INTRODUCTION

The mammalian gastrointestinal tract is considered a metabolically active organ containing a diversity of microbial species. This commensal intestinal microbiota is essential as they protect the host against infections and maintain the body’s homeostasis under normal circumstances (Gaggia et al., 2010). Among the numerous intestinal microbes, Lactobacillus, Bifidobacterium and Enterococcus have been demonstrated to affect the host beneficially by improving the intestinal microbial balance and hence are categorized as probiotics (Ishibashi & Yamazaki, 2001). Among several enterococcal species, Enterococcus faecium and Enterococcus faecalis are the two predominant species in the human intestine (Giraffa, 2003). Enterococci are added to fermented foods as they contribute to organoleptic properties (Bhardwaj et al., 2009). They are used extensively as starter cultures in food products such as cheeses, as probiotics for humans and animals, and as silage additives (Foulquie Moreno et al., 2006). The beneficial effects of probiotic Enterococcus spp. in different hosts, such as mice, piglets and humans, to treat various gastrointestinal disorders have been well studied (Szabó et al., 2009). E. faecium NCIMB 10415 is widely used as a feed additive for animals (Franz et al., 2011), and evidence has
co corroborated the probiotic benefits of such strains. Furthermore, oral administration of *E. faecium* was shown to affect the intestinal microbiota and modulate the immune function of mice and dogs (Benyacoub et al., 2003; Sun et al., 2010). *E. faecium* SF68 possesses inhibitory effects against enteropathogens, including enterotoxigenic *Escherichia coli*, and is used as an antiangioreetal agent (Lewenstein et al., 1979). The efficacy of SF68 to treat antibiotic-associated acute diarrhea in humans has been demonstrated (Wunderlich et al., 1989). A recent study reported that enteric-coated capsules of live *Bacillus subtilis* and *E. faecium* could ameliorate murine experimental colitis by downregulating macrophages (Chen et al., 2014). However, experimental evidence on the possible effect of probiotics on the psychological state of the host is rather limited. Previously, administration of *Bifidobacterium infantis* to rats was reported to result in neurochemical alterations (Desbonnet et al., 2008). In human volunteers, as well as in rat models, administration of a probiotic formulation consisting of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175A significantly attenuated psychological distress and also reduced anxiety-like behaviour (Messouidi et al., 2011). These limited findings suggest the potential of probiotics to modulate the host psychological state, which would have profound clinical applications.

In our earlier work, we isolated and characterized *E. faecium* CFR 3003 from indigenous fermented food (Divyashri & Prapulla, 2015a). In the present investigation, we addressed three major issues related to the biological attributes of these probiotics employing *in vitro* and *in vivo* approaches. Although probiotic live cultures are increasingly being used in a wide spectrum of diseases to modulate a number of biological processes, studies with regard to their propensity to influence levels of endogenous defence molecules is rather limited. Accordingly, we evaluated the antioxidant potential of *E. faecium* 3003 metabolites and the anti-inflammatory properties of this strain in the J774A.1 cell line. Furthermore, we sought to understand the propensity of *E. faecium* CFR 3003 and *Lactobacillus rhamnosus* GG MTCC 1408 supplements to modulate the behavioural phenotype, and the response of endogenous oxidative markers, antioxidant defences and neurotransmitter levels in various regions of the brain in mice.

**METHODS**

**Bacterial strains and growth conditions**

*E. faecium* strain CFR 3003 was isolated from indigenous fermented food and identified by its 16S rRNA gene and internal transcribed spacer region (Divyashri & Prapulla, 2015a). *L. rhamnosus* GG MTCC 1408 was used as positive control for cell line and in *vivo* studies. The strains were maintained at −80 °C in 10 % (v/v) glycerol with de Man, Rogosa and Sharpe (MRS, HiMedia laboratories) broth and were cultured normally in MRS broth at 37 °C.

**Experiment 1: gastrointestinal stress tolerance and evaluation of biological and probiotic characteristics**

**Acid resistance.** MRS broth inoculated with *E. faecium* (10 %, v/v) was incubated at 37 °C for 24 h and the cells were harvested by centrifugation at 5366 g for 15 min at 4 °C. Harvested cells were suspended in an equal volume of sterile MRS broth, adjusted to pH 1.5, 2.0 or 3.0. Hydrochloric acid (5 M) was used to adjust the pH of the MRS broth. Viable cell counts were determined after 0, 30, 60 and 120 min on MRS agar plates and the results expressed as log c.f.u. ml⁻¹.

**Bile tolerance.** MRS broth containing 0.15, 0.3 or 0.45 % Oxgall (HiMedia) was inoculated with *E. faecium* (10 %, v/v) and incubated at 37 °C for 24 h. Viable cell counts were determined after 0, 12 and 24 h on MRS agar plates and the results expressed as log (c.f.u. ml⁻¹) (Lei et al., 2014).

**Tolerance to pepsin and trypsin.** Sterile PBS (pH 3.0) containing 0, 2.5, 5.0, 7.5 or 10 g pepsin l⁻¹ was inoculated with *E. faecium* (10 %, v/v) and incubated at 37 °C for 6 h. Viable cell counts were determined on MRS agar plates and the results expressed as log c.f.u. ml⁻¹. The trypsin tolerance method was the same but the pH of the sterile PBS was changed to 6.8 (Lei et al., 2014).

