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In vitro neuroprotective activities of two distinct probiotic consortia

D.R. Michael[1*], T.S. Davies[1], K.E. Loxley[1], M.D. Allen[1], M.A. Good[2], T.R. Hughes[3] and S.F. Plummer[1]

[1]Cultech Limited, Unit 2 Christchurch Road, Baglan Industrial Park, Port Talbot, SA12 7BZ, United Kingdom; [2]School of Psychology, Tower Building, Cardiff University, Cardiff CF10 3AT, United Kingdom; [3]Systems Immunity Research Institute, Henry Welcome Building, Cardiff University, Cardiff CF14 4XN, United Kingdom; darynm@cultech.co.uk

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RESEARCH ARTICLE

Abstract

Neurodegeneration has been linked to changes in the gut microbiota and this study compares the neuroprotective capability of two bacterial consortia, known as Lab4 and Lab4b, using the established SH-SY5Y neuronal cell model. Firstly, varying total antioxidant capacities (TAC) were identified in the intact cells from each consortia and their secreted metabolites, referred to as conditioned media (CM). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Crystal Violet (CV) assays of cell viability revealed that Lab4 CM and Lab4b CM could induce similar levels of proliferation in SH-SY5Y cells and, despite divergent TAC, possessed a comparable ability to protect undifferentiated and retinoic acid-differentiated cells from the cytotoxic actions of rotenone and undifferentiated cells from the cytotoxic actions of 1-methyl-4-phenylpyridinium iodide (MPP+). Lab4 CM and Lab4b CM also had the ability to attenuate rotenone-induced apoptosis and necrosis with Lab4b inducing the greater effect. Both consortia showed an analogous ability to attenuate intracellular reactive oxygen species accumulation in SH-SY5Y cells although the differential upregulation of genes encoding glutathione reductase and superoxide dismutase by Lab4 CM and Lab4b CM, respectively, implicates the involvement of consortia-specific antioxidative mechanisms of action. This study implicates Lab4 and Lab4b as potential neuroprotective agents and justifies their inclusion in further *in vivo* studies.

Keywords: neurodegeneration, anti-oxidant, neurone, rotenone, *in vitro*

1. Introduction

Aging effects the physiology of every cell of the human body leading to changes and/or loss of function. The brain is particularly susceptible to deterioration and neurodegeneration; the progressive loss of structure or function of neurones, can be found to varying degrees in the brains of all age groups (Wyss-Coray, 2016). Such changes are irreversible, non-curative, contribute to age-related declines in cognitive function and memory and are a major risk factor for neurological disorders, including Parkinson's and Alzheimer's disease (Wahl *et al.*, 2017; Wyss-Coray, 2016). As the age of the population increases, so does the socio-economic burden of neurodegeneration thus highlighting the need to identify neuroprotective agents (Gillette-Guyonnet *et al.*, 2013; Lalkovičová and Danielisová, 2016; Wahl *et al.*, 2017; Wyss-Coray, 2016).

The mechanisms driving neurodegeneration are complicated and not fully understood although it is widely accepted that disturbances in hormonal balance, inflammation, metabolism and oxidative stress contribute. The relationship between the gut microbiota and the brain (the gut-brain-axis) and neurodegeneration is related to changes/imbalances in the composition of the gut microbiota causing decreases in population diversity and functionality (Dinan and Cryan, 2017). The gut-brain axis is a dynamic bi-directional system consisting of neurological, endocrine and immunological elements. If the microbial balance of the gut is disrupted, communication with the brain is disturbed and this has been associated with low grade inflammation, increased oxidative status, unbalanced energy homeostasis and increased cellular degeneration (Westfall *et al.*, 2017). Alterations of the gut microbiota composition can affect many brain biological processes (e.g. development and neurogenesis) and behavioural traits (e.g.

anxiety, depression, learning and memory). Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2006) and have been demonstrated to play a role in maintaining microbial homeostasis within the gut (Kumar *et al.*, 2016; Umbrello and Esposito, 2016). Indeed, probiotic organisms have shown beneficial effects on neurodegenerative disorders (Akbari *et al.*, 2016; Bonfili *et al.*, 2017, 2018; Kobayashi *et al.*, 2017; Nimgampalle and Kuna, 2017) and animal studies with probiotics have shown clear neuroprotective effects (Ait-Belgnaoui *et al.*, 2014; Distrutti *et al.*, 2014; Möhle *et al.*, 2016; Park *et al.*, 2017). The aetiology of neurodegeneration is complex and the functional diversity of probiotics may facilitate the simultaneous targeting of multiple disease-related mechanisms (Elufioye *et al.*, 2017; Faden and Stoica, 2007). Of particular interest are the antioxidant properties of such bacteria (Mishra *et al.*, 2015; Wang *et al.*, 2017) that can target the susceptibility of neurones to oxidative damage, a key driver of neurodegeneration (Liu *et al.*, 2017).

The Lab4 and Lab4b probiotic consortia comprise distinct populations of *Bifidobacterium* spp. and *Lactobacillus* spp. Supplementation with Lab4b has been shown to reduce the incidence of allergy in infants (Allen *et al.*, 2010, 2014) whilst Lab4 impacted on both inflammation (Davies *et al.*, 2018; Garaiova *et al.*, 2015; Hepburn *et al.*, 2013) and metabolism (Michael *et al.*, 2016, 2017). Some probiotic bacteria are known producers of metabolites with neuromodulatory capability (Sarkar *et al.*, 2016) and Lab4 has been shown to improve memory and alter brain metabolite profiles in aging rats (O'Hagan *et al.*, 2017). The aim of this *in vitro* study was to compare the neuroprotective potential of two distinct microbial populations (Lab4 and Lab4b) by assessing their ability to protect SH-SY5Y neuronal cells against oxidative stress and investigate the potential modes of action of the two different consortia.

