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Lactobacilli-fermented cow's milk attenuated lipopolysaccharide-induced neuroinflammation and memory impairment in vitro and in vivo

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Nutritional interventions are now recommended as strategies to delay Alzheimer's disease (AD) progression. The present study evaluated the neuroprotective effect (anti-inflammation) of lactic acid bacteria (either *Lactobacillus fermentum* LAB9 or *L. casei* LABPC) fermented cow's milk (CM) against lipopolysaccharide (LPS)-activated microglial BV2 cells in vitro. The ability of CM-LAB in attenuating memory deficit in LPS-induced mice was also investigated. ICR mice were orally administered with CM-LAB for 28 d before induction of neuroinflammation by LPS. Learning and memory behaviour were assessed using the Morris Water Maze Test. Brain tissues were homogenised for measurement of acetylcholinesterase (AChE), antioxidative, lipid peroxidation (malondialdehyde (MDA)) and nitrosative stress (NO) parameters. Serum was collected for cytokine analysis. CM-LAB9 and CM-LABPC significantly ($P < 0{\cdot}05$) decreased NO level but did not affect CD40 expression in vitro. CM-LAB attenuated LPS-induced memory deficit in mice. This was accompanied by significant ($P < 0{\cdot}05$) increment of antioxidants (SOD, GSH, GPx) and reduction of MDA, AChE and also pro-inflammatory cytokines. Unfermented cow's milk (UCM) yielded greater cytokine lowering effect than CM-LAB. The present findings suggest that attenuation of LPS-induced neuroinflamation and memory deficit by CM-LAB could be mediated *via* anti-inflammation through inhibition of AChE and antioxidative activities.

Keywords: Alzheimer's disease, neuroinflammation, probiotics, fermented cow's milk, microglia.

Alzheimer's disease (AD), a neurodegenerative disease characterised by progressive decline in memory and cognitive function, affects more than 37 million individuals worldwide (Matthews, 2010). The pathogenesis of AD involves microglia activation and neuroinflammation. In mild cognitive impaired and AD brain, oxidative stress is reflected by increased oxidations of DNA, RNA, lipid and protein, and decreased total plasma antioxidant activity. Nutraceuticals with high antioxidant content like vitamin E (Alzoubi et al. 2012), ginger (Ha et al. 2012) and *Gingko biloba* (Mansour et al. 2011) have all demonstrated neuroprotection against neuroinflammation and cognitive impairment in vivo. Probiotics are also known to exhibit high antioxidant activity. Their role in protecting the CNS, however, is still not well-documented.

Probiotics are live microorganisms which confer health benefit to the host when administered in adequate amounts (FAO/WHO, 2001). There is increasing focus on how the reversed pathway of gut microbiota can affect CNS. Previous studies have demonstrated the effect of probiotics

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in preventing stress-induced memory deficits (Gareau et al. 2011), regulating emotional behaviour (Bravo et al. 2011), ameliorating memory impairment and inflammation (Woo et al. 2014), as well as improving memory (Yeon et al. 2010). Recently, fermented milk containing *L. helveticus* IDCC3801 was found to improve cognitive function in healthy older adults (Chung et al. 2014).

The present study addressed the hypothesis that Malaysian LAB-fermented cow's milk (CM-LAB) acting as a probioitic would provide cognitive benefit by attenuating neuroinflammation and memory impairment. The anti-inflammatory effect of fermented milk against activated BV2 microglial cells and the effect of orally administrated fermented milk against LPS-induced memory impairment in mice were determined. The mechanisms underlying the neuroprotective effect of CM-LAB were also elucidated.

Materials & methods

Bacterial strains and growth condition

Lactobacillus plantarum (LAB1, LAB11, LAB12) and *L. fermentum* (LAB9, LAB10) were previously isolated from Malaysian fermented food and milk products (Ramasamy et al. 2012). *L. casei* (LABPC) was isolated from Yakult cultured milk. All LAB were maintained in MRS broth (OXOID, Basingstoke, Hampshire, UK), and incubated at 37 °C. Stock cultures were stored in 15 % glycerol at −80 °C.

