

Isolation and characterisation of new putative probiotic bacteria from human colonic flora

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The present study describes a novel bacterial isolate exhibiting high ability to synthesise and secrete butyrate. The novel isolated bacterium was obtained from human faeces and grown in selective liquid intestinal microflora medium containing rumen fluid under microaerobic conditions. Its probiotic properties were demonstrated by the ability of the isolate to survive high acidity and medium containing bile acids and the ability to adhere to colon cancer cells (Caco-2) *in vitro*. Phylogenetic identity to *Enterococcus durans* was established using specific primers for 16S rRNA (99% probability). PCR analyses with primers to the bacterial gene encoding butyrate kinase, present in the butyrogenic bacteria *Clostridium*, showed that this gene is present in *E. durans*. The *in vivo* immunoprotective and anti-inflammatory effects of *E. durans* were assessed in dextran sodium sulfate (DSS)-induced colitis in Balb/c mice. Administration of *E. durans* ameliorated histological, clinical and biochemical scores directly related to intestinal inflammation whereas the lactic acid bacterium *Lactobacillus delbrueckii* was ineffective in this regard. Colonic cDNA concentrations of IL-1 β and TNF- α were significantly down regulated in DSS-treated *E. durans*-fed mice but not in control or DSS-treated *L. delbrueckii*-fed mice. Fluorescent *in situ* hybridisation analyses of colonic tissue from mice fed *E. durans*, using a butyrate kinase probe, demonstrated that *E. durans* significantly adheres to the colonic tissue. The novel isolated bacterium described in the present paper, upon further characterisation, can be developed into a useful probiotic aimed at the treatment of patients suffering from ulcerative colitis.

***Enterococcus durans*: Butyrate: Butyrate kinase: Intestinal microflora: Probiotics: Short-chain fatty acids**

SCFA such as butyrate, propionate and acetate are the end-products of dietary carbohydrate breakdown by anaerobic bacteria in the large bowel. Butyrate is utilised by colonocytes for energetic purposes.

Topical therapy with butyrate appears to be a promising development in the treatment of inflammatory bowel disease. The molecular mechanisms leading to the response to SCFA, and especially towards butyrate, are poorly understood. The mechanisms do not seem to be limited to the view that butyrate only supplies the energy for colonocytes. Recent studies have demonstrated that butyrate exerts immunomodulatory effects, such as down regulation of T-cell responses, induction of Th1 cell energy and modulation of antigen-presentation-associated molecules (Diakos *et al.* 2002). Additionally, a recent study has demonstrated that butyrate decreases pro-inflammatory cytokine expression via inhibition of NF κ B activation and inhibitory κ B α degradation (Luhrs *et al.* 2002). Less consideration has been given to the possible use of butyrate-producing bacteria as probiotics. A number of studies focusing on culturable human intestinal microflora (Frankel *et al.* 1994; Wilson *et al.* 2000; Duncan *et al.* 2002, 2003,

2004a,b; Pereira & Gibson, 2002; Vine *et al.* 2002; Louis *et al.* 2004) have demonstrated that the colon harbours significant populations of butyrate-producing bacteria, such as *Clostridium*, *Eubacterium*, *Fusobacterium*, etc. Recent studies have concentrated on combining molecular and cultural approaches to investigate the dominant butyrate-producing bacteria of the human colon and have identified a large number of butyrate-producing isolates based on sequencing and restriction fragment length polymorphism analyses of 16S rRNA genes.

A probiotic is considered a live microbial feed supplement that has beneficial effects on the host by improving its intestinal microbial balance (O'Sullivan *et al.* 2005) or 'a live microbial food ingredient that is beneficial to health' (Otlés *et al.* 2003). The criteria for a micro-organism to be defined as probiotic include that the strain be of human origin, be safe for human use, be stable in acid and bile acids, and adhere to the intestinal mucosa (Chen & Walker, 2005). Probiotic strains are often selected on an empirical basis from bacterial pools and include human and traditional fermented food isolates. Probiotic bacteria are shown to promote the

Abbreviations: cfu, colony-forming unit; DAI, disease activity index; DSS, dextran sodium sulfate; FISH, fluorescent *in situ* hybridisation; IMM, intestinal microflora medium; MPO, myeloperoxidase; MRS, De Man–Rogosa–Sharpe; SSC, saline sodium citrate.

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host's endogenous defence mechanisms by enhancing the humoral immune responses and thus promoting the intestine's immunological barrier (Saggiaro, 2004). Moreover, probiotic bacteria have been shown to stimulate non-specific host resistance to microbial pathogens (Resta-Lenert & Barrett, 2003), and thereby aid in immune elimination, and modulate the host's immune responses to potentially harmful antigens with the potential to down regulate hypersensitivity reactions (Snelling, 2005). A previously reported study (Djouzi *et al.* 1997) demonstrated an increase in SCFA in the caeca of germ-free rats inoculated with human intestinal microflora and fed fermented milks without a concomitant change in lactate concentration. The authors concluded that the increase in SCFA was beneficial for the host since they were absorbed from the colon and provided an important source of energy for tissues, especially of butyrate, which is the preferred energy source for colonocytes.

The aim of the present study was to isolate and characterise in-depth a putatively functional butyrate-producing facultative anaerobe, a bacterium belonging to the species *Enterococcus durans*. We tested the ability of this novel bacterial isolate to suppress inflammation and concomitantly ameliorate the symptoms of dextran sodium sulfate (DSS)-induced colitis in mice.

Materials and methods

Faecal sample preparation

The bacterium described in the present paper was isolated from the fresh faeces of a healthy human vegetarian. The volunteer had not consumed any antibiotics or other drug known to affect faecal flora for at least 6 months before taking the faecal sample. Sterile peptone solution (9 ml; 0.1%, w/v) in water was added to 1 g faeces and then mixed with a stomacher. A dilute faecal sample was incubated in liquid intestinal microflora medium (IMM) containing 15 ml rumen fluid, 0.2 g peptone, 0.2 g bacto soytone, 0.1 g yeast extract, 1×10^{-4} g resazurin, 0.4 g Na_2CO_3 , 0.05 g cysteine hydrochloride, 0.5 g carboxymethylcellulose, 5 ml of a mineral solution containing (per litre): 0.004 g KH_2PO_4 , 0.09 g NaCl, 0.009 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.014 g $(\text{NH}_4)_2\text{SO}_4$, 15×10^{-6} g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 18×10^{-7} g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 ml of a vitamin solution containing per litre: 0.1 mg B_{12} , 20 mg thiamine hydrochloride, 20 mg Ca-D-pantothenate, 20 mg pyridoxine hydrochloride, 20 mg nicotinamide, 20 mg riboflavin, 1 mg *p*-aminobenzoic acid, 0.25 mg biotin and 0.25 mg folic acid, essentially as previously described by Barcenilla *et al.* (2000). The dilute faecal sample was incubated in IMM broth under microanaerobic conditions (using anaerobic sachets AN-35 (Oxoid Unipath Ltd, Basingstoke, Hants, UK) in anaerobic chambers) at 37°C, and at different pH: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.6.