2. Methods and reagents

Materials and reagents

All materials and reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Preparation of bacterial cultures

DeMan Rogosa Sharpe (MRS) broth (10 ml) was inoculated with freeze dried powder (10 mg) of Lab4 (composed of *Lactobacillus acidophilus* CUL21 (NCIMB 30156), *L. acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis* subsp. *lactis* CUL34 (NCIMB 30172)) or Lab4b (composed of *Lactobacillus salivarius* CUL61 (NCIMB 30211), *Lactobacillus paracasei* CUL08 (NCIMB 30154), *Bifidobacterium bifidum* CUL20 (NCIMB 30153), *B. animalis* subsp. *lactis* CUL34 (NCIMB 30172)) and incubated anaerobically (10% carbon dioxide, 10% hydrogen and 80% nitrogen) without shaking at 37 °C for 18 h.

Preparation of Lab4 and Lab4b conditioned media

Bacteria were harvested from 18 h cultures by centrifugation (2,500×*g* for 20 min), suspended in phosphate buffered saline (PBS), and centrifuged (2,500×*g* for 20 min). The resulting bacterial pellet was adjusted to a concentration of 1×10^9 cfu/ml in a 1:1 mix of Dulbecco's Modified Eagle's medium and Ham F-12 medium (DMEM/F12) and incubated under anaerobic conditions at 37 °C for 5 h, centrifuged (2,500×*g* for 20 min) and the supernatant passed through a 0.22 µm filter (Gilson, Bedfordshire, UK) to produce the conditioned media (CM) preparation. The CM were adjusted to pH 7.4 using 1 M NaOH and supplemented with 100 U/ml penicillin and 100 U/ml streptomycin (Labtech, Heathfield, UK). Enumeration at the start and end of the 5 h incubation period confirmed that no change in bacterial number occurred during this period (data not shown). DMEM/F12 supplemented with 100 U/ml penicillin and 100 U/ml streptomycin was used as a diluent for the preparation of CM doses and as a control for CM.

Total antioxidant capacity

The total antioxidant capacity (TAC) of intact cells and CM was determined using the Total Antioxidant Capacity Assay Kit (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions. Prior to measurement, intact cells were washed twice in phosphate buffered saline (PBS) to remove all traces of culture media and the TAC value for PBS subtracted from experimental values. The TAC of serum-free DMEM/F12 was subtracted from CM values. Data are presented as the mean Trolox equivalent antioxidant capacity (mM).

SH-SY5Y cell culture, differentiation and stimulation

SH-SY5Y cells were maintained in DMEM/F12 (Labtech) supplemented with 10% (v/v) heat inactivated foetal bovine serum (Labtech), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in 5% CO_2 and 95% humidity. Approximately 24 h before experimentation, cells of ~80% confluency were seeded at 5×10^5 cells/cm^2 into appropriate tissue culture plates (Costar, Cambridge, UK). Cells between passage 21 and 30 were used in this study. Where differentiation was required, SH-SY5Y were incubated with DMEM/F12 supplemented with 1% (v/v) heat inactivated foetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and 10 µM all-trans retinoic acid (ATRA) for 5 days. Differentiating cells were incubated under light-free conditions and the media changed every 2 days. Rotenone was dissolved in dimethyl sulfoxide (DMSO) and applied

to SH-SY5Y cells at a concentration of 250 nM for viability and apoptosis/necrosis assays (Supplementary Figure S1A) or 10 µM for intracellular reactive oxygen species (ROS) assays (Supplementary Figure S1B). DMSO was also included at the relevant concentration in the controls for these experiments and is referred to as the vehicle control. 1-Methyl-4-phenylpyridinium iodide (MPP+) was dissolved in water that was included at the relevant concentration in the controls for these experiments and is referred to as the vehicle control.

MTT-assay

Following exposure to experimental conditions, SH-SY5Y cells were washed with 200 µl of warm PBS (pH 7.4) before incubation with 100 µl of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (500 µg/ml in DMEM/F12) for 2 h in 5% CO_2 and 95% humidity. The cells were then washed in 200 µl of PBS (pH 7.4, 37 °C) before the addition of 100 µl of DMSO. The absorbance at 570 nm was read using a colorimetric spectrophotometer and viability was expressed as percentage survival compared to untreated cells or cells treated with the appropriate vehicle that have been arbitrarily assigned as 100%.

Crystal violet assay

Following exposure to experimental conditions, SH-SY5Y cells were washed with 200 µl of PBS (pH 7.4, 37 °C) before incubation with 0.2% (w/v) crystal violet solution (in 10% (v/v) ethanol) for 5 min at room temperature. The cells were then washed three times in PBS (pH 7.4, 37 °C) and left to dry at room temperature overnight before 150 µl of 10% acetic acid solution (v/v) was added to each well and the absorbance read at 595 nm using a colorimetric spectrophotometer. Viability is expressed as percentage survival compared to untreated cells or cells treated with the appropriate vehicle that have been arbitrarily assigned as 100%.

Apoptosis and necrosis assay

Levels of apoptosis and necrosis were measured in SH-SY5Y cells exposed to experimental conditions using the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

Intracellular reactive oxygen species assay

Intracellular ROS levels were measured in SH-SY5Y cells exposed to experimental conditions using the DCFDA Cellular ROS Detection Assay Kit (Abcam) in accordance with the manufacturer's instructions. SH-SY5Y cells were cultured in phenol red-free DMEM/F12 (Gibco, Paisley, UK) to eliminate assay interference by phenol red. Intracellular ROS is expressed as a percentage compared to cells treated with the appropriate vehicle that have been arbitrarily assigned as 100%.

