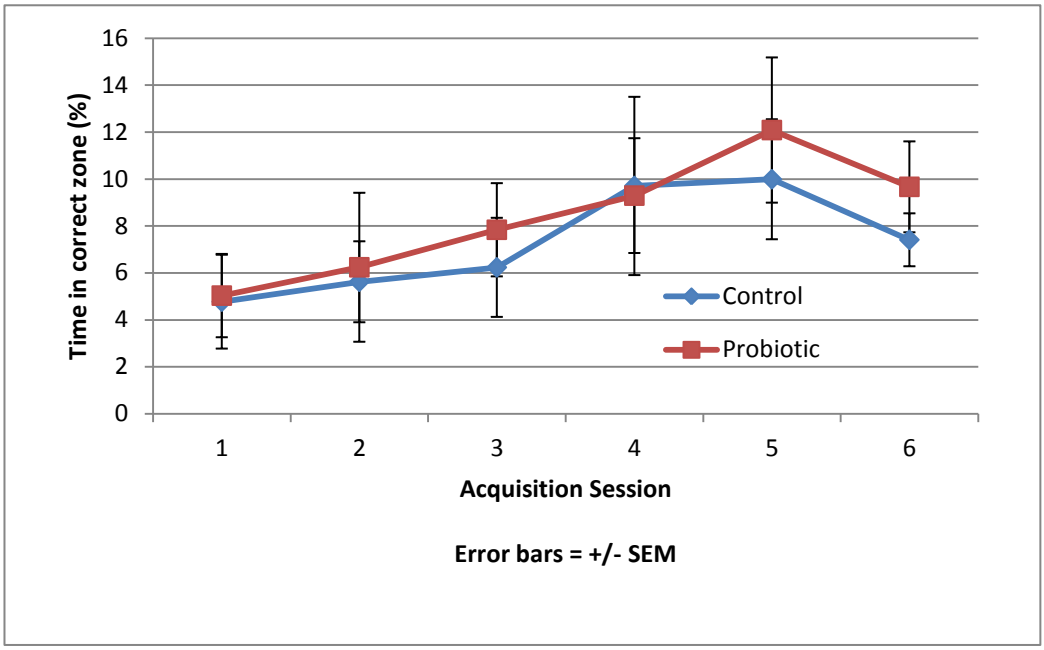

Figure 16. Mean time spent in correct zone during acquisition sessions.

The mean time to reach the platform during the acquisition phase is presented in Figure 17. Repeated measure ANOVA indicated that there was no main effect of group ($F(1,30)=1.528$, p=NS) or session by group interaction ($F(5,150)=0.429$, p=NS). However, there was a significant main effect of session ($F(5,150)=10.175$, $p<0.001$).

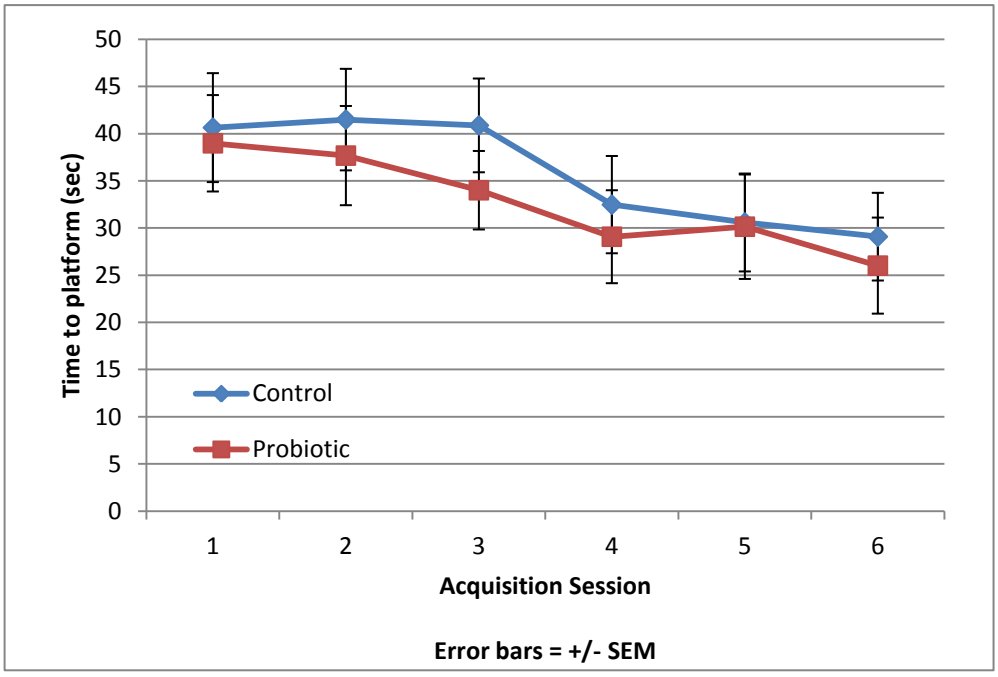

Figure 17. Mean time taken to reach the platform in the acquisition phase.

The mean velocity of swimming in the acquisition phase is presented in Figure 18. Repeated measures ANOVA indicated there was no significant main effect of group ($F(1,30)=1.009$, p=NS) or group by session interaction ($F(5,150)=2.062$, p=NS). However, there was a significant main effect of session ($F(1,30)=21.381$, $p<0.001$).

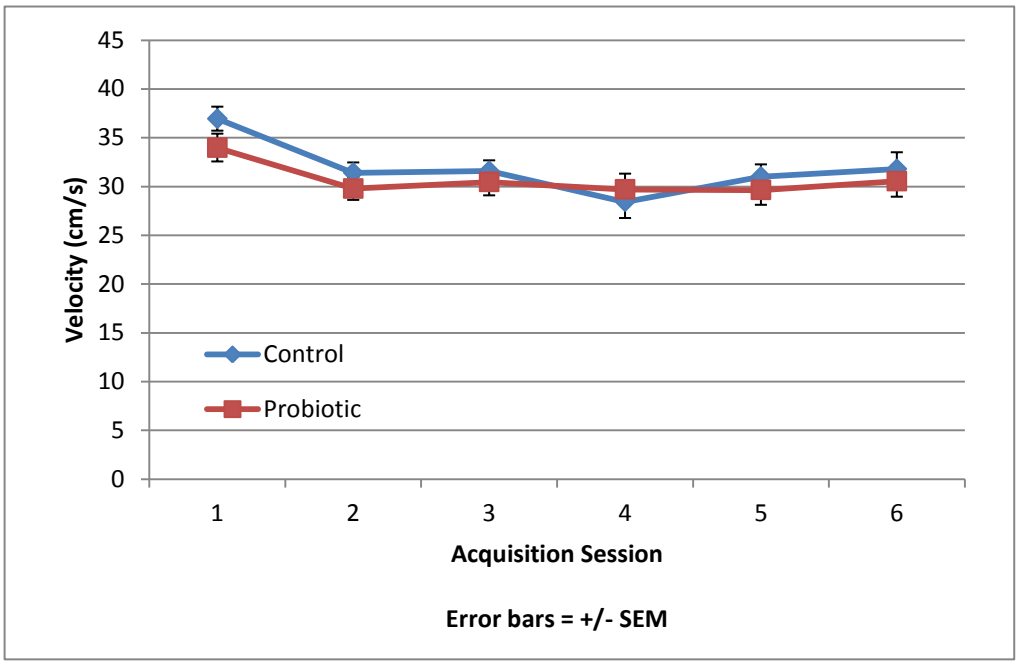

Figure 18. Mean velocity of swimming in the acquisition phase.

During the probe trial in the last session of the acquisition phase the platform was removed and the number of platform crossings during the trial was calculated (Figure 19). One-way ANOVA of the number of platform crossings in the probe trial showed no significant difference between the groups ($F(1,30)=0.339$, p=NS).

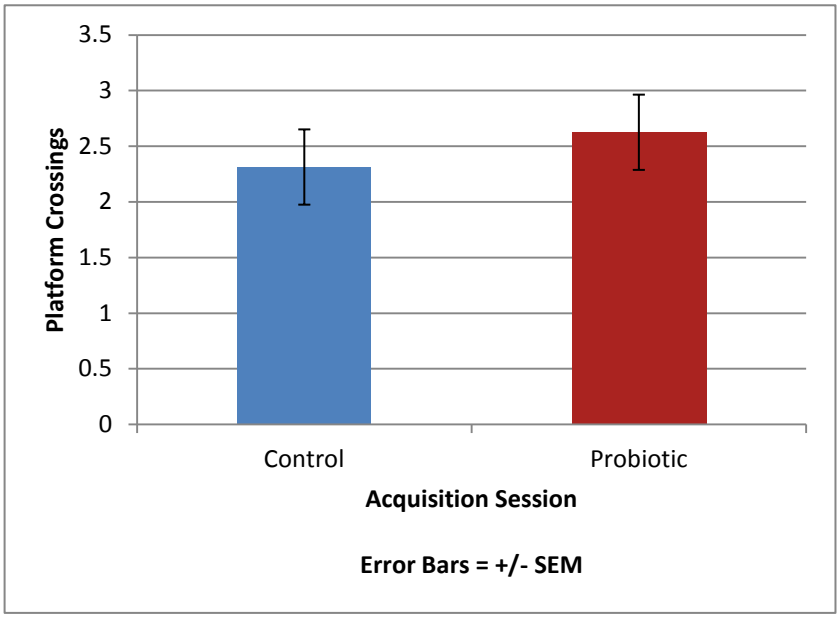

Figure 19. Mean number of platform crossings in probe trial of acquisition phase session 6.

Reversal Phase

The percentage of time spent in the correct quadrant of the maze during the reversal phase is presented in Figure 20. Repeated measures ANOVA indicated there was no significant main effect of group ($F(1,30)=0.807$, p=NS) or interaction across the sessions ($F(2,60)=0.280$, p=NS). However, there was, a significant main effect of session ($F(2,60)=12.561$, $p<0.001$).

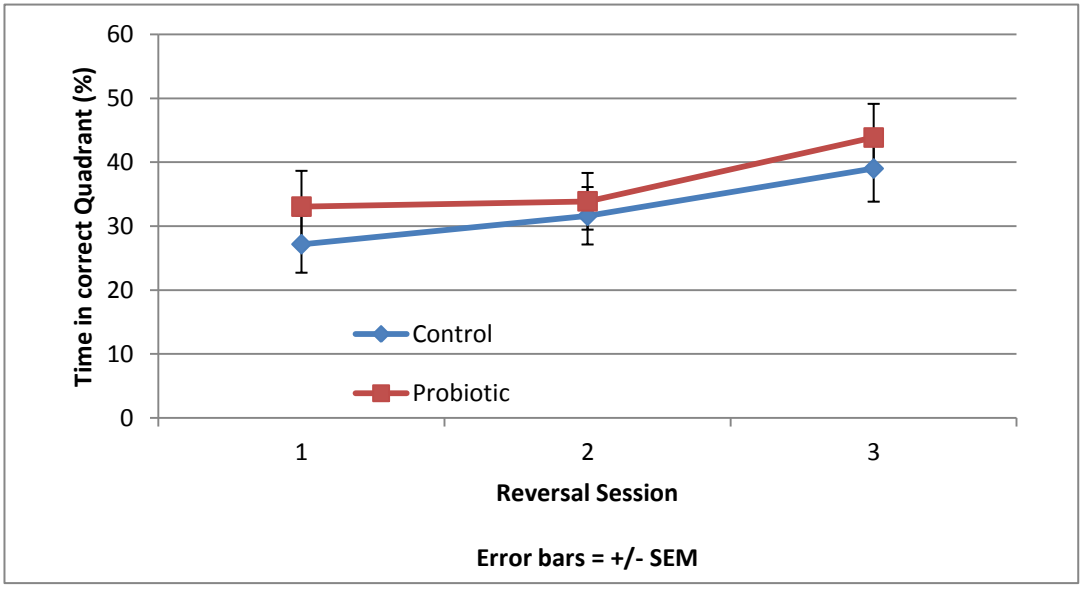

Figure 20. Mean time spent in correct quadrant during reversal sessions.

The percentage of time spent in the correct zone is presented in Figure 21. Repeated measures ANOVA indicated that there was no significant main effect of group ($F(1,30)=1.464$, p=NS). There was also no significant session by group interaction ($F(2,60)=0.852$, p=NS). However, there was a significant main effect of session ($F(2,60)=7.565$, $p<0.01$).

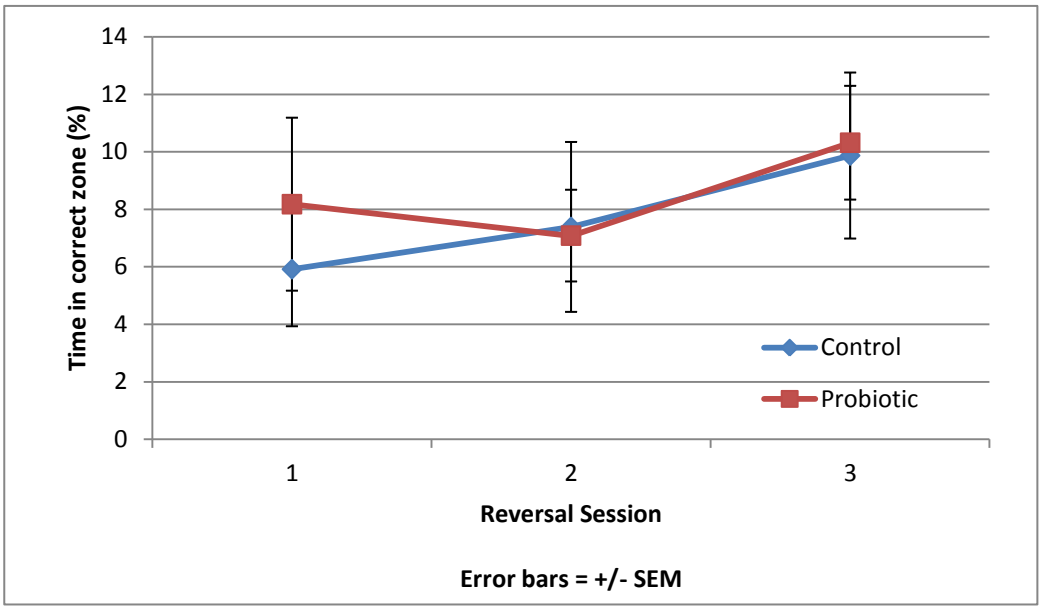

Figure 21. Mean time spent in correct zone during reversal sessions.

The mean time to reach the platform in the reversal phase is presented in Figure 22. Repeated measures ANOVA showed there was no significant main effect of group ($F(1,30)=0.255$, p=NS) or group by session interaction ($F(2,60)=0.796$, p=NS). There was also no significant main effect of session ($F(2,60)=1.603$, p=NS).