**Organic acid production profile.** The production of lactic, acetic, butyric and formic acid as a result of glucose fermentation was studied. *E. faecium* was grown in MRS broth at 37 °C for 24 h and the cells were harvested by centrifugation at 8000 r.p.m. for 15 min at 4 °C. The resulting supernatant was filter sterilized (0.22 μm; Millipore) and analysed for organic acids using HPLC (LC-6A; Shimadzu) equipped with an SPA-20A detector. Sample aliquots (20 μl) were injected using an HPLC injector syringe (Hamilton) and analysis was carried out at room temperature (30 ± 2 °C) using an analytical column (Hypersil ODS; Thermo Scientific). The flow rate of the mobile phase [2.5 mM NH₄H₂PO₄ with 1 % methanol, pH 2.7 (adjusted using H₃PO₄)] was 0.5 ml min⁻¹ and the wavelength for detection was 210 nm (Lei et al., 2014).

**Antibiogram.** An antibiogram for *E. faecium* was determined using antibiotic diffusion discs. *E. faecium* was inoculated in MRS broth and incubated at 37 °C for 24 h. A sample of the culture (100 μl) was streaked onto MRS agar and antibiotic discs were applied to the surface using a disc dispenser. The plates were incubated at 37 °C and evaluated after 24 h inoculation. The antibiotics (HiMedia) tested were used at the following concentrations (μg): tetracycline (30), polymyxin B (30), vancomycin (30), ampicillin (10), chloramphenicol (30), streptomycin (10), kanamycin (30), rifamycin (5), erythromycin (15), metronidazole (5), co-trimoxazole (25) and penicillin G (10). The results were interpreted according to the cut-off levels proposed in the interpretative chart.

**Antimicrobial activity.** An antimicrobial assay was performed to determine the antimicrobial property of *E. faecium* against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*. *E. faecium* was grown in MRS broth at 37 °C for 24 h, after which the broth was sterile filtered (0.22 μm; Millipore) and the pH was adjusted to 7.0 with NaOH. Brain–heart infusion agar plates were initially seeded with indicator micro-organisms (10⁵ c.f.u. ml⁻¹), and culture supernatant (75 μl) was added to the wells (2 mm diameter). The plates were incubated at 37 ºC for 24 h and the zone of inhibition was measured (Lei et al., 2014).
Probiotic attributes of Enterococcus faecium CFR 3003

Experiment 2: measurement of the antioxidant properties of E. faecium metabolites

Preparation of lyophilized cell-free culture supernatant (LCS). MRS broth inoculated with E. faecium was incubated at 37 °C for 24 h. Cells were harvested and supernatant was collected by centrifugation at 5366 g for 15 min. The supernatant was filter sterilized (0.22 μm; Millipore) and the metabolites produced as a result of fermentation were analysed as described above using HPLC. The supernatant was lyophilized using a lyophilizer (Heto Dry winner; Thermo Scientific, ScanVac Coolsafe), operated at 0.08 Pa at −45 °C for 8 h. The lyophilized supernatant was suspended in PBS buffer (pH 7.4) to varying concentrations (5, 10 and 20 mg ml⁻¹) and stored at −80 °C until further use.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. DPPH (Sigma-Aldrich) radical scavenging ability, an indicator of hydrogen-donating ability, was measured. A volume of 0.8 ml DPPH solution (0.1 mM in 95 % methanol) was mixed with 0.2 ml of varying concentrations of LCS and incubated at 30 ± 2 °C for 30 min. The samples were then centrifuged at 8385 g for 5 min and the absorbance was measured at 517 nm (A517; UV:-1601; Shimadzu). Ascorbic acid (20 mg l⁻¹) was used as a standard. The antioxidant activity was expressed as:

DPPH scavenging activity (%) = [1 − (Absorbance sample / Absorbance control)] × 100

Reducing power assay. The reducing ability of LCS was assayed as described previously (Tsai et al., 2006). Varying concentrations of LCS (200 μl) were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1 % (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid [TCA; 10 % (w/v), 500 μl] was added to the mixture and then centrifuged at 755 g for 10 min. The upper phase of the solution was mixed with deionized water and 0.1 % FeCl₃ (w/v) at a ratio of 1 : 1 : 1 (v/v/v) and A₇₀₀ was determined. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid (20 mg l⁻¹) was used as a standard.

Ascorbate auto-oxidation inhibition. Varying concentrations of LCS were mixed with 0.1 ml ascorbate solution (5 mM) and 9.7 ml phosphate buffer (0.2 M, pH 7). The reaction mixture was incubated at 37 °C for 10 min and A₂₆₅ was measured. Ascorbate auto-oxidation inhibition (%) was calculated as:

Ascorbate auto-oxidation inhibition (%) = [(Absorbance sample / Absorbance control) − 1] × 100

Oxygen radical absorbance capacity (ORAC) assay. The ORAC values of LCS were evaluated according to the method of Huang et al. (2002). For calibration, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich; 6.25–50 μM) was used as a standard and was prepared fresh by diluting the stock solution (2 mM). Various concentrations of LCS (20 μl) and Trolox were placed into 96-well plate. A working solution of fluorescein sodium salt (200 μl, 81.6 mM) was obtained by dilution of a stock solution (8.16 μM) with PBS buffer (75 mM, pH 7.0). After the fluorescein solution had been added, fluorescence (maximum excitation and emission spectra at 485 and 528 nm) at 0 min was measured in a microplate reader (Varioskan Flash Multimode Reader; Thermo Scientific). 2,2′-Azobis (2-aminopropane, Sigma-Aldrich) dimethylformamide (AAHP, 75 μl) was prepared fresh at a concentration of 200 mM and used for automatic injection. Fluorescence was recorded every 5 min over 40 min. Quantification of the antioxidant activity was based on calculation of the area under the curve (AUC), as proposed by Cao & Prior (1999). The antioxidant activity using the ORAC assay was calculated as TE (μmol Trolox g⁻¹ LCS) μmol Trolox equivalents (TEs) (g sample)⁻¹ using the formula:

Relative ORAC value = [(AUC_LCS − AUC_blank)/(AUC_Trolox − AUC_blank)] × (TE/g sample)

Experiment 3: anti-inflammatory studies using the J774A.1 cell line

Bacterial sample and LCS preparation. MRS broth inoculated with E. faecium and L. rhamnosus (10 %, v/v) was incubated at 37 °C for 24 h. Cells were harvested by centrifugation at 5366 g for 15 min and resuspended in PBS buffer (pH 7.4) after washing twice using the same buffer. The washed cells were lyophilized and stored at −80 °C until further use. LCS of E. faecium and L. rhamnosus was prepared as described above. L. rhamnosus was used as a positive control in the study.