RT-qPCR

Total RNA was isolated using Ribozol (Amresco LLC, Solon, OH, USA) and cDNA generated using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley, UK) in accordance to the manufacturer's instructions. Real-time qPCR amplification was performed as previously described (Michael *et al.*, 2016) using the oligonucleotide primers shown in Supplementary Table S1. Ratios of gene expression compared to untreated cells were determined using $2^{-(\Delta Ct1 - \Delta Ct2)}$, where ΔCt represents the difference between the threshold cycle (CT) for each target gene and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin mRNA transcript levels.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) of the assigned number of independent experiments. The normality of all data was assessed using histograms and Q-Q plots and data transformations were applied where appropriate. For single comparisons, values of P were determined using a Student's t-test. For multiple comparisons, P-values were determined using one-way analysis of variance (ANOVA) with Tukey's or Dunnett's T3 post-hoc analysis or, where the data was not normally distributed, Kruskal-Wallis ANOVA with Dunns post-hoc analysis. All statistical tests were performed using SPSS statistical software package version 22 (IBM, Armonk, NY, USA). Differences were considered significant when $P<0.05$.

3. Results

Lab4 and Lab4b consortia possess antioxidant capacity

Antioxidant activity was observed in three independent cultures of whole cells of the Lab4 and Lab4b consortia (0.105±0.010 and 0.233±0.023 mM, respectively) and CM preparations (0.735±0.052 and 0.084±0.057 mM, respectively). Significant differences were observed in whole cell preparations of Lab4 compared to Lab4b (P=0.042) and in CM from Lab4 compared to Lab4b (P=0.026).

Lab4 CM and Lab4b CM can impact upon the viability of undifferentiated SH-SY5Y cells

Undifferentiated SH-SY5Y cells were exposed to various doses of Lab4 and Lab4b CM for 48 h and viability was assessed using the MTT assay (Figure 1A). There was a trend of improved viability at all doses that reached significance for the 10% Lab4 CM dose (14.8%, P=0.031) compared to the control. Similar results were obtained using the CV

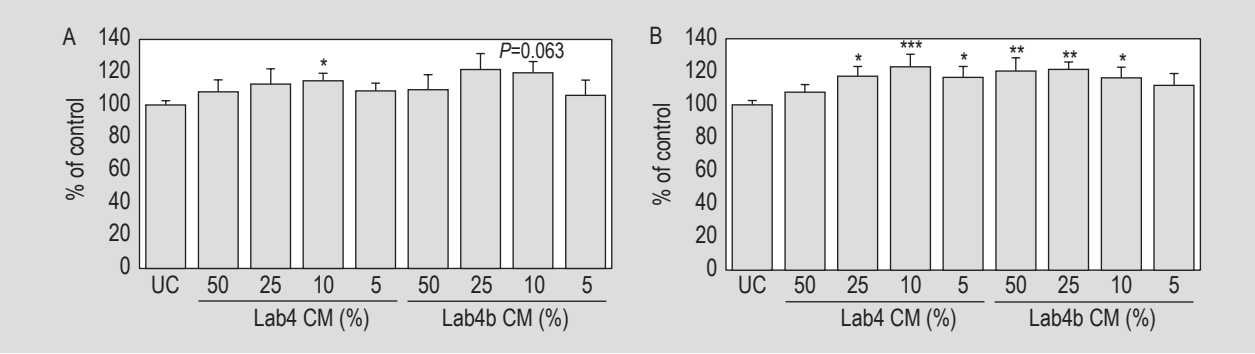

Figure 1. The effect of Lab4 and Lab4b CM on the viability of undifferentiated SH-SY5Y cells. The viability of undifferentiated SH-SY5Y cells that were treated with either serum-free DMEM/F12 (untreated control, UC) or treated with various doses of Lab4 or Lab4b CM for 48 h was assessed by (A) MTT assay or (B) Crystal Violet assay. Data are expressed as a percentage of the control that has been arbitrarily assigned as 100%. The data are presented as the mean ± standard error of the mean of four independent experiments. * *P*<0.05, ** *P*<0.01, * *P*<0.001 or as stated vs untreated cells.**

assay (Figure 1B) where significant improvements were observed for 25% (17.4%, *P*=0.018), 10% (23.1%, *P*<0.001) and 5% (16.6%, *P*=0.031) CM from Lab4 and 50% (20.5%, *P*=0.002), 25% (21.4%, *P*=0.001) and 10% (16.4%, *P*=0.034) CM from Lab4b compared to the control. No statistically significant differences were observed between doses or between Lab4 and Lab4b in either assay.

Lab4 CM and Lab4b CM protect undifferentiated SH-SY5Y cells against rotenone-induced loss of viability

The toxicity of doses of rotenone in undifferentiated SH-SY5Y cells was measured (Supplementary Figure S1A). Survival rates in SH-SY5Y cells exposed to 250 nM rotenone were 53.4% (*P*<0.001) of the control (Figure 2A) and survival rates in the presence of rotenone were improved up to 72.1% (*P*=0.006) and 67.0% (*P*=0.130) with Lab4 and Lab4b CM, respectively (see Supplementary Figure S2 for optimisation experiments). This neuroprotective capability was confirmed in CV assays (Figure 2B) where the survival rate of 75.9% (*P*<0.001) in rotenone exposed cells increased to 91.9% (*P*=0.062) in the presence of Lab4 CM and 96.9% (*P*=0.007) in the presence of Lab4b CM. No statistically significant differences were observed between Lab4 and Lab4b in these experiments.