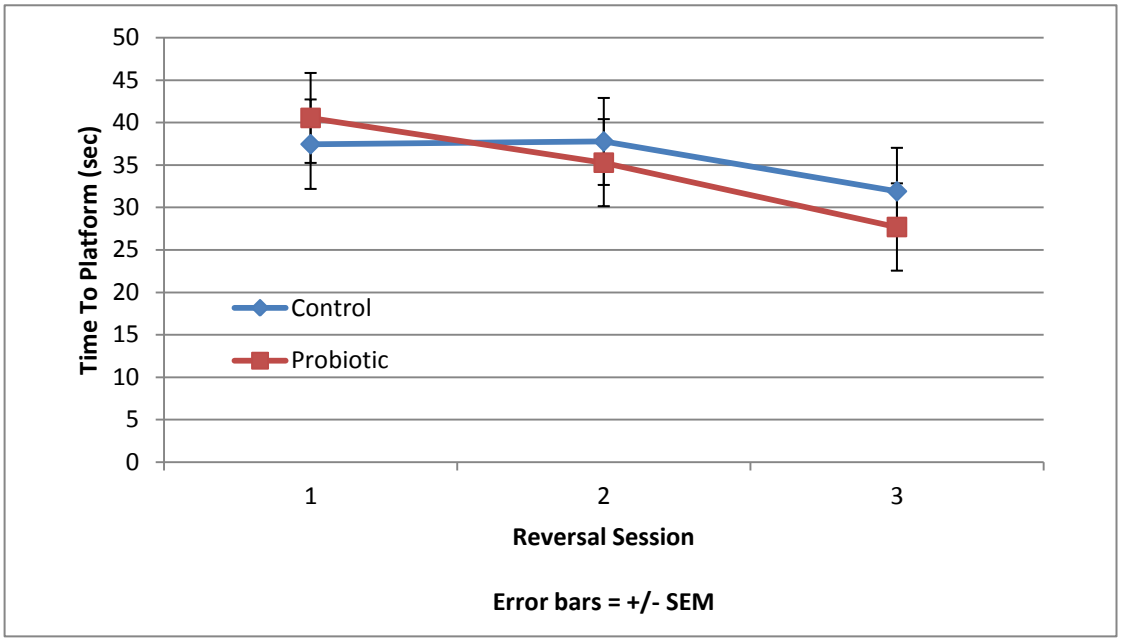

Figure 22. Mean time taken to reach the platfrom in the reversal phase.

The mean velocity in the reversal phase is presented in Figure 23. Repeated measures ANOVA indicated that there was no significant main effect of group ($F(1.30)=2.493$, p=NS) and no session by group interaction ($F(2,60)=0.027$, p=NS). However, here was a significant main effect of session ($F(2,60)=21.927$, $p<0.001$).

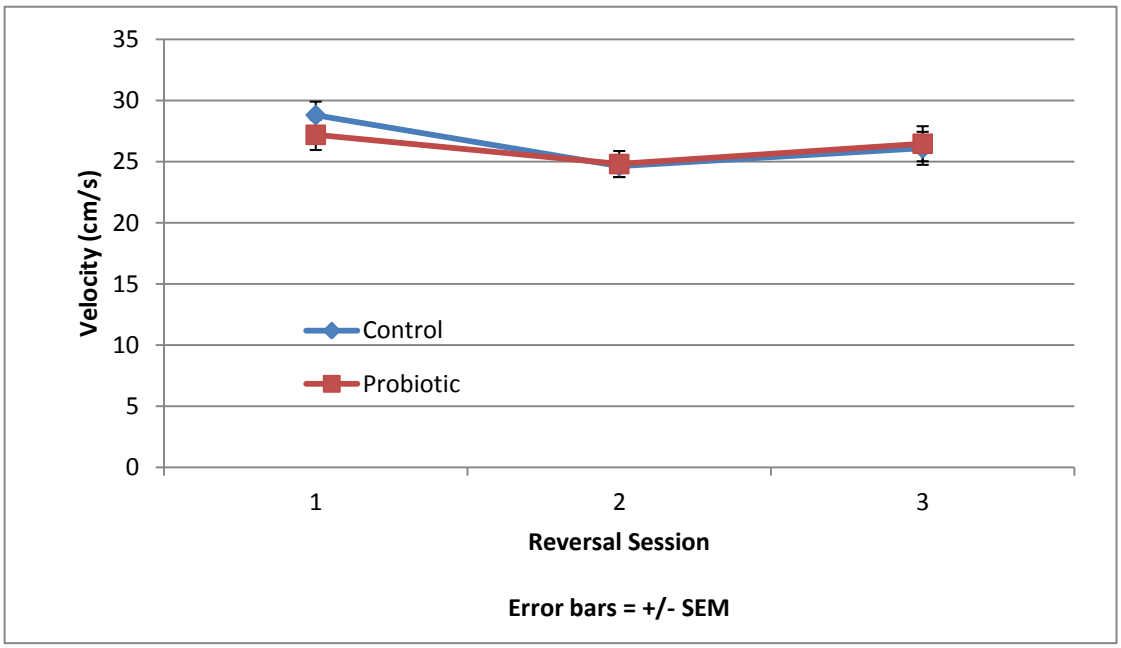

Figure 23. Mean velocity of swimming in the reversal phase.

Figure 24 shows the mean number of platform crossings in the reversal phase. Repeated measure ANOVA indicated a significant session by group interaction ($F(1,30)=4.347$, $p<0.05$). However, there was no significant main effect of group ($F(1,30)=2.033$, p=NS) or main effect of session ($F(2,60)=2.292$, p=NS). Pairwise comparison of the sessions show the probiotic group had a significantly higher number of platform crossings in the third reversal session ($F(1,30)=5.232$, $p<0.05$), however, there was no significant difference between the groups on the first reversal session ($F(1,30)=0.260$, p=NS) or the second reversal session ($F(1,30)=0.108$, p=NS).

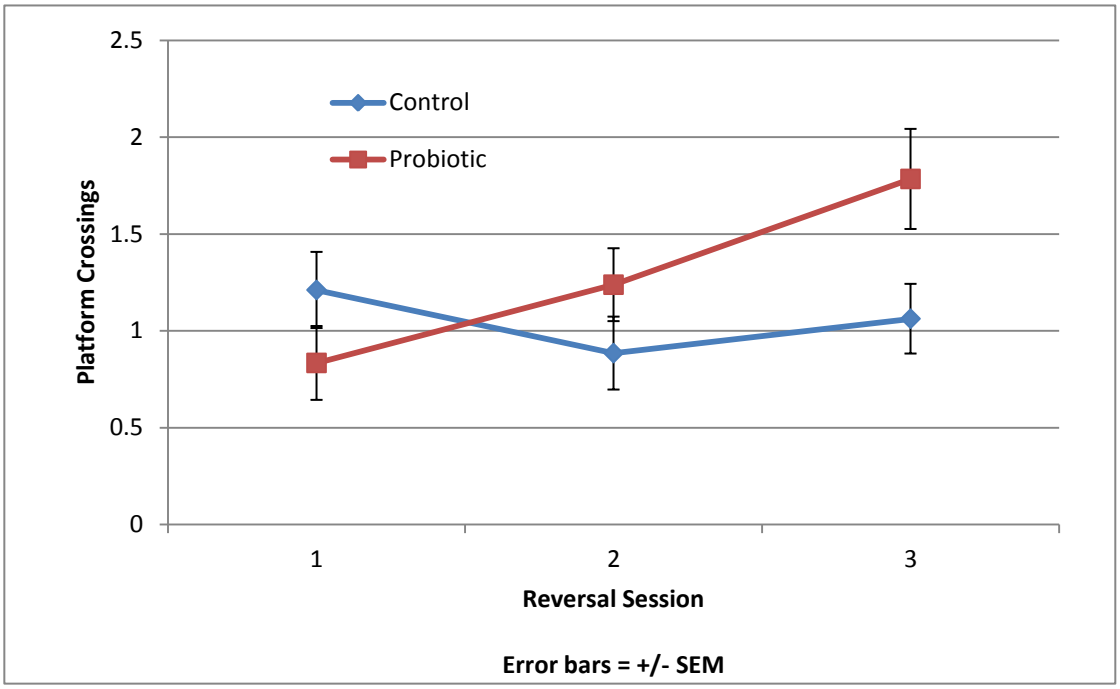

Figure 24. Mean number of platform crossings in probe trials of reversal sessions.

The results for the watermaze reversal task indicated that during acquisition the probiotic group showed less time spent in the correct quadrant in the first 3 sessions. However, results showed a higher percentage of time spent in the correct quadrant in the last 3 sessions. This was reflected in the probe trial of session 6 where the probiotic group showed a higher number of platform crossings compared to the control group. Time spent in the correct zone, time taken to reach the platform and velocity in the acquisition phase did not differ between the groups. In the reversal phase the

probiotic group had a significantly higher number of platform crossings in the third session. This was the only measure that showed differences between the groups in the reversal phase.

4.5. *Discussion*

The elevated plus maze (EPM) and open field test (OFT) are behavioural tests often used to assess the efficacy of pharmacological treatments for anxiety (Ramos *et al,* 2008). The results from the current study indicate conflicting results with no significant difference found between the groups in the EPM. However, in the OFT the control group showed significantly more entries into the middle of the maze, duration of time spent there, total and maximum distance travelled in that area. The probiotic group showed a preference for the corners of the maze demonstrating increased frequency of entrances into the corners as well as higher duration and total distance travelled on the second day. The results from the OFT are indicative of greater anxiety-like behaviour displayed by the probiotic group.

This is the first time this has been found in 'normal' probiotic treated animals. However, of the existing evidence of anxiety-like behaviour in 'normal' probiotic treated animals, none of the studies examined behaviour in the OFT. Results from a study by Bravo *et al* (2011) indicated that in the EPM, *lactobacillus rhamnosus* treated mice had significantly greater frequency of entries to the open arms of the maze. Duration of time spent in the open arms did not significantly differ between the two groups suggesting that it was a mild effect. This gives further support to the theory that the anxiolytic effects of probiotic administration are more profound in animals that have either been exposed to stress, (Desbonnet *et al,* 2010) infection (Bercik *et al,* 2010) or suffering induced depression (Arseneault-Bréard *et al,* 2012). However, this is in contrast to the current results from the OFT. Exploration into the middle potion of the maze was mostly a behaviour exhibited by the control group. However, when examining the differences between the groups on behaviour in the corners and edges, some of the differences between the groups were only found on the second day, suggesting differential adaptive behaviour between the groups. The probiotic group showed higher total distances travelled and duration of time spent in the corners on the second day. The control group demonstrated greater exploration of the middle and edges of the maze. Of the previous

studies showing an anxiolytic effect of probiotics in 'normal' animals (Bravo *et al,* 2011; Messaoudi *et al,* 2011) the rats were treated with probiotics for 28 days and 14 days, respectively. Neural adaptations are evident in pharmacological interventions for anxiety, for example serotonin selective reuptake inhibitors (SSRIs) (Muraki *et al,* 2008; Portella *et al¸* 2011; Romero *et al*, 1996). Therefore, it is possible that chronic treatment compared with acute treatment with probiotics may induce differential neurochemical changes resulting in contradicting behaviours.

Desbonnet *et al*, (2008) demonstrated that probiotics reduced degradation of frontal lobe serotonin levels. Increased serotonin levels with SSRIs are known to cause desensitisation of the 5-HT1a autoreceptor in the dentate gyrus (Blier, 2001), and prolonged exposure inducing serotonin signalling to the frontal cortex (Kreiss & Lucki, 1994, 1995). If the impact of probiotic treatment on serotonin functioning is limited to the frontal cortex, it may inhibit serotonergic functioning in the dentate gyrus via a negative feedback mechanism thereby reducing activation of serotonergic signalling pathways to other areas of the brain, of which may be implicated in anxiety-like behaviour. Further inspection of the effect of probiotics on serotonergic receptors in the frontal cortex and the dentate gyrus are required before conclusions can be drawn on whether chronic probiotic treatment influences adaption in this system.

The results from the locomotor activity test again showed differential adaptive behaviour between the two groups. On the first day the probiotic group were breaking the same beam repeatedly indicating that they did not actively move around in the cage as much as the control group. On the third day this was reversed with fewer consecutive breaks in the probiotic rats. Further analysis of this showed that the differences occurred in the latter bins of the third day indicating that adaption to the chamber was different between the groups. The probiotic group also had fewer breaks of beams on opposite ends of the cage on the third day. When considered collectively this pattern indicates that the probiotic increased activity, as represented by the significantly higher number of breaks. On the third day the number of consecutive breaks and runs were lower in the probiotic

group indicating that there was a general decline in locomotor activity over the three days. This reduction in activity may account for some of the results from the OFT. Much of the group differences in the OFT were only found to be significant on the second day. Although the OFT results indicate that the probiotic group showed initial preference for the edges and corners of the maze, their lack of exploration beyond those areas may be as a result of reduced activity following adaption on the second day. The results from these three tests combined produce a challenging pattern of results to interpret. The OFT and EPM are both used a standard behavioural tests of anxiety but have produced contrasting results. The changes in locomotor activity may confound the results of the OFT test. Given that there was a smaller area for exploration in the EPM than the OFT, the differences in LMA may account for the lack of between group differences in the EPM.

Results from the PRL task indicate that probiotic treatment had no effect on probabilistic reversal learning, during either acquisition or reversal and no effect on sensitivity to positive or negative reinforcement.

In the nosepoke training phase of the PRL task, 8 rats failed to complete this stage. This is a much greater proportion of rats unable to complete this stage of the task compared with previous cohorts (see Chapter 2 Experiment 1 and 2 and Chapter 3 Experiment 4). In the current study, the rats were 24 months old when being tested. With the previous cohorts the rats were tested from 3 months to 7 months. The greater age of the probiotic and control groups could explain the performance in the PRL task. As cognitive abilities diminish with age this may have reduced the margin whereby one group could have out-performed the other.

The probabilistic reversal learning task is a more demanding procedure than classical reversal learning paradigms, as the response-reward contingency is less than 100%. This requires greater attention to the reward contingency, as is found to be reliant on the mPFC (see Chapter 2 Experiment 2). It is possible that the effects of probiotic administration reported by Desbonnet *et al* (2008) do not impact serotonergic function in areas of the frontal cortex required for probabilistic

reversal learning, namely, the mPFC. In the watermaze reversal learning paradigm, accurate identification of the 'correct' response was always rewarded by escape from the water. In this task the 100% rewarded correct response bears similarity with 'classical' reversal learning paradigms, which is reliant on OFC functioning. It is possible that the differentiation of the effect of probiotic treatment in these two tasks identifies dissociable brain areas as a locus of impact of probiotics. However, a comparative study controlling for age differences would be required in order to examine this hypothesis.

