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**Long-term multi-species *Lactobacillus* and *Bifidobacterium* dietary supplement  
enhances memory and changes regional brain metabolites in middle-aged rats**

Abbreviated title: Memory and gut bacteria

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## **Abstract**

Ageing is associated with changes in the gut microbiome that may contribute to age-related changes in cognition. Previous work has shown that dietary supplements with multi-species live microorganisms can influence brain function, including induction of hippocampal synaptic plasticity and production of brain derived neurotrophic factor, in both young and aged rodents. However, the effect of such dietary supplements on memory processes has been less well documented, particularly in the context of aging. The main aim of the present study was to examine the impact of a long-term dietary supplement with a multi-species live *Lactobacillus* and *Bifidobacteria* mixture (*Lactobacillus acidophilus* CUL60, *L. acidophilus* CUL21, *Bifidobacterium bifidum* CUL20 and *B. lactis* CUL34) on tests of memory and behavioural flexibility in 15-17-month-old male rats. Following behavioural testing, the hippocampus and prefrontal cortex was extracted and analysed *ex vivo* using  $^1\text{H}$  nuclear magnetic resonance ( $^1\text{H}$ -NMR) spectroscopy to examine brain metabolites. The results showed a small beneficial effect of the dietary supplement on watermaze spatial navigation and robust improvements in long-term object recognition memory and short-term memory for object-in-place associations. Short-term object novelty and object temporal order memory was not influenced by the dietary supplement in ageing rats.  $^1\text{H}$  NMR analysis revealed diet-related regional-specific changes in brain metabolites; which indicated changes in several pathways contributing to modulation of neural signalling. These data suggest that chronic dietary supplement with multi-species live microorganisms can alter brain metabolites in aging rats and have beneficial effects on memory.

## Introduction

An increasing body of evidence indicates that the bacterial constituents of the **gut microbiota** can influence cognition and mood behaviours, although the exact nature and mechanisms of action remains to be determined (Tillisch et al., 2013; Slyepchenko et al., 2014; Burokas et al., 2015). Communication between the gut and the brain appears to be bidirectional and includes neural, endocrine, immune and microbial metabolite pathways (Carabotti et al., 2015). The gut microbiome is established in early life and can be influenced by diet, infection, stress, medication and aging (Hopkins et al., 2001; Yatsunenko et al., 2012; Distrutti et al., 2014; Scott et al., 2017). Indeed, dysregulation of the composition of the gut microbiome has been linked with psychiatric disorders, for example, major depressive disorder (e.g., Kelly et al., 2016; Logan & Katzman, 2005; Zheng et al., 2016). These findings have led to the hypothesis that manipulation of the gut flora, for example by “probiotic” supplements (i.e., consumption of gut bacteria that may convey health benefits), can influence brain function in psychiatric conditions and aging individuals (Benton et al., 2007; Messaoudi et al., 2011; Tillisch et al., 2013; Distrutti et al., 2014; Mello et al., 2016).

The gut microbiome changes with maturation, especially in the elderly (Claesson et al., 2011; Leung & Thuret, 2015; Zapata & Quagliarello, 2015; Aboleya et al., 2016) and this change may influence aspects of brain function and behaviour, for example by promoting inflammatory changes (Dinan & Cryan, 2017; Thevaranjan et al., 2017). The mechanism by which changes in gut microbiota may influence brain activity, however, remains to be clarified. However, they include changes in HPA activity, brain neurotrophic factors (BDNF), and neurotransmitter activity, including GABA receptor expression and serotonin levels (Desbonnet et al., 2008; Patterson et al., 2014). Recent work by Distrutti et al., (2014) showed that consumption of multiple *Streptococcus*,

*Bifidobacterium* and *Lactobacillus* species (VSL#3) by 20-22 month old rats ameliorated an age-dependent impairment in hippocampal synaptic plasticity (LTP), and increased BDNF expression. The dietary supplement also reduced brain markers of inflammation and dramatically altered gene expression profiles relative to aged-matched and young control rats. Taken together this evidence indicates that manipulation of the gut microbiome in aging rats should have beneficial effects on memory (c.f., Vaiserman et al., 2017).

*Bifidobacterium* and *Lactobacillus* are the main genera of bacteria that convey beneficial effects, for example, on anxiety- and depression-like behaviours (Akkasheh et al., 2016; Steenbergen et al., 2015). For example, Bravo et al., (2011) showed that *Lactobacillus* strains improved emotional behaviour and influenced the expression of brain GABA receptors in a region-specific manner. Similarly, Desbonet et al., (2010) showed that *Bifidobacterium infantis* bacteria influenced emotional behaviour and reduced depressive-like behaviours in early-life stressed rats. However, the impact of these bacterial strains on memory function has not been examined.

The present study therefore exposed rats chronically to four strains of bacteria: *Lactobacillus acidophilus* CUL60, *L. acidophilus* CUL21, *Bifidobacterium bifidum* CUL20 and *Bifidobacterium animalis* subsp.*lactis*., CUL34. The use of these specific strains was based on evidence that *Bifidobacterium bifidum* in combination with lactobacillus strains improved measures of depression (Akkasheh et al., 2016; see also Steenbergen et al., 2015). *Lactobacillus acidophilus* enhanced glutamine+glutamate and myo-inositol ratios in the brain of patients with minimal hepatic encephalopathy (Ziada et al., 2013). Tillisch et al., (2013) reported that a multispecies fermented milk product (including *Bifidobacterium animalis* subsp *lactis*) influenced fMRI network activity during an emotional face attention task; the regions influenced included the prefrontal cortex and parahippocampal cortex. Thus, *bifidobacterium* and *lactobacillus* strains

(including those used in the present study) have been shown to affect brain network activity, gene expression and synaptic plasticity in brain regions implicated in cognition.

In the present study, the selection of the behavioural tests was based on evidence from human fMRI studies and rodent studies that gut bacteria influenced the frontal cortex, hippocampus and parahippocampal gyrus (e.g., Tillisch et al., 2013; Distrutti et al., 2014; regions that are also associated with age-related cognitive changes). Consequently, Experiment 1 examined acquisition of a spatial bias in the watermaze, a task that is sensitive to manipulation of the hippocampus (more specifically, the dorsal hippocampus; Moser et al., 1993; Bannerman et al., 2004) and is often disrupted by normal aging in rodents (Kennard & Woodruff-Pak, 2011). Flexible learning of new platform locations is also sensitive to medial prefrontal cortex damage in rats (e.g., McDonald et al., 2008; see also Hernandez et al., 2017). Therefore, Experiment 1 tested the hypothesis that a live multi-strain *Bifidobacterium* and *Lactobacillus* dietary supplement would promote both the acquisition and “reversal” of a spatial bias in the watermaze.

To assess cognitive function across different motivational and sensorimotor requirements, the effect of the supplement on behaviour was also evaluated using a battery of object recognition memory tests. Object memory involves the integration of information about not only an object’s identity, but also information about the visuo-spatial and temporal contexts in which it was presented. Furthermore, this integrated representation of object information relies on a well-characterised integrated neural network involving the perirhinal cortex (processing object identity familiarity and novelty), the hippocampus (processing the spatial location of objects) and the medial frontal cortex, which contributes to memory for the temporal order of objects (see Warburton & Brown, 2015). Consistent with age-related changes in this neural network, recognition memory involving object-place information and temporal order is sensitive

to ageing (Diniz et al., 2016; Hernandez et al., 2015) and perturbation of this memory network is associated with increased risk of dementia (Hirni et al., 2016).