Pre-incubation with Lab4 CM or Lab4b CM protects undifferentiated SH-SY5Y cells against rotenone-induced loss of viability

Undifferentiated SH-SY5Y cells were incubated with Lab4 or Lab4b (50% CM) for 2 h prior to the exposure of the neuronal cells to 250 nM rotenone. A survival rate of 70.9% (*P*<0.001) was observed in cells treated with rotenone and this increased to 86.7% (*P*=0.053) in Lab4 and 90.3% (*P*=0.033) in Lab4b pre-treated cells (Figure 2C). These findings were corroborated using CV assays (Figure 2D) where the neuronal survival rate increased from 56.2% (*P*<0.001) in rotenone-treated cells to 69.4% (*P*=0.035) and 62.5% (*P*=0.523) in the Lab4 and Lab4b pre-treated cells, respectively. No statistically significant differences were observed between Lab4 and Lab4b in these experiments.

Protection of ATRA-differentiated SH-SY5Y cells against rotenone-induced loss of viability

SH-SY5Y cell were incubated with 10 μM ATRA for 5 days to induce differentiation as described elsewhere (Kovalevich and Langford, 2013) and changes to cell morphology and gene expression levels of tropomyosin receptor kinase B (Kaplan *et al.*, 1993) confirmed the responsiveness of SH-SY5Y cells to these conditions (Supplementary Figure S4). In ATRA-treated SH-SY5Y cells (Figure 2E), 84.8% (*P*<0.001) of cells remained viable after rotenone exposure compared to the control and the survival rate increased to 104.9% (*P*<0.001) and 98.5% (*P*=0.029) as a result of co-incubation with Lab4 and Lab4b CM respectively. Pre-incubation for 2 h with Lab4 and Lab4b CM prior to rotenone challenge (Figure 2F) significantly improved the survival rate from 86.2% (*P*<0.001) in rotenone-treated cells to 109.3% (*P*<0.001) and 107.2% (*P*<0.001), respectively. These improvements in survival rate were also significantly different compared to the control (*P*<0.001 and *P*=0.013 respectively). No statistically significant differences were observed between Lab4 and Lab4b in these experiments.

Lab4 CM and Lab4b CM protect undifferentiated SH-SY5Y cells against MPP+ induced loss of viability

The toxicity of doses of MPP+ in undifferentiated SH-SY5Y cells was measured (Supplementary Figure S3) and exposure to 2 μM MPP+ reduced viability to 62.4% (*P*<0.001) of the control in these cells (Figure 3A). In the presence of CM from Lab4 and Lab4 there was indication of improved survival rates at all concentrations tested compared to rotenone-treated cells although statistical