Growth and maintenance of cell culture. Mouse macrophage J774A.1 cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco), supplemented with 10 % FBS (Gibco), penicillin (100 U ml⁻¹; Sigma-Aldrich) and streptomycin (100 μg ml⁻¹; Sigma-Aldrich). The cells were routinely grown in 25 cm² cell culture flasks at 37 °C in 5 % CO₂ humidified incubator. Viable cells were enumerated using 0.5 % trypan blue dye in PBS. Cell suspension (200 μl) at a concentration of 2.5 × 10⁶ cells ml⁻¹ was placed in 96-well culture plates and allowed to adhere for 48 h at 37 °C at 5 % CO₂ in an incubator. The spent culture medium was removed and the cell surfaces were washed with PBS and fresh DMEM complete medium (500 μl) with antibiotics was then added to each well.

Culture of J774A.1 cells with bacterial strains. J774A.1 cells were treated with different concentrations of test samples and LPS from Escherichia coli O55 : B5 (Sigma-Aldrich) at 1 μg ml⁻¹. After 24 h incubation, cleared supernatants were collected by centrifugation and assayed for TNF-α and IL-10.

Measurement of TNF-α and IL-10. The cell culture supernatants were assayed to determine the concentrations of TNF-α and IL-10 by ELISA (Sigma-Aldrich) according to the manufacturer’s guidelines.

Experiment 4: in vivo studies of the modulation of endogenous oxidative markers in mice brains

Animals and care. Male mice (CFT-Swiss; 6 weeks old) were obtained from the Council of Scientific & Industrial Research/Central Food Technological Research Institute animal facility for the study. Mice were acclimatized for 1 week prior to the start of the experiment and maintained on a commercial diet and tap water ad libitum. Mice in different experimental groups were maintained separately and housed in standard (27 × 21 × 14 cm; three per cage) polypropylene cages with standard air. In our laboratory, we have been using this strain of Swiss inbred mice for all our studies, with the aim of assessing the neuromodulatory potential of extracts of traditional medicinal plants, spice bioactives, etc. In the present study, we used a mouse model as one of the main objectives was to assess the effect of probiotics on the behavioural phenotype. In general, males are employed in studies of this nature in order to eliminate any possible influence of hormonal factors.

All experimental test paradigms were approved by the Institutional Animal Ethics Committee and were conducted in adherence with the guidelines stipulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment, Forests and Climate Change, Government of India, India.
Experimental design. Mice were randomly assigned to four groups (control and three treatment groups) with a total of 24 mice (n=6 per group) and were orally gavaged as follows. Group I, the control (CTR) group, received saline, groups II and III received *E. faecium* (10^8 and 10^9 c.f.u. day^{-1}, respectively), and group IV mice received *L. rhamnosus* GG (10^8 c.f.u. day^{-1}) for consecutive 28 days (Carasi et al., 2014). Briefly, reaction mixture (1 ml) containing H2DCF-DA, Sigma-Aldrich) assay. H2DCF-DA is de-esterified to its DCF formed min^{-1} (molar extinction coefficient of DCFH (e) = 22 000 M^{-1} cm^{-1}).

Activities of enzymes: catalase (CAT) and glutathione S-transferase (GST). CAT activity was estimated by adding an aliquot of cytosol (0.25 mg protein) to phosphate buffer (0.1 M, pH 7.4, containing H2O2). The decomposition of H2O2 was monitored at 240 nm and the activity expressed as nmol H2O2 hydrolysed min^{-1} (mg protein)^{-1} (e = 44.2 M^{-1} cm^{-1}) (Aebi, 1984). GST activity was assayed by measuring the rate of enzyme-catalysed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich) (Flohé & Günzler, 1984) and enzyme activity was expressed as nmol S-2,4-dinitrophenyl glutathione formed min^{-1} (mg protein)^{-1}.

Acetylcholinesterase (AChE) activity. AChE activity was determined as described previously (Ellman et al., 1961). To the reaction mixture containing 1 ml phosphate buffer (0.1 M, pH 8.0), 50 μl 3',5'-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich, 10 mM), cytosolic sample (0.01 mg protein) and 20 μl acetylcholine iodide (Sigma-Aldrich, 78 mM) were added and the change in A_{412} was monitored over 3 min. Enzyme activity was expressed as nmol substrate hydrolysed min^{-1} (mg protein)^{-1}.

Quantification of γ-aminobutyric acid (GABA) and dopamine (DA) levels. Tissue homogenate was analysed for GABA and DA levels. The samples (20 μl) were passed through a microbore LC-18 analytical column (150 x 4.6 mm, 5 μm particle size, SUPELCOIL; Sigma-Aldrich) connected to a UV detector (280 nM). The mobile phase consisted of 0.2 % trifluoroacetic acid and methanol (70 : 30, v/v, pH 3.5) in deionized water. The mobile phase was subsequently filtered and degassed, following which the pH was adjusted to 3.5. A flow rate of 1 ml min^{-1} was maintained. DA levels in the sample were calculated from an external standard and expressed as μg DA (mg protein)^{-1} (Dalpiaz et al., 2007).