Sample preparation

Fresh cow's milk (CM) containing (per 100 ml) 3·2 g fat, 4·9 g carbohydrate, 3·2 g total sugar and 3·5 g protein was purchased from a local supplier in Kuala Lumpur, Malaysia and the nutritional information was analysed by Permulab Sdn. Bhd. The milk was autoclaved at 105 °C for 5 min, and stored (after cooling down) at 4 °C. Lactobacilli [5 % (v/v)] were sub-cultured in milk and incubated at 37 °C for 24 h. Prior to experimental use, each strain was sub-cultured three times. CM-LAB was then centrifuged (10 000***g***; 10 min). The supernatant was collected and filter-sterilised (0·2 µm). The resultant cell-free supernatant (CFS) was used for subsequent assays.

Cell culture

BV2 (an immortalised microglia cell line), originally a generous gift from A/Prof Dr Thameem Dheen of the National University Singapore, were provided by Dr Sharmili Vidyadaran (UPM). The cells were maintained in DMEM (high glucose) supplemented with 5 % heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 mg/ml gentamicin, 250 µg/ml fungizone (Invitrogen, Carlsbad, CA), 1 % nonessential amino acid (Sigma-Aldrich, St. Louis, MO), 6·25 µg/ml insulin (Sigma-Aldrich, St. Louis, MO). Cell cultures were incubated at 37 °C and 5 % CO_2.

Griess assay

BV2 cells were seeded onto the 96-well plate at $2{\cdot}5 \times 10^4$ cells/well and left to attach overnight. Cells were treated with CFS of each CM-LAB at 5–20 % (v/v) in triplicates for 24 h before exposure to 1 µg/ml LPS (*Escherichia coli* serotype O26:B6; Sigma-Aldrich, St. Louis, MO) in DMEM without phenol red (Invitrogen, Carlsbad, CA) supplemented with 5 % FBS. After incubation for 24 and 48 h in the presence of LPS, nitric oxide (NO) level was determined using the Griess reaction. Culture supernatants (50 µl) of the six CM-LAB were incubated with 50 µl of Griess reagent [1 % sulfanilamide and 0·1 % naphthylene diamine dihydrochloride with 2·5 % phosphoric acid (all Sigma-Aldrich, St. Louis, MO)] for 10 min. The absorbance was read at 530 nm using a microplate reader (MRXll® Dynex Technologies, USA).

MTT assay

Cell viability was determined using the MTT assay (Hazalin et al. 2009). After 48 h incubation with treatments, 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO) was added to each well and incubated at 37 °C for 4 h. The absorbance was read at 570 nm using a microplate reader (MRXll® Dynex Technologies, USA).

CD40 immunophenotyping

The expression of CD40 on BV2 cells was determined using flow cytometry. Only CFS-CM-LAB9 and LABPC that had exhibited significant inhibition of LPS-induced NO production in BV2 were used. Cells were trypsinised and washed with PBS/0·1 % bovine serum albumin, and incubated with 0·5 µg/ml anti-CD40-FITC (BD Biosciences, San Jose, CA) at 4 °C for 1 h. Finally, cells were resuspended in PBS/0·1 % BSA and analysed using LSRFortessa (BD Biosciences, San Jose, CA).

Animal study design

The in vivo experiment was approved by the Committee on Animal Research and Ethics (CARE), UiTM [reference number: 600-FF (PT.5/2)]. A total of 30, two months-old male ICR mice, weighing 25–35 g were used. All mice, in groups of six, were housed in polypropylene mouse cages (30 × 20 × 16 cm) and maintained at ambient temperature (22 °C). The rodents were allowed access to food pellet and water *ad libitum* and acclimatised for 7 d. The mice were randomly assigned to groups of untreated control (saline only, 0·2 ml), LPS-treated (LPS-saline), UCM (unfermented cow's milk, 0·2 ml), CM-LAB9 (cow's milk fermented with *L. fermentum,* 10^9 CFU/0·2 ml), and CM-LABPC (cow's milk fermented with *L. casei*, 10^9 CFU/0·2 ml). Mice were administered with treatments *via* oral gavage for 28 d daily. Except for untreated control, all groups were injected with LPS (0·25 mg/kg, 0·2 ml) (*Escherichia*

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coli, serotype 055:B5, Sigma, St. Louis, MO, USA) intraperitoneally for four consecutive days (days 29–32).