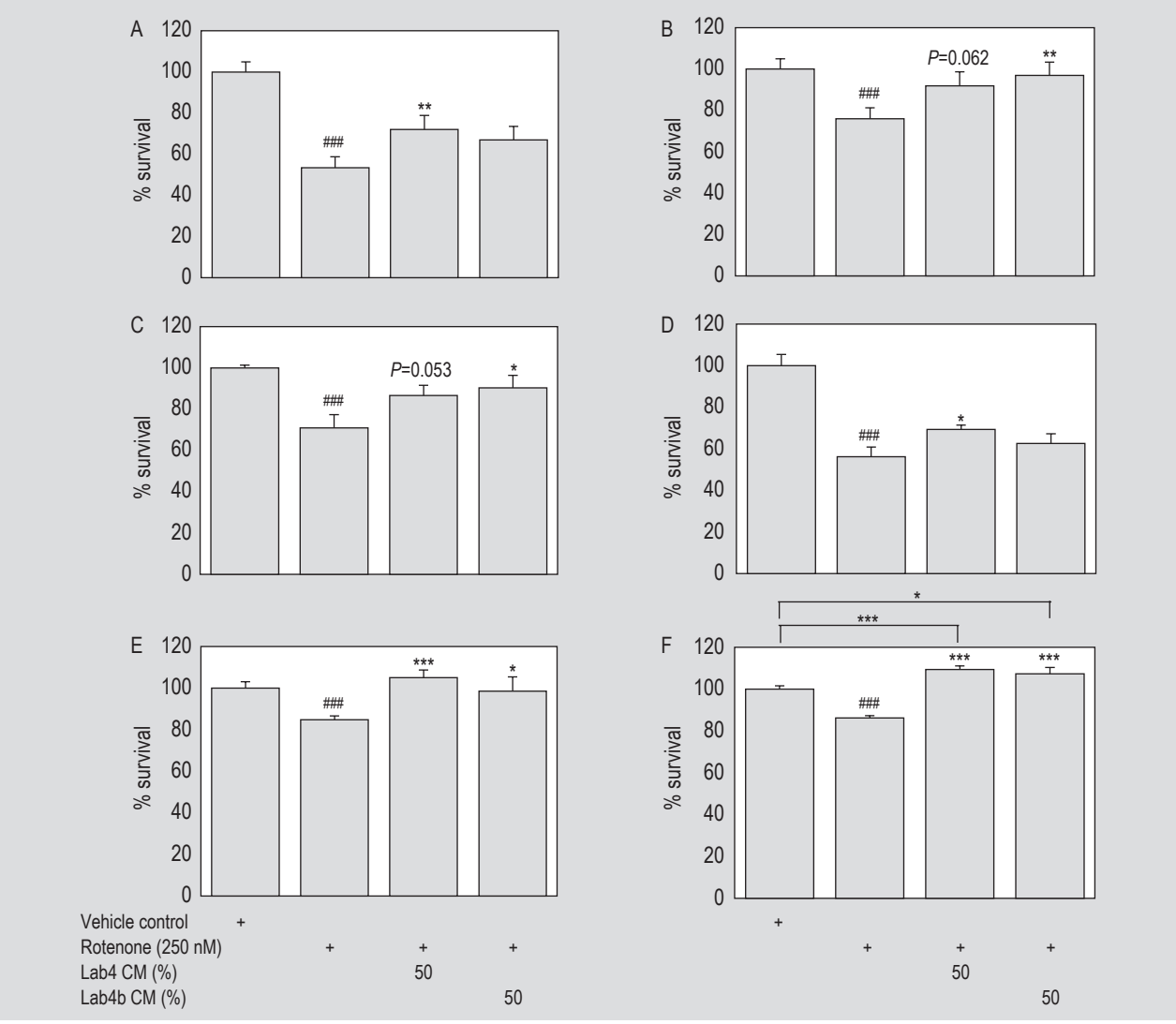

Figure 2. The effect of Lab4 and Lab4b CM on rotenone-induced toxicity in undifferentiated and ATRA-differentiated SH-SY5Y cells. The viability of undifferentiated SH-SY5Y cells that were treated with the vehicle control (DMSO) or 250 nM rotenone or 250 nM rotenone with Lab4 or Lab4b CM for 24 h was assessed by (A) MTT assay or (B) Crystal Violet assay. The viability of undifferentiated SH-SY5Y cells that were pre-incubated with serum-free DMEM/F12 or CM from Lab4 or Lab4b for 2 h prior to the addition of the vehicle control (DMSO) or 250 nM rotenone for 24 h was assessed by (C) MTT assay or (D) Crystal Violet assay. The viability of differentiated SH-SY5Y cells (10 µM ATRA and 1% FCS for 5 days) that were treated with (E) vehicle control (DMSO) or 250 nM rotenone or 250 nM rotenone with Lab4 or Lab4b CM or (F) pre-incubated with serum-free DMEM/F12 or 250 nM rotenone for 24 h was assessed by MTT assay. Data are expressed as percentage survival compared to the vehicle control that has been arbitrarily assigned as 100%. The data are presented as the mean ± standard error of the mean of at least three independent experiments. * *P*<0.05, ** *P*<0.01, * *P*<0.001 or as stated vs the rotenone-treated cells or ### *P*<0.001 vs the vehicle control.**

significance was not achieved. In CV assays, exposure to 2 µM MPP+ reduced viability to 82.6% ($P<0.001$) of the control (Figure 3B) and indication of improved survival was observed upon the addition of Lab4 and Lab4b at all concentrations tested that, in the case of 25 and 50% CM from Lab4b, were significantly improved to 108.2% ($P=0.033$) and 99.9% ($P=0.010$), respectively. No statistically significant differences were observed between Lab4 and Lab4b in these experiments.

Lab4 and Lab4b CM protect undifferentiated SH-SY5Y cells against rotenone-induced apoptosis and necrosis

Exposure of undifferentiated SH-SY5Y cells to 250 nM rotenone for 24 h resulted in a significant 1.74-fold ($P<0.001$) increase in apoptosis compared to the untreated control (Figure 4A) and in the presence of Lab4 and Lab4b CM, reduced apoptotic activity was observed (1.54-fold, $P=0.040$ and 1.18-fold, $P<0.001$ respectively). After 48 h exposure (Figure 4B), the levels of apoptosis were lower but similar

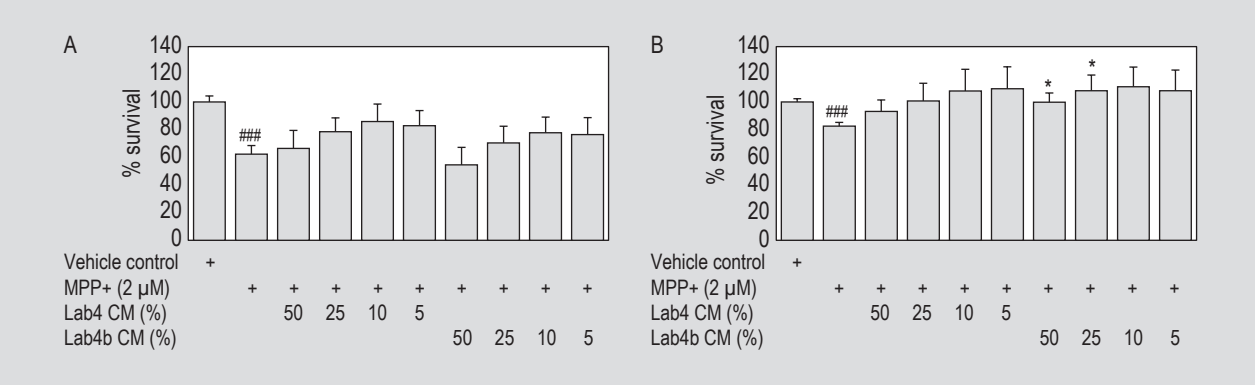

Figure 3. The effect of Lab4 and Lab4b CM on 1-methyl-4-phenylpyridinium iodide (MPP+)-induced toxicity in undifferentiated SH-SY5Y cells. The viability of undifferentiated SH-SY5Y cells that were treated with the vehicle control (water) or 2 µM MPP+ or 2 µM MPP+ plus the stated concentration of Lab4 or Lab4b CM for 24 h was assessed by (A) MTT assay or (B) Crystal Violet assay. Data are expressed as percentage survival compared to the vehicle control that has been arbitrarily assigned as 100%. The data are presented as the mean ± standard error of the mean of at least five independent experiments. * *P*<0.05 vs the MPP+-treated cells or ### *P*<0.001 vs the vehicle control.