Quantitative detection of butyrate

Detection and quantification of butyrate concentration was performed following injection of a sample of growth medium into a gas chromatograph with a flame ionisation detector and capillary column (HP-FFAP 30 m \times 0.53 mm \times 1.0 μm). Running conditions were as described in the capillary column

instructions (Agilent Technologies, Palo Alto, CA, USA). Briefly, the samples were injected at 240°C, He was run at 15 ml/min and the flame ionisation detector was brought to 240°C. The initial temperature was 40°C for 2 min, and then rose gradually at 30°C/min or 35°C/min, until the desired temperature was reached.

Selection of high-butyrate-producing bacterial isolates

The faecal sample bacteria were inoculated on IMM agar for 48 h and fifty colonies were then randomly chosen. The colonies were incubated in IMM broth and those which secreted the highest quantities of butyrate were selected for further characterisation.

Identification of the faecal isolate producing butyrate

Gram staining was performed as described in the Gram stain kit instructions (Difco Laboratories, Detroit, MI, USA).

The selected bacterial isolate was grown in 10 ml De Man–Rogosa–Sharpe (MRS) medium (Difco Laboratories) for one night. Putative bacterial plasmids were isolated using the Qiagen Plasmid Mini purification kit (Hilden, Germany) according to the instruction manual. Isolated and purified plasmids were monitored on an agarose-analytical gel.

Genomic DNA was isolated from the bacterial isolate by the genomic DNA purification kit (Promega, Madison, WI, USA). The nucleotide sequence of the reverse primer used in the PCR was 1392r universal 5'-ACGGGCGGTGTGTAC-3' and the nucleotide sequence of the forward sequence was 11f universal 5'-GTTTGATCCTGGCTCAG-3' (Sigma Chemicals, St Louis, MO, USA). PCR amplification was performed by a PCR thermal cycler under the following conditions: denaturation at 95°C for 1 min, followed by twenty-nine cycles of denaturation at 94°C for 30 s, annealing at 44°C for 40 s, and extension at 72°C for 1 min and 40 s. After completion, an additional extension step was performed at 72°C for 4 min, and the samples were then chilled to 4°C. The final mixture volume was 50 μl , and contained deoxynucleotide triphosphates (2 mmol/l), 4 mM-MgCl₂, bovine serum albumin (250 $\mu\text{g/ml}$), 30 μl PCR water, forward primer (50 pmol/ μl), reverse primer (50 pmol/ μl), 1.5 μl red *Taq* DNA polymerase and its 5 μl buffer (Sigma) and purified bacterial genomic DNA (30 ng/ μl). The primers were tested in PCR amplification with *E. coli* genomic DNA. Analyses were performed by Blast on the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Butyrate kinase sequence analysis

The nucleotide sequences of the PCR primers were chosen from the genomic DNA sequence of the butyrate-producing bacterium *Clostridium acetobutylicum* taken from the website of the National Center of Biotechnology Information. The nucleotide sequence of the forward primer used in PCR was 5'-TGCGTCAAATCTTGGTGGAA-3', which is located at 1385–1404; that of the reverse sequence was 5'-AAGTACC-TCCACCCAGGTGT-3', which is located at 1615–1635 (MWG-Biotech AG, Reinach, Switzerland). PCR amplification was performed by a gradient-PCR thermal cycler under the following conditions: denaturation at 95°C for 5 min,

followed by thirty-five cycles of denaturation at 95°C for 30 s, annealing at 40°C to 60°C (with 2°C jumps) for 1 min, and extension at 72°C for 1 min. After completion, an additional extension step was performed at 72°C for 5 min, and the samples were then chilled to 4°C. The final mixture volume was 50 µl, and contained deoxynucleotide triphosphates (10 mmol/l), MgSO₄ (25 mmol/l), 31 µl PCR water, forward primer (10 mmol/l), reverse primer (10 mmol/l), 1.5 µl Tfl Taq DNA polymerase and its 10 µl buffer (RT PCR kit; Promega) and purified bacterial genomic DNA (25 ng/µl).

All PCR products were visualised in ethidium bromide-stained agarose gels, purified by gel extraction kit (Qiagen; Westburg, Leusden, The Netherlands) and nucleotide sequences were determined by automated DNA sequencer (ABI 3100) at the DNA analysis laboratory, Faculty of Science of the Hebrew University of Jerusalem (Israel).

Gelatin zymography

We examined the bacterial isolate's ability to synthesise some of the harmful factors previously described to be produced by pathogenic *Enterococcus* strains, such as gelatinases. To this end zymographic analyses using gelatin gels were performed on concentrated medium samples from bacterial cultures essentially as described by Kawalec *et al.* (2005) with modifications. Samples were treated with Laemmli SDS-PAGE sample buffer for 30 min at 37°C, subjected to electrophoretic separation on a gelatin-impregnated (1 mg/ml; Difco) SDS-8% polyacrylamide gel under non-reducing conditions, followed by 30 min of shaking in 2.5% Triton X-100 (BDH, Poole, Dorset, UK). The gels were then incubated for 16 h at 37°C in tri(hydroxymethyl)-aminomethane (50 mmol/l), NaCl (0.2 mol/l), CaCl₂ (5 mmol/l) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% (w/v) Coomassie Blue G 250 (Bio-Rad, Richmond, CA, USA) in methanol-acetic acid-water (30:10:60, by vol.).

Effects of low pH and bile acids on growth rate of selected bacterial isolate

Selected bacterial colonies were grown to mid log phase (up to 0.4–0.5 optical density units at 600 nm) and then incubated for 1, 2, 3 and 4 h at 37°C in MRS medium, adjusted to pH 2 or 3 with HCl (5 mol/l). The number of surviving bacteria was determined by a plate-count procedure on MRS agar at pH 6.0. The sensitivity of the selected bacterial colonies to bile was tested by incubating the bacteria in MRS medium containing 0.05% (w/v) ox-gall (Oxoid Unipath Ltd) for 2, 4 and 6 h. MRS medium at pH 6.0 was used as a control. The number of surviving bacteria was again determined by the plate-count procedure as above. The number of colonies was counted after each incubation time. All the experiments were performed in quadruplicate.

E. durans was grown in IMM as described previously. MRS medium was used to maintain the *Enterococcus* isolate. *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 was grown in MRS broth at 37°C without shaking. Bacterial isolates were stored in appropriate media containing 50% (v/v) glycerol at –80°C.