Morris water maze test

The spatial memory test was performed as previously described (Lee et al. 2008). Briefly, habituation trials (once a day) were performed on days 26, 27 and 28 before the LPS challenge. The maximum trial length was 120 sec. Mice were injected with LPS over three consecutive days (day 29–31). Four hours after LPS treatment, treated mice were allowed to swim until they found the escape platform. Escape latency, escape distance, time spent in target quadrant and swimming speed of each mouse was monitored for three days. Data were analysed using a video tracking system (ANY-Maze, San Diego Instruments, San Diego, CA). On day 32, a probe trial was conducted four hours after LPS injection. During this trial, the platform was removed from the maze and the time spent by animals in the platform quadrant was recorded.

Biochemical analyses

After behavioural tests (on day 32), the mice were euthanized and whole brains were carefully removed, homogenised using a glass WiseStir Homogeniser (Daihan Scientific, Korea) and centrifuged (2700***g*** ; 4 °C; 10 min). Supernatant of the brain homogenate was collected and kept at −80 °C for biochemical analyses (AChE, catalase, SOD, GSH, GPx, MDA and NO). All biochemical analyses were measured using a microplate reader (InfiniteM200, Tecan, Switzerland). The QuantiChrom™ Acetylcholinesterase Assay Kit (BioAssay Systems, CA, USA) was used to measure AChE level. The Catalase, SOD, Glutathione and GPx Assay Kits (all Cayman Chemical, Ann Arbor, MI) were used to measure antioxidant activities. The TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI) was used to measure MDA (malondialdehyde). The QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA) was used to determine NO levels.

BioPlex cytokine measurement

The collected blood (cardiac puncture) was centrifuged (2700***g*** and 4 °C for 10 min), serum was collected and stored (−80 °C) for cytokines analyses [Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Monocyte Chemoattractant Protein-1 (MCP-1)] using the Procarta® Immunoassay Kit (Panomics, Santa Clara, CA).

Statistical analysis

Data was analysed using the One-way ANOVA procedure in GraphPad InStat3 (GraphPad Software Inc. La Jolla, CA). When there was a difference, Tukey–Kramer post hoc test was used to identify pairs that differed significantly. Significance level was $P < 0{\cdot}05$ unless stated otherwise.

Results

CFS from lactobacilli-fermented cow's milk (CM-LAB) inhibited LPS-induced NO production in BV2

Both CFS-CM-LAB9 and CFS-CM-LABPC exhibited significant inhibition ($P < 0{\cdot}05$) of LPS-induced NO production in BV2 at the highest concentration (Fig. 1a). CFS-CM of LAB1, LAB10, LAB11 and LAB12 also exhibited inhibition of LPS-induced NO production but not to a significant extent (Supplementary table). Longer exposure to CFS from CM-LAB, however, did not further inhibit NO, indicating that 48 h was no more effective than 24 h. CFS-CM-LAB9 (20 %) significantly ($P < 0{\cdot}05$) reduced NO level by 79 and 55 % at 24 and 48 h, respectively. CFS-CM-LABPC (20 %), on the other hand, inhibited ($P < 0{\cdot}05$) NO by 83 and 73 % at 24 and 48 h, respectively. The other CM-LAB also inhibited NO but not to a significant extent. Short exposure (24 h) to CM-LAB was more superior in inhibiting NO when compared to L-NAME. It is noteworthy that NO inhibition by CSF-CM-LAB was independent of cytotoxicity (Fig. 1b).

CFS from CM-LAB did not affect CD40 expression in BV2

Resting BV2 expressed only 9·9 ± 2·1 % CD40The expression of CD40 on activated BV2, however, increased drastically to 85·9 ± 2·8 %. CFS-CM-LAB9 and CFS-CM-LABPC did not significantly alter CD40 expression in either resting (35·9 ± 4·0 and 16·4 ± 1·8 %) or activated (88·9 ± 3·2 and 95·9 ± 1·4 %) BV2 microglia cells.