Based on evidence from Savignac et al., (2015) and Distrutti et al., (2014) that a live microorganism dietary supplement facilitated object recognition and altered hippocampal synaptic plasticity in rodents, we hypothesised that the functional properties of the recognition memory network in ageing rats would be positively influenced by the dietary supplement. Experiment 2, therefore, examined spontaneous object familiarity/novelty discrimination; Experiment 3 examined memory for object-in-place associations and, finally, Experiment 4 assessed memory for object temporal order memory.

After the completion of behavioural testing (at approximately 17-18 months of age), we examined the impact of the dietary supplement on regional brain metabolite activity using <sup>1</sup>H-NMR spectroscopy. As recently reviewed by Harris et al., (2015; see also Shankar 2010), aging is associated with changes in various brain metabolites, including myo-inositol, a component of membrane phospholipids, and glutathione, which is thought to be a marker of astrocyte antioxidant status. Furthermore, the hippocampus displays age-related metabolite changes in both humans (e.g., Shuff et al., 2001; Gruber et al., 2008; and rodents (Zhang et al., 2009; Paban et al., 2010; Harris et al., 2014, 2015; see also Haga et al., 2009). The impact of gut bacteria supplements on brain metabolites in ageing rodents is not known. However, a recent study has shown that a *Lactobacillus* dietary supplement enhanced hippocampal and frontal cortex GABA, NAA and glutamate+glutamine metabolite levels in mice (Janik et al., 2016; see also Ziada et al., 2013). This suggests that the *Lactobacillus* and *Bifidobacterium* dietary supplement used in the present study would alter the metabolite profiles of the hippocampus and frontal cortex in middle-aged rats.

## **1.0 Methods**

### 1.1 Subjects

Thirty-two, experimentally naïve adult male Lister Hooded rats (bred in house from females supplied by Harlan, UK) were used in Experiment 1. Each group of rats (control and diet supplemented) was formed from the litters of 16 dams. The weight of the rats ranged from 400g to 560g when behavioural testing started at 15 months of age. Two animals from both the bacterial supplement and control groups became unwell and were culled and did not contribute to the object recognition studies. Experiments 2 and 3 therefore used 14 rats in the control and diet groups, respectively. In Experiment 4, one diet supplemented rat did not make contact with the objects and was excluded from the analysis (N= 14, control, N= 13 diet, respectively) All animals received *ad libitum* access to food and water throughout the study. 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°C and humidity at 55% ±10.

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and associated guidelines, as well as European Union directive 2010/63/EU. A local ethical review committee at Cardiff University, UK also approved the programme of work.

### 1.2 Feeding Procedure

All rats had their food placed into their cages in glass dishes. The diet treated group had their food top-dressed daily with the multi-species live bacteria powder extracted from the Lab4 capsules (i.e., the powder was sprinkled on standard laboratory food pellets, which was then placed in a bowl inside the home cage). The Lab4 capsule (Cultech Ltd,

U.K., Port Talbot) contained four strains of bacteria, *L. acidophilus* CUL60 (NCIMB 30157), *L. acidophilus* CUL21 (NCIMB 30156), *B. bifidum* CUL20 (NCIMB 30153) and *B. lactis* CUL34 (NCIMB 30172). A dose rate of  $10^8$  cfu (colony forming unit)/capsule per rat was used. As rats were housed in pairs, 2 capsules per cage were used daily. The capsules were stored at 7°C and the food was prepared with fresh *Lactobacillus* and *Bifidobacterium* powder each morning. Rats continued to receive fresh supplement powder daily after weaning until the end of the experiment. Colonization of the infant gut commences at birth when delivery exposes the infant to a complex microbiota and its initial microbiome has a maternal signature. Thus, in order to ensure maximum exposure to the *Lactobacillus* and *Bifidobacterium* bacteria, 8 female dams received the same dose in their food (using the same method of delivery as describe above) during both the gestation and post-natal period prior to weaning. The diet treated group and the control group were kept in separate holding rooms to avoid cross-contamination. Testing commenced when the rats were 15 months old.

## 2.0 Apparatus

2.1 Watermaze. The watermaze was constructed from a white circular arena 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 (3.5m x 3m). The pool was filled with water (23-25°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 every day. A circular wooden ceiling was suspended 1m above the top of the pool. A video camera with wide-angled lens was placed in the center of the suspended ceiling. The camera was connected to a video monitor and fed input into a RM PC running Windows XP. The data were analysed using WaterMaze software (Actimetrics, Inc., U.K., Edinburgh). The room was illuminated by 8, 45 watts lights with a diameter of 20cm in a 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 towards the ceiling.

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 a black and white striped plastic rod of 2 cm in diameter, which rose 10cm above the surface of the platform.

On the walls of the room were three large distinct shapes made from differently coloured 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 objects were hung at a distance of 60cm above the top of the pool and approximately 20cm outside the perimeter of the pool and at east and west positions.

### 2.2 Object Recognition Arena

The apparatus was based on that described by Ennaceur and Delacour (1988). The arena was constructed from 1m x 1m walls that were 40cm high. Sawdust was placed on the floor of the arena at the start of each test session. Illumination was provided by a 70-Watt florescent tube lighting in the centre of the room (approximately  $38 \text{ cd/m}^2$  at the arena surface). 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.

## **3.0 Procedure**

### *3.1 Experiment 1: Watermaze*

The watermaze 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 dark holding 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 each of the four start locations during each training session. The animals were released by gently placing them into the pool facing the maze wall.

During pre-training, the platform was located in one of the four platform positions (NE, SE, SW, NW), the order of locations cross trials in a training session was random and was changed after each rat had performed one trial at its specified location. The order of platform locations was the same for each group of four rats. The platform was positioned in the middle of the quadrant, 20cm from the edge of the pool. A beacon was attached to the platform to encourage the rats to swim and locate the submerged platform. Each trial lasted a maximum of 60 seconds. After mounting the platform, the rats were left in place for 20 seconds. If after 60 seconds, the rats failed to locate the platform, the experimenter guided the animal to the location, where, once on the platform, they remained for 20 seconds. After this interval, the rat was removed from the maze, dried with a warm towel and returned to the holding box. Each of the four rats in a group completed the first trial before moving on to the second trial. The inter-trial interval was approximately 4-5 minutes. All rats were required to locate and climb on to the platform, without assistance, by the fourth trial before moving on to the acquisition phase.

During the acquisition phase, platform locations (either SE or NW quadrants) were counter-balanced between and within groups. The beacon was removed from the platform for the remainder 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 remained there

for 20 seconds. Rats were trained on acquisition of the platform location for 6 sessions. On the 6<sup>th</sup> day a probe or test trial was conducted to evaluate the extent of a learned spatial bias. During the probe trial, the platform was removed from the pool and the rats allowed to search for the platform freely for a maximum of 60 seconds. The reversal phase of training started on the 7<sup>th</sup> session. During these sessions, the platform location was switched to the quadrant opposite that used for acquisition. The procedure was, otherwise, identical to that used in during acquisition, except that a probe trial was completed at the end of each training session to track the acquisition of a new spatial bias.

### *3.2 Experiment 2: Object recognition memory*

#### *3.2.1 Habituation*

Habituation to the apparatus was carried out across four days. Rats were carried from their holding room in a dark carrying box to the testing room. On days 1-4, the rat was introduced to the arena facing a wall, released and allowed to explore the area for 15 min. After 15 mins, the rat was returned to the carrying box and any faecal matter removed from the maze, the walls were cleaned with alcohol wipes, to remove any olfactory cues.

After 4 days of habituation, rats were exposed to a pair of identical objects for 4-mins and their object contact time was recorded over two successive days. The purpose of this stage was to evaluate potential object contact time differences between the two groups. 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.

The analysis of contact time data indicated that diet treated rats displayed higher contact times with objects and so a yoking procedure was introduced and used through out each subsequent experiment to ensure that both groups received comparable exposure to the sample objects.