Assays in tissue cultures

A Caco-2 cell line, originally isolated from a human colon adenocarcinoma, was obtained from ATCC (number: HTB-38). Cells were routinely grown at 37°C in a 5% CO₂–95% air atmosphere in Dulbecco's modified Eagle's medium with 1% (v/v) L-glutamine, 10% (v/v) fetal calf serum and 0.2% (v/v) penicillin (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were seeded at a concentration of 10⁵ cells/ml. The culture medium was replaced every 2 d.

In vitro adhesion assay. Caco-2 monolayers at late stages of post-confluence (after 14 d of culture) were prepared on plastic coverslips, which were placed in eight-well tissue culture plates. The selected *E. durans* was compared with additional established probiotic bacteria, *L. rhamnosus* GG and *L. casei* Shirota, which were included as high- and low-adhesive strains (Tuomola & Salminen, 1998), respectively. Overnight bacterial cultures (50 µl) in 5 ml of MRS medium and 200 µl Dulbecco's modified Eagle's medium without additions were added to each well of the tissue culture plate and incubated. After 0.5, 1, 1.5 and 2 h of incubation, the monolayers were washed twice with sterile PBS, fixed with 95% (v/v) methanol, stained with Giemsa stain solution (1:20) (Sigma), washed until no colour was observed in the washing solution, dried and examined microscopically at 1000× magnification under oil immersion. For each monolayer on a coverslip well, the number of cells with adherent bacteria was evaluated compared with the number of cells without adherent bacteria in ten random microscopic areas. This comparison represents the adherence percentage. The adhesion capacity was determined by counting Giemsa-stained Caco-2 and bacterial cells under a microscope at 1000× magnification.

Effect of Enterococcus durans on dextran sodium sulfate-induced colitis

The experiments were conducted in full compliance with the strict guidelines of the Hebrew University policy on animal care and use. Colitis was induced in male 10- to 12-week-old Balb/c mice (Harlan Laboratories Ltd, Rehovot, Israel), which were randomly divided into three different groups of six mice each. The DSS group received 2.5% (w/v) DSS (Sigma) in the drinking water and was fed only Purina chow (Harlan Laboratories Ltd, Rehovot, Israel), whereas the control group received tap water and Purina chow. There were two experimental groups: one received 5% (w/v) DSS in the drinking water and fed Purina chow containing 1 × 10⁸ colony-forming units (cfu) *L. delbrueckii*/g; the other also received 5% DSS in the drinking water as above and fed Purina chow containing 1 × 10⁸ cfu *E. durans*/g.

We choose as control the food-grade lactic acid bacterium *L. delbrueckii* subsp. *lactis* ATCC 4797, since its measured ability to produce butyrate as assessed by gas chromatograph methodology resulted in negligible concentrations as compared with *E. durans* (data not shown).

We evaluated animal body weight, stool consistency and the presence of blood in the stools daily. After 8 d of DSS treatment and feeding with the different diets, mice were killed and tissue samples were harvested from the colon. The tissue samples were removed and divided. One portion was

snap-frozen in liquid N₂ for evaluation of myeloperoxidase (MPO) activity. Another was fixed in buffered formalin (4%, v/v) at 4°C and embedded in paraffin, and then sections (5 µm thick) stained with haematoxylin and eosin were subjected to histological evaluation or fluorescent *in situ* hybridisation (FISH) analysis to assess the presence of *E. durans* adhered to the colonic mucosa.

The extent of clinical activity in DSS-induced colitis was determined and scored according to typical clinical signs of intestinal inflammation (disease activity index; DAI). Scores were defined as follows: loss in body weight, 0 = no loss; 1 = 5 to 10%; 2 = 11 to 15%; 3 = 16 to 20%; 4 = over 20%; haemocult, 0 = no blood; 2–3 = positive; 4 = gross blood.

Histological findings were also graded according to stage of intestinal inflammation, using a previously described grading scale (Schultz *et al.* 2004) with the following criteria: no inflammation = 0; slightly disturbed mucosal architecture = 1; more disturbed mucosal architecture = 2; marked mucosal hyperplasia = 3; severe hyperplasia and mucosal ulcerations = 4.

Determination of myeloperoxidase activity

Colonic tissue samples (about 100–120 mg) were homogenised in 50 mM-potassium phosphate buffer (pH 6.0, Sigma) and centrifuged at 41 400 g for 10 min; pellets were suspended in 50 mM-potassium phosphate buffer containing 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma). After three freeze-and-thaw cycles, with sonication between cycles, the samples were centrifuged at 41 400 g for 10 min. Samples (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM-potassium phosphate buffer, *o*-dianisidine (Sigma), and H₂O₂ solution (20 mmol/l). One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Expression analyses of colonic interleukin-1β and tumour necrosis factor-α

Total RNA was isolated from colonic tissue samples homogenised in Tri Reagent solution (MRC, Cincinnati, OH, USA). RT-PCR assay was performed using the Promega kit assay. The selected primers were as follows: IL-1β (sense) 5'-ATGAA-GCTTTGTACAAGGAGAACCA-3'; (antisense) 5'-TTAGGA-AGACACAGATTCCATGGT-3'; TNF-α (sense) 5'-ATCAGT-TCTATGGCCCAGACCT-3'; (antisense) 5'-TCACAGAGCA-ATGACTCCAAAGTA-3'. Glyceraldehyde-3-phosphate dehydrogenase primers were used for RNA control loading: (sense) 5'-ACCACAGTCCATGCCATCAC-3'; (antisense) 5'-TCCACCACCTGTTGCTGTA-3'. The PCR protocol was 5 min at 94°C, then twenty-eight cycles (1 min, 94°C, 1 min, 68°C, 1 min, 72°C) for the test genes and twenty cycles at similar conditions for glyceraldehyde-3-phosphate dehydrogenase, and a final 10 min at 72°C for all samples.

Localisation of butyrate-producing bacteria by fluorescent *in situ* hybridisation analyses

The FISH method was used to determine whether the bacterial isolate adhered to the large intestinal tissue thereby affecting the composition of the mouse intestinal microflora.

Fluorescent *in situ* hybridisation analysis

The 250 bp PCR product from the butyrate kinase gene was labelled with digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-11-UTP) under the PCR conditions described earlier but with the addition of 0.5 µl (1 nmol/µl) DIG-11-UTP (Roche Diagnostics GmbH, Mannheim, Germany).

The slides containing large-intestinal sections were incubated at 56°C overnight. Sections were then deparaffinised in xylene for 10 min while shaking at 60°C. This process was repeated three times and then the slides were washed twice with 100% ethanol for 5 min each time. The slides were then incubated in 2 × saline sodium citrate (SSC) buffer at room temperature for 2 min. This process was repeated three times and then the slides were dehydrated for 1 min in 70%, then 85% and finally 100% (v/v) cold ethanol (aqueous solution) at -20°C. The slides were then incubated for 1 h in tri(hydroxymethyl)-aminomethane (100 mmol/l), EDTA (50 mmol/l) buffer containing lysozyme (0.1 mg/ml), at 37°C, to dissolve the bacterial wall. Then the slides were dehydrated again in cold graded cold ethanol solutions as before.

