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

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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}H nuclear magnetic resonance (^{1}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 aging rats. ^{1}H NMR analysis revealed diet-related regional-specific changes in brain metabolites; which indicated changes in several pathways contributing to modulation of neural signaling. 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.

1. 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 (Burokas, Moloney, Dinan, & Cryan, 2015; Slyepchenko, Carvalho, Cha, Kasper, & McIntyre, 2014; Tillisch et al., 2013). Communication between the gut and the brain appears to be bidirectional and includes neural, endocrine, immune and microbial metabolite pathways (Carabotti, Scirocco, Maselli, & Severi, 2015). The gut microbiome is established in early life and can be influenced by diet, infection, stress, medication and aging (Distrutti et al., 2014; Hopkins, Sharp, & Macfarlane, 2001; Scott et al., 2017; Yatsunenko et al., 2012). 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, Williams, & Brown, 2007; Distrutti et al., 2014; Mello, Paroni, Daragjati, & Pilotto, 2016; Messaoudi et al., 2011; Tillisch et al., 2013).

The gut microbiome changes with maturation, especially in the elderly (Arboleya, Watkins, Stanton, & Ross, 2016; Claesson et al., 2011; Leung & Thuret, 2015; Zapata & Quagliarello, 2015) and this change may influence aspects of brain function and behaviour, for

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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, Garrett, Clarke, Bienenstock, & Dinan, 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, Koliada, & Marotta, 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, Sellaro, van Hemert, Bosch, & Colzato, 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, Desbonnet et al. (2010) showed that *Bifidobaterium 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-insoitol ratios in the brain of patients with minimal hepatic encephalopathy (Ziada, Soliman, El Yamany, Hamisa, & Hasan, 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., Distrutti et al., 2014; Tillisch et al., 2013; 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; Bannerman et al., 2004; Moser, Moser, & Andersen, 1993) 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, King, Foong, Rizos, & Hong, 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 aging (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, Tramullas, Kiely, Dinan, and Cryan (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 aging 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 ^{1}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., Gruber et al., 2008; Schuff et al., 2001; and rodents (Harris et al., 2014, 2015; Paban, Fauvelle, & Alescio-Lautier, 2010; Zhang et al., 2009; see also Haga, Khor, Farrall, & Wardlaw, 2009). The impact of gut bacteria supplements on brain metabolites in aging 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.

2. Methods

2.1. 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 400 g to 560 g 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 12 h light-dark cycle with lights on at 7 am. Testing occurred during lights on hours. The tempera-