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ORIGINAL ARTICLE

Investigation of the impact of feeding *Lactobacillus plantarum* CRL 1815 encapsulated in microbially derived polymers on the rat faecal microbiota

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Keywords
exopolysaccharide, fluorescence *in situ* hybridization, *Lactobacillus*, microencapsulation, temporal temperature gradient gel electrophoresis.

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Abstract

Aims: The aim of this study was to evaluate the impact of the administration of microencapsulated *Lactobacillus plantarum* CRL 1815 with two combinations of microbially derived polysaccharides, xanthan : gellan gum (1%:0·75%) and jamilan : gellan gum (1%:1%), on the rat faecal microbiota.

Methods and Results: A 10-day feeding study was performed for each polymer combination in groups of 16 rats fed either with placebo capsules, free or encapsulated *Lact. plantarum* or water. The composition of the faecal microbiota was analysed by fluorescence *in situ* hybridization and temporal temperature gradient gel electrophoresis. Degradation of placebo capsules was detected, with increased levels of polysaccharide-degrading bacteria. Xanthan : gellan gum capsules were shown to reduce the *Bifidobacterium* population and increase the *Clostridium histolyticum* group levels, but not jamilan : gellan gum capsules. Only after administration of jamilan : gellan gum-probiotic capsules was detected a significant increase in *Lactobacillus-Enterococcus* group levels compared to controls (capsules and probiotic) as well as two bands were identified as *Lact. plantarum* in two profiles of ileum samples.

Conclusions: Exopolysaccharides constitute an interesting approach for colon-targeted delivery of probiotics, where jamilan : gellan gum capsules present better biocompatibility and promising results as a probiotic carrier.

Significance and Impact of Study: This study introduces and highlights the importance of biological compatibility in the encapsulating material election, as they can modulate the gut microbiota by themselves, and the use of bacterial exopolysaccharides as a powerful source of new targeted-delivery coating material.

Introduction

The human intestinal tract is an active and complex ecosystem where the microbiota plays an important role in health and disease. It is widely accepted that the intestinal microbiota is involved in host nutrition, mucosal defence and host immunity (Delzenne and Cani 2011; Molloy *et al.* 2012). However, various disease states have been associated with an imbalance of the intestinal microbiota. These can include susceptibility to pathogens such as *Clostridium difficile*, chronic diseases such as Crohn's disease and ulcerative colitis, acute gastroenteritis, food hypersensitivity and allergies, and colon cancer. The intake of probiotics as dietary supplements has been used to reinforce and/or modify the microbiota (Rochet *et al.* 2006).

Food and pharmaceutical industries want to profit from the benefits provided by probiotics, and therefore, different formulations and dairy products have incorporated them (Vasiljevic and Shah 2008; Sanz *et al.* 2010; Turcotte and Huynh 2010). However, cell viability in these products is often low, and the ability to survive and multiply in the digestive tract after ingestion strongly influences the benefits that probiotics can produce (Ozer *et al.* 2009). Moreover, ingestion of dairy products or oral formulations includes exposure of said products (including probiotics) to the highly acidic conditions of the stomach, enzymes and bile salts of the small intestine – harsh conditions that reduce probiotic viability (Kailasapathy 2002; Muthukumarasamy *et al.* 2006; Ding and Shah 2007).

Consequently, efforts have been directed to enhance the survival and resistance of probiotics during processing, as well as inside the food matrix and during transit of the upper gastrointestinal tract (GIT) (Del Piano *et al.* 2006). Over the last few years, research has focused on microencapsulation for probiotic survival improvement as it is a technological process that aims to protect probiotic bacteria, offering great potential in delivery of viable cells (Rodrigues *et al.* 2011). Numerous studies have been conducted in this area, mainly aimed towards the improvement of probiotic bacteria survival within the food product or following oral delivery (Crittenden *et al.* 2006; Kailasapathy 2006; Del Piano *et al.* 2010; Saxelin *et al.* 2010). For probiotic microencapsulation, polysaccharides are widely employed as encapsulating material. They constitute a matrix that can be degraded by microorganisms of the intestinal microbiota and, thus, allow targeted delivery of the probiotics to the human intestine (de Vos *et al.* 2010). Although a variety of polysaccharides has been described for lactic acid bacteria encapsulation, by far, the most commonly used polysaccharide is alginate as it provides nontoxic matrices with calcium chloride, suitable for sensitive bacteria (Kailasapathy 2002). However, the gel is susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+}-chelating agents and harsh chemical environments such as low pH (Smidsrod and Skjak-Braek 1990). Exopolysaccharides (EPS) obtained from different bacteria constitute an invaluable source of polysaccharidic material and can be employed as an alternative encapsulating material. In this regard, xanthan and gellan gum, being EPS, have been employed widely in the food industry and have been proven safe for human consumption (Freitas *et al.* 2011; Palaniraj and Jayaraman 2011). Previous studies accomplished with several microbial polymers found that combinations of gellan gum with either xanthan gum or jamilan were suitable for capsule formation and lactobacilli encapsulation, providing also protection against simulated gastrointestinal conditions (Sun and Griffiths 2000; Jiménez-Pranteda *et al.* 2012). Despite the demonstrated effectiveness of polymeric materials for *in vitro* protection of probiotics against adverse conditions (Sun and Griffiths 2000; Adhikari *et al.* 2003; Krasaekoopt *et al.* 2003; Chandramouli *et al.* 2004; Iyer and Kailasapathy 2005; Chavarri *et al.* 2010), very little is known as to whether this type of microencapsulation protects probiotics *in vivo*. In addition, little is known as to the effect of administration of polysaccharide encapsulated probiotics on the faecal microbiota (Wang *et al.* 1999; Del Piano *et al.* 2010).