The slides were warmed in a slide plate for 5 min at 37°C following 5 min incubation, at 75°C, in 60 ml denaturation buffer containing 49 ml 70% (v/v) formamide, 7 ml 20 × SSC and 14 ml sterile water. The slides were washed in graded cold ethanol solutions as before.

Hybridisation buffer was prepared to contain 250 µl 70% formamide, 100 µl 10 × SSC phosphate buffer, 100 µl 50% (w/v) dextran sulfate and 20 µl of salmon sperm DNA (10 mg/ml). The hybridisation buffer was warmed at 70°C for 10 min and 9 µl warm hybridisation buffer and 1 µl of probe (25 ng/µl) were applied to the treated slides and then covered with cover slips and sealed with Fixogum rubber cement. The slides were then incubated at 37°C in an incubator overnight.

The rubber cement was removed and the slides were washed in 20 × SSC at 72°C and twice for 1 min in PBS containing Tween 20 (50 µl Tween 20 in 200 ml PBS). The slides were incubated for 10 min at room temperature with Reagent A from a Spot-light CISH translocation detection kit (Zymed Laboratories Inc., San Francisco, CA, USA). Anti-digoxigenin-rhodamine (50 µl; from a stock solution containing anti-digoxigenin-rhodamine (200 µl/ml) with 10% (v/v) 1:250 horse serum) was added to the slides and incubated for 40 min at 37°C. The slides were then washed twice in PBS-Tween 20 for 2 min each time and the slides were dried. Finally, slides were counterstained with 4',6-diamidino-2-phenylindole (1 mg/ml) in anti-fade solution and examined with an Olympus U-RFL-T microscope (Olympus Europe GmbH, Hamburg, Germany) suitable for FISH analyses.

Statistical analysis

Every experiment was conducted under a definite experimental design and specific statistical models were used to discern differences among treatments. Values are expressed as mean values with their standard errors. Data analyses were performed by *t* test or by the Tukey-Kramer test. Differences were considered significant at *P* < 0.05.

Results

Butyrate detection and quantification

A diluted fresh faecal sample was grown in IMM broth prepared at a wide range of different pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.6) and incubated for 3 weeks. Butyrate concentration was determined weekly by GC. The control consisted of IMM solution without faeces prepared at pH 9.6. Different pH were used to mimic the intraluminal pH of the different compartments of the gastrointestinal tract. The highly acid pH in the stomach is changed to about pH 6 in the duodenum and increases to about pH 7.4 in the terminal ileum and then the pH drops to 5.7 in the caecum, but again gradually increases, reaching pH 6.7 in the rectum. Therefore we concentrated on pH from 5.0 to 9.6. The highest amount of butyrate was recorded in samples incubated at pH 5.0, 5.5 and 6.0 following 2 weeks of incubation, relative to the control; however, the optimal pH for butyrate production was 6.0 (Table 1). We elected the optimal and not the maximal butyrate secretion, i.e. at pH 6.0 a steady butyrate secretion is achieved already at 1, 2, 3 and 4 h. The faecal culture producing the optimal amount of butyrate was spread on an IMM agar plate and colonies were isolated.

Bacterial characterisation of high-butyrate producing selected isolate

Gram staining of the bacterial isolates demonstrated that it is a Gram-positive coccus. The PCR product obtained following amplification with the 16S rRNA sequence was 1000 bp in size. The 1000 bp sequence exhibited phylogenetic identity to *E. durans* category (GenBank accession no. DSM20; 99 % probability).

Since the selected bacterium produced butyrate, it was tested for the expression of genes encoding one of the key enzymes participating in the butyrate biosynthesis pathway, butyrate kinase. Using the primers described earlier (p. 726), a fragment of 250 bp was amplified and sequenced (data not shown). The sequence was found to have 100 % similarity (at the amplified sequence) to the gene encoding the bacterial

enzyme butyrate kinase expressed in the genome of other additional butyrogenic bacteria such as *Clostridium* (Duncan *et al.* 2002). The selected bacterial isolate was devoid of gelatinase activity (Franz *et al.* 1999), as obtained by gelatin-zymography assay (data not shown).

As indicated in Fig. 1 (A), the selected bacterial isolate was able to grow at pH 2 and 3 for 4 h. Although the number of surviving bacteria following 1 h of incubation at pH 2 and 3 was significantly lower than in the control at pH 6, a significant number still survived and kept growing after 4 h incubation under the acidic conditions. It fulfilled additional probiotic criteria such as resistance to bile acids (Fig. 1 (B)), and survived 4 h in 0.5 % ox-gall (bile acid mixture).

Percentage adhesion of *E. durans* to Caco-2 cells was compared with the well-known probiotic strains *L. rhamnosus* GG and *L. casei* Shirota, which are considered to be high- and low-adhesive strains, respectively (Tuomola & Salminen, 1998). The percentage of Caco-2 cells in each field with adhering bacteria and demonstrated that the number of adhered *E. durans* cells was comparable with the highly adhesive strain *L. rhamnosus* GG but significantly higher ($P < 0.005$ as calculated by the Tukey–Kramer test) than the low-adhesive strain *L. casei* Shirota (data not shown).

The selected bacterial isolate was devoid of gelatinase activity according to the gelatin-zymography assay (data not shown).

Effect of Enterococcus durans on dextran sodium sulfate-induced colitis

Acute colitis was induced in mice via administration of DSS in the drinking water. At the end of the experiment, significant differences were observed among the different groups in terms of body-weight gain and extent of rectal bleeding, measurements that were all scored as DAI. Daily administration of 1×10^8 cfu *E. durans*/g chow to DSS-treated mice induced a significant lowering effect in DAI, i.e. *E. durans* prevented weight loss in the DSS-treated mice and concomitantly the administered bacteria prevented colonic bleeding. In contrast, the food-grade bacteria *L. delbrueckii* subsp. *lactis* ATCC

Table 1. Determination of optimal intestinal microflora medium (IMM) conditions for faecal butyrate production†
(Mean values with their standard errors)

Time (weeks) ...	Butyrate concentration (mmol/l)							
	0		1		2		3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control‡	1.9	0.2	2.1	0.3	2.0	0.5	1.3	0.7
pH 5.0	1.8	0.4	4.7*	0.8b	6.8**	1.2	5.3**	1.1
pH 5.5	2.1	0.2	5.1*	0.9	4.8*	0.4	5.6**	0.8
pH 6.0	2.3	0.3	5.1*	0.5	5.8**	0.4c	5.6**	0.6
pH 6.5	1.9	0.2	2.8	0.2	2.8	0.1	3.5*	0.3
pH 7.0	1.7	0.4	3.0	0.6	2.5	0.3	3.4*	0.5
pH 7.5	1.7	0.2	2.7	0.4	2.7	0.5	4.2*	0.4
pH 8.0	2.0	0.1	2.9	0.2	2.7	0.2	3.8*	0.4
pH 9.6	2.2	0.4	3.4	0.5	3.8*	0.7	3.8*	0.6

Mean value was significantly different from that of the control condition: * $P < 0.05$, ** $P < 0.01$ (Tukey–Kramer test).