CM-LAB improved spatial learning and memory of LPS-induced mice

When compared to saline group, LPS saline-treated group took significantly ($P < 0{\cdot}05$) longer time (Fig. 2a) and travelled significantly ($P < 0{\cdot}05$) further (Fig. 2b) before finding the platform, indicative of impaired spatial learning and memory abilities. Oral CM-LAB ameliorated the effects of LPS. Both CM-LAB9 and CM-LABPC significantly ($P < 0{\cdot}05$) reduced escape latency and escape distance of LPS-challenged mice. Mice treated with UCM also exhibited reduced escape latency and distance, both of which were not significantly different from CM-LAB except for escape distance exhibited by CM-LAB9 on day 1 and CM-LABPC on day 3. Probe test, which reflects memory consolidation (Fig. 2c), showed that LPS saline-treated mice spent significantly lesser time ($P < 0{\cdot}05$) in target quadrant when compared to saline group. This corroborated the spatial memory deficit in LPS saline-treated rodents. All other treatments (UCM, CM-LAB9 and CM-LABPC) showed improved consolidated memory in LPS-treated animals. In terms of swimming speed, the presence of LPS resulted in no significant changes when compared to LPS-free mice (Fig. 2d). This confirmed that LPS did not affect motor functions. Subsequent treatments (UCM, CM-LAB9 and CM-LABPC) also did not bring about significant changes amongst mice exposed to LPS when compared to control.

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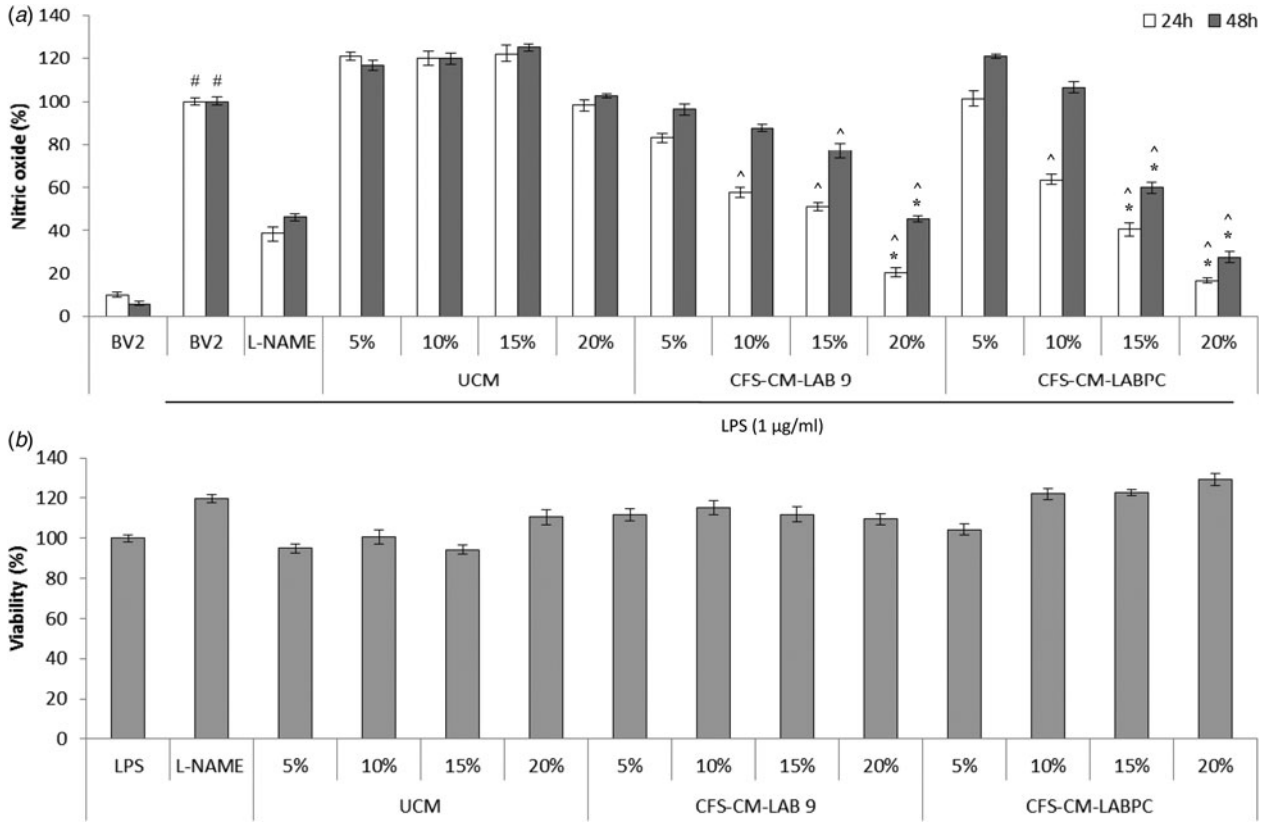