Ability to effectively use extramaze cues was fundamental to the watermaze reversal learning task. The results indicated that acquisition of the task was significantly different between the groups with the probiotic group showing less time spent in the correct quadrant of the pool in the first two sessions. This pattern was then reversed in the last three sessions. This differential adaption to the task may be indicative of the same underlying effects responsible for the differential adaption to environments found in the OFT and the LMA test. As the watermaze task is an anxiety inducing paradigm it is possible that an increased state of anxiety may have increased sensitivity to the probiotic treatment, therefore facilitating faster learning of the platform location. An index of the accuracy of the animals representation of the platform location was provided by the number of platform crossings. During the reversal phase the probiotic group learned the new platform location faster than the control group and by the third reversal session this group had a significantly higher number of platform crossings than the control group. Interpretation of this data is difficult as the data for platform crossings in the incorrect quadrant was not analysed therefore it cannot be concluded as to whether the probiotic treated rats learned the new location of the platform with better accuracy than the control group or they showed improved memory for locations in general.

The watermaze reversal learning paradigm arguably relies to a greater degree on spatial memory than the PRL task. In order to examine the sensitivity of spatial memory to probiotic administration

in the absence of an explicit navigation requirement the subsequent chapter will examine the effects of probiotic treatment on spatial recognition memory.

The effects of probiotic administration on novel object recognition, object-in-place and object recency and the effect of citalopram on novel object recognition.

5.1. Summary

This chapter presents a series of experiments that examined the effects of probiotic administration on object novelty, object-place associations and object recency memory processes. The purpose of this was to examine the effects of probiotics on memory. This chapter also presents a series of experiments that examined the extent to which altered serotonergic function (citalopram administration) paralleled the behavioural effects of probiotic administration on recognition memory. The results from the novel object recognition task indicated that probiotics improved memory when a longer delay of one hour occurred between the sample and test phase. This was not found when a shorter delay of 5 minutes was used. When normal rats were administered with 5 mg/kg of citalopram an opposing pattern of results were obtained. Again, no difference with found in the novel object recognition task with the shorter delay of 5 mg/kg however, when there was a longer delay of one hour been the sample a test phases the citalopram group showed impaired memory for the familiar objects. Spatial recognition memory was assessed using an object in place task and found that probiotic treatment improved memory for the spatial arrangement of object. An object recency task was employed to examine the effect of probiotics on temporal discrimination. The results from this showed that there was no effect of probiotics on this type of memory.

5.2. Introduction

Novel object recognition (NOR) is a one-trial test of object memory developed by Ennaceur and Delacour in 1988 for use in neurobiological studies (Akkerman *et al,* 2012). The assumption that underpins NOR is that rodents show an instinctual preference for novel objects over familiar objects.

This is expressed as an increase in exploratory behaviour towards the novel versus familiar objects. NOR utilises spontaneous behaviour and allows for examination of learning and memory without the use of food or water deprivation, rule learning or training of response-reward associations (Dere *et al,* 2007).

The most commonly used version of the NOR task consists of a sample trial (using sample exposure times ranging from 2-10 minutes) during which the rat or mouse is allowed to explore two identical copies of the same object spaced a few centimetres apart. This stage is followed by a delay (in which the animal is confined to a holding cage) and then a test phase, during which one of the original objects is replaced with an object that the animal has not seen before. Normal animals demonstrate a preference for exploring the novel object exploration during the test phase that is typically modified by increasing the delay between the sample and test phases. In order to examine not only encoding of the main features of the object but its spatial location object-in-place tasks are used (Ozawa *et al,* 2014). This involves switching the location of two familiar objects, while leaving two equally familiar objects in the locations they occupied during the sample stage. A further assessment of recognition memory focuses on object recency. In a task involving a temporal discrimination presentation of a pair of objects is followed by a delay after which there is the presentation of another pair of objects. The test phase consists of the presentation of an object from each of the pairs. In this task, recognition of the object from the most recently presented pair and greater exploration of the most recent object is found in control animals (Barker, 2007).

Studies have shown functional dissociations in the brain systems responsible for different aspects of recognition memory. In NOR tasks, lesions to the rhinal (perirhinal and entorhinal) cortices impair performance, specifically the deficit exhibited is delay-dependent (Eacott *et al,* 1994; Meunier *et al,* 1993). Lesions disrupt performance when the task involves longer delays (>60 seconds) but result in no deficits at shorter delays (10 seconds) (Eacott *et al,* 1994; Meunier *et al,* 1993). Conversely, lesions to the hippocampus and amygdala which leave the rhinal cortices intact produce no deficit in

NOR performance (Murray and Mishkin, 1998; Tam *et al,* 2013). Further studies have shown that impairment of the perirhinal cortex produces the most substantial deficits in object novelty recognition with impairments of the entorhinal cortex producing only a mild and transient deficit (Buffalo *et al,* 1999; Meunier *et al,* 1993). This is supported by findings showing that bilateral lesions to the perirhinal cortex induce deficits in object recognition (Barker *et al*, 2007). Whilst there exists an consensus of opinion that role of the hippocampus in encoding of information about the object in the NOR task is limited (Baxter *et al*, 2001; Prusky *et al,* 2004), studies have found a double dissociation in the role of the hippocampus and the rhinal corticies in spatial recognition memory and NOR (Bussey *et al,* 2000; Ennaceur *et al,* 1996). Fornix lesions impair spatial memory but not NOR (Bussey *et al*, 2000). A study by Winters *et al* (2004) demonstrated that specific neurotoxic lesions of the hippocampus impaired performance in a standard radial maze spatial task but not the NOR task, conversely lesions to the perirhinal cortex impaired NOR but not spatial memory tasks (Barker *et al*, 2007; Winters *et al*, 2004). NOR tasks using the same objects but in different spatial arrangements are impaired following hippocampal lesions although NOR performance is unaffected (Barker & Warburton, 2011). This heterogeneity of function between these brain areas has been reported in other studies using immediate-early gene expression (Aggleton & Brown, 2005; Wan *et al,* 1999). Much of the recent research on the contribution of specific brain areas to recognition memory focus on *c-fos* levels. There are several benefits to this methodology over lesion studies. It allows for an assessment of the contribution of the targeted brain area as opposed to focussing solely on the aberrant behaviour as a consequence of lesions that may produce more widespread systems effects. Due to the difficulty in achieving highly selective lesions without impacting the adjacent brain areas, *c-fos* studies allow for examination of adjacent and subregions of the target site (Aggleton *et al,* 2012). *C-fos* studies have consistently reported that the perirhinal cortex is fundamental to recognition memory independent of hippocampal and entorhinal cortex function (Zhu *et al,* 1995, 1996, 1997). Adaptations of recognition memory tests include the bow-tie maze

(Albasser, 2010); which is a running recognition procedure. Perirhinal lesions also produced a deficit in novel object discrimination in this task (Albasser, 2010). In addition, increased levels of *c-fos* in the perirhinal cortex are associated with object novelty detection (Aggleton *et al,* 2012). Unlike previous *c-fos* and lesion studies, the bow tie maze, as a test of novel object discrimination, also implicated a role for the hippocampus in recognition. Increased levels of *c-fos* were also reported in the hippocampus. However, the authors did not attribute the hippocampal activation to object novelty *per se* rather the behavioural demands of the task, active exploration and navigation through the maze utilises object-in-place or object recency learning (Aggleton *et al,* 2012*).* Furthermore, spatial rearrangements of familiar objects resulted in significantly higher *c-fos* levels in the hippocampus but not the perirhinal cortex (Jenkins *et al,* 2004; Wan *et al,* 1999). When object-in place recognition was tested in the bow-tie maze, precisely the same pattern of *c-fos* increases were found in hippocampal subreqions (Dentate gyrus, CA1 and CA3) as in the object novelty task (Albasser *et al,* 2010). This gives support to the theory that the behavioural demands of the object novelty task in the box-tie maze employ hippocampal resources but that these are not fundamental for discrimination of novelty specific to the objects.

The relative contribution of hippocampal resources in recognition memory following preoperative exposure was examined by Broadbent *et al,* (2010). The results from this study indicated that when several tests of NOR, larger groups sizes, larger lesions and shorter delays between exposure phase and surgery are used hippocampal lesions produce deficits in NOR. Furthermore, in NOR tests where spatial and contextual cues are limited hippocampal lesions produce a deficit (Broacdbent *et al,* 2004). When considered with the previous work on IEG expression and the factors required to produce a deficit highlighted by Broadbent *et al* (2004,2010), it is possible that larger hippocampal lesions may impair function of adjacent brain areas such as the rhinal corticies thereby explaining the mild impairment found with multiple tests of NOR.

This research implies that performance on NOR tasks and object-in-place tasks have differential dependence on the function of different components of the memory system. However, due to the anatomical interconnectedness of these regions various forms of recognition memory testing have varying degrees of dependence on different components of this system. Temporal order recognition memory, where performance is based on the relative recency of objects, is reliant on both the perirhinal cortex and hippocampus with lesions to either or contralateral lesions to both areas severely impairing performance (Barker *et al*, 2007; Barker and Warburton, 2011; Fortin *et al,* 2002; Hannesson *et al,* 2004). Another structure found to be fundamental for memory for the sequence of events is the medial prefrontal cortex (mPFC). Bilateral lesions to this area impair temporal order memory tasks as does contralateral lesions to the mPFC and perirhinal cortex (Barker *et al*, 2007, Baker & Warburton, 2011). This suggests a functional interdependence of these two brain regions in processing sequence information. A functional interdependence of the hippocampus and mPFC was also found in this task with contralateral lesions producing a deficit in memory for temporal order (Barker & Warburton, 2011). This research provides evidence that the hippocampus, perirhinal cortex and mPFC form a functional network in order to successfully process sequential information.

The current literature on recognition memory including lesion studies and IEG expression provide a comprehensive framework on which to test the effects of probiotic administration on memory function.

Of particular interest in the current study is the effect of serotonergic manipulations on recognition memory. The reasoning behind this stems from research published by Desbonnet *et al* (2008) who showed that probiotic administration reduced degradation of serotonin (5-HT) in the frontal cortex of rats. The present study used serotonin reuptake inhibitors (SSRIs) to explore the effects of direct serotonergic manipulation on NOR. This experiment provides important baseline data to determine if behavioural changes in novel object recognition following probiotic administration may reflect (at least in part) changes in serotonergic function.

In order to examine the effect that 5-HT manipulation has on NOR the current study used citalopram, a SSRI. This class of antidepressant drugs prevents reuptake of 5-HT from the synaptic cleft back into the presynaptic membrane increasing the concentration of 5-HT in the cleft. Much of the research on the role of 5-HT function in NOR has focussed on the expression of the 5-HT1A receptor and subsequent effects following agonism and antagonism of this receptor. Systemic application of the 5-HT1A receptor antagonist, WAY100635, was found to improve NOR with a 24-hr delay when administered prior to the sample trial (Pitsikas *et al,* 2003). Furthermore the administration of WAY100635 was also shown to ameliorate deficits induced by the AMPA receptor blocker, NBQX (Schiapparelli *et al,* 2006). Other studies have focussed on the effect of selective 5-HT lesions. Lesion of the dorsal raphe nucleus with 5,7-DHT impairs NOR with a 1-hour delay (Lieben *et al,* 2006). Given the results with the perirhinal lesion studies previously discussed, this result implies serotonin manipulation in the dorsal raphe nuclei may affect perirhinal cortex function.

Detrimental effects of serotonergic manipulation on performance in the Morris watermaze, a test of spatial memory, have been reported following administration of the serotonergic depletor, p-chloroamphetamine (PCA) (Santucci *et al,* 1995) and citalopram (Schaefer *et al,* 2013). Performance in this task has shown to be dependent on hippocampal functioning as with object-in-place tasks.

Manipulation of 5-HT levels though either 5,7-DHT lesions, tryptophan depletion, 5-HT receptor agonism/antagonism or SSRI administration have effects on other neurotransmitter concentrations. Some of the behavioural effects found on NOR and spatial memory maybe mediated by the effect of 5-HT manipulation on other neurotransmitter concentrations. For example, WAY 100635 antagonises acetylcholine release in the neocortex and hippocampus, and blockade of cholinergic function using scopolamine has been shown to impair NOR (Koyama *et al,* 1999). Similarly, agonism of the 5-HT6 receptor improves recognition memory via modulation of cholinergic and glutaminergic neurotransmission (Kendell *et al,* 2001).

The pattern of results summarised above suggests a role for 5-HT function in NOR. This effect may occur either directly by altering serotonergic functioning of fundamental brain areas such as the perirhinal cortex or indirectly though attenuated function of other neurotransmitters such as dopamine or acetylcholine (Koyama *et al,* 1999; Nelson *et al,* 2012).

The current experiment examined the effect of probiotic administration on NOR, object-in-place and temporal recognition memory. The rats in the current study were chronically exposed to probiotics from birth in order to examine the effects of probiotics on the brain over an extended period. Although there is a lack of data available on probiotic treatment and monoaminergic concentrations, it is hypothesised that chronic exposure will lead to more pronounced affects on neurotransmitter function similar to those reported by Desbonnet *et al* (2008). The effects citalopram on NOR were also examined to provide a reference condition involving a direct manipulation of 5HT function to establish their effects on NOR with short and long delays . As acute treatment of citalopram treatment has been shown to activate the auto-inhibitory properties of 5-HT1a receptor it is expected citalopram will impair NOR.

5.3. *Methods and Procedure*

Subjects

Probiotic treated group

The cohort of rats were used in the current experiments as those in Chapter 4, section 4.3. Twenty from this cohort were randomly selected to participant in these experiments. The rats were 17 months old when being tested. Their weights ranged from 400g to 560g. The rats were housed in pairs in a holding room with a 12h light-dark cycle with lights on at 7am. Testing occurred during lights on hours. The temperature of the room was maintained at 19-23 ^{o}C and humidity at 55% ±10.

Feeding Procedure

The feeding procedure is identical to that described in Chapter 4, section 4.3.

Citalopram treated group

Ten male adult Lister hooded rats (Harlan, UK) weighing 400-550g were used in this experiment. This group were housed under the same conditions as the probiotic treated rats and were also 17 months old when being tested.

Injection Procedure

The citalopram treated groups were injected intraperitoneally (i.p) with 5mg/kg of Citalopram Hydrobromide (Tocris, Bristol, UK). Citalopram was dissolved in 0.9% injectable saline at a concentration of 1mg/ml and administered 30 minutes prior to testing. Vehicle treated animals were injected with 0.9% injectable saline at a volume of 1ml/kg administered 30 minutes prior to testing. The animals were injected in a separate room and were returned to their holding room for 30mins before testing.

All animals were thoroughly habituated to handling before the study began. The experiment was performed in accordance with Home Office under Animals Scientific Procedures Act 1986.