A sample (10 μl) was derivatized using O-phthalaldehyde–mercaptoethanol (20 μl; prepared by dissolving 10 mg O-phthalaldehyde and 10 μl mercaptoethanol in 2.5 ml acetonitrile) reagent and boric acid buffer (0.4 M, pH 10.4; 100 μl) for GABA quantification. Derivatization was carried out at 30 ± 2 °C for 5 min and the reaction mixture was filtered using a microfilter (0.45 μm; Millipore), and analysed by HPLC on an LC-20 AD (Shimadzu Corp.) equipped with a UV detector (SPA-20A). The samples (20 μl) were injected using an HPLC injector syringe (Hamilton) and analysis was carried out at room temperature (30 ± 2 °C) using an analytical column (Hypersil ODS). The flow rate of the mobile phase [sodium acetate (1.64 g) and triethylamine (200 μl) in 1000 ml 20 % (v/v) acetonitrile] was 0.8 ml min^{-1} and the wavelength for detection was 338 nm (Gangaraju et al., 2014).
**Protein estimation.** The protein concentration in the samples was determined using BSA as a standard (Lowry et al., 1951).

**Statistical data analysis**

The experimental data obtained were expressed as means ± SD. For animal experiments, the differences between the control and treated groups were analysed by one-way ANOVA followed by a post-hoc Dunnett test. Statistical analysis was performed using InStat3 software (v3.36).

**RESULTS**

**Experiment 1: gastrointestinal stress tolerance and evaluation of biological and probiotic characteristics**

**Gastrointestinal stress tolerance.** The ability of *E. faecium* to withstand acid stress at pH 1.5, 2 and 3 was evaluated (Fig. 1a). Better viability was observed at pH 2 and 3. *E. faecium* could maintain viability above 10⁴ c.f.u. ml⁻¹ at pH 2.5 and 3 for 120 min but had lower viability at pH 1.5 and could not survive at pH 1.5 for 120 min. Viability was greater than 10⁵, 10⁶ and 10⁷ c.f.u. ml⁻¹ at bile salt concentrations of 0.45, 0.3 and 0.15 %, respectively (Fig. 1b). *E. faecium* showed a better survival rate upon exposure to pepsin and trypsin (Fig. 1c). A decrease in viability was observed with increases in pepsin and trypsin concentration. However, viability was maintained at greater than 10⁵ c.f.u. ml⁻¹ at the maximum pepsin and trypsin concentrations tested.

**Organic acid production profile.** The concentrations of organic acid produced from *E. faecium* at various fermentation times are shown in Fig. 2. Lactic acid constituted a large proportion of the organic acids, followed by butyric acid. Acetic acid and formic acid were not detected in the fermentation broth within a fermentation time of 48 h.

**Antibiogram.** The antibiotic susceptibility of *E. faecium* is as shown in Table 1. It was found to be highly sensitive to vancomycin, tetracycline, ampicillin, chloramphenicol, rifamycin, co-trimoxazole and penicillin but was resistant to polymyxin B, streptomycin, kanamycin, erythromycin and metronidazole.

**Experiment 2: measurement of the antioxidant properties of *E. faecium* metabolites**

LCS exhibited significant DPPH scavenging, reducing ability and ascorbate auto-oxidation inhibition effects (Fig. 3). DPPH scavenging and reducing ability increased with an increase in LCS concentration. While the DPPH scavenging and reducing ability of LCS (20 mg ml⁻¹) was not significantly different from that of ascorbic acid (20 mg l⁻¹), LCS (20 mg ml⁻¹) exhibited 16.50 ± 0.16 % of the ascorbate

![Fig. 1. Survival of the probiotic *E. faecium* at various pH values (a), varying bile salt concentrations (b) and varying pepsin and trypsin concentrations (c). Data represent the means ± SD of three separate experiments.](http://jmm.microbiologyresearch.org)
auto-oxidation inhibition. Data on the TEs of E. faecium determined by the ORAC method are presented in Table 2. LCS (20 mg ml\(^{-1}\)) showed a value of 128.31 ± 3.34 µmol Trolox (g LCS\(^{-1}\)).

Experiment 3: anti-inflammatory studies using the J774A.1 cell line

An increase in TNF-\(\alpha\) level induced by LPS was significantly inhibited upon exposure to E. faecium and L. rhamnosus (Fig. 4). Furthermore, the TNF-\(\alpha\) level was also reduced significantly in the L. rhamnosus (100 µg ml\(^{-1}\)) and E. faecium (100 µg ml\(^{-1}\)) samples. In contrast, this effect was not observed for LCS-treated samples. While a significant increase in IL-10 concentration was observed between the samples treated with LPS and with E. faecium at 50 and 100 µg ml\(^{-1}\) and L. rhamnosus at 50 and 100 µg ml\(^{-1}\), no significant increase in IL-10 levels was observed between the negative control and all other treated samples.

Experiment 4: neuromodulatory studies in a mouse model

Mice administered oral supplements of E. faecium and L. rhamnosus showed no significant alterations in body weight gain (Table 3). Furthermore, the mice did not develop any signs of behavioural abnormalities assessed in terms of gait/posture by morphological evaluation during the experimental period.

Effect on exploratory behaviour and elevated plus maze activity. At the end of the treatment (when the mice were 10 weeks old), the mice were measured for their exploratory behaviour and anxiety response in the control and probiotic-supplemented groups. In the open field test, two fundamental indices of exploratory measures were analysed: the number of entries into the centre zone and the total time spent in the centre of the open field arena. No significant change was observed in the frequency of entries to the centre arena (Fig. 5a) and number of times the mice reared (not shown) between control and all the treated groups. In contrast, mice supplemented with E. faecium and L. rhamnosus at the high dose spent relatively more time in the centre arena (Fig. 5b; \(P<0.05\)). In the elevated plus maze test, mice given the high dose of E. faecium (\(P<0.05\)) and L. rhamnosus (\(P<0.01\)) supplements had a significantly higher number of entries to the open arms than the controls (Fig. 5c). This effect was also evident with regard to the time spent in the open arms among mice given the high dose of E. faecium and L. rhamnosus supplements (Fig. 5d; both \(P<0.01\)).