† Four different independent experiments were conducted.

‡ Control culture contained IMM at pH 9 without faecal bacterial samples.

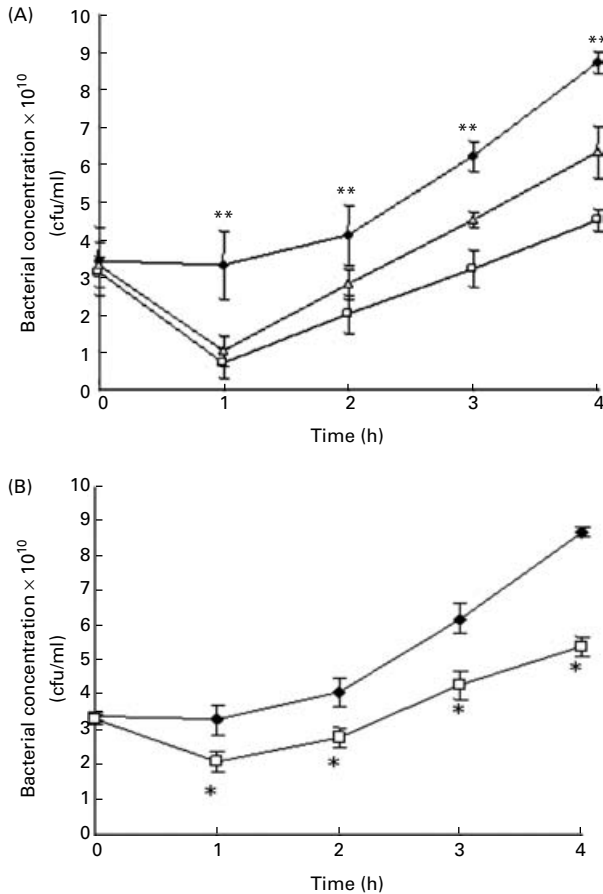


Fig. 1. (A) The effect of pH on bacterial growth rate. (—◆—), Control (pH 6); (—□—), pH 2; (—Δ—), pH 3. (B) The effect of bile acids on bacterial growth rate. (—◆—), Control (—□—), bile acids. Data are means of four independent cultures, with standard errors represented by vertical bars. Mean values were significantly different: * $P < 0.05$, ** $P < 0.001$.

4797 was completely ineffective at ameliorating DAI in mice treated with DSS.

Histological analyses demonstrated that DSS-induced colitis caused severe mucosal damage that included massive necrosis of the crypt cells (see Fig. 2 (B)); the control mice showed well-organised colonic mucosa (Fig. 2 (A)). A significant beneficial effect was observed following dietary administration of *E. durans* (see Fig. 2 (D)); it significantly inhibited the histological damage induced by DSS; however, this was not the case for *L. delbrueckii* subsp. *lactis* ATCC 4797 (see Fig. 2 (C)). The DAI (Fig. 3 (A)) mirrored the histological conditions of the different mouse groups. Histological scores for signs of colitis are summarised in Fig. 3 (C) and mirror the photomicrographs presented in Fig. 2. As shown in Fig. 3 (B), the intestinal inflammation induced by DSS and measured in terms of MPO activity was significantly ameliorated by oral administration of the selected high-butyrate-secreting bacteria *E. durans*. In contrast, *L. delbrueckii* subsp. *lactis* ATCC 4797 was completely ineffective in this regard and thus was further used as control bacteria.

To verify the anti-inflammatory role of *E. durans* in DSS-induced colitis, we measured colonic cDNA expression of the inflammatory cytokines IL-1 β and TNF- α in control, DSS-treated animals or DSS-treated animals fed

with *E. durans* or *L. delbrueckii* as control bacteria (Fig. 4). In DSS-treated animals, high IL-1 β and TNF- α cDNA levels were observed. In *E. durans*-fed animals a striking down regulation in cytokine transcription was observed. In contrast, in *L. delbrueckii*-fed animals, the concentrations of