### *3.2.2 Sample Phase*

The total object contact time (summed across all objects) for each diet treated rat was yoked with that of a randomly assigned control rat. Thus, during each exposure phase, for each pair of control and diet treated rats, the control rat was first given 4 minutes to explore the objects. Then the yoked diet rat in each pair was given access to the same objects and allowed to accumulate the same total contact time as its paired control. The diet rat remained in the arena until it accumulated the same contact time as its paired control animal or for a maximum of 4 minutes. Note, no attempt was made to match exploration times for individual objects. After the object exposure phase, each rat was placed either in a carrying box (for the 5-min interval) or its home cage (for the 1-hour delay), in preparation for the test phase.

### *3.2.3 Test Phase*

During the delay interval, one of the objects was replaced with a novel object. Both objects were cleaned with 30% alcohol wipes to remove any olfactory cues. The position of the novel object (left or right) was counterbalanced within and across the groups. During the test phase, the animal was reintroduced in to the arena and contact times with the familiar and novel objects were recorded for 4 mins. 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 on two consecutive days with a novel set of objects used on each day. The data from the two days was averaged for statistical analysis.

### *3.3 Experiment 3: Object-in-place memory*

#### *3.3.1 Sample Phase*

The rats had two days in their home cages without behavioural testing before the start of Experiment 3. The protocol used for this experiment followed a similar pattern as Experiment 2. The same yoking procedure and trial parameters were used in the sample 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 used in any other phase of the study.

#### *3.3.2 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 that were diagonally opposite each other were switched. The pair of objects that were switched was counterbalanced within and between the two groups. After five minutes, the rat was returned to the arena for four minutes and contact time with each of the objects was recorded. The contact time with the objects that had switched locations was used as a measure of mismatch detection between the current visuo-spatial array and memory for the prior object-location associations. At the end of this phase, the animals 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. The experiment was run on two consecutive days, with novel sets of objects used on each day. The data for the two days was averaged for statistical analyses.

### *3.4 Experiment 4: Object temporal order*

#### *3.4.1 Sample Phase 1 and 2*

The rats were given a further two days free of testing before the start of Experiment 4. The protocol used for this experiment was identical to that used for Experiment 2. In Phase 1, a pair of identical objects was placed in the arena and control rats were given four minutes to explore the objects, during which their contact times were recorded. Following this, the paired diet rat was given a maximum of four minutes to achieve the same total object contact time. After this phase, each rat was removed from the arena and returned to its home cage. In phase 2, after a 1 hour delay, the rats were returned to the arena, which contained a novel pair of identical objects and the same yoking procedure was used to match exposure to the objects. After this, the rats were returned to their home cages for a three-hour delay.

#### *3.4.2 Test Phase*

During the test phase, one object from each pair presented in sample phase 1 and 2 was placed in to the arena in the location they had occupied during the initial sample phase. Object locations were counterbalanced between the groups so that the most recent object was placed equally often on the right or the left side of the arena from the release point. The rats were given four minutes to explore the objects and their contact time with the ‘old object (from sample phase 1) and ‘recent’ 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 procedure was carried out on two consecutive days, with novel sets of object on each day. The data for the two days was averaged for statistical analysis.

### *3.5 Experiment 5: $^1\text{H}$ NMR spectroscopy*

#### *3.5.1 Brain dissection*

After the completion of behavioural testing, five rats from the control and diet treated groups were sampled randomly for brain tissue collection and culled by decapitation. The hippocampus and frontal cortex were snap-frozen immediately in liquid nitrogen and stored at -80°C until the tissue was processed.

### *3.5.2 $^1\text{H}$ NMR Spectroscopic Analysis*

The  $^1\text{H}$ - NMR spectroscopic method was similar to that used by Yap et al., (2008). 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 g at 4°C, the aqueous phase was transferred to a fresh microcentrifuge tube and dried using a speed vacuum. The sample was suspended with 600  $\mu\text{l}$  of deuterium oxide ( $\text{D}_2\text{O}$ ) and sonicated for 10 min. Fifty micro litres of 0.2 M phosphate buffer (pH=7.4) containing 100%  $\text{D}_2\text{O}$  for the magnetic field lock, 0.01% 3-(trimethylsilyl)- [2,2,3,3- $^2\text{H}_4$ ]-propionic acid sodium salt (TSP) for the spectral calibration and 3mM sodium azide ( $\text{NaN}_3$ ) to prevent bacterial contamination was added and thoroughly vortexed for 15 sec followed by spinning at 10,000 g for 5 minutes. A total of 600  $\mu\text{l}$  of the supernatant was transferred to an NMR tube (5mm outer diameter) for  $^1\text{H}$  NMR spectral acquisition using a Bruker 600MHz spectrometer (Bruker; Rheinstetten, Germany). A  $^1\text{H}$  frequency of 600.13 MHz was applied to the samples at a temperature of 27°C. A standard NMR pulse sequence (recycle delay[RD]-90 $^\circ$ - $t_1$ -90 $^\circ$ - $t_m$ -90 $^\circ$ - acquisition) was applied to acquire 1-dimensional (1-D)  $^1\text{H}$  NMR spectral data with  $t_1$  set to 3  $\mu\text{s}$  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.

### *3.5.3 Statistical analysis*

The behavioural data were analysed by analysis of variance. Significant interaction terms were further analysed by tests of simple main effects using the pooled error term. Where appropriate group comparisons were carried out using t-tests. Multivariate data analysis was performed based on pre-processed NMR spectroscopic datasets. <sup>1</sup>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 a 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 <sup>1</sup>H 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, were calculated using students t-test between the two group.

## **4.0 Results**

### 4.1 Experiment 1: Watermaze

#### 4.1.1 Acquisition Phase

The mean latency to reach the platform during the acquisition phase is presented in Table 1. Inspection of these means indicates that there were no overall group differences in performance during acquisition. A repeated measure ANOVA indicated that there was no main effect of group ( $F_{(1,30)}=1.52$ ,  $p>0.05$ ) or session by group interaction ( $F<1$ ). However, there was a significant main effect of session ( $F_{(5,150)}=10.17$ ,  $p<0.001$ ).

The mean velocity of swimming in the acquisition phase is presented in Table 1. An ANOVA indicated there was no significant main effect of group ( $F_{(1,30)} =1.00$ ,  $p>0.05$ ) or group by session interaction ( $F_{(5,150)} =2.06$ ,  $p>0.05$ ). However, there was a significant main effect of session ( $F_{(5,150)} =21.38$ ,  $p<0.001$ ), that reflected an overall reduction in swim speed during training.

#### 4.1.2 Probe trial

The mean percentage time spent in the training and remaining three quadrants during the probe trial are shown in Table 1. Although rats treated with the dietary supplement spent more time, numerically, than control rats in the training quadrant, the ANOVA revealed no main effect of group ( $F_{(1,30)} =2.95$ ,  $p>0.05$ ), a main effect of quadrant, ( $F_{(3,90)} =6.64$   $p <0.001$ ), nor a significant interaction between these factors ( $F<1$ ).

The number of target platform crossing during the probe trial is shown in Table 1. Inspection of these data suggests that both groups performed similarly during the probe trial and this was confirmed by a t-test ( $t<1$ ).

## 4.2 Reversal learning

### 4.2.1 Training

The mean latency to reach the platform for the control and diet treated rats during training in the reversal phase is shown in Table 1. Both groups acquired the new platform location at approximately the same rate. This was confirmed by a repeated measures ANOVA that revealed no significant main effect of group ( $F<1$ ) or group by session interaction ( $F<1$ ). There was also no significant main effect of session ( $F_{(2,60)} = 1.60$ ,  $p>0.05$ ).