Effect on caecum characteristics. The effect of E. faecium and L. rhamnosus on caecum characteristics was assessed by evaluating the bacterial population in the

Table 1. Antibiotic susceptibility of the probiotic E. faecium

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (µg)</th>
<th>Sensitivity (S)/resistance (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>30</td>
<td>S</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>S</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>300</td>
<td>R</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30</td>
<td>R</td>
</tr>
<tr>
<td>Rifamycin</td>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>R</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>5</td>
<td>R</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>25</td>
<td>S</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10</td>
<td>S</td>
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</table>

Fig. 2. Organic acid profile in E. faecium fermented MRS broth. Data represent the means ± SD of three separate experiments.
intestine. Data on caecum wet weight, pH and the number of bifidobacteria and lactobacilli are presented in Table 4. While no significant change in caecum weight was evident among the treated groups, *E. faecium* supplements at the higher dose resulted in a significant decrease ($P < 0.01$) in caecum pH, which was also apparent in mice receiving *L. rhamnosus* ($P < 0.01$). An increase in lactobacilli numbers was also discernible among mice given the higher dose of *E. faecium* and *L. rhamnosus* ($P < 0.01$).

**Effect on oxidative markers and antioxidant defences.** At the lower dose, *E. faecium* caused a marginal decrease in basal ROS levels, while the higher dose significantly diminished the levels in the cortex ($P < 0.05$) (Table 5). *L. rhamnosus* administration resulted in a significant reduction (25%, $P < 0.01$) in levels of ROS in the hippocampus, whereas no change was evident in other brain regions. Levels of protein carbonyls were significantly reduced in the cortex (33%, $P < 0.01$) but only marginally in the hippocampus and striatum in mice supplemented with *L. rhamnosus* (Table 5). No significant change in superoxide dismutase activity in mice brains was evident with either of the probiotic supplements (data not shown). However, with *L. rhamnosus* supplements CAT activity was significantly increased in the cortex (27.9%, $P < 0.05$) and hippocampus (28.6%, $P < 0.05$) (Table 6). In the striatum, the activity was moderately (albeit non-significantly) increased. Furthermore, mice supplemented with the lower dose of *E. faecium* showed significantly enhanced GST activity in the hippocampus (15.8%, $P < 0.05$), and it was further increased with the higher dose in the hippocampus (37.8%, $P < 0.01$), cortex (14.0%, $P < 0.05$) and striatum (44.2%, $P < 0.01$). Likewise, *L. rhamnosus* supplements also enhanced the GST activity in hippocampus (18.2%, $P < 0.01$) and striatum (21.3%, $P < 0.01$) but had no effect in the cortex region.

**Effect on AChE activity.** *E. faecium* supplements to mice resulted in differential effects on the AChE activity in brain regions (Table 7). The lower dose of *E. faecium* supplements significantly diminished the basal activity levels of AChE in the cortex (13.9%, $P < 0.05$) and striatum (14.4%, $P < 0.05$), while the activity remained unchanged in the

### Table 2. Trolox values of LCS for probiotic *E. faecium*

<table>
<thead>
<tr>
<th>Sample</th>
<th>TE (μmol Trolox g⁻¹ LCS)</th>
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</thead>
<tbody>
<tr>
<td>LCS5</td>
<td>56.56 ± 2.34</td>
</tr>
<tr>
<td>LCS10</td>
<td>96.38 ± 5.09</td>
</tr>
<tr>
<td>LCS20</td>
<td>128.34 ± 3.34</td>
</tr>
</tbody>
</table>

LCS5, LCS at 5 mg ml⁻¹; LCS10, LCS at 10 mg ml⁻¹; LCS20, LCS at 20 mg ml⁻¹.
hippocampus. However, *L. rhamnosus* supplements to mice did not appreciably influence the cholinesterase activity.

**Effect on DA and GABA levels.** In general, mice given *E. faecium* and *L. rhamnosus* showed enhanced cytosolic brain levels of DA and GABA. While the levels were marginally increased at the lower dose of *E. faecium*, significant elevation (*P*<0.05) of DA levels was evident at the higher dose but only in the cortex (Fig. 6a). DA levels were enhanced (*P*<0.05) in the striatum among mice given *L. rhamnosus* (Fig. 6a). GABA levels were also significantly enhanced with the higher dose of *E. faecium* in all regions (cortex, *P*<0.05; hippocampus, *P*<0.01; striatum, *P*<0.05). Interestingly, among mice given *L. rhamnosus*, higher levels of GABA were observed in the hippocampus only (*P*<0.05) (Fig. 6b).

**DISCUSSION**

*E. faecium* is the most common *Enterococcus* sp. isolated from cheese, and the high prevalence of this strain in processed foods is attributed to their resistance to heat, extreme salinity and harsh conditions during ripening of fermented foods (Pieniz *et al*., 2014). *E. faecium* CFR 3003, isolated from a fermented traditional indigenous ayurvedic preparation, was found to be a potent producer of the neurotransmitter GABA (Divyashri & Prapulla, 2015a, b). There are many reports on the in vitro properties of lactobacilli, whereas studies pertaining to the probiotic characteristics of enterococci are scarce (Bhardwaj *et al*., 2009). Accordingly, the present study aimed to evaluate the antioxidant, anti-inflammatory and neuromodulatory properties of *E. faecium*.