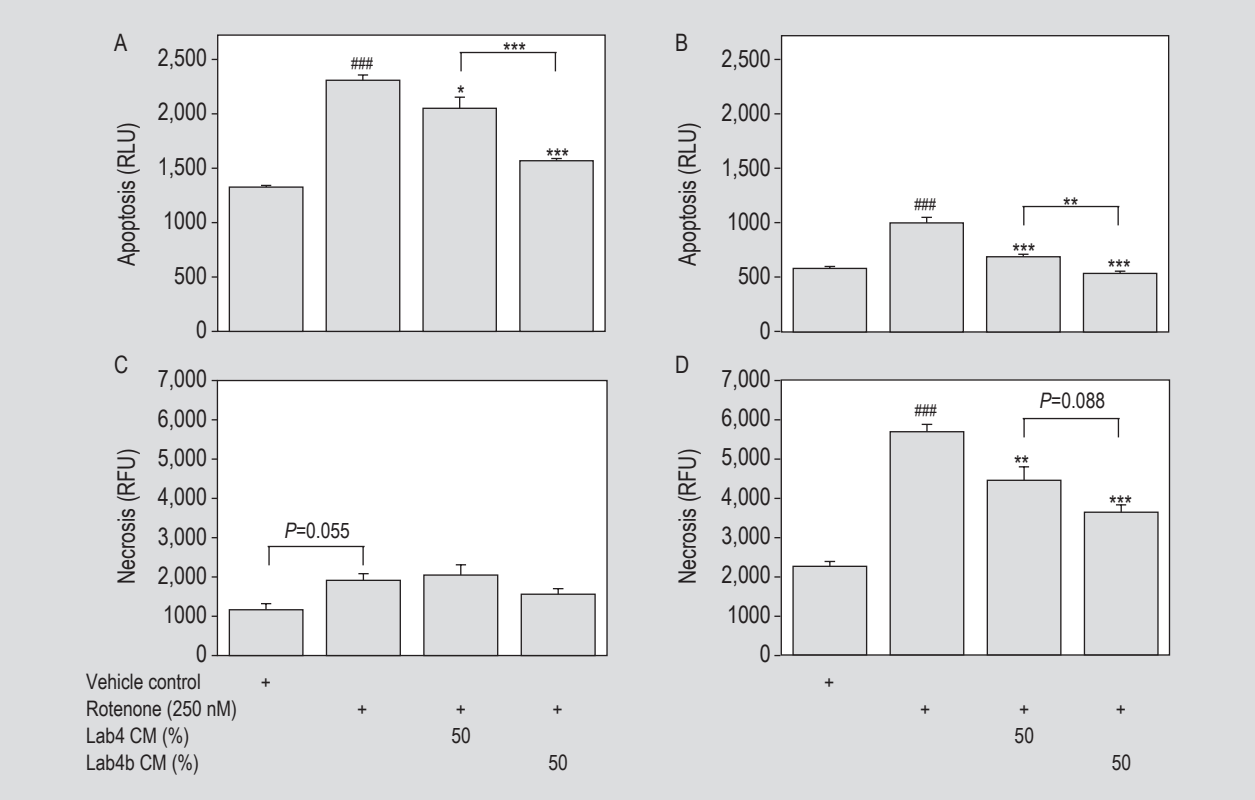

Figure 4. The effect of Lab4 and Lab4b CM on rotenone-induced apoptosis and necrosis in undifferentiated SH-SY5Y cells. Levels of apoptosis (A+B) or necrosis (C+D) were assessed in undifferentiated SH-SY5Y cells that were treated with the vehicle control (DMSO) or 250 nM rotenone or 250 nM rotenone with Lab4 or Lab4b CM for 24 h (A+C) or 48 h (C+D). The data are presented as the mean ± standard error of the mean of relative luminescent units (RLU, A+B) or relative fluorescent units (RFU, C+D) from five independent experiments. * *P*<0.05, ** *P*<0.01 and * *P*<0.001 vs rotenone-treated cells or ### *P*<0.001 vs the vehicle control.**

patterns of change were observed with rotenone inducing a 1.71-fold increase ($P<0.001$) that was reduced to 1.18-fold ($P<0.001$) and 0.92-fold ($P<0.001$) in the presence of Lab4 and Lab4b CM, respectively. Reductions in apoptosis were significantly greater with Lab4b compared to Lab4 at both 24 h ($P<0.001$, Figure 4A) and 48 h ($P=0.001$, Figure 4B).

Apoptosis precedes secondary necrosis and at 24 h low levels of necrosis were detected (Figure 4C) with a trend towards increased levels of necrosis in rotenone-treated cells when compared to the control (1.64-fold, P=0.055, Figure 4C) that remained unchanged in the presence of Lab4 and Lab4b. Higher levels of necrosis were detected at 48 h and rotenone exposure induced a significant increase compared to the control (2.52-fold, P<0.001, Figure 4D). In the presence of Lab4 or Lab4b necrosis rates were 1.97-fold (P=0.006) and 1.60-fold (P<0.001), respectively, in line with the reduced apoptotic activity. Lab4b showed a trend towards increased capability compared to Lab4 (P=0.088, Figure 4D) similar to the effects observed during apoptosis.

Lab4 and Lab4b CM protect undifferentiated cells against ROS and induce genes involved in antioxidant defence

Exposure of undifferentiated SY-SY5Y cells to 10 µM rotenone for 6 h increased cellular ROS levels by 1.43-fold (P<0.001) compared to the control and the degree of this effect was significantly reduced to 1.14-fold (P<0.001) in the presence of Lab4 CM and 1.23-fold (P=0.002) in the presence of Lab4b CM (Figure 5). No significant differences were observed between Lab4 and Lab4b. At rotenone concentrations between 250 nM and 10 µM, no increase in intracellular ROS levels was detected (Supplementary Figure S1B).