The mean swim speeds during the reversal phase are presented in Table 1. Once again, ANOVA indicated that there was no significant main effect of group ( $F_{(1,30)} = 2.49$ ,  $p>0.05$ ) and no session by group interaction ( $F<1$ ).

### 4.2.2 Probe trials

The mean percentage times spent in each quadrant during the probe trial at the end of each reversal training session are shown in Table 1. Inspection of these means indicates that there were no systematic differences between the groups in terms of preference for the new quadrant. An ANOVA conducted on these data revealed a non-significant main effect of group ( $F<1$ ), a non-significant main effect of session ( $F_{(2,60)} = 2.71$ ,  $p>0.05$ ), and no significant interaction between these factors ( $F<1$ ). There was, however, a main effect of quadrant ( $F_{(3,90)} = 34.85$ ,  $p<0.001$ ), but no significant group x quadrant interaction ( $F<1$ ). There was a significant test day x quadrant interaction, ( $F_{(6,180)} = 2.78$ ,  $p < 0.02$ ) and a significant three-way interaction ( $F_{(6,180)} = 5.14$ ,  $p<0.001$ ). The interaction reflected a bias to the adjacent quadrant in the control, but not the diet rats on

day 1 of reversal training ( $p<0.05$ ).

Table 1 shows the mean number of platform crossings during the probe trial conducted at the end of each reversal session. Inspection of these means indicate that the diet supplemented rats appeared to become more accurate in their search patterns with training. A repeated measures ANOVA indicated there was no significant main effect of group ( $F_{(1,30)} = 2.03$ ,  $p>0.05$ ) or main effect of session ( $F_{(2,60)} = 2.29$ ,  $p>0.05$ ). There was, however, a significant session by group interaction ( $F_{(2,60)} = 3.30$ ,  $p<0.05$ ). Tests of simple main effects revealed a significant difference between the groups on the final day of training ( $F_{(1,87)} = 7.69$ ,  $P <0.01$ ). In addition, there was a main effect of training session for the diet group ( $F_{(2,60)} = 5.68$ ,  $p<0.01$ ), but not for the control group ( $F<1$ ).

#### 4.3 Experiment 2: Object recognition memory

Figure 1 shows the mean total contact times summed across the two days of testing with the novel and familiar objects after a 5 min or 1 hour retention interval. Inspection of this figure suggests that rats receiving the diet supplement displayed greater preference for exploring the novel versus familiar objects. An ANOVA revealed a non-significant main effect of group ( $F<1$ ), a non-significant main effect of delay ( $F<1$ ) and a non-significant interaction between these two factors ( $F<1$ ). There was a main effect of object type (novel versus familiar;  $F_{(1,26)} = 63.02$ ,  $p <0.01$ ) and a significant interaction of this factor with group ( $F_{(1,26)} = 7.31$ ,  $p <0.05$ ). There was no delay x object type interaction ( $F_{(1,26)} = 2.38$ ,  $p > 0.10$ ) and no significant three-way interaction, ( $F_{(1,26)} = 1.65$ ,  $p >0.10$ ). Tests of simple main effect revealed a significant difference between the groups in contact with the novel object ( $F_{(1,43)} = 4.87$ ,  $p <0.05$ ), but not with the familiar object ( $F<1$ ).

The mean discrimination ratios for control rats for the 5 min and 1 hour delays were 0.67 (SE = 0.042) and 0.57 (SE = 0.041), respectively. For rats administered the diet supplement, the discrimination ratios were 0.72 (SE = 0.029) and 0.69 (SE=0.034), respectively. In order to assist in interpretation of Experiment 3, in which a 5-minute delay was used, individual t-tests were carried out at each delay and revealed no significant difference between the diet and control rats at the 5-minute delay ( $t<1$ ), but a significant difference between the groups at the 1 hour delay ( $t_{(26)} = 2.29$ ,  $p<0.05$ ).

#### 4.4 Experiment 3: Object-in-place Memory

Figure 2 (left hand side) shows the mean contact times with the target (Different) and the remaining (Same) objects in the test trial. Inspection of this figure indicates that rats receiving the diet supplement displayed a greater preference than control rats for the objects in the different spatial locations. An ANOVA conducted on the contact times revealed a non-significant main effect of group ( $F_{(1,26)} = 1.78$ ,  $p>0.10$ ) a main effect of Same v Different location ( $F_{(1,26)} = 11.61$ ,  $p<0.01$ ) and a significant interaction between these factors, ( $F_{(1,26)} = 6.87$ ,  $p <0.02$ ). Tests of simple main effects revealed a significant group difference in contact times with the objects in the different location ( $F_{(1,43)} = 6.33$ ,  $p <0.02$ ), but not with the objects in the same location ( $F<1$ ). The discrimination ratio for the diet treated rats, 0.64 (SE= 0.033), was significantly higher than that of the controls 0.53 (SE =0.041;  $t_{(26)} = 2.10$ ,  $p<0.05$ ).

#### 4.5 Experiment 4: Object temporal order

Figure 2 (right hand side) shows the average contact times with the old and recent objects in the temporal order task for control and diet treated rats. The data for one diet

treated rat was excluded because of a failure to make contact with the objects. Inspection of this figure suggests that both groups of rats performed the task at a comparable level. An ANOVA revealed a non-significant main effect of group ( $F<1$ ), a main effect of old v recent, ( $F_{(1,25)} = 9.21$ ,  $p <0.01$ ) and no significant interaction between these factors ( $F<1$ ). The mean discrimination ratio for control and diet treated rats was 0.63 (SE= 0.045) and 0.59 (SE 0.058), respectively. There was no significant different between these means ( $t<1$ ).

#### 4.6 Experiment 5: $^1\text{H}$ NMR spectroscopy

##### 4.6.1 Frontal Cortex

Spectral data from the frontal cortex was aligned using a recursive segment-wise peak alignment method due to the heavy shift of peaks (Veselkov et al, 2009). Figure 3A shows an unsupervised multivariate principal component analysis (PCA) score plot for the samples from the frontal cortex. The purpose of this analysis was to observe intrinsic similarities or dissimilarities in metabolic profiles among the samples and identify any outliers. The PCA score plot indicates segregation of the two groups based on their metabolic profiles acquired using  $^1\text{H}$  NMR spectroscopy. The  $R^2$  value of the PCA model was 0.43, i.e., 43% of the variation in the dataset explained by the first two principal components of the PCA model.

Figure 3B shows the scores plot of the supervised multivariate analysis, orthogonal partial least-squares discriminant (OPLS- DA). This method is a predictive model that assigns the samples to two classes, i.e., control vs. diet, and seeks the metabolites (or signals in the metabolic profiles) that are predictive to the sample classes. The  $R^2X$  value, representing the fraction of variation in the NMR spectral data modeled by the

components in the model, was 0.38. The  $Q^2$  value is indicative of the quality of the model in terms of its prediction ability. The  $Q^2$  value in this model was 0.45. The metabolite profiles from the frontal cortex of control and diet treated rats were thus markedly different.

#### 4.6.2 Hippocampus

Figure 3C shows an unsupervised PCA score plot for the samples taken from the hippocampus. The PCA score plot indicates clear segregation of the two groups. The  $R^2X$  value of the model was 0.41, i.e. 41% of the variation in the dataset were explained by the first two principal components of the PCA model. Figure 3D shows the scores plot of the supervised OPLS- DA analysis. The  $R^2$  value in the model was 0.34 and the  $Q^2$  value in this model was 0.72. This analysis demonstrates that the metabolite profiles of the hippocampus from control and diet treated rats were markedly different.