**Table 3.** Effect of probiotic supplements on mouse body weight

Mice were weighed daily before gavage and were orally administered *E. faecium* and *L. rhamnosus* supplements daily for 28 days. CTR, control; EF4, 10⁵ c.f.u. *E. faecium* day⁻¹; EF8, 10⁶ c.f.u. *E. faecium* day⁻¹; LGG8, 10⁶ c.f.u. *L. rhamnosus* day⁻¹. Values are means ±sd (n=6). Data were analysed by one-way ANOVA.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>CTR</td>
<td>21.60 ± 0.76</td>
</tr>
<tr>
<td>EF4</td>
<td>21.65 ± 0.44</td>
</tr>
<tr>
<td>EF8</td>
<td>21.45 ± 0.68</td>
</tr>
<tr>
<td>LGG8</td>
<td>20.30 ± 0.59</td>
</tr>
</tbody>
</table>
Table 4. Effect of probiotic supplements on total caecum weight, caecum pH and the number of bifidobacteria and lactobacilli

Mice were orally administered *E. faecium* and *L. rhamnosus* supplements daily for 28 days. Values are mean ± SD (*n* = 6). Data were analysed by one-way ANOVA followed by a post-hoc Dunnett test (*P*, *0.05; **P*, *0.01). Significant differences (bold) were determined by comparing CTR versus EF4, EF8 or LGG8. See Table 3 for abbreviations.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total caecum weight (g)</th>
<th>Caecum pH</th>
<th>Bifidobacteria*</th>
<th>Lactobacilli*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>0.44 ± 0.01</td>
<td>6.85 ± 0.10</td>
<td>8.05 ± 0.83</td>
<td>8.09 ± 1.01</td>
</tr>
<tr>
<td>EF4</td>
<td>0.44 ± 0.01</td>
<td>6.78 ± 0.04</td>
<td>8.47 ± 1.45</td>
<td>9.57 ± 0.98</td>
</tr>
<tr>
<td>EF8</td>
<td>0.44 ± 0.03</td>
<td>6.34 ± 0.03**</td>
<td>8.69 ± 1.19</td>
<td>9.68 ± 1.24*</td>
</tr>
<tr>
<td>LGG8</td>
<td>0.45 ± 0.02</td>
<td>6.30 ± 0.11**</td>
<td>8.89 ± 0.91</td>
<td>9.95 ± 0.82*</td>
</tr>
</tbody>
</table>

*Log_{10}[c.f.u. (g^{-1} caecum)^{-1}]* (wet content).

Fig. 5. (a, b) Effect of probiotic supplements on mice exploratory behaviour in an open field test. The number of entries into the centre zone (a) and the time spent (s) in the centre zone (b) of the open field box were assessed. Mice were orally administered *E. faecium* and *L. rhamnosus* supplements daily for 28 days. Data are expressed in absolute values collected during a 10 min testing session under ambient lighting conditions. (c, d) Effect of probiotic supplements on mice behaviour in elevated plus maze. The number of entries into the open and closed arms (c) and the time spent (s) in the open and closed arms of the test were determined. Mice were orally administered *E. faecium* and *L. rhamnosus* supplements daily for 28 days. Data are expressed in absolute values (*n* or s). CTR, control; EF4, 10^4 c.f.u. *E. faecium* day^{-1}; EF8, 10^8 c.f.u. *E. faecium* day^{-1}, LGG8, 10^8 c.f.u. *L. rhamnosus* day^{-1}. All values are means ± sd (*n* = 6). Data were analysed by one-way ANOVA followed by post-hoc Dunnett test (*P*, *0.05; **P*, *0.01). Significances were determined by comparing CTR versus EF4, EF8 and LGG8.
Table 5. Effect of probiotic supplements on the endogenous levels of oxidative markers in mice brain regions

Mice were orally administered with *E. faecium* and *L. rhamnosus* supplements daily for 28 days. Values are means ± SD (*n* = 6). Data were analysed by one-way ANOVA followed by a post-hoc Dunnett test (*P*<0.05; **P**<0.01; values in bold type). Significances were determined by comparing CTR versus EF4, EF8 and LGG8. See Table 3 for abbreviations.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ROS [nmol DCF (mg protein)^−1]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>4.2 ± 0.01</td>
<td>3.2 ± 0.11</td>
<td>2.38 ± 0.28</td>
</tr>
<tr>
<td>EF4</td>
<td>4.0 ± 0.10</td>
<td>3.3 ± 0.23</td>
<td>2.42 ± 0.24</td>
</tr>
<tr>
<td>EF8</td>
<td>3.7 ± 0.15*</td>
<td>3.5 ± 0.16</td>
<td>2.47 ± 0.12</td>
</tr>
<tr>
<td>LGG8</td>
<td>4.1 ± 0.12</td>
<td>2.4 ± 0.13**</td>
<td>2.37 ± 0.15</td>
</tr>
<tr>
<td><strong>Protein carbonyls [nmol carbonyls (mg protein)^−1]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>22.9 ± 1.3</td>
<td>21.8 ± 0.9</td>
<td>15.7 ± 1.0</td>
</tr>
<tr>
<td>EF4</td>
<td>23.1 ± 1.2</td>
<td>22.7 ± 1.4</td>
<td>15.6 ± 0.8</td>
</tr>
<tr>
<td>EF8</td>
<td>20.1 ± 2.6</td>
<td>22.1 ± 1.9</td>
<td>16.1 ± 1.1</td>
</tr>
<tr>
<td>LGG8</td>
<td>15.3 ± 0.7**</td>
<td>19.4 ± 0.7</td>
<td>14.4 ± 1.1</td>
</tr>
</tbody>
</table>