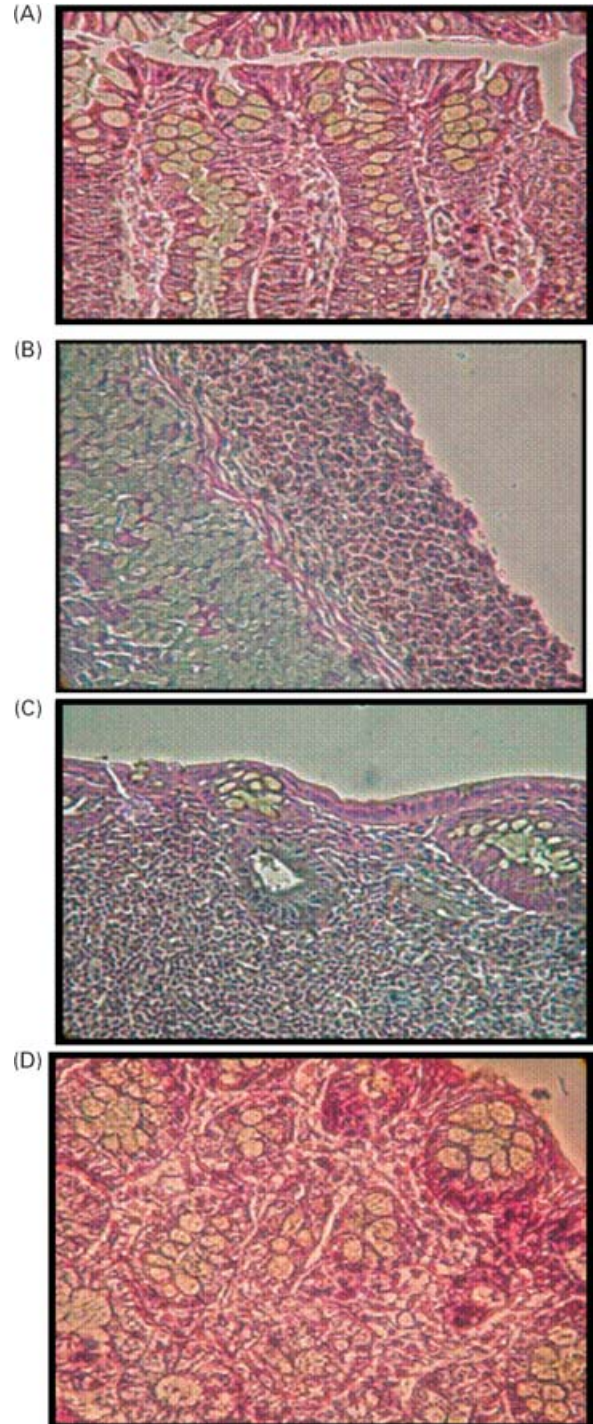


Fig. 2. Intestinal histology of murine sections of the distal colon shown by haematoxylin and eosin staining. (A) Control mice; (B) dextran sodium sulfate (DSS)-treated mice; (C) DSS-treated mice fed 1×10^8 colony-forming units (cfu) *Lactobacillus delbrueckii* subsp. *lactis*/g feed; (D) DSS-treated mice fed 1×10^8 cfu *Enterococcus durans*/g feed.

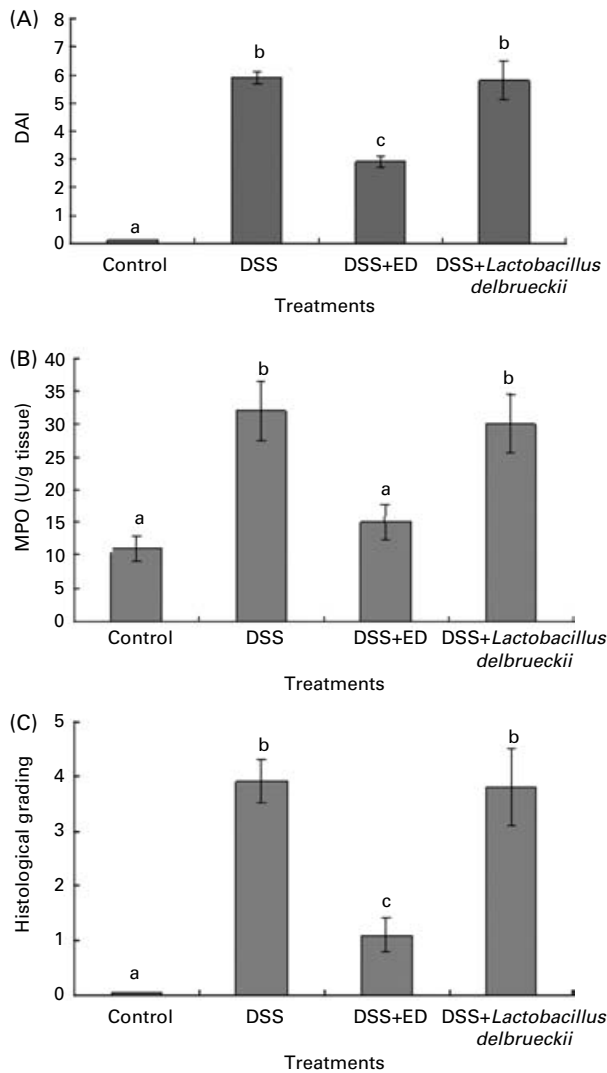


Fig. 3. Effect of *Enterococcus durans* (ED) on dextran sodium sulfate (DSS)-induced colitis. (A) Effect of ED on DSS-induced colitis, as reflected by disease activity index (DAI). Scores were defined as: loss in body weight, 0 = no loss; 1 = 5 to 10% loss; 2 = 11 to 15% loss; 3 = 16 to 20% loss; 4 = over 20% loss; haemoccult, 0 = no blood; 2 = positive; 3–4 = gross blood. (B) Effect of ED on DSS-induced colitis, as reflected by myeloperoxidase (MPO) activity. (C) Effect of ED on DSS-induced colitis, as reflected by histological grade. Histological findings were also graded according to stage of intestinal inflammation. The grading scale is described on p. 730. ^{a,b,c} Mean values with unlike letters were significantly different as calculated by the Tukey–Kramer test ($P < 0.01$).

IL-1 β and TNF- α were similar to those recorded in DSS-treated animals.

Localisation of butyrate-producing bacteria in colon by fluorescent in situ hybridisation method

FISH analysis was used to detect the presence of *E. durans* in different colonic and caecal tissue sections of mice from different experimental groups. Probes specific for the bacterial butyrate kinase gene were used for PCR amplification as part of the characterisation process of *E. durans*. Fig. 5 (D) exemplifies a colonic section of DSS-treated mice fed with *E. durans*. These mice revealed a very high concentration of butyrate kinase-positive enterobacterial colonies relative to

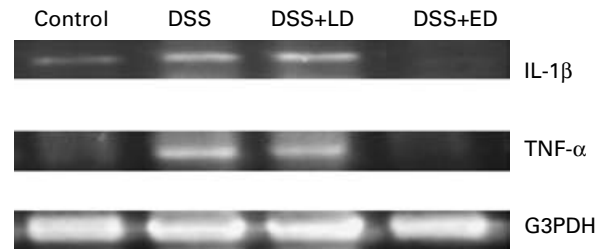


Fig. 4. Effect of *Enterococcus durans* (ED) on dextran sodium sulfate (DSS)-induced colitis on colonic IL-1 β and TNF- α expression. RNA extracts were obtained from colonic sections of control mice, DSS-treated mice, DSS-treated mice fed 1×10^8 colony-forming units (cfu) *Lactobacillus delbrueckii* (LD)/g feed or DSS-treated mice fed 1×10^8 cfu ED/g feed. Colonic RNA extracts were analysed for IL-1 β and TNF- α cDNA expression by RT-PCR and compared with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcription (a constitutive enzyme of mammalian cells, a catalytic enzyme involved in glycolysis, and therefore used as a loading control). One representative experiment from three identical ones is shown.

control mice (Fig. 5 (A)) or to mice treated with DSS (Fig. 5 (B)) or to those treated with DSS and fed *L. delbrueckii* subsp. *lactis* (Fig. 5 (C)). The results indicate that *E. durans* species do not normally populate the large intestine of mice; however, when they are orally administered they can colonise the large intestine, proliferate and inhibit DSS-induced colitis.

Discussion

Butyrate arises from microbial fermentation, it is important for the energy metabolism and normal development of colonic epithelial cells and has a mainly protective role in colonic disease (Avivi-Green *et al.* 2000, 2002). Butyrate has anti-inflammatory effects that result from inhibition of activation of the transcription factor NF κ B, and consequent reduced formation of pro-inflammatory cytokines (Luhrs *et al.* 2001, 2002). These interactions have important consequences for the health of the colonic epithelium. Recent studies have concentrated on combining molecular and cultural approaches to investigate the dominant butyrate-producing bacteria of the human colon and have identified a large number of butyrate-producing isolates based on sequencing and restriction fragment length polymorphism analyses of 16S rRNA genes (Barcenilla *et al.* 2000).