The expression of key genes involved in endogenous antioxidant defence in undifferentiated SH-SY5Y cells exposed to Lab4 and Lab4b for 24 h can be seen in Table 1 and demonstrates a significant 1.51-fold (P=0.022) induction of glutathione reductase (GSR) expression with Lab4 and an 18.12-fold (P<0.001) induction in superoxide dismutase 2 (SOD2) expression in those treated with Lab4b. No changes in expression were observed for superoxide dismutase 1 (SOD1), glutathione peroxidase (GPx1) or catalase nor any differences between Lab4 and Lab4b for any gene.

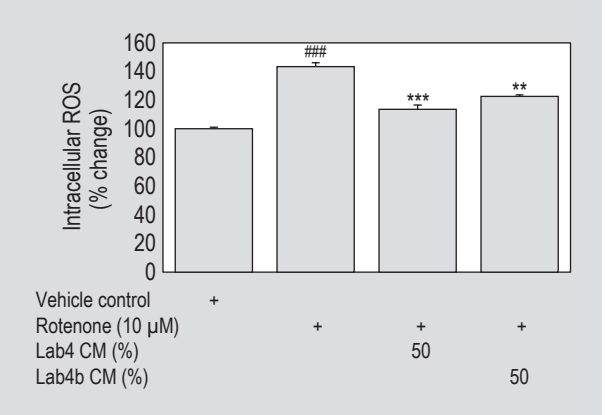

Figure 5. The effect of Lab4 and Lab4b CM on rotenone-induced cellular ROS accumulation in undifferentiated SH-SY5Y cells. Intracellular ROS levels in undifferentiated SH-SY5Y cells that were treated with the vehicle control (DMSO) or 10 µM rotenone or 10 µM rotenone with Lab4 or Lab4b CM for 6 h. Data are expressed as a percentage compared to the vehicle control that has been arbitrarily assigned as 100%. The data is presented as the mean ± standard error of the mean of four independent experiments. ** P<0.01, *** P<0.001 vs rotenone-treated cells or ### P<0.001 vs the vehicle control.

4. Discussion

In an SH-SY5Y neuronal model, both Lab4 and Lab4b populations of microorganisms induced neuroprotective effects on challenged cells. Both consortia were found to possess antioxidant activity and were able to induce proliferation in SH-SY5Y cells. When either undifferentiated or retinoic acid-differentiated SH-SY5Y cells were challenged with the neurotoxin, rotenone, pre- or co-exposure of the cells to Lab4 or Lab4b partly prevented rotenone-induced loss of viability. There were also protective effects against the development of apoptosis and necrosis and the intracellular accumulation of ROS.

Neurodegeneration occurs as a result of oxidative damage (Westfall *et al.*, 2017) and there is a growing interest in the role of probiotic bacteria with the ability to impact upon the antioxidant capacity of the host (Wang *et al.*, 2017). Improvements in oxidative status alongside improved behaviour have been observed in an Alzheimer's disease mouse model supplemented with a multi-strain probiotic composed of strains of *Bifidobacterium* and *Lactobacillus* (Bonfili *et al.*, 2017, 2018). Probiotics are thought to impart antioxidant effects by modulating endogenous antioxidant defence mechanisms and/or via the secretion of metabolites with antioxidant properties (Wang *et al.*, 2017). Antioxidant activities were detected in both viable cells and conditioned media of Lab4 and Lab4b, albeit at differing levels, and

Table 1. Changes in gene expression of key antioxidant genes in undifferentiated SH-SY5Y cells exposed to CM from Lab4 and Lab4b.[1]

Gene	Lab4	Lab4b
Superoxide dismutase 1	1.19±0.40	0.85±0.21
Superoxide dismutase 2	1.41±0.15	18.12±5.75###
Glutathione peroxidase	1.06±0.15	0.80±0.12
Glutathione reductase	1.51±0.07#	1.18±0.08
Catalase	1.09±0.11	0.80±0.17

[1] Gene expression is represented as a ratio in relation to an untreated control and is presented as the mean ± standard error of the mean of three independent experiments. # P<0.05 and ### P<0.001 vs the untreated control.

highlighted both consortia as potential neuroprotective candidates.

Neurodegenerative disorders and age-related cognitive decline are associated with impaired neurogenesis; the process by which stem cells differentiate and proliferate into new neurones and other brain cells (Winner and Winkler, 2015). In our study, Lab4 and Lab4b induced proliferation in both undifferentiated SH-SY5Y cells (Figure 1) and rotenone-challenged ATRA differentiated SH-SY5Y cells (Figures 2F). The multi-strain probiotic, VSL#3, has been shown to fully restore defective neurogenesis in antibiotic-treated mice (Möhle *et al.*, 2016) and a probiotic comprising *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 supported mice exposed to chronic stress (Ait-Belgnaoui *et al.*, 2014). Furthermore, other dietary antioxidants, such as the catechins found in green tea, have been shown to induce proliferation in undifferentiated SH-SY5Y cells and improve cognition in a murine model of brain aging (Pervin *et al.*, 2017).