The viability and activity of probiotic bacteria are important for survival in food during its shelf life and transit through the acidic conditions of the stomach (Pieniza et al., 2014). To exert their effect on the host, these bacteria need to be able to withstand stomach acid and resist degradation by hydrolytic enzymes, bile salts, pepsin and trypsin in the small intestine (Playne, 1994). In the present study, *E. faecium* showed robust resistance to gastrointestinal stress conditions, demonstrating its ability to survive at various pH conditions. The results clearly indicated that *E. faecium* has acid and bile tolerance capacity, surviving at the tested pH and in all concentrations of bile salts. Furthermore, *E. faecium* exhibited tolerance towards both pepsin and trypsin. These data are consistent with our previous report in which we demonstrated the viability of *E. faecium* following exposure to simulated gastric and intestinal conditions (Divyashri & Prapulla, 2015b). Although *Enterococcus* spp. are considered an asset in food technology, some isolates of this genus have emerged as opportunistic pathogens (Araújo & Ferreira, 2013), and thus it is of prime importance to evaluate their virulence factors (gelatinase production, hyaluronidase activity, cytolysin and aggregation substance). We found no zone of inhibition on gelatin-incorporated MRS agar plates, clearly suggesting

Table 6. Effect of probiotic supplements on the activities of antioxidant enzymes and GST in brain regions of mice

Mice were orally administered *E. faecium* and *L. rhamnosus* supplements daily for 28 days. Values are means ± SD (*n* = 6). Data were analysed by one-way ANOVA followed by a post-hoc Dunnett test (*P*<0.05; **P**<0.01; values in bold type). Significances were determined by comparing CTR versus EF4, EF8 and LGG8. See Table 3 for abbreviations.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAT [nmol H₂O₂ hydrolysed min⁻¹ (mg protein)^−1]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>4.33 ± 0.2</td>
<td>3.95 ± 0.4</td>
<td>3.29 ± 0.3</td>
</tr>
<tr>
<td>EF4</td>
<td>4.53 ± 0.2</td>
<td>3.88 ± 0.3</td>
<td>3.20 ± 0.2</td>
</tr>
<tr>
<td>EF8</td>
<td>4.99 ± 0.1</td>
<td>4.25 ± 0.2</td>
<td>3.48 ± 0.1</td>
</tr>
<tr>
<td>LGG8</td>
<td>5.54 ± 0.3*</td>
<td>5.08 ± 0.3*</td>
<td>3.66 ± 0.2</td>
</tr>
<tr>
<td><strong>GST [nmol conjugate formed min⁻¹ (mg protein)^−1]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>135 ± 2.7</td>
<td>82 ± 2.1</td>
<td>70 ± 2.2</td>
</tr>
<tr>
<td>EF4</td>
<td>130 ± 5.3</td>
<td>95 ± 0.3*</td>
<td>75 ± 4.0</td>
</tr>
<tr>
<td>EF8</td>
<td>157 ± 3.4*</td>
<td>113 ± 4.9**</td>
<td>101 ± 5.3**</td>
</tr>
<tr>
<td>LGG8</td>
<td>140 ± 4.8</td>
<td>97 ± 2.0*</td>
<td>89 ± 5.3*</td>
</tr>
<tr>
<td><strong>GSH [µg GSH (mg protein)^−1]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>17.9 ± 1.2</td>
<td>15.1 ± 0.1</td>
<td>10.8 ± 0.3</td>
</tr>
<tr>
<td>EF4</td>
<td>17.5 ± 0.4</td>
<td>15.0 ± 0.6</td>
<td>10.9 ± 0.9</td>
</tr>
<tr>
<td>EF8</td>
<td>17.5 ± 0.4</td>
<td>14.8 ± 0.7</td>
<td>11.7 ± 1.0</td>
</tr>
<tr>
<td>LGG8</td>
<td>17.7 ± 0.9</td>
<td>14.9 ± 0.6</td>
<td>12.3 ± 0.7</td>
</tr>
</tbody>
</table>
Recently reported findings on probiotic resistance to erythromycin, and these results are in agreement with clinically relevant antibiotics vancomycin, penicillin G, rifampicin, streptomycin, and tetracycline. In contrast, E. faecium is the most commonly isolated species in hospitals on a global scale (Franz et al., 2009). Vancomycin-resistant enterococci are causing problems in hospitals, and animal consumption of vancomycin-resistant enterococci is an important prerequisite to considering it safe for human health (Dixit et al., 2014). The use of vancomycin-resistant enterococci is a controversial issue because the multiple antibiotic traits of some strains may contribute to their pathogenicity (Lei et al., 2013). The use of vancomycin-resistant enterococci is a controversial issue because the multiple antibiotic traits of some strains may contribute to their pathogenicity (Lei et al., 2013). Specifically, vancomycin-resistant enterococci are causing problems in hospitals on a global scale (Franz et al., 2011). In the present study, E. faecium showed no evidence of resistance to the clinically relevant antibiotics vancomycin, penicillin G, rifampicin, and tetracycline. In contrast, E. faecium showed resistance to erythromycin, and these results are in agreement with recently reported findings on probiotic E. faecium YF5 isolated from sourdough (Tan et al., 2013). We are conducting further studies to explore the genetic basis of E. faecium antibiotic resistance.

Free radicals are formed during normal metabolism and in higher fluxes when exposed to xenobiotic agents from foods and environment. They cause cellular oxidative damage that is related to many diseases (Ahire et al., 2013). DPPH is a relatively stable organic radical that has been widely used in the determination of the antioxidant activities of probiotic cell-free extracts (Afify et al., 2012). LCS exhibited the highest reducing ability as evidenced by an absorbance of 0.647 ± 0.01 % at 700 nm. This suggests the reducing ability of E. faecium to decompose hydroperoxides to hydroxyoctadecadienoic acids (Gardner, 1975). The value obtained for ascorbate auto-oxidation inhibition (16.50 ± 0.16 %) was higher than that for the strains of Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus and Bifidobacterium longum reported previously (Lin & Yen, 1999). Accurate determination of antioxidant capacity requires the measurement of both inhibition degree and inhibition time as the reaction among free radicals, substrate and antioxidant is very complicated, which makes it impossible to use a fixed equation to express the kinetic order (Ramalakshmi et al., 2009). The ORAC value of LCS obtained in this study was found to be higher than values reported for probiotic Bifidobacterium adolescentis ATCC 15703, Bifidobacterium animalis subsp. lactis BB-12, Bifidobacterium longum subsp. infantis ATCC 15702, Bifidobacterium longum subsp. longum PRO 42-2 and in line with values reported for Bifidobacterium longum subsp. longum PRO42-8 and Bifidobacterium longum subsp. longum RW 023 (Gagnon et al., 2015).