Figures 4 and 5 are the loadings plots of the OPLS-DA analysis of the frontal cortex and hippocampus, showing the key metabolites that contribute to the separation between the sample classes, i.e., diet treated rats relative to the control group. Peaks pointing upwards represent higher concentrations of these metabolites in the diet-treated (probiotic) group compared with control group, and *vise versa*. The colour of the peaks represents the correlation ( $r^2$ ) between the metabolites and the classification, red peaks meaning a high correlation coefficient value and blue peaks meaning a low correlation ( $r^2$  is close to 0).

Tables 2 and 3 summarise the Pearson's correlation coefficient ( $r$ ) of the metabolites in the frontal cortex and hippocampus, respectively, with the classification (e.g. control vs. diet-treated) and the p-value of the statistical difference between diet and control rats. This comparison was performed using a student's t-test. The positive correlation means

that the concentrations of these metabolites are higher in the diet group in comparison to the control group. The negative correlation values, such as the r values for hypoxanthine and succinate, represent higher concentrations of these metabolites in the control group compared with the diet group. It should be noted that the presence of blood contamination in the brain tissue could have influenced the metabolite profiles. However, given there were differences in metabolite changes between the hippocampus and prefrontal cortex, it is unlikely this results from a common blood contamination of the sample.

### 5.0 Discussion

Rats receiving a long-term mix of *Lactobacillus* and *Bifidobacterium* species in a dietary supplement showed a small improvement in spatial accuracy during acquisition of a new platform location in a watermaze task. The same rats, however, showed a robust improvement in object novelty detection following a 1-hour delay and improved memory for object-in-place associations. In contrast, memory for object temporal order was not influenced by the dietary supplement.

The present study provided only relatively weak evidence for a change in watermaze navigation in rats receiving the dietary supplement. Indeed, similar patterns have emerged in other studies of watermaze learning following gut bacteria supplements. Jeong, Kim, Ahn et al., (2015) examined acquisition of a watermaze task in 18-month-old Fischer 344 rats fed *Lactobacillus curvatus* (HY7601) and *Lactobacillus plantarum* (KY1032). There was no statistical evidence for a diet-related benefit on performance, although the treated rats did show improved spontaneous alternation in a Y-maze. A similar study reported by Jeong, Woo, Kim et al., (2015), reported improved Y-maze alternation and acquisition of a watermaze task in 18-month old Fischer 344 rats fed

*Lactobacillus pentosus* var. *plantarum* (C29). In addition, these authors also reported increased hippocampal doublecortin, BDNF and phosphorylated CREB expression and reduced pro-inflammatory cytokines, TNF $\alpha$  and IL-6. These results indicate that gut multi-species live microorganism supplements positively influenced hippocampal neurotrophic and inflammatory markers. Nevertheless, the effect of gut microbiota on watermaze performance, both in middle-aged rats (current study) and aged Fisher rats (see also Wang et al., 2015) is clearly variable. This raises the possibility that different gut bacteria (or their combination) may interact with the emotional, cognitive and performance related components of the watermaze tasks in different ways. One advantage of assessing the impact of gut microorganism manipulation on tasks that differ in terms of their motivational and motor requirements is that it can help reveal similarities and differences in the cognitive systems influenced by the manipulation across different motor and sensory requirements.

In this respect, the task specific improvements in recognition memory observed in the present study indicate that the probiotic supplement had a relatively circumscribed effect on brain systems supporting object novelty detection and spatial, object-place, associations. The improvement in object memory is similar to that reported by Liang et al., (2015) who showed that *L. helveticus* (NS8) improved object novelty detection and promoted object-location memory after a 3 hour delay. In the latter test, a familiar object was moved to a novel location, unlike the present study. Nevertheless, performance on this task is still reliant on the same network of brain regions. Improvements in object recognition were also reported by Smith et al., (2014) using immunodeficient mice administered *Lactobacillus rhamnosus* (R0011) and *L. helveticus* (R0052). Administration of a mix of live gut bacteria, therefore, appears to have a positive effect on memory for object familiarity and object-location associations in rodents following a number of different challenges that compromise cognitive function.

The above pattern of results suggests an interaction between the microbiota supplement and brain systems supporting object memory (Warburton & Brown, 2015). The perirhinal cortex primarily supports memory for object familiarity/novelty, but also interacts with the medial prefrontal cortex in support of object-place associations (Warburton and Brown, 2015; Barker et al., 2007). Similarly, the hippocampus is also required for object-place associations and interacts both with the perirhinal cortex and medial prefrontal cortex in support of this form of memory (Warburton & Brown, 2015). The fact that the *Lactobacillus* and *Bifidobacterium* mixture influenced both object novelty and object-in-place associations indicates that the bacteria influenced the activity of this integrated neural system.

Before considering the diet-related changes in regional brain metabolites, it is worth considering the fact that object temporal order memory was not influenced by the dietary supplement. The absence of a behavioural effect was perhaps surprising given the evidence for metabolite changes in the frontal cortex (discussed below; see also Desbonnet et al., 2008). One possibility is that the test may not have been optimised to reveal subtle changes in object temporal order memory. For example, a longer interval between the final sample trial and test may have challenged memory more robustly, particularly given the improvement in long-term object memory observed in the diet treated rats. However, the absence of a diet-related change in temporal order memory is not sufficient to conclude that the metabolite changes in the frontal cortex were of no functional significance. The interactive nature of the network involving the medial prefrontal cortex - perirhinal cortex and medial prefrontal cortex - hippocampus in support of object-in-place memory (Barker et al., 2007; Barker & Warburton, 2015) raises the possibility that metabolite changes in the prefrontal cortex influenced the integrated activity of this recognition memory network in the diet treated rats.

One of the major metabolite changes observed in the frontal cortex, but not the

hippocampus, of rats fed *Lactobacillus* and *Bifidobacterium* supplement was an increased level of myo-inositol. Myo-inositol is a metabolite frequently linked to astrocyte activity and one that often changes as a function of brain injury and ageing (Harris Choi & Brookes, 2015). Myo-inositol is reduced in fronto-limbic regions in depressed patients and elevated in patients with mania (Levine, 1997). Thus, an elevation in myo-inositol may underpin the reported anti-depressive actions of some “probiotic” supplements. However, the functional significance of the increase in myo-inositol in the frontal cortex of rats remains uncertain. In terms of a potential mechanism for promoting cognitive function, one possibility (although speculative) is that increased myo-inositol may have improved the regulation of synaptic plasticity processes through the modulation of intracellular Ca<sup>2+</sup> release (Baker et al., 2013). Alternatively, the increase in myo-inositol may reflect the sensitivity of glia to changes in GABA activity (see below; Mariotti et al., 2016). Interestingly, increased frontal cortex myo-inositol levels in elderly patients has been linked with poorer episodic memory performance (Schreiner et al., 2016). It is possible, therefore, that increased myo-inositol in the frontal cortex could have a negative impact on memory, especially those involving integrative, e.g., episodic, memory. Clearly, further work is required to examine the impact of multi-species bacteria on different memory processes and their neural substrates (c.f., Barker et al., 2017; Good et al., 2007).

Differences in peak intensities in GABA in the frontal cortex, but not hippocampus, suggest that the *Lactobacillus* and *Bifidobacterium* supplement influenced the GABA/glutamate-glutamine cycle. This finding is consistent with Ziada et al, (2013) who reported that *Lactobacillus acidophilus* reduced levels of glutamine/glutamate in hepatic patients. Alternatively, the increase in GABA signal may reflect increased production of GABA by the bacteria (Marques et al., 2016); although one might expect the signal to be increased in the hippocampus as well if that were a system wide effect.

Taken together, it seems likely that the increase in myo-inositol and GABA concentration may reflect increased modulation of glutamate neurotransmission (and potentially synaptic plasticity) in the frontal cortex of rats receiving the microorganism supplements.