Apparatus

An adaption of the apparatus originally used by Ennaceur and Delacour (1988) was used in this study. A 1m x1m arena with walls 40cm high was used. Sawdust was placed on the floor of the arena at the start of each test session. Illumination was provided by 70 watt florescent tube lighting in the centre of the room. All phases of the study were recorded by a camera in the ceiling placed directly above the centre of the maze. The visual feed was sent directly to a monitor from where the experimenter observed the behaviour and recorded it in real time. When the objects were placed in the arena they were 40cm apart and 25 cm from the walls.

Procedure

Habituation

Habituation was carried out for four days. During habituation each rat was placed in the arena for 15 minutes. Rats were carried from their holding room in a blacked out carrying box to the testing room. They were introduced to the arena facing the wall. They were given 15 minutes to explore the arena after which they were removed and any faecal matter was removed and the walls of the maze were cleaned with alcohol wipes to remove any olfactory cues. They were placed back into the carrying box and placed back into their home cages.

Yolking

After four days of habituation, during which the animals became familiar with the arena, their average exploration time was recorded over two days. The purpose if this phase was to control for contact time with the object between the two groups. Each rat was introduced to the arena in the same way as the habituation phase. During this phase two identical objects were placed into the

arena. Each rat was given four minutes to explore the objects. During this time the contact time with the objects was recorded. Contact time was defined as the nose directed towards the object at a distance of two centimetres or less. Climbing or sitting on the object was not considered to be object exploration.

After two days of data collection the average exploration time for each rat was calculated. From this the rats from the group which explored the most were paired with a rat from the group which explored the least so that they were matched for exploration time in the next phase of the experiment. (The data from the yolking phase is not available)

Experiment 1 (Probiotic) & 5 (Citalopram) – Novel object recognition with 5 minute delay

Sample Phase

During this phase the rats were introduced to the arena in the same way as in the habituation phase. A pair of identical objects, different from the pair used in the yolking phase, was placed in the arena. The rats from the group which explored the least were the first from each pair to take part in this phase. They were given four minutes to explore the objects during which their contact time was recorded. Following this their paired rat in the other group was given four minutes to reach the same amount of contact time. Therefore, each pair of rats had different lengths of contact time to other pairs.

Test Phase

After the sample phase the rat was removed from the arena and placed into the carrying box for five minutes. During this time one of the objects was replaced with a new, novel object. Both objects were wiped with alcohol wipes to remove any olfactory cues. The object which was replaced was counterbalanced across the groups. After five minutes the animal was reintroduced to the arena

and contact time with the familiar and novel objects was recorded. At the end of this phase the animal was removed from the arena and placed back into their home cages and the arena was cleared of faecal matter and the walls cleaned with alcohol wipes. The test phase lasted for 5 minutes.

This experiment was run on two consecutive days with new objects used on the second day. The data from the two days was then averaged for analysis.

Experiment 2 (Probtiotic) & 6 (Citalopram) –Novel Object Recognition with 1 hour delay

The protocol for this experiment follows the same procedure as experiment 1 however instead of a five minute delay between the sample and test phase, a one hour delay was given. During the one hour delay the rats were removed from the testing room in the carrying box and placed back into their home cages. This was to minimise anxiety levels during the longer delay. The experiment was run of two consecutive days with new objects used on the second day. The data from the two days was then averaged for analysis.

Experiment 3 – Object in place with five minute delay

Sample Phase

The rats had two days free of testing before the second experiment. The protocol used for this experiment followed a similar pattern as experiment 1. The rats from the group which had lower exploration times in the yolking phase were the first from each pair to take part in this phase. Rats were handled and introduced to the arena in the same way as during the habituation phase. Four different objects were placed into the arena 40 cm from each other and 25 cm from the walls. None

of these objects had been previously used in any other phases of the study. The rats were given four minutes to explore all the objects during which their contact time was recorded. Following this their paired rat in the other group was given four minutes to reach the same amount of contact time. Therefore, each pair of rats had different lengths of contact time to other pairs.

Test Phase

After the sample phase the rat was removed from the arena and placed into the carrying box for five minutes. During this time two of the objects which were diagonally opposite each other were switched. The pair of objects that were switched was counterbalanced across the two groups. After five minutes the rat was reintroduced to the arena for four minutes and contact time with each of the objects was recorded. The contact time with the objects which had been switched was complied to give a contact time for 'novel' exploration, the same was done with the data recorded for the 'familiar' pair of objects. At the end of this phase the animals were removed from the arena and placed back into their home cages and the arena was cleared of faecal matter and the walls cleaned with alcohol wipes.

The experiment was run twice, on two consecutive days, the pairs of objects used on the second day had never been used in any phase of this, or any previous experiment. The data for the two days was complied to give one value.

Experiment 3 – Object Recency

Sample Phase 1

The rats were given a further two days free of testing before the third experiment. The protocol used for this experiment followed a similar patter as experiment one. The rats from the group which had lower exploration times in the yolking phase were the first from each pair to take part in this phase. A pair of objects was placed in the arena. These objects had not been previously used in any experiment. Rats were introduced to the arena in the same way as in the habituation phase. They were given four minutes to explore all the objects during which their contact time was recorded. Following this their paired rat in the other group was given four minutes to reach the same amount of contact time. Therefore, each pair of rats had different lengths of contact time to other pairs. After this phase the rat was removed from the arena and returned to its home cage.

Sample Phase 2

After one hour the rats were returned to the arena. A new pair of objects had been placed into the arena. Again, this pair of objects had never been used in any of the previous experiments. The rats were given four minutes to reach the same amount of contact time as they had with the previous pair of objects. After this phase the rats were returned to their home cages for three hours.

Test Phase

After three hours the rats were reintroduced to the arena. In the arena one of each of the pairs of objects were placed. These were counterbalanced between the groups so that the most recent object was placed on the right an equal number of times as placed on the left. The rats were given four minutes to explore the objects and their contact time with the 'primary' object (object from sample phase 1) and 'recent' object (object from sample phase 2) were recorded. At the end of this

phase the rats were removed from the arena and placed back into their home cages. The arena was cleared of faecal matter and the walls cleaned with alcohol wipes.

This experiment was run twice, on two consecutive days, the pairs of objects used on the second day had never been used in any phase of this, or any previous experiment. The data for the two days was complied to give one value.

Statistical Analysis

The data was analysed using an investigation ratio (IR). Raw exploration times of each object were calculated as a ratio of exploration times of both objects. The difference in IRs for the familiar and novel objects was then used for statistical analysis. Each of the experiments were run twice, a mean was calculated for each rat and the data complied. The data from the probiotic and control groups were analysed separately to the citalopram and vehicle treated groups. One-way ANOVA were carried out on the data using SPSS version 20.0.

The results are expressed as mean ± 1 standard error. The threshold for significance was $p<0.05$. Where data was non-spherical given the output from *Malchly's Test of Shericity* ,the *Greenhouse-Geisser* adjustment was used to give a corrected *F* ratio. Where interactions were significant pairwise comparisons were used to examine the simple effects of this interaction.

5.4. Results

5.4.1. Experiment 1-4: Probiotic administration

Figure 1 shows the raw scores of time spent exploring the novel and familiar objects during the test phase of novel object discrimination with a 5 minute delay, a one hour delay, object in place discrimination and object recency. Analysis of this data indicated that the probiotic group spent significantly longer investigating the novel object in the novel object discrimination tasks with both a 5-minute delay ($t(13)=8.06$, $p<0.001$) and one hour delay ($t(13)=7.43$, $p<0.001$) as well as the object in place task ($t(13)=3.534$, $p<0.01$) and the object recency task ($t(12)=2.368$, $p<0.05$) (one animal was excluded in the object recency as it did not explore any of the objects in the test phase). Analysis of performance of the control animals indicated that they explored the novel object significantly longer than the familiar object in the novel object discrimination task with a 5 minute delay ($t(13)=3.576$, p=NS) and object in place recognition ($t(13)=2.702$, $p<0.05$). However, with 1 hour delay in the novel object recognition task ($t(13)=1.706$, p=NS) and the object in place task the control rats did not significantly discriminate between the objects ($t(13)=0.754$, p=NS).

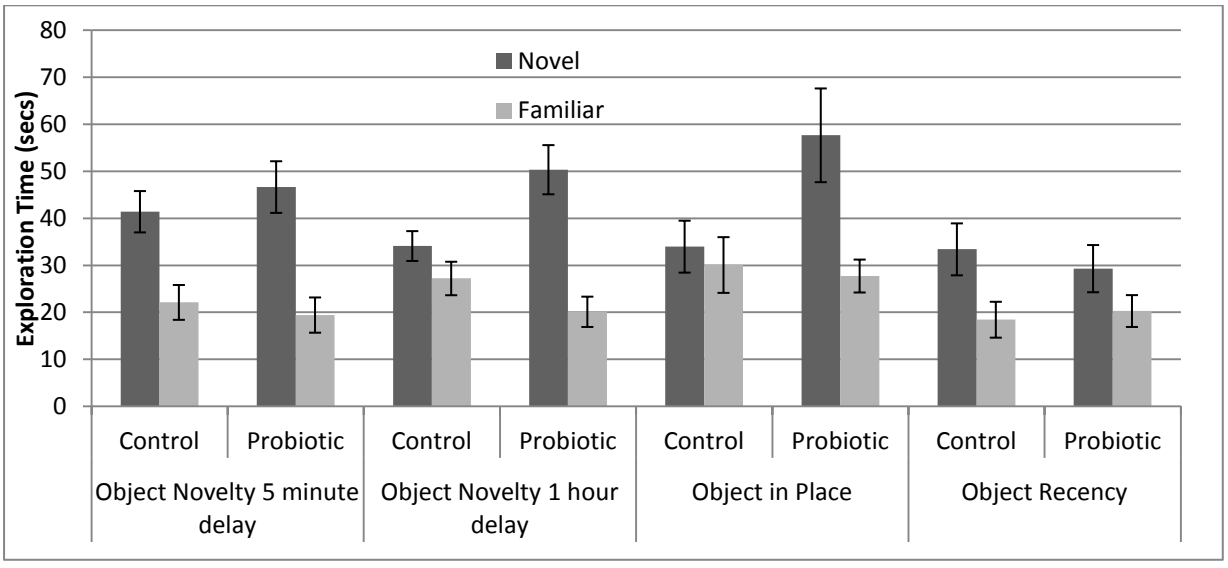

Figure 1. Experiment 1-4: Exploration time of novel and familiar objects (raw scores) by control and probiotic groups.

Figure 2 shows the investigation ratio (IR) of exploration time in the test phase of novel object discrimination with a 5 minute delay, one hour delay, object in place discrimination and object recency. Statistical analysis of the IR of exploration time in novel object discrimination test with a 5 minute delay showed no significant difference between the groups ($F(1,27)=4.545$, p=NS). However, a 1 hour delay showed a significantly higher IR in the probiotic group ($F(1,27)=7.615$, $p<0.05$). Analysis of the data from the test phase of the object in place experiment as showed a significantly higher IR in the probiotic group ($F(1,27)=6.876$, $p<0.05$). Analysis of the object recency indicated that there was no significant difference between the groups ($F(1,27)=0.624$, p=NS).

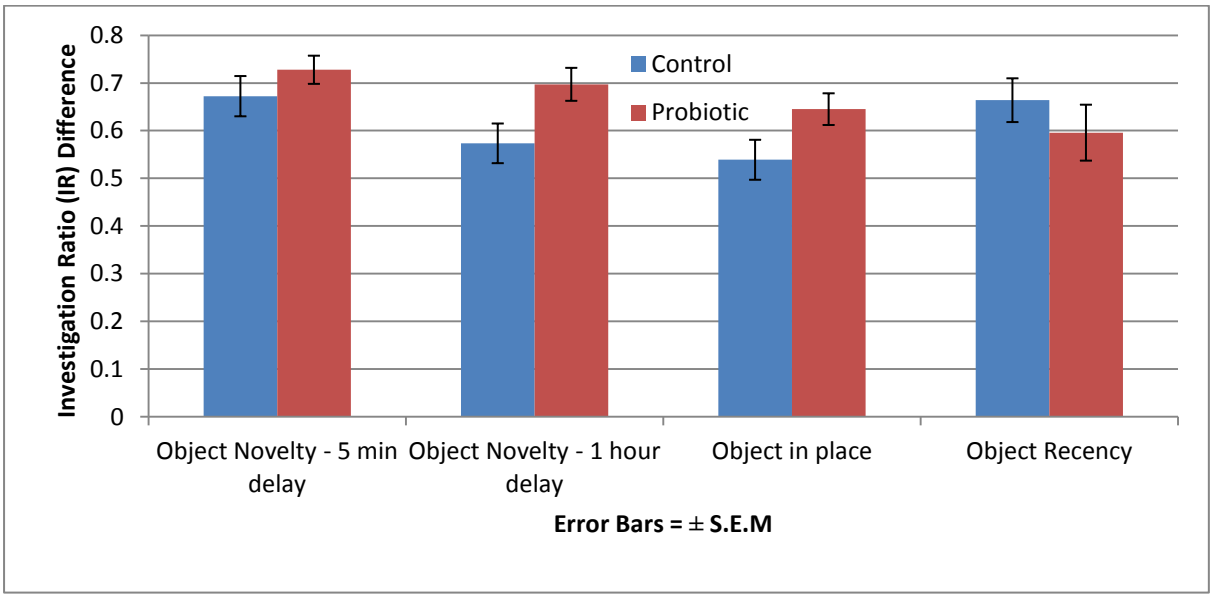

Figure 2. Experiment 1-4: Investigation ratios (IR) of object novelty with a 5 minute delay, a one hour delay, object in place discrimination and object recency in probiotic and control groups.

5.4.2. *Experiment 5 & 6: Citalopram Administration*

Figure 3 shows the raw scores of exploration time of the novel and familiar objects in the test phase by the control and citalopram treated groups. Statistical analysis of the exploration times indicated that both the control ($t(9)=8.643$, $p<0.001$) and citalopram ($t(9)=3.331$, $p<0.01$) treated groups were able to discriminate between the two objects with a significantly longer exploration times of the novel object in the novel object discrimination task with a 5 minute delay between the sample and test phase. With one hour delay between the two phases, only the control group ($t(9)=3.202$, $p<0.05$) showed significantly longer exploration times of the novel object.