In the present study, using a cultural-restriction technique, a novel butyrate-producing bacterium that inhabits the human large intestine was identified. We characterised one bacterial isolate from a human faecal sample harvested from a vegetarian volunteer and showed that this bacterium can synthesise significant amounts of butyrate under anaerobic conditions at pH 6.0. We chose pH 6.0 and not pH 5.0, the pH that allowed peak butyrate production, since pH 6.0 is the ambient pH found in the caecum and proximal large intestine, and at pH 6.0 additional bacteria which are part of the large-intestinal flora are functionally active (Collado *et al.* 2005). The level of production of butyrate at the chosen pH seems to be similar to the activity reported for butyrogenic bacteria (Duncan *et al.* 2002; Walker *et al.* 2005).

The purpose of the present research was to find a way to create microanaerobic conditions and be able to isolate butyrate-producing bacteria from human faeces. These non-restrictive anaerobic conditions should allow their putative use in the food industry. To exert a beneficial effect

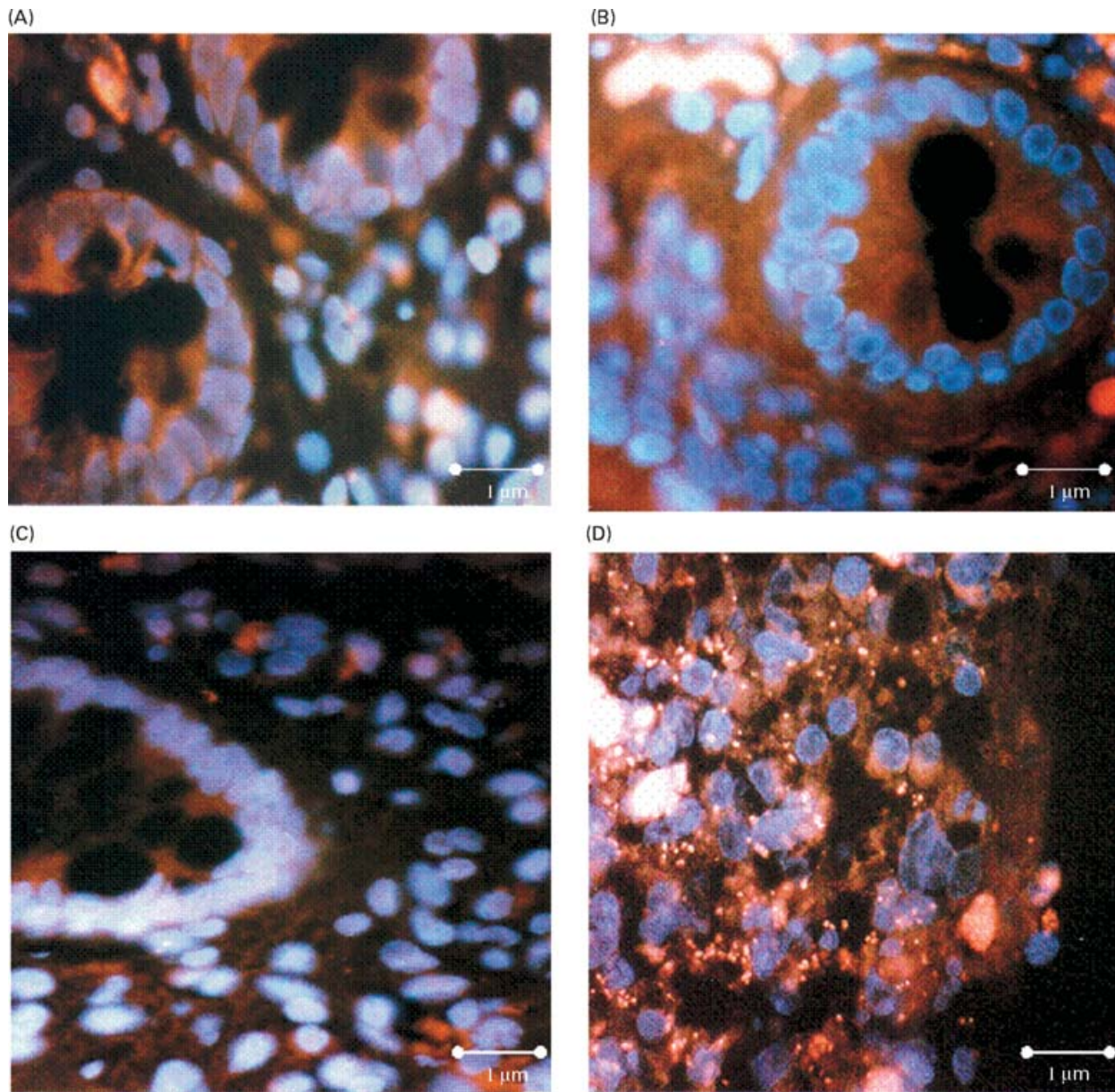


Fig. 5. Localisation of *Enterococcus durans* butyrate-producing bacteria by fluorescent *in situ* hybridisation (FISH) analyses for the bacterial butyrate kinase gene in colonic tissue areas of mice. (A) Control mice; (B) dextran sodium sulfate (DSS)-treated mice; (C) DSS-treated mice fed 1×10^8 colony-forming units (cfu) *Lactobacillus delbrueckii* subsp. *lactis*/g feed; (D) DSS-treated mice fed 1×10^8 cfu *E. durans*/g feed. FISH analyses utilising the butyrate kinase gene probe allowed us the identification of *E. durans* tightly attached to the mouse colonic mucosa, shown as bright spotted fluorescent staining (D). The blue staining represents the colonocyte's nuclei. Only in colonic mucosa from mice fed *E. durans* was the presence of the bacteria evident (D). In all other sections, from control mice (A), DSS-treated mice (B), DSS-treated mice fed with 1×10^8 cfu *L. delbrueckii*/g feed (C), no *E. durans* was identified.

in the gut, probiotic cultures must survive passage through the stomach and be tolerant to the concentrations of bile present in the small intestine (Otlés *et al.* 2003) in order to reach the colon. Survival at pH 3.0 for 2 h and growth in medium containing 500–1000 mg bile acids per litre are considered standard acid and bile tolerance in probiotic cultures (Snelling, 2005). The present bacterial isolate fulfilled these requirements. Both of these traits are prerequisites for efficacious probiotics, and variability in survival rates may help explain the controversy over their beneficial aspects.