The analysis of hippocampal metabolites revealed increased levels of inosine (a metabolite of the neuromodulator adenosine) and decreased levels of hypoxanthine in rats receiving the dietary supplement. The opposing changes in concentration of these two metabolites is unsurprising given that both of these metabolites are part of the purine nucleotide cycle (Schultz & Lowenstein, 1978). Increased inosine, in particular, has been shown to have beneficial effects on the CNS. For example, Muto et al., (2014) showed that a single oral dose of inosine enhanced neurogenesis in the dentate gyrus and enhanced phosphorylation of MAPK in mice. These changes were also accompanied by an anti-depressive action of inosine. In terms of cognitive function, there is also evidence that inosine can enhance recognition memory in rodent models of traumatic brain disorder (Dachir et al., 2014). Thus, the increase in hippocampal inosine levels in the rats receiving the dietary supplement is likely to have contributed to the improvement in object-place associative memory.

A metabolite change that was common to both the hippocampus and frontal cortex was increased levels of lactate. This change is perhaps unsurprising given the major metabolic end product of the bacteria is lactic acid (Klein et al, 1998). Nevertheless, lactate serves as a precursor to alanine, which was found to be elevated in the hippocampus, but not significantly elevated in the frontal cortex. Lactate is thought to be an important source of energy and signaling in the brain (Dienel, 2012; Tang et al., 2014). Furthermore, lactate (or more specifically, lactate-mediated metabolic coupling of astrocytes and neurons) also plays an important role in the establishment of long-term, but not short-term memories (Steinman et al., 2016). Thus, the increased levels of

lactate in the hippocampus (and frontal cortex) of rats receiving the microorganism supplement may have promoted neuronal and synaptic plasticity processes that underpin memory.

In summary, the present study has shown that the long-term administration of the Lab4 multi-species live microorganism supplement to rats throughout aging resulted in relatively circumscribed improvement in cognition and regional changes in brain metabolites. The behavioural data indicates that the supplement influenced frontal-hippocampal-perirhinal circuits involved in object and place information processing. The assessment of metabolites in the frontal cortex and hippocampus indicate changes in GABA and glutamate signaling through a number of pathways, including glial-neuronal interactions. Further work is required to specify the precise mechanism(s) for these metabolite changes and the nature of the signaling, synaptic and brain network changes influenced by manipulation of the gut flora. Although the method of administering the supplements used in the present study clearly does not lend itself to clinical applications, the findings represent proof of principle that manipulation of the gut microbiome can have an impact on brain activity and memory. Further work will establish whether a more clinically relevant delivery method can have similar effects on brain metabolites and memory in aged animals. In conclusion, this study showed that long-term dietary supplement with a mixture of live *Lactobacillus* and *Bifidobacterium* microorganisms in middle aged rats changed brain metabolite profiles in the frontal cortex and hippocampus and caused task-specific improvements in memory.