Fig. 1. NO inhibition by CFS from lactobacilli (LAB)-fermented cow's milk in activated BV2 cells. The CFS-induced NO inhibition, which occurred in a dose-dependent manner (a), was independent of cytotoxicity (b). BV2: untreated BV2 cells; UCM: unfermented cow's milk (vehicle control); CFS-CM-LAB: cell free supernatant from LAB-fermented cow's milk; CFS: cell-free; cow's milk; supernatant; CM NO: nitric oxide; LPS: lipopolysaccharides. Percentage was normalised to that of the control of LPS-activated BV2 cells. Data represent mean ± standard deviation ($n = 3$). $^{\#}P < 0{\cdot}05$ when compared with control BV2 group; $^{*}P < 0{\cdot}05$ when compared with LPS control, $^{\wedge}P < 0{\cdot}05$ when compared with UCM.

Biochemical analyses

CM-LAB reduced acetylcholinesterase (AChE) activity in brain tissue of LPS-induced mice. LPS significantly ($P <$ 0·001) increased AChE activity in LPS saline-treated group by 39 % when compared to saline group (Table 1). Administration of CM-LAB9 and CM-LABPC significantly ($P < 0{\cdot}001$) reduced AChE activity by 35 and 41 %, respectively when compared to LPS saline group. It is noteworthy that treatments had lowered AChE to levels that were comparable to that of the saline group. Given UCM did not show significant reduction, the attenuation of AChE was an effect solely contributed by lactobacilli fermentation.

CM-LAB increased antioxidants but reduced lipid peroxidation and nitrosative stress in brain tissue of LPS-induced mice. When compared to saline group, peripheral administration of LPS resulted in significant reduction ($P < 0{\cdot}05$) of catalase (−44 %), SOD (−25 %), GSH (−58 %), and GPx (−61 %). Administration of UCM and CM-LAB significantly restored and enhanced ($P < 0{\cdot}05$) catalase activity to that of the baseline (saline group) (Table 1). Increased SOD activity ($P < 0{\cdot}001$) was also observed in mice treated with CM-LAB and the level was even higher than the saline group ($P < 0{\cdot}05$). UCM, on the other hand, did not show any significant differences when compared to LPS saline group (Table 1).

The concentrations of GSH and GPx were significantly increased ($P < 0{\cdot}001$) when treated with CM-LAB (Table 1). GSH activity in CM-LAB-treated groups was significantly higher when compared to LPS saline and UCM groups. The GSH level was restored to that of the baseline (saline group). GPx activity, on the other hand, was significantly increased in the following groups: CM-LAB9 (+121 %, $P < 0{\cdot}001$), CM-LABPC (+92 %, $P < 0{\cdot}001$), and UCM (+87 %, $P < 0{\cdot}05$).

Lipid peroxidation was indicated by level of MDA in brain tissue. MDA level was significantly increased ($P < 0{\cdot}001$) by 57 % in LPS saline-treated mice as opposed to the saline group (Table 1). Whilst UCM decreased ($P < 0{\cdot}001$) the MDA level to almost that of the saline group, CM-LAB reduced MDA levels more effectively.

Nitrosative stress was indicated by NO level in the brain tissue. Whilst LPS saline group increased ($P < 0{\cdot}001$) NO, treatment with UCM or CM-LAB significantly reduced ($P < 0{\cdot}001$)