Primary characterisation analyses demonstrated that the isolated bacteria are Gram-positive cocci. We tested whether the

bacterial isolate could produce antimicrobial materials such as bacteriocins in analogy to other probiotic bacteria (Edelman *et al.* 2003; Collado *et al.* 2005) and found that they do not (data not shown). There are additional pathways via which putative probiotic bacteria can act against pathogens. A case in point is the stimulation of cytokine secretion (Erickson & Hubbard, 2000; Kalliomaki & Isolauri, 2004; Schultz *et al.* 2004) or the production of butyrate which is used as a major energy source for the colon cells (Frankel *et al.* 1994; Duncan *et al.* 2002) and as an inhibitor of pro-inflammatory factors (D'Argenio & Mazzacca, 1999; Luhrs *et al.* 2001, 2002). In the present study, the bacterial isolate demonstrated

adherence to Caco-2 cells *in vitro*. In the colon, the bacteria need to adhere to the epithelial cells and form active colonies. The colonised bacteria can influence the host via their metabolic activity and can compete with pathogenic bacteria (Jacobsen *et al.* 1999).

We genetically characterised the bacterial isolate by PCR amplification of the 16S rRNA. The sequence obtained following 16S rRNA amplification was 99% identical to an *E. durans* isolate in the GeneBank. *Enterococcus* bacteria such as *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus* and *E. gallinarum* are known to be significant for the gastrointestinal ecology as well as for that of some foods (Klein, 2003). *Enterococcus* bacteria exist in soil, water and in human and animal intestines (10^5 – 10^7 cfu/g faeces). *E. faecalis* and *E. faecium* are the most dominant strains found in the human intestine. *Enterococcus* strains are already known as probiotic bacteria and, to a certain extent, are used to treat gastroenteritis in human patients and animals (Franz *et al.* 1999). In this regard, Pereira & Gibson (2002) have recently identified *E. durans* DSM 20 633 as a potential probiotic isolated from faecal material.

Sequencing the 250 bp PCR product obtained following amplification with specific primers to bacterial butyrate kinase indicated that in the isolated butyrate-producing *E. durans* this gene exists in its genome, as judged from 100% identity of the 250 bp amplified to the respective sequence in the gene present in the anaerobic butyrate-producing strain *C. acetobutylicum*. Two key enzymes have been found to be involved in the last stage of the butyrate-synthesising process in bacteria; butyrate kinase and butyryl CoA:acetate CoA transferase (Duncan *et al.* 2002, 2003, 2004a,b; Louis *et al.* 2004). The butyryl CoA:acetate-CoA transferase gene has been found in most butyrate-producing bacteria from the human intestine, whereas butyrate kinase is less common (Duncan *et al.* 2002, 2004a,b; Louis *et al.* 2004). Nonetheless, both pathways can be expressed at once. The significance of these metabolic differences has yet to be established.

An additional important issue to be addressed relates to bacterial safety for human use. We analysed whether the isolated *E. durans* has a plasmid and found none. This suggests that if the bacteria become resistant to any antibiotics, transfer of this trait to other bacteria would be impossible. We found that the bacterial isolate cannot survive in the presence of ampicillin, tetracycline and chloramphenicol. We also examined the ability of the bacterial isolate to synthesise gelatinase, an activity associated with harmful factors such as adhesins, invasins and haemolysin (Franz *et al.* 1999; Kawalec *et al.* 2005). We showed that *E. durans* isolated in the present study does not bear gelatinase activity. Cumulatively these qualities further support the view that the bacterial isolate can be safe for human use.

An additional important finding of the present study relates to the ability of the bacterial isolate to prevent DSS-induced colitis. We tested the effect of *E. durans* on a murine model of ulcerative colitis simultaneously induced by DSS. Several animal models of experimental ulcerative colitis have been described and, of these, colitis induced in mice by oral administration of DSS has been widely used because of its many similarities to human ulcerative colitis (Cooper *et al.* 1993). *E. durans* was able to prevent disease induction almost completely as reflected by DAI, MPO activity and histological grade, while *L. delbrueckii* subsp. *lactis* ATCC 4797 bacteria was completely ineffective in this regard.

Using a preventive setup in the present study, we demonstrated that following daily feeding of DSS-treated mice with 1×10^8 *E. durans* per g feed, there is significant inhibition in the development of chronic experimental colitis. The mechanism by which *E. durans* exerts its protection in DSS-induced colitis may reside in the bacteria's effect on immune-system regulation, or on the ability of the *E. durans* isolate to produce high concentrations of butyrate. Butyrate's role in preventing and/or ameliorating conditions such as ulcerative colitis has been studied (Hallert *et al.* 2003; Vernia *et al.* 2003; Galvez *et al.* 2005). It has been proposed that an inadequate supply of energy to colonocytes, 70% of which is normally obtained from butyrate, can be a causative factor in colitis. DSS, which inhibits butyrate oxidation without affecting glucose metabolism, induces colitis when given orally to mice (Ahmad *et al.* 2000), and it has been proposed that sulfide toxicity results largely from inhibition of the butyrate energy supply to colonocytes. It is surmised that DSS changes in epithelial cell metabolism are secondary to mucosal inflammation. *E. durans* can supply sufficient butyrate concentrations to counteract the effect of DSS or inhibit intestinal mucosal inflammation, or both concomitantly. Present work in our laboratory is addressing each pathway and its significance. We conclude that *E. durans* prevents the induction of ulcerative colitis by DSS, significantly improving the clinical conditions. This could have important biological consequences for human health.

We used FISH analysis in order to localise the fate of *E. durans* in the mouse intestinal tissue. We used a 250 bp probe obtained by PCR amplification of the bacterial butyrate kinase gene to localise the *E. durans* in the mice colonic tissue. A high concentration of fluorescent dots on the colonic tissue surfaces of mice fed *E. durans* were obtained, whereas no such fluorescent dots were detected in control mice or in those fed with *L. delbrueckii*. This suggests that *E. durans* does not normally populate the mouse caecal and large-intestinal tissue surface; however, when these bacteria colonise the rodent intestine they are not only harmless to the mice, but also prevent DSS-induced colitis.

In conclusion, we described in the present paper the characterisation of a bacterial isolate exhibiting 99% homology to *E. durans* and expressing typical probiotic features. Oral administration of the bacterial isolate prevented and/or ameliorated experimental colitis, suggesting that this isolate provides a unique oral anti-inflammatory tool that deserves further studies specifically addressing the pathogenesis and treatment of human inflammatory bowel disease. Additional *in vivo* and *in vitro* studies are needed, to determine the mechanism by which this bacterial isolate exerts its preventive effect on ulcerative colitis. Moreover, its influence on the indigenous microflora and overall metabolic activity of the gut should be further assessed *in vitro* in mixed-culture and mixed-substrate environments before the design of any clinical intervention trials. We believe that the putative probiotic bacteria described in the present paper could potentially make a significant contribution to the food and medicinal industries.

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