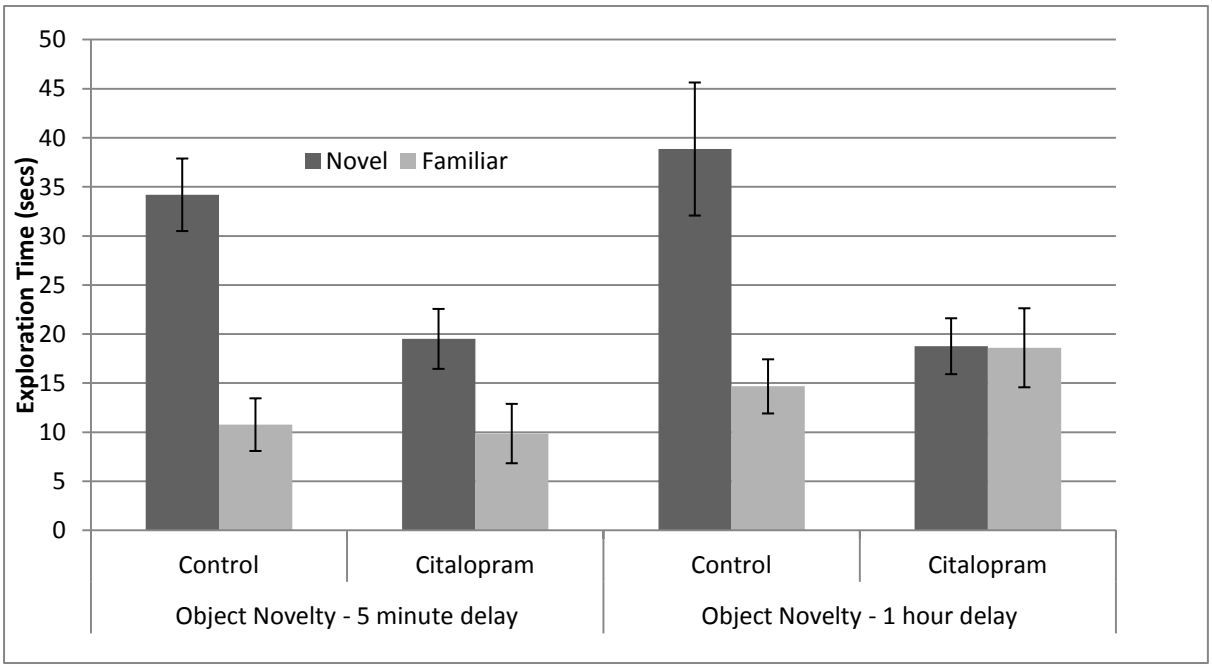

Figure 3. Experiment 5 & 6: Exploration time of novel and familiar objects (raw scores) by control and citalopram groups.

Figure 4 shows IR of exploration times in the test phase of the novel object discrimination with 5 minutes delay in vehicle treated and citalopram treated rats. Statistical analysis of the results indicated that there was significant difference between the group ($F(1,18)=2.423$, p=NS). Statistical

analysis of the novel object discrimination with a one hour delay showed that the citalopram treated group had a significantly lower IR than the vehicle treated group ($F(1,19)=8.601$, $p<0.01$).

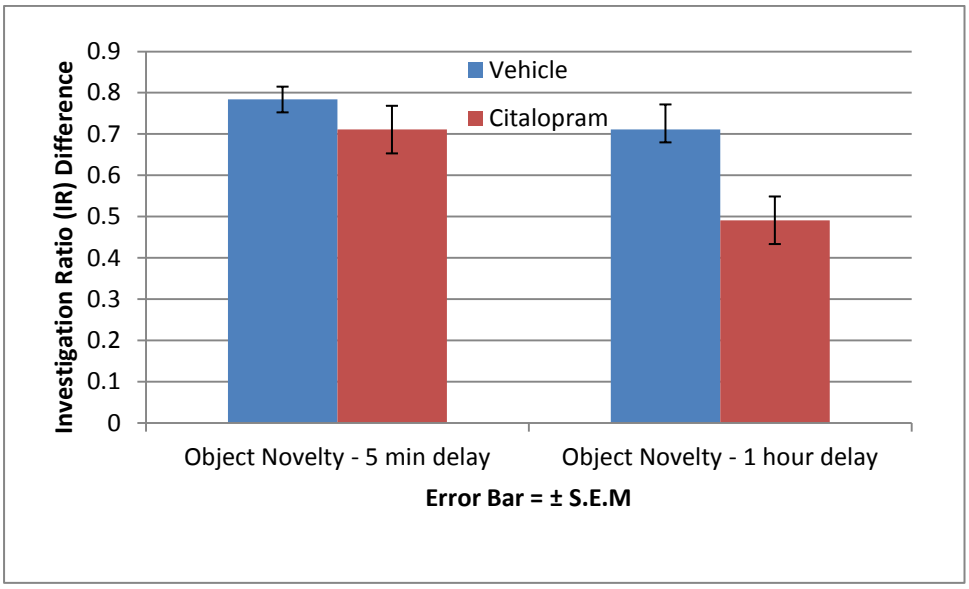

Figure 4. Experiment 5 and 6: Investigation ratio (IR) of novel object recognition with a 5 minute day and a one hour delay in citalopram and vehicle treated groups.

5.5. *Discussion*

The beneficial effects of probiotic administration on recognition memory presented here is to my knowledge the first demonstration of such an effect. The changes found in frontal lobe serotonergic function and hippocampal noradrenalinergic function reported by Desbonnet *et al* (2008) provided the rationale for the present study. The results from the probiotic study where novel object discrimination was examined indicated that following a 5 minute delay between the sample phase and the test phase there was no significant effect of treatment type despite the probiotic group having higher mean exploration time of the novel object. However, with a longer delay this effect became statistically significant. The memory for object place associations was significantly improved by probiotic treatment following a 5 minute delay between sample and test phase. Processing of sequential information tested in the object recency task was clearly unaffected by probiotic administration following a 3 hour delay period. Of particular interest in the results from analysis of the raw scores is that the probiotic group were able to significantly discriminate between the novel and familiar objects during the test phase of all the tasks however the control group were unable to do this with the longer delay in the object novelty task or the object in place task. These results indicate that the probiotic group had superior recognition ability over the control group. In contrast to probiotic administration, rats given an acute administration of citalopram revealed a deficit in object novelty discrimination following a 1 hour delay but no significant difference was found following a 5 minute delay. Analysis of the raw scores indicated that citalopram treatment diminished the rats ability to discriminate between the objects following a longer delay. Given the functional dissociations found in lesion and IEG studies the results indicate that the locus of action for probiotic treatment is the perirhinal cortex (Eacott *et al,* 1994; Meunier *et al,* 1993; Zhu *et al,* 1995, 1996, 1997), accounting for the improvement in the NOR task, and the hippocampus (Barker & Warburton, 2011) indicated by improved performance in the object in place task. However, both of these regions are implicated in temporal order recognition tasks (Barker *et al*, 2007; Barker and

Warburton, 2011; Fortin *et al,* 2002; Hannesson *et al,* 2004). In the current study no improvement is temporal order processing was found following probiotic treatment. The medial prefrontal cortex (mPFC) has also been reported to be fundamental to this task (Barker *et al*, 2007, Baker & Warburton, 2011) suggesting a functional interdependence of this brain area and the perirhinal cortex. Implications from this study may indicate anatomical specificity for the action of probiotics limited to the perirhinal cortex and the hippocampus. Furthermore, the contribution of the mPFC may prove to be a limiting factor in temporal order processing following probiotic treatment.

The results from the citalopram study are consistent with those found in previous studies using SSRIs in NOR. Fluoxetine was found to impair novel object discrimination following delays from 15 minutes to 24 hour between the sample phase and the test phase (Valluzzi & Chan, 2007, Sass & Wortwein, 2012). Both of these studies involved administration of fluoxetine over an extended period (14 days and 32 days). Given the acute dosing procedure used in the present study and the selective affinity of citalopram for the 5-HT transporter protein (Friedman *et al,* 2005), the present study indicates that NOR is sensitive to transient alterations in global 5-HT levels. Acute treatment of citalopram has been shown to activate the auto-inhibitory properties of the 5-H1a receptor (Ceglia *et al*, 2004*)*. The results found here are consistent with studies that have shown antagonism of this receptor improves NOR performance (Pitsikas *et al* 2003). This suggests that the 5-HT1a receptor is implicated in NOR. As the acute dosing regime used in the current study produced similar results to the studies using longer dosing periods of SSRIs, it suggests that either fluoxetine has a lower affinity for the 5-HT1a receptor, therefore not inducing desensitisation following prolonged expose, or the adaptive response of this receptor requires longer exposure to the drug than that used in these studies. The relevance of this information to the current study is that the effects of probiotic treatment on NOR could be explained by prolonged activation of 5-HT1a receptors causing desensitisation and therefore improving performance. In order to clarify the involvement of the 5-HT1a receptor in the effects of SSRIs on NOR a study using much longer dosing

periods is required. Results by Leiben *et al* (2006) found that acute tryptophan depletion and 5,7-DHT lesions to the dorsal raphe nuclei both impaired novel object recognition. The inhibitory effect of 5-HT1a receptor activation in the raphe nuclei on 5-HT stimulation in other areas of the brain by acute citalopram treatment further supports the theory that this receptor is implicated in the effect of SSRIs on NOR. Whether the effect of citalopram on NOR is direct by altering 5-HT transmission or indirect through 5-HT1a receptor activation altering transmission of other neurochemicals remains speculative. Blockade of cholinergic function through administration of scopolamine has been found to impair NOR, furthermore it has been demonstrated that 5-HT1a receptor antagonism has been found to attenuate the deficit. In order to examine the impact of citalopram administration on choleringic neurotransmission in NOR a study using co-administration of citalopram and scopolamine should be conducted. Given the results of previous studies showing that the perirhinal cortex is fundamental for NOR, it suggests that citalopram may compromise the function of this region. Previous studies have demonstrated that lesions of the perirhinal cortex impair NOR performance in a delay-dependant manner, similar to the pattern of results shown in the present study by citalopram-treated rats.

In summary, the results from this study indicate that probiotic treatment facilitates specific aspects of recognition memory, object novelty detection over long delays and object-place associations. In contrast, temporal order memory was not influenced by probiotics. This pattern of results contrasts with those following acute administration of citalopram and suggest that alterations in the 5-HT neurotransmitter system may not provide the main mechanism by which probiotics influence memory (although further work is required to examine the effects of chronic alterations in 5-HT activity). As the perirhinal cortex is fundamental to NOR following delays lasting longer than 10 minutes the results of the present study suggest that probiotics may influence processes supported by this region. However, the mechanism of action for this effect remains unclear. The opposite effect found with acute citalopram treatment , which may reflect activity at the 5-HT1a receptor,

provide an interesting avenue for further research. Should the action of citalopram on NOR be mediated by acute 5-HT1a receptor activation, prolonged exposure to the drug may presumably produce an opposing effect, similar to that found with probiotic treatment. Co-administration of 5-HT1a receptor agonists and probiotics would provide further insight into this theory. As with citalopram, the effect of probiotics it not limited to one neurotransmitter. Desbonnet *et al* (2008), reported alterations in dopamine metabolite concentrations in the amygdaloidal cortex. Furthermore, acetylcholine, GABA, NMDA and AMPA have all been implicated in NOR (Kim *et al,* 2014; Rogoz, 2013; Yamaguchi *et al,* 2013; Zheng *et al,* 2011). In order to understand the mechanism by which probiotics have a beneficial effect on memory, further research is required to understand the putative neurotransmitter systems affected by this dietary manipulation.

Nuclear Magnetic Resonance (NMR) identifies alterations in metabolite profile in bifidobacterium bifidum (CUL20), bifidobacterium lactis (CUL34) and lactobacillus acidophilus (CUL21 & CUL60) treated rats.

6.1. Summary

Nuclear magnetic resonance spectroscopy (NMRS) was used to examine the metabolic profile of the frontal cortex and hippocampus of probiotic treated and control rats. ^{1}H nuclear magnetic resonance spectral analysis indicated peaks with significantly higher correlation coefficients for inosine, alanine, succinate and lactate in the hippocampus of probiotic treated rats and a significantly higher correlation coefficient for hypoxanthine in control rats. In the frontal cortex significantly higher correlation coefficients were found for lactate, myo-inositol, GABA and fumarate in the probiotic group. The results from this study indicate that dietary probiotic administration alters intracellular metabolism in the neurons of both brain areas.

6.2. Introduction

Nuclear magnetic resonance spectroscopy (NMRS), as used in the present context, falls under the heading of metabolomics. It represents a powerful assessment tool that detects alterations in the metabolome, a pool of metabolites that are end products of cellular processes (Fiehn, 2002). The metabolite profile serves as a chemical fingerprint reflecting downstream genomic, transcriptomic and proteomic fluctuations and provides insight into cellular processes in the context of health and disease (Trushina and Mielke, 2013). To my knowledge NMRS has never been conducted on animals administered probiotics. It has been used in the current study to examine the effects of probiotic treatment on the intracellular metabolism in brain tissue. As discussed in the previous chapters, probiotics modulate the function of several putative signalling pathways between the gut and brain, including enteric nerve stimulation, possibly through neurotransmitter production, vagus nerve

stimulation, immune system activation and hormonal stimulation of the HPA axis. However, the effect of dietary probiotic administration on intracellular metabolism has yet to be explored. Interestingly, a recent study used NMRS to examine the neurometabolites of patients with minimal hepatic encephalopathy (MHE) (Ziada *et al,* 2013). This disease results in perturbation in cognitive function caused by peripheral ammonia levels accumulating in the brain. Probiotics were administered to a group of patients based on the rational that improving gut flora and composition would inhibit urease-producing bacteria, thus resulting in decreased ammonia absorption. The MRS data showed increased brain metabolite ratios of myo-inositol/choline and myo-inositol + choline/glutamine and decreased glutamine/creatine (Ziada *et al,* 2013). Given that previous NMRS studies on MHE patients have indicated significant decreases in myo-inositol and choline and increases in glutamine levels as a result of perturbed liver function (Kreis *et al,* 1991), probiotic treatment could potentially be used as therapeutic intervention for this disorder.

The aim of the current study was to use NMRS to evaluate the potential effects of probiotic treatment on the metabolic profile in the hippocampus and frontal cortex. These two brain areas have been implicated in the behavioural studies carried out in the present thesis and in other published biochemical studies. Given the results of the study by Ziada *et al* (2013) it was expected that probiotic administration in rats would produce a change in the metabolic profile. Although the nature and direction of changes is difficult to predict, prior work in humans would suggest potential increases in myo-inositol, choline and decreased glutamine/glutamate peak intensities in rats administered with probiotics.