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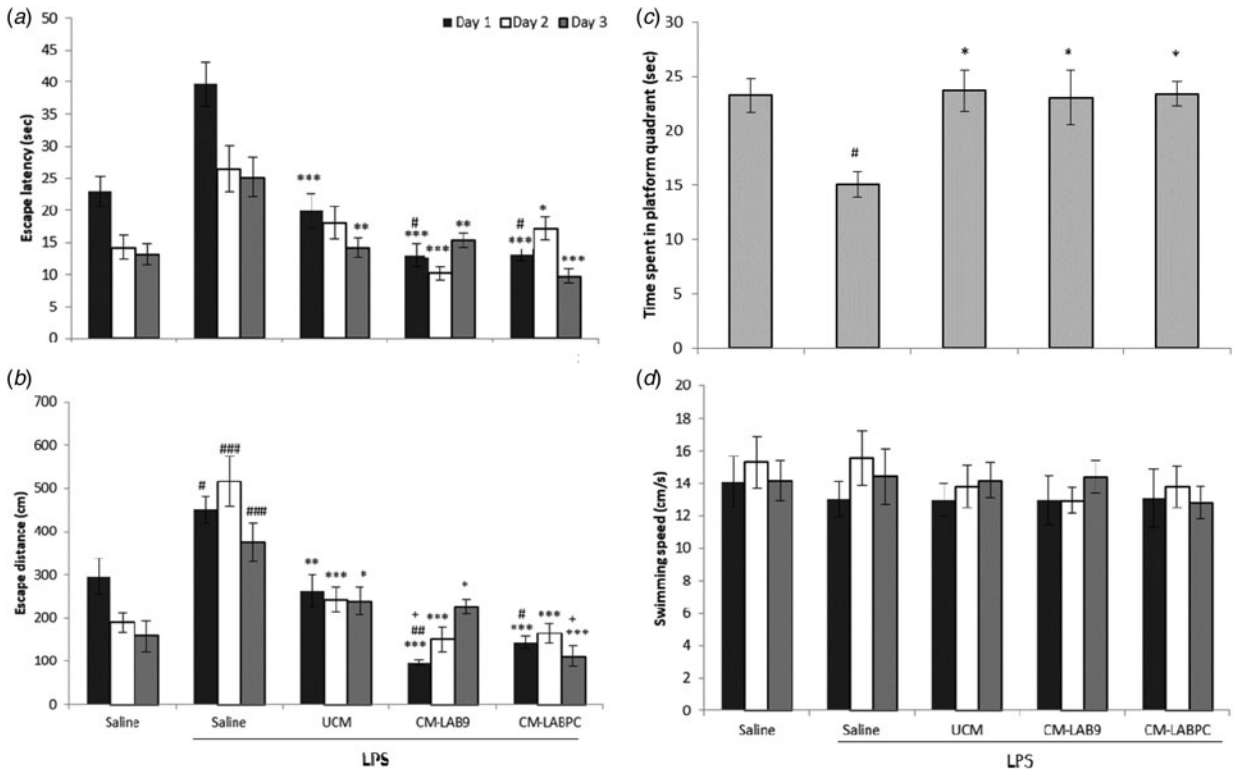

Fig. 2. Effects of CM-LAB on LPS-induced spatial learning and memory impairment. LPS (0·25 mg/kg, *i.p.*) or saline-treated mice were subjected to Morris Water Maze Test for 3 consecutive days. (a) Escape latency, (b) escape distance, (c) time spent in platform quadrant and (d) swimming speed were measured. UCM: unfermented cow's milk (vehicle control); CM-LAB9: LAB9-fermented cow's milk; CM-LABPC: LABPC-fermented cow's milk; LPS: lipopolysaccharides. Data represent mean ± SEM ($n = 6$). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ when compared to LPS saline group; $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ when compared to saline group; $^{+}P < 0{\cdot}05$ when compared to UCM group.

NO concentration by 39, 25 and 36 %, respectively (Table 1). The reduced NO level was equivalent to that of saline group (2·6 μM).

CM-LAB inhibited serum cytokines in LPS-induced mice. Peripheral LPS administration resulted in significant increase ($P < 0{\cdot}01$) of serum MCP-1, IL-1β and IL-6 by 125, 12 and 65 %, respectively when compared to saline-treated group (Table 1). Generally, oral administration of CM-LAB significantly ($P < 0{\cdot}05$) inhibited LPS-induced cytokines. CM-LAB9 inhibited LPS-induced MCP-1, IL-1β and IL-6 by 33, 10 and 22 %, respectively. Except for IL-1β (no significant reduction), CM-LABPC reduced level of LPS-induced MCP-1 and IL-6 by 32 and 24 %, respectively. The inhibitory effect elicited by CM-LAB, however, was likely co-contributed by UCM.

Discussion

Nutraceuticals like cinnamon extracts (Ho et al. 2013) and resveratrol (Bi et al. 2005) have been found to significantly decrease NO in LPS-activated BV2 cells. To the best of our knowledge, this is the first study that has demonstrated NO scavenging effect of CFS from CM-LAB in LPS-activated BV2 cells lines without affecting cell viability.