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Figure 1

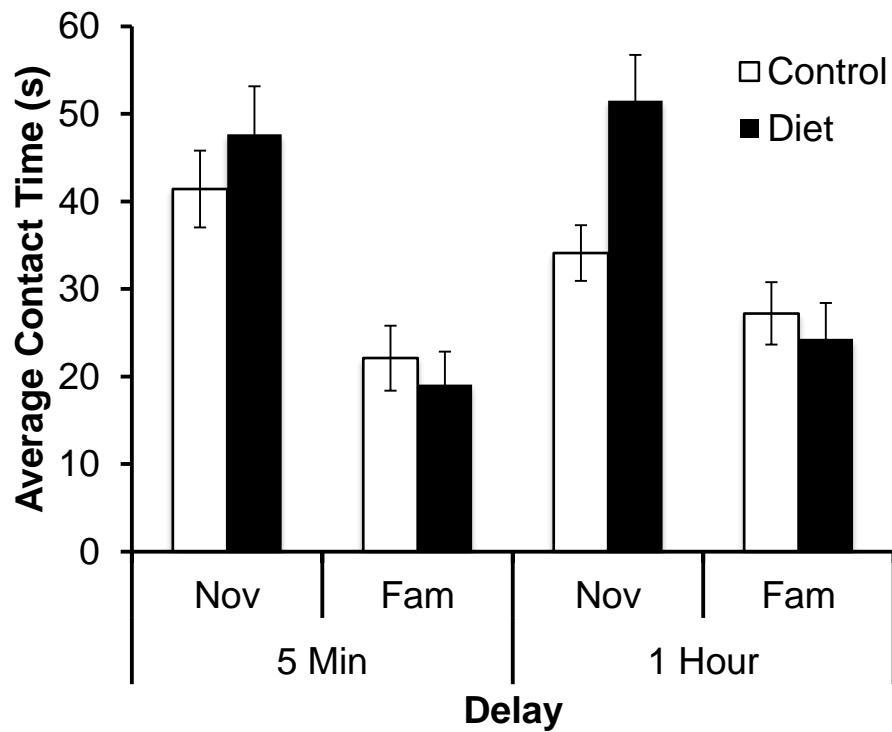


Figure 2

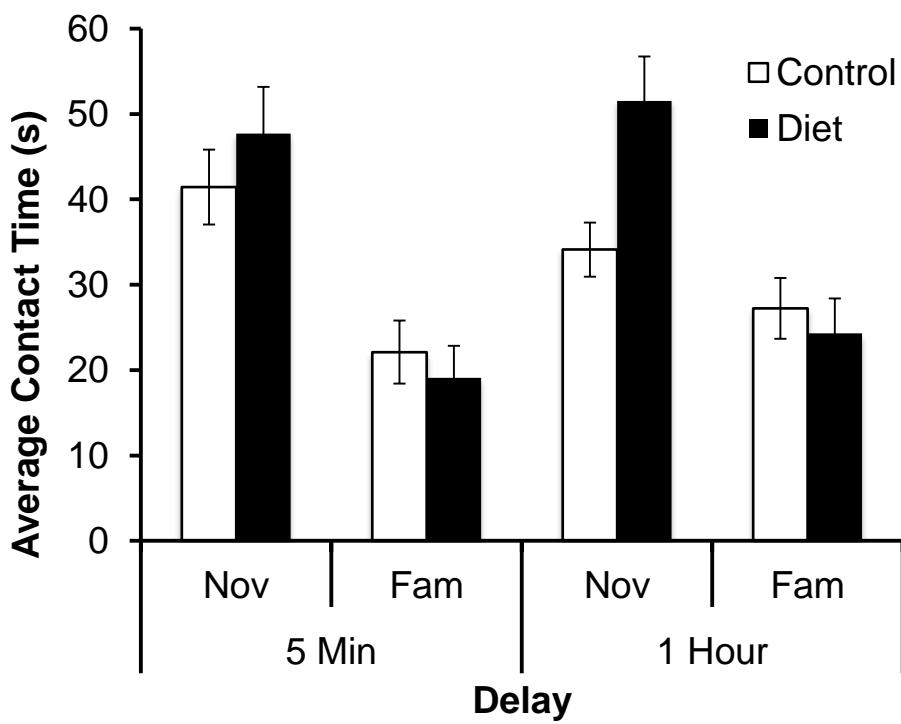


Figure 3 A-D

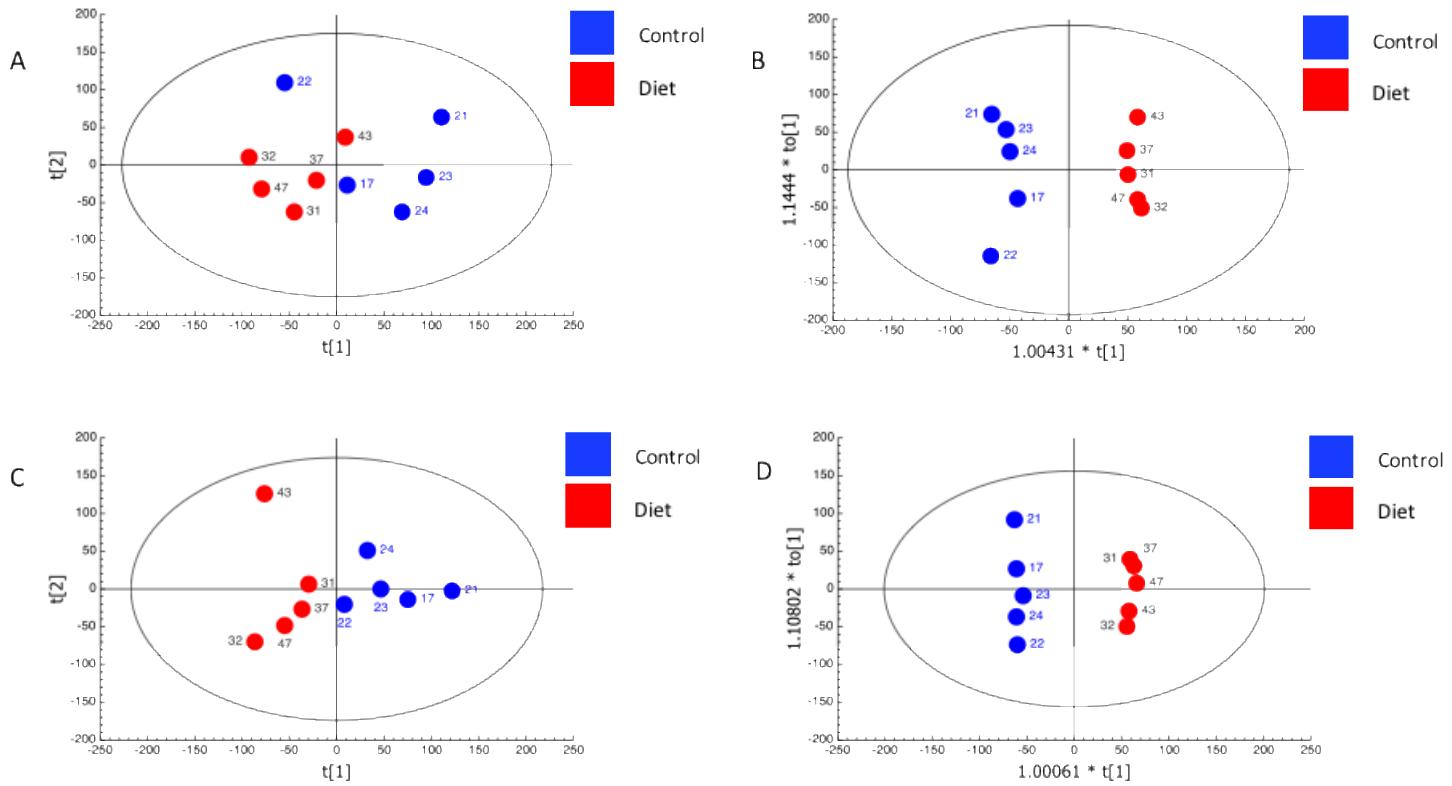


Figure 4

A

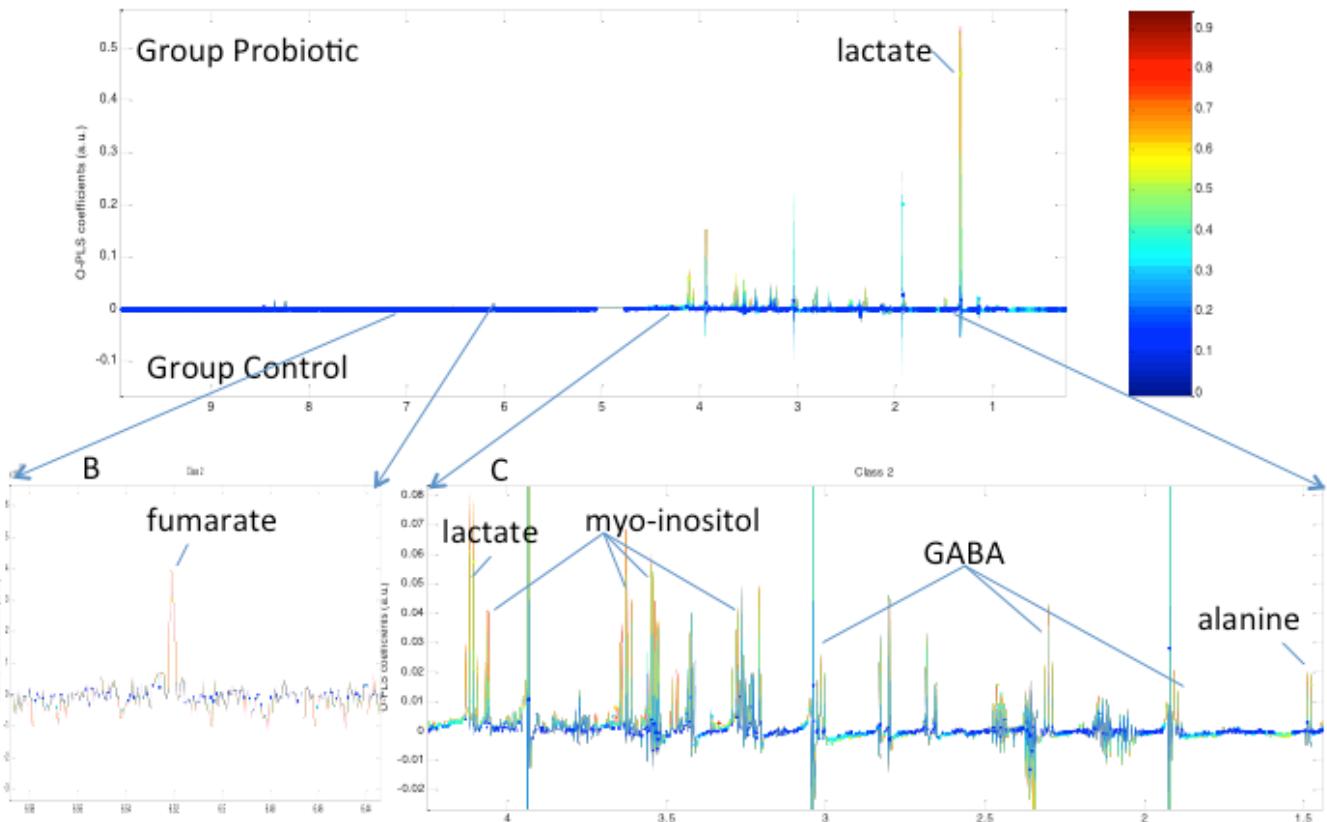
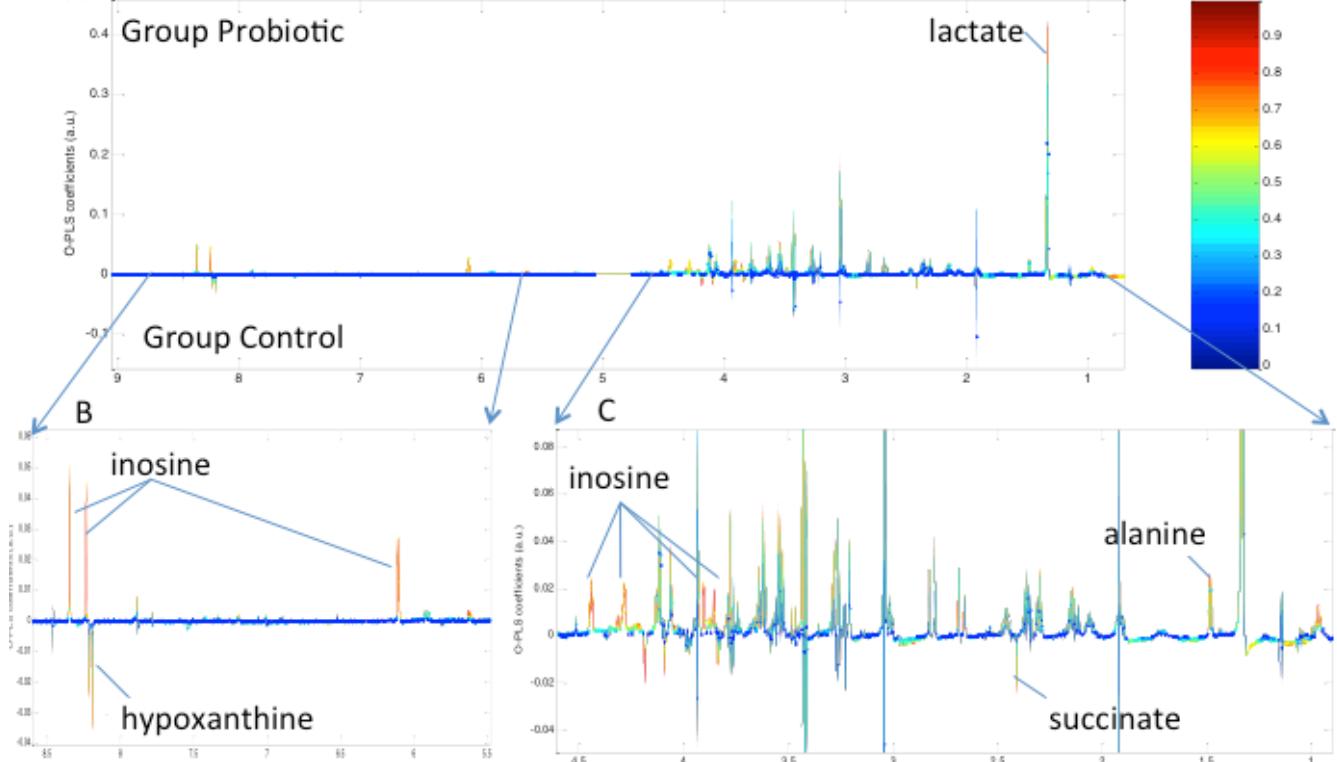