Myo-inositol is a naturally occurring isomer of nine possible inositol isomers (Parthasarathy & Eisenberg, 1986). It comprises 95% of the total free inositols in the human body (Petroff *et al,* 1988) and is a natural dietary component found in a range of food stuffs, fruits beans, grains in nuts (Clements & Darnell, 1980). There are three main sources of myo-inositiol; recycling in the phosphate-phosphonositide (PIP) cycle, *de novo* synthesis from D-glucose-6-phosphate and as

previously mentioned, extracelluar dietary sources. However, only 3% of plasma myo-inositol passes the blood-brain barrier (Spector, 1988). At an intracellular level, myo-inositol is a precursor for the inositol PIP cycle, a metabolic cycle that serves as a neuronal second messenger system. PIP cycle turnover is directly related to neurotransmitter functioning. Incubation of cortical tissue with varying concentrations of 5-HT induces accumulation of intracellular myo-inositol. Treatment with resperine, a mono-amine depletor, induced upregulation of the 5-HT2C receptor which in turn increased myo-inositol concentrations (Lee and Wei, 2013). Reduced immobility time in the forced swim test following inositol treatment was found to be abolished following co-administration of inositol and PCPA, a serotonergic metabolism inhibitor. This was not the case following co-administration of inositol and the noradrenergic neurotoxin, DSP-4. This observation suggests a common pathway for inositol and serotonergic functioning in influencing behaviour (Einat *et al,* 2001). Products of the PIP cycle have also been coupled with dopaminergic systems (D_1 and D_2 receptors) (Frégeau *et al,* 2013; Medvedev *et al* 2013; Lezcano *et al,* 2000). Analysis of monoamines and their metabolites via high performance liquid chromatography (HPLC) following acute and chronic myo-inositol administration indicates that myo-inositol does not directly impact monoamine synthesis, metabolism or reuptake (Einat *et al,* 1999b). However, coupled with the results of receptor studies it is evident that a complex interaction occurs between monoamine function and components of the PIP cycle.

The behavioural effects of myo-inositol treatment in clinical trials have presented this compound as a potential treatment for patients with depression, panic disorder and obsessive-compulsive disorder (OCD) but not patients with schizophrenia, Alzheimer's disease, attention-deficit-hyperactivity disorder (ADHD) or autism (Einat and Belmaker, 2001). Lower levels of myo-inositol have been found in prefrontal and anterior cingulate cortical areas of depressed patients (Coupland *et al,* 2005). Post-mortem studies of the frontal cortex in bipolar patients and suicide victims have also shown lower levels of myo-inositol with no differences in myo-inositol levels in the cerebellum or occipital cortex relative to controls (Shimon *et al,* 1997). Animal studies of affective disorders

have reflected these results. Chronic myo-inositol treated (2 weeks) rats showed significantly reduced immobilisation time and increased struggle time in the forced-swim test (FST) (Einat *et al,* 1999), an established pre-clinical model of depression (Porsolt *et al,* 1978; Borsini and Meli, 1988). Genetic models of depression, Flinders Sensitive Line rats (Overstreet, 1986), have demonstrated that inositol treatment ameliorates exaggerated immobility in the FST (Einat *et al,* 2001). Locomotor activity levels has also been reported to be significantly affected by myo-inositol oral administration, a 30% increase in ambulation was observed as well as a 60% increase in rearing (Kofman *et al,* 1998). A similar result was reported with i.p. myo-inositol administration, which significantly increased vertical activity (rearing) and induced a similar but non-significant trend in horizontal activity (Kofman *et al,* 1993). Reserpine- induced hypoactivity, used as a model of depression, is also sensitive to myo-inositol administration by reducing immobility time in locomotor activity tests (Einat *et al,* 1999a). Myo-inositol administration is also implicated in anxiety-related behaviours, anxiety-like behaviour in the elevated plus maze (EPM) is reduced following myo-inositol treatment (Cohen *et al,* 1997). An acute dose of 1.25g/kg administered i.p. has been shown to significantly increase the number of entries into the open arms of the maze and the duration of time spent in the open arms of the EPM relative to control animals (Einat *et al,* 1998).

The effects of probiotic treatment on anxiety-like behaviour in the open field test in the current thesis were inconclusive. However, further analyses of the metabolites myo-inositol and choline concentrations in these animals may provide an insight into the possible mechanisms responsible for the alterations in anxiety-like behaviour found in other animal models treated with probiotics. The improved memory demonstrated by the probiotic group in the spatial memory tasks (Chapter 5 experiment 3) may be accounted for by increasing in choline levels previously reported as a function of probiotic treatment (Ziada *et al,* 2013).

6.3. *Method and Procedure*

Subjects

The rats used in this study were also used in the studies described in chapter 4 & 5. 5 rats from the control group and 5 from the probiotic group were used for NMRS analysis.

Brain dissection for NMRS

Animals were sacrificed by decapitation. The brain was removed and extirpated; the hippocampus and frontal cortex were snap-frozen immediately in liquid nitrogen. Samples were stored at -80 ^{o}C until tissue was processed.

NMRS method

Tissue samples were defrosted and extracted with a mixture of water, chloroform and methanol (v:v:v, 3:2:1) in a glass test tube. Following centrifugation at 5,000 rpm at 4 ^{o}C, the aqueous phase was separated into an Eppendorf tube and then dried using a speed vacuum. The sample was then suspended with 600 µl of deuterium oxide (D_2O) and sonicated for 10 min. Fifty micro litre of 0.2 M phosphate buffer (pH=7.4) containing 100% D_2O for the magnetic field lock, 0.01% 3-(trimethylsilyl)-[2,2,3,3-$^{2}H_4$]-propionc acid sodium salt (TSP) for the spectral calibration and 3mM sodium azide (NaN_3) to prevent bacterial contamination was added and thoroughly vortex for 15 sec followed by spinning at 10,000 *g* for 5 minutes. A total of 600 µl of the supernatant was transferred to an NMR tube (5mm outer diameter) for ^{1}H NMR spectral acquisition using a Bruker 600MHz spectrometer (Bruker; Rheinstetten, Germany*).* A ^{1}H frequency of 600.13 MHz was applied to the samples at a temperature of 27 ^{o}C. A standard NMR pulse sequence (recycle delay[RD]-90^{o}-t_1-90^{o}-t_m-90^{o}-acquisition) was applied to acquire 1-dimensional (1-D) ^{1}H NMR spectral data with t_1 set to 3 µs and t_m (mixing time) set to 100 ms. Using selective irradiation during RD of 2s and t_m the water peak suppression was achieved. A total of 128 scans were collected into 64 k data points.

Statistical analysis

Multivariate data analysis was performed based on pre-processed NMRS datasets. ^{1}H NMR spectra were phased, referenced and baseline corrected manually in TopSpin 3.0.b.60 software (Bruker). The entire spectral data (0-10ppm) were imported into MATLAB R2012a and the water signal region (4.75-5.05 ppm) was removed to avoid water suppression-induced baseline distortion. The remaining spectral data was normalised using median fold change normalisation method and subsequently analysed using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

Each metabolite may have multiple peaks in the 1H NMR spectra. A peak, which is least overlapping with other signals, was selected and values of correlation (r, Pearson's correlation coefficient) and significant differences in correlations of these variables, calculated using students t-test, between the two group.

The brain tissue was collected and stored at Cardiff University. NMR analysis was conducted at Imperial College London by Dr. Jia Li, Faculty of Medicine, Department of Surgery and Cancer. Production of PCA and OPLS-DA score plots and statistical analysis was also conducted by Dr. Jia Li.

6.4 Results

6.4.1. Frontal Cortex

Spectral data of frontal cortex was aligned using recursive segment-wise peak alignment method due to the heavy shift of peaks (Veselkov *et al,* 2009).

Figure 1 shows an unsupervised multivariate principal component analysis (PCA) score plot for the frontal cortex. The purpose of this was to observe intrinsic trends in metabolites between the samples and identify any outliers. The PCA score plot indicates strong segregation of the two groups based on the correlation of their main metabolites. The R^2 value for main metabolites in this in the samples is 0.43, i.e. 43% of the variance between the samples is accounted for by the main metabolites.

Figure 2 shows supervised multivariate analysis orthogonal partial least-squares discriminant (OPLS-DA). This is a predication regression model which assumes there are two groups and separates the samples based on correlation between its main components. The R^2 value in the model was 0.38. The Q^2 value is indicative of the quality of the model in its prediction ability. The Q^2 value in this model was 0.45.

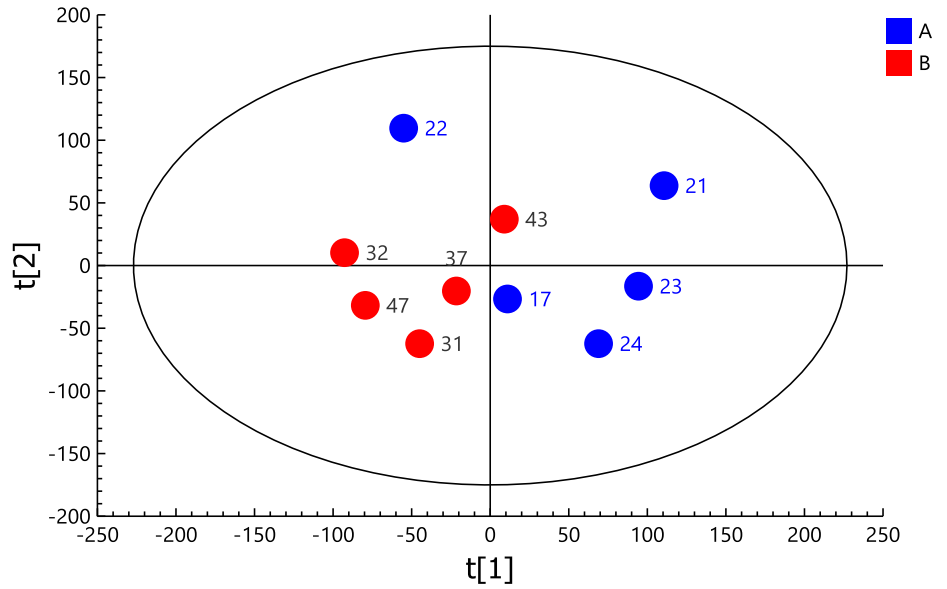

Figure 1. Principal Component Analysis (PCA) score plot of metabolites in the frontal cortex. A = Control group, B = Probiotic Group.

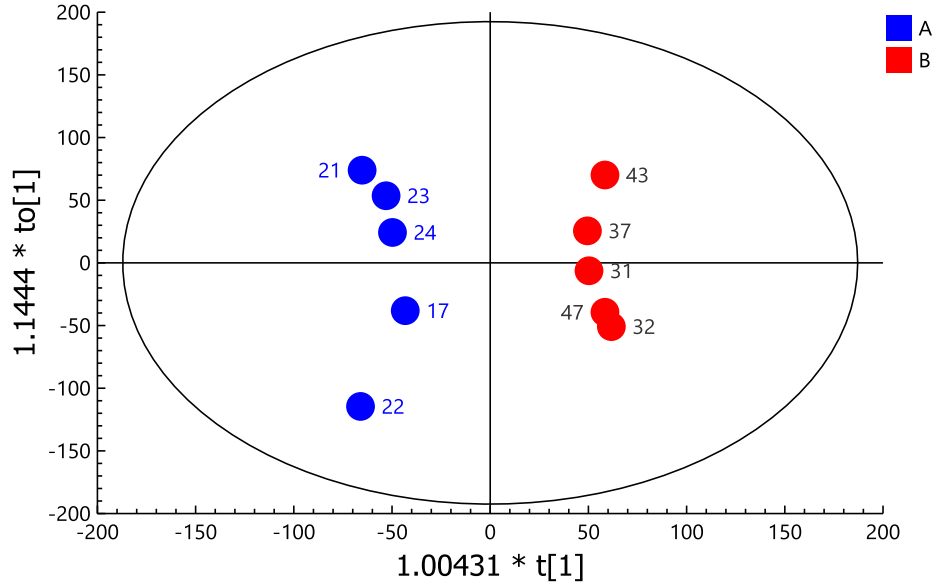

Figure 2. OPLS-DA score plots of metabolites in the frontal cortex. A = Control group, B = Probiotic group.

Table 1 shows the Pearson's correlation coefficient (r) of the metabolites which produced the largest peaks in NMR spectral output and the significant value (*p*) of theses metabolites between the groups. This was conducted using a student's t-test.

Table 1. Correlation coefficients (r) of the main metabolites in frontal cortex of the probiotic group and significance values (*p*) when compared with control group.

Front cortex metabolites	**Selected chemical shift**	**r**	***p***
lactate	1.34 ppm	0.76	0.01
myo-inositol	4.06 ppm	0.73	0.015
GABA	1.89 ppm	0.59	0.02
fumarate	6.52 ppm	0.79	0.01
alanine	1.47 ppm	0.60	0.06

6.4.2. Hippocampus

Figure 3 shows an unsupervised multivariate principal component analysis (PCA) score plot for the hippocampus. The PCA score plot indicates strong segregation of the two groups based on the correlation of their main metabolites. The R^2 value for main metabolites in the samples is 0.41, i.e. 41% of the variance between the samples is accounted for by the main metabolites.

Figure 4 shows supervised multivariate analysis orthogonal partial least-squares discriminant (OPLS-DA). The R^2 value in the model was 0.34. The Q^2 value in this model was 0.72.

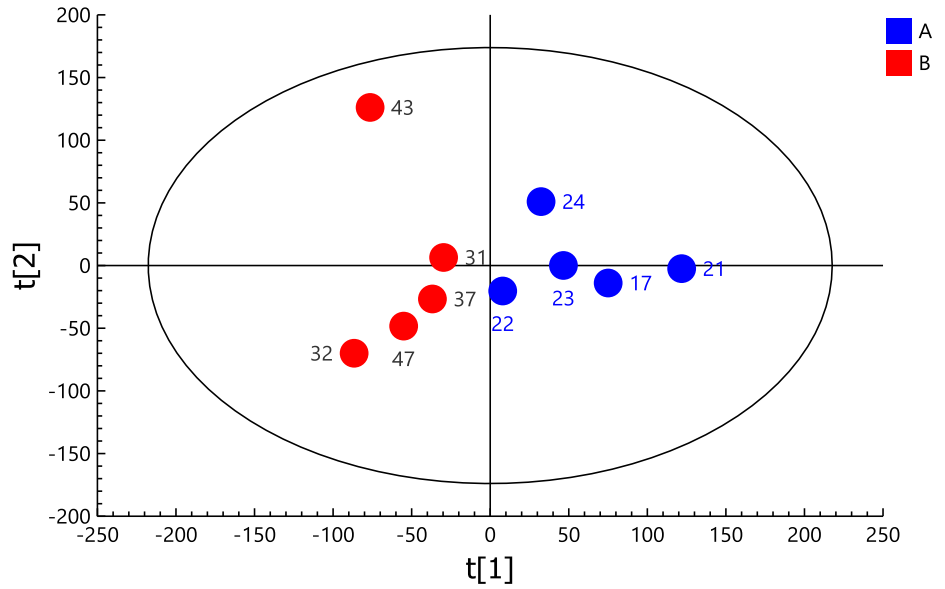

Figure 3. Principal Component Analysis (PCA) score plot of metabolites in the hippocampus. A = Control group, B = Probiotic Group.