Pathogenic microbes in the intestine have been demonstrated to play a role in exacerbation of inflammatory bowel diseases (IBDs), such as Crohn’s disease and ulcerative colitis in both animal models and humans (Balish & Warner, 2002). Live probiotic bacteria residing in the intestine and their metabolites such as organic acids are reported to be effective in treating IBD (Okada et al., 2009; Wu et al., 2010). Previous evidence suggests that probiotic bacteria may have potential use as anti-inflammatory agents in...
chronic inflammatory conditions (Lorea Baroja et al., 2007). Beneficial roles of probiotic lactobacilli and bifidobacteria under the conditions of inflammation have been reported previously (Okada et al., 2009). However, data on the anti-inflammatory role of the probiotic E. faecium is limited. The bacterial endotoxin LPS is a representative molecule of the outer membrane of Gram-negative bacteria and is detected in the plasma of IBD patients (Caradonna et al., 2000). Macrophages stimulated by enteric bacteria and/or their constituents (LPS) release pro-inflammatory cytokines and chemokines into the bloodstream (Okada et al., 2009). IL-10 is a 17–20 kDa glycoprotein and is a potent inhibitor for the production of pro-inflammatory cytokines such as TNF-α (Wu et al., 2010). In the present study, E. faecium, L. rhamnosus and LCS showed significant anti-inflammatory effect, as evidenced by the suppression of LPS-induced TNF-α levels and upregulation of IL-10 levels (Okada et al., 2009).

Growing evidence now supports the role of probiotics in influencing various psychotropic behavioural aspects in animals (Messaoudi et al., 2011). The results obtained in the elevated plus maze test among mice given E. faecium and L. rhamnosus supplements are in line with previous reports on the potential of probiotics to reduce an anxiety-like response in animal models (Bravo reports on the potential of probiotics to reduce an anxiety-like response in animal models (Bravo et al., 2009)). Macrophages stimulated by enteric bacteria and/or their constituents (LPS) release pro-inflammatory cytokines and chemokines into the bloodstream (Okada et al., 2009). IL-10 is a 17–20 kDa glycoprotein and is a potent inhibitor for the production of pro-inflammatory cytokines such as TNF-α (Wu et al., 2010). In the present study, E. faecium, L. rhamnosus and LCS showed significant anti-inflammatory effect, as evidenced by the suppression of LPS-induced TNF-α levels and upregulation of IL-10 levels (Okada et al., 2009).

The tight regulation of the cellular redox balance is due to the presence of several endogenous cytoprotective proteins such as antioxidant and detoxifying enzymes. These results suggest the potential of probiotics to enhance the activity levels of enzymes involved in oxidative free-radical scavenging, suggesting an in vivo antioxidant property. These findings emphasize the need for further studies to understand the role of bacteria in the expression of antioxidant defence genes. It is likely that probiotic-mediated modulation of endogenous protective systems may play a vital role in counteracting the pro-oxidative insult in the brain (Calabrese et al., 2012). Taken together, the present findings in mice support the contention that probiotics significantly reduce the basal levels of several oxidative stress markers and enhance the activities of antioxidant enzymes, suggesting an antioxidant capacity in vivo. It would be interesting to examine whether chronic supplementation of probiotics can offset toxicant-mediated behavioural phenotype and oxidative perturbations in vivo employing well-established chemical neurotoxin models. Such studies would be more relevant to exploit the therapeutic potential of probiotics against oxidative stress-mediated neurodegenerative conditions.

AChE is an enzyme found in the synaptic cleft of cholinergic synapses and plays a key role in terminating neuronal transmission by hydrolysing the neurotransmitter acetylcholine. Consistent with these findings, previous studies have reported that certain Lactobacillus spp. have the capacity to produce neuropeptidolites such as acetylcholine and GABA leading to enhanced cholinergic neurotransmission (Lytte, 2011). Recent studies have also documented that gut microbes influence amino acid metabolism and play an important role in neurotransmitter regulation (owing to their ability to produce neuroactive metabolites) (Cryan & Dinan, 2012), which seems to lend credence to the presently observed enhanced levels of GABA and DA and probiotic supplements. Thus, it could reasonably be presumed that the probiotics administered chronically may also provide a viable approach to enhance neurochemical production in vivo and thus may serve as an innovative approach for clinical application.

### CONCLUSION

Taken together, our findings suggest the efficacy of probiotic E. faecium in exerting a free-radical scavenging effect coupled with reductions in the levels of inflammatory cytokines. More importantly, the data in our mice model clearly suggest the propensity of short-term oral supplements of E. faecium to modulate behavioural phenotypes and endogenous antioxidant defences in the brain. Although the underlying molecular mechanisms need further study, it is reasonable to speculate that the intake of these probiotic bacteria is likely to confer...
protective benefits in vivo against oxidative damage-mediated neurodegenerative conditions. However, we cannot preclude the possible involvement of other functional processes and systems that may contribute to the beneficial effects of probiotics. Our future studies are directed towards understanding the effect of probiotics in mitigating neurotoxin-induced oxidative dysfunctions and neurotoxicity.

REFERENCES