Figure 5

A



Experiment 1: Watermaze						
Acquisition						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Latency (s)						
Control	40.63(3.21)	41.48(2.28)	40.87(2.49)	32.47(3.67)	30.58(2.97)	30.41(2.70)
Diet	39.98(2.65)	37.67(2.54)	34.00(2.27)	29.07(2.68)	30.13(3.27)	27.26(3.18)
Swim-Speed (cm/s)						
Control	36.94(0.67)	31.41(0.69)	31.59(0.61)	28.43(1.04)	31.01(0.88)	32.21(1.35)
Diet	33.97(1.26)	29.78(1.07)	30.46(0.98)	29.70(1.20)	29.64(1.34)	31.69(1.33)
Probe trial						
	Target %Time	AdL %Time	AdR %Time	Opp %Time		Target Platform crossings
Control	45.96 (3.98)	23.34(2.54)	17.87(2.61)	12.81(2.05)		2.31(0.32)
Diet	51.25(4.26)	17.35(1.83)	17.53(2.08)	13.85(2.60)		2.62(0.41)
Reversal training						
	Day 1 Latency(s)	Day 2 Latency(s)	Day 3 Latency(s)	Day 1 Swimspeed (cm/s)	Day 2 Swimspeed (cm/s)	Day 3 Swimspeed (cm/s)
Control	37.45(5.27)	41.48(2.28)	40.87(2.49)	28.89(1.09)	24.63(0.89)	26.09(1.35)
Diet	39.98(2.65)	37.67(2.54)	34.00(2.27)	27.19(1.23)	24.80(1.06)	26.46(1.43)
Probe trial						
	Day 1 Control	Day 1 Diet	Day 2 Control	Day 2 Diet	Day 3 Control	Day 3 Diet
% Time						
Target	30.15(3.21)	32.59(1.82)	36.59(3.95)	38.45(4.05)	42.27(3.57)	47.94(3.53)
AdL	30.33(1.73)	19.69(2.98)	21.65(2.39)	24.44(3.05)	20.80(3.36)	19.58(2.52)
AdR	14.04(2.08)	18.40(2.39)	21.65(3.47)	18.83(3.16)	21.22(3.34)	18.26(1.51)
Opp	25.57(2.87)	29.31(2.22)	20.33(2.95)	18.26(2.45)	15.70(1.68)	14.20(1.51)
Target Platform Crossing	1.00(0.30)	0.81(0.22)	1.12(0.22)	1.25(0.30)	0.93(0.26)	2.12(0.44)*

Table 1. Performance of normal and probiotic treated rats during acquisition and reversal training

in a water maze hidden platform task. Numbers in parenthesis represent the +/- standard error of the mean. \* indicates a significant difference between groups, p<0.05.

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

Table 2. Coefficients (r) of the main metabolites in frontal cortex of the probiotic group and significance values (p) when compared with the control group. These metabolite exhibit higher concentrations in the diet group compared with the control group.

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. Coefficients (r) of the main metabolites in hippocampus of the probiotic group and significance values (p) when compared with the control group. Metabolites with positive correlation values exhibit higher concentrations in the diet group compared with the control group, whereas the ones with negative correlation values show lower concentrations in the diet group in comparison with the control group.

## Figure legends

Figure 1. Average contact times (seconds) with objects in the novelty test trial conducted either 5 min or 1 hour after the sample trial. Control refers to rats fed a normal chow and Diet refers to rats fed the mix of *Lactobacillus* and *Bifidobacteria* dietary supplement.

Figure 2. Average contact times (seconds) with objects in same or different spatial locations in the object-in-place task (left side of figure) and “old” versus “recently” presented objects in the temporal order task (right side of figure). Control refers to rats fed a normal chow and Diet refers to rats fed the mix of *Lactobacillus* and *Bifidobacteria* dietary supplement.

Figure 3. **A** and **C**, unsupervised Principal Component Analysis (PCA) score plot of metabolites in the frontal cortex and hippocampus, respectively, showing intrinsic differences in metabolic profiles. **B** and **D**, supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots of metabolites in the frontal cortex and hippocampus, respectively, showing predictive metabolic profiles associated with the sample class (i.e., Control versus Diet). Control refers to rats fed a normal chow and Diet refers to rats fed the mix of *Lactobacillus* and *Bifidobacteria* dietary supplement. The metabolites profiles of control and diet treated rats was markedly different in both brain regions.

Figure 4. **A**, **B** and **C**, O-PLS-DA correlation coefficient loadings plots derived from <sup>1</sup>H-NMR spectra of frontal cortex indicating discrimination between probiotic treated and control rats. Peaks pointing upwards represent higher concentrations

of these metabolites in the diet-treated (probiotic) group compared with control group, and vice versa. The colour code corresponds to the correlation coefficient between the variables and the classification. The unit of the colour bar is  $r^2$ . Red peaks mean a high correlation coefficient value and blue peaks mean a low correlation ( $r^2$  is close to 0).

Figure 5. **A**, **B** and **C**, O-PLS-DA scores and coefficient plots derived from  $^1\text{H}$ -NMR spectra of the hippocampus indicating discrimination between probiotic treated and control rats. Peaks pointing upwards represent higher concentrations of these metabolites in the diet-treated (probiotic) group compared with control group, and vice versa. The colour code corresponds to the correlation coefficient between the variables and the classification. The unit of the colour bar is  $r^2$ . Red peaks mean a high correlation coefficient value and blue peaks mean a low correlation ( $r^2$  is close to 0).