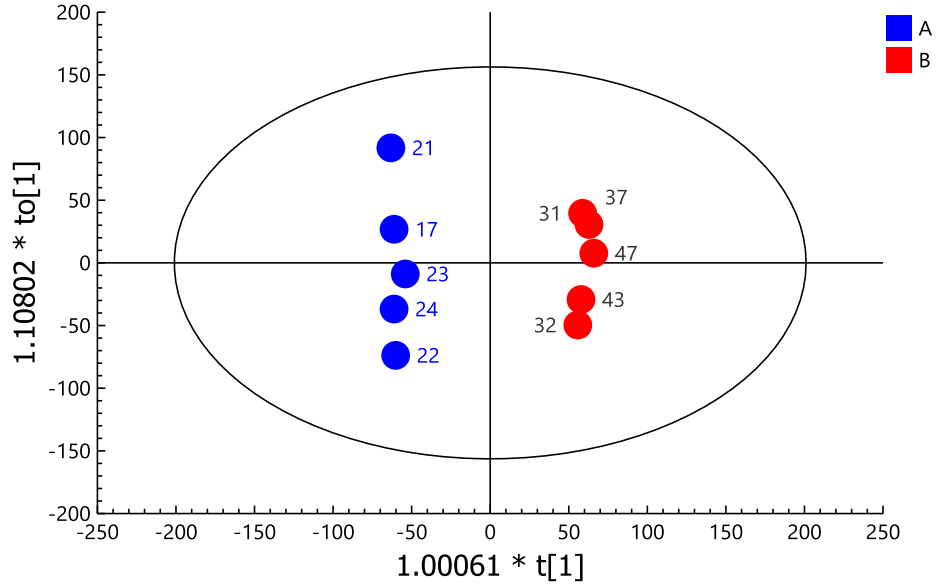

Figure 4. OPLS-DA score plots of metabolites in the hippocampus. A = Control group, B = Probiotic group.

Table 2 shows the Pearson's correlation coefficient (r) of the metabolites which produced the largest peaks in NMR spectral output and the significant value (*p*) of theses metabolites between the groups. This was conducted using a student's t-test. The NMR spectral output identifies hypoxanthine as the only metabolite to produce a higher peak in the control group than the probiotic group. Therefore, the r and *p* values reported for this metabolite are the correlation coefficient in the control group and difference from the probiotic group, respectively.

Hippocampus metabolites	**Selected Chemical shift**	**r**	***p***
Inosine	6.10 ppm	0.76	0.01
alanine	1.47 ppm	0.72	0.02
succinate	2.41 ppm	0.67	0.03
hypoxanthine	8.19 ppm	0.71	0.02
lactate	1.34 ppm	0.81	0.005

Table 3. Correlation coefficients (r) of the main metabolites in hippocampus of the probiotic group and significance values (*p*) when compared with control group, with the exception of hypoxanthine (see above).

Summary

The results from the NMRS output, PCA, OPLS-DA score plots and statistical analysis of the metabolites in the frontal cortex indicate that the metabolites which produced the main differences between the groups were lactate, myo-inositol, GABA, fumarate and alanine. The concentrations of these metabolites were all significantly different between the groups with the exception of alanine ($p=0.06$). In the hippocampus the main metabolites were inosine, alanine, succinate, hypoxanthine and lactate. The concentrations of these metabolites were all found to significantly differ between the groups. The correlation coefficient of hypoxanthine was found to produce a strong peak in the NMR spectral output of the control group which does not occur in the probiotic group. This difference was also found to be significant.

6.5. *Discussion*

As expected probiotic treatment significantly increased myo-inositol concentrations in the frontal lobe however this was not observed in the hippocampus. The differentiation between the brain areas indicate that myo-inositol is regulated differentially in the brain by probiotic administration Myo-inositol transport from extracellular sources across the blood-brain barrier is a low-capacity saturable system and thus it is unlikely that this is the sole explanation for high peak intensities in the probiotic treated group. Myo-inositol is a precursor for the PIP cycle and is synthesised from D-glucose-6-phosphate therefore it is reasonable to assume that probiotic treatment is altering this process or another process in the PIP cycle resulting in increased recycling. Either way, it is hypothesised that probiotic treatment affects endogenous levels of myo-inositol as opposed to facilitating an extracellular source. Furthermore, studies have shown that levels of myo-inositol, when in the environment of probiotic microbiota, are reduced by the bacteria that utilise it in their own metabolic pathways (Yebra *et al,* 2007). However, the current *in vitro* study produces contrasting results to systemic studies and may fail to identify this as a saturable process. Other studies focussing on the effects of route of administration have found that oral administration of myo-inositol is not as effective in altering brain levels of the metabolite as intraperitoneal (i.p.) administration (Einat *et al,* 1999a). This gives some weight to the hypothesis that gut bacteria also utilise this compound. As regards to the current study, the increase in frontal lobe myo-inositol indicated a net increase in myo-inositol production from extracellular processes or more likely, altered PIP cycle functioning. There is need to further examine the metabolite profiles of the blood from control and probiotic treated rats in order to determine the extent of the effect of extracellular processes on intracellular myo-inositol concentrations.

Direct oral consumption of myo-inositol has been found to significantly increase cortical and hippocampal inositol levels by 36% and 27%, respectively (Kofman *et al,* 1998). However, due to the

differential alteration in the metabolic profile between the two brain areas in this study, this provides support for the hypothesis that probiotic treatment alters intracellular metabolic pathways.

The differences in peak intensities in GABA, alanine and lactate in the frontal cortex and alanine, lactate and succinate in the hippocampus strongly implicate alteration of the GABA/glutamate-glutamine cycle in the probiotic group. This is the first time this has been reported in animal models. However, it is consistent with the findings of Ziada *et al* (2013) who reported a significant reduction in glutamine ratios following treatment with *lactobacillus acidophilus* in patients with minimal hepatic encephalopathy (MHE). In the current study, an increased level of GABA, an inhibitory neurotransmitter, was found in the frontal cortex. Production of GABA occurs in a cycle known as the GABA/glutamate-glutamine cycle. Glutamate is a major excitatory neurotransmitter in the brain while GABA acts an inhibitory neurotransmitter. The balance of these two neuroactive substances is maintained by the GABA/glutamate- glutamine cycle (Bak *et al,* 2006). When glutamate is released into the synaptic cleft it activates the receptors on the post-synaptic membrane, this reduces some of the glutamate present. However, some is reabsorbed into the presynaptic neuron while most is taken up into astrocytes where it is converted to glutamine by glutamine synthetase using ammonia and returned to the neuron where it is converted back to glutamate and the ammonia transported back to the astrocyte. Glutamate may be released in the synaptic cleft or converted to GABA by glutamate decarboxylase and released to act as an inhibitory neurotransmitter. As with glutamate, some GABA is taken up post-synaptically, some undergoes reuptake but some is also absorbed by astrocytes. In the astrocyte, GABA is converted to succinate in the tricarboxylic acid (TCA) cycle and then into α-ketoglutarate before being converted into glutamate. From there it undergoes the same process as glutamate; directly absorbed by the astrocyte, aminated to glutamine and transported to the neuron where it is degenerated back into glutamate and ammonia (Bak *et al,* 2006). The role of alanine in this system is to balance the pH disturbance caused by the movement of ammonia between the neuron and the astrocyte. When glutamine is transported to the neuron it is converted back into glutamate and ammonia. The ammonia is then used in the *lactate-alanine shuttle* which

converts pyruvate (derived from lactate) into alanine and then exported to the astrocyte where this process is reversed (alanine converted back into pyruvate and then lactate) and an ammonium cation produced and used for the amination of glutamate. Lactate is then exported back to the neuron where it is used to produce more alanine. In the TCA cycle, some succinate is converted into fumarate as part of the process to process energy (ATP) from carbohydrates, fats and proteins (Krebs & Weitzman, 1987). In the current study increased concentrations of GABA were found in the frontal cortex of probiotic treated rats. This suggests a shift in the GABA/glutamate-glutamine cycle functioning to favour the production of the inhibitory neurotransmitter GABA over the excitatory neurotransmitter glutamate. This is consistent with reports by Ziada *et al,* (2013) were probiotic treatment reduced levels of glutamine/glutamate.

The present study also found no difference in choline levels between the two groups. The difference in choline concentrations reported in the study by Ziada *et al* (2013), indicated an increase in myo-inositol + choline: glutamine/glutamate ratios. The present study has also found an increase in myo-inositol and GABA concentrations (which may account for lower glutamine/glutamate levels previously reported). Therefore, the previously reported increase in myo-inositol +choline: glutamine/glutamate ratio may have primarily driven by changes in myo-inositol and glutamine/glutamate levels as opposed to any net change in choline concentrations which would be consistent with the current study.

In the hippocampus of probiotic treated rats, there was increased inosine and decreased hypoxanthine concentrations relative to controls. The opposing concentrations of these two metabolites are unsurprising given that both of these metabolites are part of the purine nucleotide cycle (Schultz & Lowenstein, 1978). A precursor to both of these metabolites is adenosine. Adenosine is a potent neuromodulator in the brain and inhibits neurotransmitter release and neuronal excitability (Dunwiddie, 1985; Dunwiddie & Masino, 2001). Adenosine concentrations in the hippocampus have been implicated in long-term potentiation (LTP) with increased levels

associated with inhibition of LTP via activation of adenosine A_1 receptors (A_1Rs) (Arai *et al,* 1990, Alzheimer *et al,* 1991; Forghani & Krnjevic, 1995). A_1R agonist administration was found to impair spatial memory acquisition in a watermaze paradigm while antagonists produced no behavioural alteration (Von Lubitz *et al,* 1993). In a study examining the role of adenosine in impaired hippocampal LTP and spatial memory following chronic opiate exposure, it was reported that accumulation of adenosine contributed to impaired performance in spatial memory and induced inhibition of hippocampal CA1 LTP. Administration of an A_1R antagonist was found to reverse the deficit in the watermaze. Furthermore, adenosine deaminase, which converts adenosine into inosine, also reversed the impaired CA1 LTP inhibition (Lu *et al,* 2010). The results from the study by Lu *et al* (2010) indicated that the presence of inosine acts as a neuroprotective agent and facilitates LTP in the hippocampus. Concentrations of inosine are regulated by conversion to hypoxanthine by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) where it is either converted back into inosine monophosphate (IMP) which (as well as adenosine) is a precursor to inosine or converted into the waste product uric acid. The higher concentrations of inosine found in the probiotic group along with the higher concentration of hypoxanthine found in the control group in the hippocampus suggest probiotic treatment impacts this pathway. As there was no increase in adenosine found in the hippocampus and control animals showed higher levels of hypoxanthine it is likely that probiotic treatment reduces metabolism of inosine. This may have been achieved though deactivation of nucleoside phosphorylase (NP), the enzyme responsible for converting inosine to hypoxanthine or facilitation of HGPRT which converts hypoxanthine to IMP. These higher concentrations of inosine found in the hippocampus of the probiotic group indicate that LTP may be facilitated with dietary probiotic administration.

Of particular concern regarding the methodology of this experiment was the possible presence of blood in the brain tissue due to the method of collection. However given the differences in metabolite profiles between the two brains areas, it is evident that some of the alterations in metabolite profiles originate in brain tissue as opposed to the blood. Of the metabolites

demonstrated to alter between the groups, lactate is the only one which is consistent between the two brain areas. This is unsurprising given the probiotic group were fed lactic acid producing bacteria (Klein *et al,* 1998). However lactate also serves as a precursor to alanine, which was found to be elevated in the hippocampus of probiotic treated animals. Therefore, it is possible that increases in lactate are a result of both, increased exogenous production (from bacteria) and increased intracellular production from alanine. However, statistical analysis of alanine indicated that there was not a significant difference between the concentrations in the frontal cortex of the two groups despite there being a significant increase in lactate concentrations in the probiotic group. This implies that much of the increase in lactate concentrations can be attributed to exogenous production.

The results of this study can be used to explain some of the behavioural alterations reported in probiotic treated animals and provides a platform on which to continue investigations into how metabolism within the CNS is affected by probiotic treatment and how this may affect behaviour and cognition. This will be considered further in the General Discussion.

General Discussion

7.1. Summary

The purpose of the experiments presented in the current thesis was to examine the effects of dietary probiotic on central nervous system function. The basis of the research stems from a study by Desonnet *et al* (2008). Results from high performance liquid chromatography (HPLC) indicated that probiotic treatment altered metabolism of serotonin (5-HT) in the frontal cortex. Due to these effects on 5-HT transmission it has implied that probiotic treatment may be used an adjuvant therapy for affective disorders (Desbonnet *et al,* 2008, 2010). Therefore, the effects of prolonged probiotic treatment on anxiety-like behaviour were examined. The effects of direct 5-HT manipulation in anxiety-like behaviour, were also examined following administration of the serotonin selective reuptake inhibitor (SSRI) citalopram. The current studies also aimed to identify a behavioural task which is reliant on serotonergic frontal cortex functioning in order to test the effects of probiotic treatment. The probabilistic reversal learning (PRL) task was reported by Bari *et al* (2010) to be reliant on 5-HT functioning. In order to establish the role of frontal lobe functioning in this task the effects of medial prefrontal (mPFC) lesions were examined. The effects of probiotics in a task known to be dependent on the frontal cortex and hippocampus, namely a watermaze reversal learning paradigm, indicated that probiotic treatment improves functioning in this task. Identification of brain areas that show improvements following probiotic treatment are explored through object recognition tasks that rely differentially on contributions of the hippocampus and subregions of the frontal cortex. In order to decipher whether 5-HT alterations played a role in spatial recognition memory, this task was also conducted with citalopram. The effects of probiotic treatment on intracellular processes have never previously been explored. Nuclear magnetic resonance (NMR) was conducted on the frontal cortex and hippocampus to establish whether probiotics alters the metabolic profile of these two brain areas.

7.2. *The role of serotonin in probiotic treatment in the probabilistic reversal learning task*

The purpose of the experiments presented in Chapter two were firstly to establish the pattern of performance in an automated probabilistic reversal learning paradigm originally developed by Bari *et al* (2010). This task differs from classical reversal learning paradigms in that the reward schedule for 'correct' responses is probabilistic i.e. not rewarded on 100% of occasions. As such, performance requires relies on integrating feedback over a number of trials (Cools *et al,* 2002). Therefore, discrete rules cannot be adopted to guide behaviour as in classical reversal learning. Due to the probabilistic nature of the PRL task, no discrete rules exist therefore sensitivity to positive and negative reinforcement on previous trials is required to guide responding on subsequent trials. This initial task manipulated the task difficulty to determine behavioural sensitivity to changes in stimulus reward contingencies. The results indicated that when task difficulty was altered from an easier to a more difficult version of the task sensitivity to positive reinforcement was indicative of the number of reversals achieved; however, sensitivity to negative reinforcement did not alter between the tasks. The neuroanatomical specificity of this sensitivity to positive reinforcement was identified in the second experiment where lesions to the mPFC induced impairment. More specifically this was found only in the reversal phase while sensitivity to positive reinforcement remained intact during initial acquisition. This implies not only that negative and positive feedback sensitivity are neurally dissociable but also that sensitivity to positive reinforcement during initial acquisition of the PRL task is not reliant on the mPFC, only after a stimulus-reward contingency has been reversed. Having identified a distinct role for the mPFC in probabilistic reversal learning Chapter 3 examined the impact of manipulation in the serotonergic system.