CD40, a member of the tumour necrosis factor receptor family, is an important marker for activated microglial cells. Under resting conditions, CD40 expression is relatively low. It is markedly increased upon challenge with pro-inflammatory stimuli. This is consistent with the present findings. It is known that ligation of CD40 on microglia leads to production of NO, TNF-α, IL-12, MCP-1 and other unidentified mediators. As such, the present study hypothesised that the NO inhibitory activity of CSF-CM-LAB could be dependent upon changes of CD40 expression on microglia. The results, however, indicated otherwise. Given that activated microglial cells also highly express other biochemical markers like CD11c, CD68 and LN-3 (Guillemin & Brew, 2004), it may be worth exploring the correlation of the NO inhibitory activity of CSF-CM-LAB with other biochemical markers in the future.

The present in vivo study demonstrated that consumption of CM-LAB attenuated neuroinflammation and memory impairment in LPS-treated mice. The fact that not all tested LAB possess neuroprotective effect strongly indicated that neuroprotection by CM-LAB was strain-dependent. This finding is in agreement with previous reports. *L.*

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Table 1. Effects of CM-LAB on the level of AChE activity, antioxidant enzyme, lipid peroxidation, nitrosative stress and cytokines production in LPS-treated mice

	AChE (U/l)	Catalase (nmol/min/ml)	SOD (U/ml)	GSH (μM)	GPx (nmol/min/ml)	MDA (μM)	NO (μM)	MCP-1 (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
Saline	137·73 ± 6·1	10·02 ± 0·5	14·43 ± 0·9	8·33 ± 0·4	15·6 ± 2·1	18·66 ± 1·3	2·56 ± 0·1	12·18 ± 0·1	65·51 ± 0·3	6·04 ± 0·3
Saline (LPS)	191·65 ± 5·5$^{\#\#}$	5·56 ± 0·7$^{\#\#}$	10·67 ± 0·6$^{\#\#}$	3·45 ± 0·3$^{\#\#\#}$	6·12 ± 0·1$^{\#\#\#}$	29·27 ± 0·8$^{\#\#\#}$	3·66 ± 0·5$^{\#\#\#}$	27·42 ± 3·4$^{\#\#\#}$	73·26 ± 1·0$^{\#\#}$	9·93 ± 0·6$^{\#\#\#}$
UCM	170·32 ± 3·5	13·43 ± 0·7$^{\#\#, ***}$	13·08 ± 0·5	6·26 ± 0·0***	11·41 ± 0·2*	20·08 ± 0·1***	2·15 ± 0·1$^{\#\#, ***}$	12·34 ± 0·4***	66·13 ± 1·2**	6·22 ± 0·7***
CM-LAB9	123·86 ± 8·6***, $^{+++}$	8·36 ± 0·6*	17·55 ± 0·6$^{\#, ***, ++}$	8·13 ± 0·1***, $^{++}$	13·48 ± 0·3***	8·1 ± 0·4$^{\#\#\#, ***, +++}$	2·67 ± 0·0***	18·28 ± 1·6**	66·32 ± 0·9**	7·72 ± 0·1**
CM-LABPC	112·97 ± 3·1***, $^{+++}$	9·31 ± 0·4***	18·2 ± 1·0$^{\#, ***, +++}$	7·8 ± 0·6***, $^{+}$	11·67 ± 0·6***	5·77 ± 0·3$^{\#\#\#, ***, +++}$	2·25 ± 0·0***	18·66 ± 0·5**	72·18 ± 1·0	7·48 ± 0·1**

Catalase, SOD, GSH, GPx level were measured to determine antioxidant activity; MDA concentration were measured to determine lipid peroxidation, NO were measured to determine nitrosative stress and MCP-1, IL-1β and IL-6 concentrations were measured to determine cytokines level. UCM: unfermented cow's milk (vehicle control); CM-LAB9: LAB9-fermented cow's milk; CM-LABPC: LABPC-fermented cow's milk; AChE: acetylcholinesterase; SOD: superoxide dismutase; GSH: Glutathione; GPx: Glutathione peroxidase; MDA: Malondialdehyde; NO: nitric oxide; MCP-1: Monocyte Chemoattractant Protein-1; IL: interleukin. Data represent mean ± SEM ($n = 6$). *$P < 0·05$, ***$P < 0·001$ when compared to LPS saline group; $^{\#}P < 0·05$, $^{\#\#}P < 0·01$, $^{\#\#\#}P < 0·001$ when compared to saline group; $^{+}P < 0·05$, $^{++}P < 0·01$, $^{+++}P < 0·001$ when compared to UCM group

helveticus-fermented milk, for instance, reduced escape latency, swimming distance and probe test in scopolamine-induced amnesia (Yeon et al. 2010). Recently, *L. pentosus* was found to ameliorate memory impairment and inflammation in aging mouse model (Woo et al. 2014).