Neuronal cells are particularly vulnerable to oxidative damage (Lalkovičová and Danielisová, 2016) and both consortia possessed a comparable ability, as pre- and/or co-treatments, to attenuate losses in undifferentiated SH-SY5Y cell viability in response to two well characterised neurotoxins and mediators of oxidative stress; rotenone and MPP+ (Giordano *et al.*, 2012). The effects against rotenone are supported by the ability of both consortia to attenuate apoptosis and necrosis with Lab4b showing more activity than Lab4. These findings are consistent with an existing study showing the ability of glyceryl 1,3-dipalmitate, purified from the fermentation products of *Lactobacillus paracasei* subsp. *paracasei* NTU 101, to protect undifferentiated SH-SY5Y cells from oxygen-glucose deprivation and reperfusion-induced oxidative stress (Cheng and Pan, 2017). Metabolites induced by the intestinal commensal, *Ruminococcus albus,* have also been shown to protect undifferentiated cells from hydrogen peroxide, MPP+ and sodium arsenate-induced oxidative damage (Park *et al.*, 2017).

Previous probiotic studies in the SH-SY5Y model have been performed on undifferentiated (naïve) cells (Cheng and Pan, 2017; Park *et al.*, 2017) and in the current study we have shown that the neuroprotective effects of the Lab4 and Lab4b consortia CM against the effects of rotenone, as pre- or co-incubations, are conserved in retinoic acid-differentiated SH-SY5Y cells. Studies with differentiated SH-SY5Y cells are often favoured as these may represent a more relevant physiological format (Korecka *et al.*, 2013; Lopes *et al.*, 2010). It is worthy of note that Jantas *et al.* (2013) found that 10 μM rotenone had little impact on the viability of undifferentiated cells that had been pre-exposed to reduced serum levels for 24 h whereas retinoic acid-differentiated cells were susceptible to rotenone under the same conditions (Jantas *et al.*, 2013). This is in contrast to our study where the susceptibility of SH-SY5Y cells to rotenone was reduced upon retinoic acid-differentiation.

The mechanisms by which probiotic bacteria may impart neuroprotective effects have not been fully elucidated. Rotenone inhibits complex I of the mitochondrial electron transport chain leading to cellular ROS accumulation (Giordano *et al.*, 2012) and our results indicate that Lab4 and Lab4b possess comparable abilities to attenuate ROS accumulation in SH-SY5Y cells. Cellular ROS levels are controlled, in part, by glutathione reductase, an enzyme that maintains intracellular pools of reduced glutathione (Espinosa-Diez *et al.*, 2015), and expression levels of the encoding gene (GSR) were found to be elevated in the presence of Lab4. Another enzyme, manganese-dependant superoxide dismutase (MnSOD), also contributes to intracellular ROS homeostasis by converting superoxide into oxygen and hydrogen peroxide (Espinosa-Diez *et al.*, 2015) and the expression of the encoding gene (SOD2) was induced in the presence of Lab4b. These data suggest the feasibility of different modes of action for Lab4 and Lab4b. These observations are supported by other studies showing reduced levels of ROS and increased levels of reduced glutathione and superoxide dismutase activity in the brains of Alzheimer's disease model mice supplemented with the VSL#3 probiotic (Bonfili *et al.*, 2018) and similar effects were seen with sodium arsenate-treated mice supplemented with *Ruminococcus albus* (Park *et al.*, 2017). Future studies would investigate the ability of Lab4 and Lab4b CM to modulate the activity of complex I of the electron transport chain. 1-methylnicotinamde has been shown to impart neuroprotective effects by increasing complex I activity in rotenone-treated SH-SY5Y cells (Parsons *et al.*, 2011).

Two potential limitations of our study involve firstly the duration of ATRA exposure used for the differentiation of SH-SY5Y cells. While there appears to be no consensus methodology for this process (Kovalevich and Langford, 2013; Xicoy *et al.*, 2017), Lopes *et al.* (2010) required 7 days incubation to achieve a dopaminergic phenotype (Lopes *et al.*, 2010) thus highlighting the possibility that 5 days incubation may be insufficient to reach terminal differentiation. Secondly, the metabolite profiles for the Lab4 and Lab4b consortia have not been determined. In the study of O'Hagan *et al.* (2017) rats supplemented daily with the Lab4 consortium from weaning through to sacrifice (18 months) had increased brain levels of lactate, myo-inositol, γ-aminobutyric acid, fumarate, inosine and alanine (O'Hagan *et al.*, 2017) for which there is reported evidence of antioxidant activity (Chen *et al.*, 2013; Grosser *et al.*, 2004; Groussard *et al.*, 2000; Gudkov *et al.*, 2006; Jiang *et al.*, 2011; Lin *et al.*, 2016). Lactate is a major metabolic end-product of lactobacilli (Klein *et al.*, 1998) which is able to pass through the blood-brain barrier (Proia *et al.*, 2016).

In summary, this *in vitro* study indicates that the conditioned media from the Lab4 and Lab4b probiotic consortia may have potential neuroprotective properties as demonstrated by the observed ability to protect SH-SY5Y cells against the actions of rotenone with distinct mechanisms of action attributed to each consortium. This study provides a basis for future targeted *in vivo* studies comparing the efficacy of these potentially beneficial probiotic consortia.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2018.0105.

Figure S1. The impact of rotenone on cell viability and intracellular ROS accumulation in undifferentiated SH-SY5Y cells.

Figure S2. Lab4 and Lab4b exert a neuroprotective effect in rotenone-treated undifferentiated SH-SY5Y cells.

Figure S3. The impact of MPP+ on cell viability in undifferentiated SH-SY5Y cells.

Figure S4. Differentiation of SH-SY5Y cells with all-trans retinoic acid.

Table S1. Oligonucleotide primer sequences.

Conflict of interest

This study was supported by Cultech Ltd, Port Talbot, UK of which DRM, TSD, KEL, MDA and SFP are or were employees.

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