Citalopram was administered in two doses 5 mg/kg and 10 mg/kg. The 5 mg/kg dose was found in increase the number of reversals on the first test session. This was not found with the higher dose and no further improvements were found in sensitivity to positive or negative reinforcement with either dose. This implies that while responsiveness to feedback is not modulated by the serontergic

system, the reversal of a learning stimulus-reward association is in part modulated by this system. While there is a convergence of evidence suggesting that 5-HT is strongly implicated in reversal learning (Bari *et al,* 2010; Clarke *et al,* 2005, 2007), other studies have focused on the 5-HT receptor subtypes that are thought to mediate the behaviour. The 5-HT2a receptor antagonist, M100907 impaired serial spatial reversal learning. In contrast, the 5-HT2C receptor antagonist, SB242084 improved serial spatial reversal learning (Boulougouis *et al,* 2008). The results from the current study would imply that varying doses of citalopram doses differentially impact the 5-HT2a receptor with the lower dose optimising performance. The effects of 5-HT receptor antagonism is not limited to the 5-HT system and so implicating other neurotransmitters in reversal learning. Specifically, antagonism of 5-HT2a receptor enhances dopamine (DA) 2 receptor (D2) antagonism (Bonaccorso *et al,* 2002; Liegeois *et al* ,2002; Olijslagers *et al,* 2004, 2005). Numerous studies using systemic D2 antagonist administration have found impairments in reversal learning. The results indicate that the effects of 5-HT transmission on reversal learning induced by the 5 mg/kg dose of citalopram may be mediated by cortico-striatal dompaminergic function.

The results obtained from study reported here were not consistent with those previously report by Bari *et al* (2010). Further studies are required to elucidate the role of 5-HT transmission in processing on positive and negative reinforcement in reversals of previously learned stimulus-reward associations (two effects reported by Bari *et al* (2010)).

The effects of probiotic treatment on probabilistic reversal learning (Chapter 4, Experiment 4) are partially consistent with the results from the citalopram study (Chapter 3, Experiment 3 and 4). Desbonnet *et al* (2008) reported reduced degradation of 5-HT in the frontal cortex of probiotic treated rats. Citalopram induces its effects on the 5-HT system by blocking reuptake into the presynaptic cleft therefore producing a similar neurological effect to that reported by Desbonnet *et al* (2008) following probiotic treatment. Chapter 3 experiment 3 showed that citalopram did not significantly impact sensitivity to positive reinforcement as was found following probiotic treatment.

Furthermore, Chapter 2 experiment 2 showed that the mPFC is fundamental in the processing of positive reinforcement in probabilistic reversal learning. However, the improved pharmacological effect of probiotic treatment in the frontal cortex reported by Desbonnet *et al,* (2008) did not produce an effect on this task. This indicates that improvement of 5-HT functioning in the frontal cortex of probiotic treated rats does not mediate the processing of positive reinforcement in probabilistic reversal learning.

5 mg/kg citalopram was found to increase the number of reversals achieved in the first test session of the test phase without significantly altering sensitivity to positive and negative reinforcement. The ability to reverse learned stimulus-response patterns though inhibition of responding is well established to be reliant on the orbitofrontal cortex (OFC) (Rudebeck *et al,* 2013). Intraperitoneal administration of citalopram increases global extracellular 5-HT levels therefore impacting the OFC which may have improved inhibition of responding resulting in a higher number of reversals in this session. The vehicle treated group demonstrated an increased the number of reversals across the sessions implying that learning was still occurring across the test sessions. These improvement in performance may have been due to the rats deciphering the pattern of behaviour which optimises the number of rewards. This trend was also exhibited by the 10mg/kg treated group however it did not reach statistical significance. The study by Desbonnet *et al* (2008) did not examine the effects of probiotic treatment on 5-HT metabolite concentrations in subregions of the frontal cortex, however, when the results from the current research are considered it can be assumed that these alterations do not occur in the OFC.

In summary, the probabilistic reversal learning task is a useful task for identifying mPFC dysfunction. Also the processes responsible for sensitivity to positive and negative reinforcement are neurally dissociable, with the mPFC implicated in the former. The increased number of reversals achieved by the 5 mg/kg citalopram treated group, independent of altered sensitivity to positive or negative reinforcement, indicate that SSRI administration may improve inhibition of responding. The

reduction of 5-HT degradation in the frontal cortex, which may have occurred as a result of probiotic treatment, does not reflect the pattern of results found with SSRI administration indicating that probiotic treatment does not improve 5-HT transmission in all subregions of the frontal cortex. Alternatively, the effects of probiotic treatment on frontal lobe serotonergic functioning reported by Desbonnet *et al* (2008) did not occur in rats used in the current study. Further research examining the role of 5-HT function in subregions of the frontal cortex is essential in order to elucidate the potential for probiotics to improve cognitive performance.

7.3. Effects of probiotics and citalopram on anxiety-like behaviour

The results from Chapter 3 Experiment 1 and 2 indicate that citalopram treatment did not improve behavioural measures of anxiety in both tests. Citalopram administration at two doses, 10 mg/kg and 1 mg/kg produced opposite effects in the elevated plus maze (EPM) but not the open field test (OFT). The effects found in the open field test were limited to frequency and duration in the middle of the maze. They were not found to differ significantly between the groups on either day however dose did differentially affect these measures. This is a contrast to the robust changes found in behaviour over several measures in the EPM. The incongruency between these two tests was also reported in Chapter 4 experiment 1 and 2 where probiotic treatment produced an opposing pattern of results. Probiotic treatment did not produce any effect on behaviour in the EPM however these rats showed preference for the corners and edges of the arena in OFT. These results highlight an issue in assessing the emotional state of rodents using these behavioural paradigms as they lack coherency. This inconsistency has been previously reported in pharmacological and genetic studies of anxiety (Vendruscolo *et al,* 2003) and supports the opinion that construct differences mean each test assesses only one facet of an animals' emotional profile. In Chapter 3 it was proposed that sequential testing may have attributed to the lack of effect found in the OFT, as it was the second of the two tests be administered. However, these tests were administered in the same order with the probiotic group and significant differences were reported in the second of the two tests therefore

indicating that familiarity with testing in the OFT and EPM did not impact behaviour in this study. Given that these results found an opposing pattern of results in the citalopram study in comparison with the probiotic study in can be inferred that the effects of probiotic treatment on behaviour in the OFT is not mediated by the serotonergic system. The confounding variable of locomotor activity in the OFT and EPM was assessed separately in probiotic treatment and found to produce a similar pattern of activity as the results reported by the OFT. This implies that the results from the OFT are not true indicators of anxiety-like behaviour. Therefore, whether or not the current probiotic intervention impacts anxiety-like behaviour cannot be concluded. Other studies which have reported behavioural changes as a result of probiotic treatment in the EPM found the effect in hyperammonemia rats (a disease model for hepatic encephalopathy) (Luo *et al,* 2014). These results indicated that the physiological alterations induced by probiotics are only apparent in already compromised systems. Alternatively, a test which has been found to be sensitive to the behavioural manifestations of probiotic treatment in normal rats is the defensive-burying test (Messaoudi *et al,* 2011) indicating that choice of behavioural paradigm can lead to alternative conclusions drawn on the effects of probiotics on anxiety-like behaviour. Of particular relevance to the lack of consistency between the reported effects of probiotics on anxiety-like behaviour is the choice of probiotic bacteria in preclinical studies. *Lactobacillus* and *bifidobacterium* bacteria are the primary probiotics used in preclinical studies. However, preclinical research has varied in choice of stains of these bacteria without detailed examination of potential differential effects. As previously mentioned, potential effects on anxiety-like behaviour may be reliant on initial anxiety state and choice behavioural paradigm. However, choice of bacterial strain may also be influential. *Lactobacillus helveticus* was found to improve anxiety like behaviour in normal rats (in a defensive-burying task) (Messaoudi *et al ,* 2011). However, the current study used two strains of *lactobacillus acidophilus, bifidobacterium bifidum* and *bifidobacterium lactis* in normal rats. Further research should be conducted controlling for bacterial strain in order to identify any potential differential effects of

these strains on anxiety-like behaviour. Another methodological consideration is the duration of treatment. There also exists a lack of consistency between studies on treatment duration.

7.4. Behavioural effects of metabolite profile alterations following probiotic administration.

7.4.1. Myo-inositol

Behavioural effects of intracellular metabolism alterations following probiotic treatment were examined in Chapter 6. The results from the NMR study indicate significant alterations in intracellular metabolism as a result of probiotic treatment. This was found to differentially affect the hippocampus and the frontal cortex. Increased levels of myo-inoistol were found in the frontal cortex. Of particular relevance in the current thesis is the association of myo-inositol and anxiety. Previous research has shown that direct administration of myo-inositol significantly reduces immobility time in the forced swim test (FST). The watermaze reversal learning task was an anxiety inducing paradigm using the same aversive stimuli as in the FST. The improved acquisition of the platform location in this task, as indicated by an increased number of platform crossings in the final session of the acquisition phase, as well as the increased number of platform crossings in the reversal phase may be accounted for by reduced anxiety levels therefore facilitating learning. The PRL task failed to produce significant results between the probiotic and control group. The negative reinforcement in this task was not designed to induce anxiety, therefore according to this theory the effect of increased myo-inositol levels on anxiety would not have enabled improved performance. Results from the locomotor activity (LMA) test indicated the probiotic treatment increased activity on the first day of testing. This is consistent with previous studies where oral myo-inositol administration resulted in a 30% increase in ambulation in a LMA test (Kofman *et al,* 1998). The primary effects of probiotic treatment on anxiety-like behaviour have indicated that the effect is more profound in compromised systems with models of anxiety and disease exhibiting behavioural alterations following probiotic treatment (Desbonnet *et al,* 2010; Luo *et al,* 2014). The increase in

myo-inositol reported here was found in normal rats however the behavioural impact may only manifest in anxiety inducing environments, such as the watermaze reversal learning task. Other studies have reported significant reductions in anxiety-like behaviour in other behaviour paradigms such as the defensive-burying task following probiotics treatment. Increased myo-inositol levels reported here may account for these effects on anxiety-like behaviour.

7.4.2. Fumarate

Fumarate has also been implicated in anxiety-like behaviour. However, there is limited evidence for this in the preclinical arena. Randomised placebo controlled trials have reported benefits in generalised anxiety disorder following quetiapine fumarate (Endicott *et al*, 2012; Katzman *et al,* 2011; Mezhebovsky *et al,* 2013). It is possible that increased levels of frontal lobe fumarate may contribute to the anxiogenic effects of probiotic treatment found in other studies. However given the lack of preclinical research this is not conclusive.

7.4.3. GABA

Increased levels of GABA were found in the frontal cortex of probiotic treated rats. GABA is the main inhibitory neurotransmitter in the brain. Direct infusions of Muscimol, a $GABA_A$ receptor agonist, in the mPFC was found to impair reversal learning without affecting acquisition or retention of a discrimination (Shaw *et al,* 2013). In the probiotic group reversal learning was not impaired in the probabilistic reversal learning task. Performance in this task was shown to be reliant on mPFC functioning therefore it can be concluded that the increases in GABA found in the frontal cortex were in subregions other than the mPFC. GABAergic transmission in the frontal cortex has been associated with brain-derived neurotrophic factor (BDNF) protein expression (Sakata *et al,* 2009). Mutant mice with selective disruption in activity-dependent BDNF expression (BDNF-KIV) have been shown to exhibit deficits GABA-mediated inhibition in the frontal cortex. BDNF-KIV mice also showed reduced long-term potentiation (LTP) in the CA1 of the hippocampus, which contains

projections to the PFC (Sakata *et al,* 2009). Analysis of GABAergic synapses in the hippocampus and PFC showed that BDNF expression only impacts PFC GABA and induced no alterations in hippocampal GABA (Sakata *et al,* 2013). This result is consistent with the current NMR study that found that GABA did not differ in the hippocampus therefore also implying that BDNF protein expression was altered by probiotic treatment. The behavioural effects of BDNF-KIV mutation on a watermaze reversal learning paradigm, similar to that conducted in the current thesis (Chapter 4, Experiment 5), included impairment in reversal of the platform spatial location (Sakata *et al,* 2009). Hippocampal BDNF levels have been found to be elevated in probiotic treated rats (O'Sullivan *et al,* 2011). This is consistent with the current findings where improved performance in the reversal of the platform location was a function of probiotic treatment. In conclusion, probiotic treatment may have increased BDNF expression resulting in increased LTP in the hippocampus and elevating frontal lobe GABA concentrations.

Novel object recognition has been shown to be mediated by the perirhinal cortex but not the hippocampus (Kim *et al,* 2014). Furthermore, oral GABA administration was found to significantly improve memory in a novel object recognition task (Thanappreddawat *et al,* 2013). This result supports the current findings were probiotic treatment improved novel object recognition with a 1hr delay (Chapter 5, Experiment 2).

7.4.4. Inosine and Hypoxanthine

Hippocampal CA1 LTP has been shown to be impaired following chronic opiate exposure due to an accumulation of adenosine (Lu *et al,* 2010) causing activation of adenosine A_1 receptors (Arai *et al,* 1990, Alzheimer *et al,* 1991; Forghani & Krnjevic, 1995). This has been shown to impair spatial memory. However, administration of an A_1R antagonist was found to reverse the deficit in the watermaze. Furthermore, adenosine deaminase, which converts adenosine into inosine, also reversed the impaired CA1 LTP inhibition (Lu *et al,* 2010). These results imply that increased levels of inosine facilitate LTP in the hippocampus. This is consistent with the improvement in spatial

memory in the watermaze reversal learning paradigm (Chapter 4, Experiment 5) and object in place recognition (Chapter 5, Experiment 3) found as a result of probiotic treatment. Hypoxanthine was found to be higher in the control treated group. Hypoxanthine is a product of inosine metabolism, where it is either converted into the waste product uric acid or inosine monophosphate (IMP) which (as well as adenosine) is a precursor to inosine. The higher concentrations of inosine found in the probiotic group along with the higher concentration of hypoxanthine found in the control group in the hippocampus suggest probiotic treatment impacts this pathway. Furthermore, as there was no increase in adenosine found in the hippocampus and control animals showed higher levels of hypoxanthine it is likely that probiotic treatment reduces degradation of inosine. These higher concentrations of inosine found in the hippocampus of the probiotic group indicate that LTP may be facilitated with dietary probiotic administration improving spatial memory.

The results from the studies presented here demonstrate that dietary administration of probiotic treatment impacts upon intercellular metabolism in the frontal cortex and hippocampus. These changes may account for the improvement in recognition memory and reversal learning in tasks reliant on spatial memory. Results from the H^1 nuclear magnetic resonance spectroscopy study provide a new avenue of research to examine the potential for this intervention to improve affective disorders.

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