The ability of probiotics to produce neuromodulators has already been established. It is now known that *Lactobacillus* sp produces ACh (Dinan et al. 2014). Their role in regulating AChE, however, remains poorly understood. In the present study, CM-LAB inhibited AChE activity in LPS-induced mice. Thus, the lactobacilli-induced memory improvement could be associated, at least in part, with inhibition of AChE activity. There is also increasing evidence supporting the possible relationship between neuroinflammation and the cholinergic system. Kalb et al. (2013), for example, demonstrated that AChE inhibitors reduced neuroinflammation and degeneration in the cortex and hippocampus of rodents. This has served as the basis of our suggestion that the present CM-LAB-induced anti-inflammatory activity could also be attributed to inhibition of AChE.

The strong correlation of oxidative stress with a large number of neurodegenerative diseases (including AD) has led to the use of antioxidant-rich diets as natural means for preservation of brain function. This approach appears to be highly possible as previous studies have indicated antioxidant-induced neuroprotection through attenuation of oxidative stress and cognitive deficit (Ha et al. 2012). Soymilk fermented by *Lactobacillus* sp. was reported to possess enhanced antioxidative capacity (Kang et al. 2012; Marazza et al. 2013). The present study found that CM-LAB significantly increased catalase, SOD, GSH, GPx in brain tissues of LPS-treated mice. It is established that endogenous antioxidants eliminate oxidative stressors. Whilst SOD inactivates superoxide radicals into hydrogen peroxide, catalase, GSH and GPx convert hydrogen peroxide into water and oxygen. Previous studies reported that Dahi (buffalo's milk fermented with *Lactobacillus acidophilus* and *Bifidobacterium bifidum*) alleviated age-inflicted oxidative stress in ageing mice through increased catalase and GPx and, reduced lipid peroxidation (Kaushal & Kansal, 2012).

The antioxidative activity of CM-LAB was accompanied by reduction of MDA and NO levels. It was demonstrated that oral administration of purple sweet potato added with *Bacillus subtilis*-fermented soymilk significantly decreased MDA and NO in amyloid beta-induced memory impairment (Kim et al. 2012). MDA is an end product of lipid peroxidation and its elevation has been identified as the central mechanism of neurodegeneration in AD. The lowering of NO exhibited by CM-LAB in vivo confirmed its NO scavenging properties in vitro. The present study found that the NO lowering effect of unfermented milk (UCM) was more superior when compared to fermented milk. This was possibly due to the presence of milk proteins that also possess NO inhibitory activities (Phelan et al. 2014).

Lactobacilli have been found to produce a range of secondary metabolites during milk fermentation, most notable

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being B vitamins and bioactive peptides. Besides, certain probiotic strains can also produce GABA (Hayakawa et al. 2005), conjugated linoleic acid (Gorissen et al. 2010) or angiotensin-I-converting enzyme inhibitory peptides (Ramchandran & Shah, 2008). Bioactive metabolites released in fermented milk have also been reported to activate mucosal immune system (Vinderola et al. 2007).

In the present study, systemic administration of LPS led to significant up-regulation of MCP-1, IL-1β and IL-6 cytokines in mice serum. This is consistent with reports indicating that LPS-induced cognitive impairment in mice was accompanied by increased pro-inflammatory cytokines and chemokines (Thibeault et al. 2001). Treatment with CM-LAB significantly inhibited production of these cytokines. The inhibitory effect, however, could be co-contributed by the carrier (unfermented milk) that also showed significant inhibition. Liu et al. (2011) demonstrated anti-inflammatory and immunomodulatory activities of *L. plantarum* mediated through reduction of IL-1β, IL-6 and TNF-α.

Conclusion

The present study has demonstrated the potential of CM-LAB as neuroprotective agent. The CM-LAB-induced neuroprotection was associated with restoration of the cholinergic neurotransmission and attenuation of neuroinflammation. The modulation of neuroinflammation was likely mediated through up-regulation of antioxidants, inhibition of AChE and down-regulation of proinflammatory cytokines. Current findings provide important insights into the viable use of natural means for prevention of AD.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000620.

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