The aim of this study was to evaluate the impact of the administration of microencapsulated *Lactobacillus plantarum* CRL 1815 with two different combinations of microbially derived polysaccharides on the rat faecal microbiota.

Material and methods

Animals

Thirty-two female Wistar rats (body weight of 150–174 g; 9–11 weeks old) were supplied by Harlan (Harlan Laboratories Inc., Bresso, Italy). Groups of four rats, selected randomly, were housed in plastic cages and kept in a room with 12 h light/dark cycles under pathogen-free conditions with free access to sterile commercial chow (AO4 diet; Panlab, Barcelona, Spain) and sterile water. The experiments were approved and supervised by the local ethic committee at the University of Granada according to the norm 138/2002 of the Junta de Andalucía.

Preparation of solutions of microbial polymers

Microbial polymeric mixtures of 1% (w/v) xanthan gum (Sigma, St Louis, MO, USA), 0·75% (w/v) gellan gum (Sigma) and 1% (w/v) jamilan, 1% (w/v) gellan gum, previously selected according to their probiotic encapsulation properties, were prepared as described by Jiménez-Pranteda *et al.* (2012).

Feeding procedure and study design

For the preparation of gavages, *Lact. plantarum* CRL 1815 (CERELA Culture Collection, Tucuman, Argentina) was grown in 400 ml of lactobacilli MRS broth (Merck KGaA, Darmstadt, Germany) at 37°C for 20 h. Bacteria were harvested by centrifugation (4000 ***g***, 15 min), washed twice in sterile saline solution (0·9% (w/v) NaCl) and resuspended in 5 ml of sterile saline solution (0·9% (w/v) NaCl).

Encapsulated bacteria were prepared as described by Jiménez-Pranteda *et al.* (2012). For the assessment of the

number of viable bacteria, suitable dilutions of the bacterial suspensions in phosphate-buffered saline (PBS; Sigma) were plated onto MRS agar plates and CFU were recorded after 24 h incubation at 37°C and 5% CO_2 by using CO_2 Gen® envelopes (Oxoid, Basingstoke, UK). For the enumeration of encapsulated bacteria, lactobacilli were released by dissolving the capsules in 0·05 mol l^{-1} sodium phosphate buffer (pH 7) using a stomacher for 6 min prior to preparation of the serial dilutions.

Two experimental sets were carried out according to the type of bead employed. In each test, 16 rats were employed and randomly assigned to one of the four experimental groups: (i) beads group, received beads prepared with either xanthan : gellan gum (1%:0·75%) or jamilan : gellan gum (1%:1%) (ii) probiotic group, received *Lact. plantarum* CRL 1815 suspended in sterile saline solution (iii) encapsulated probiotic group, received *Lact. plantarum* CRL 1815 encapsulated in either xanthan : gellan gum (1%:0·75%) or jamilan : gellan gum (1%:1%); and (iv) control group, received water. Rats received a daily dose of 100 μl of their test product corresponding to ~ 5×10^9 CFU of viable *Lact. plantarum* CRL 1815 and 1 : 0·75 mg of xanthan : gellan gum or 1 : 1 mg of jamilan : gellan gum, depending on the type of capsule administered. Doses were administered by the intra-oesophageal route with a stainless steel feeding needle (50/15; Harvard Apparatus, Holliston, MA, USA) and a 1-ml syringe.

Collection of samples

Faecal samples were collected from animals on days 1 (baseline), 3, 7 and 10 of daily dose administration and kept in anaerobic atmosphere until they were aliquoted immediately prior processing of samples for fluorescence *in situ* hybridization (FISH) and freezing at −80°C for temporal temperature gradient gel electrophoresis (TTGE) analysis. The rats were anaesthetized by intraperitoneal injection of ketamine and xylazine and then sacrificed after the 10-day experimental period to obtain intestinal samples. Sections of ~ 7 cm of proximal (duodenum) and distal (ileum) small intestine and colon were removed for biodiversity studies. These intestinal sections were washed with sterile PBS containing 0·05% L-cysteine hydrochloride (Scharlau, Barcelona, Spain) to discard the faecal content, weighed and stored at −80°C until DNA extraction for TTGE analysis.

Processing of samples for FISH analysis

Homogenates were prepared by diluting fresh faeces 1 : 9 (w/w) in PBS, immediately after collection. Aliquots (500 μl) of the faecal homogenates were centrifuged at 16 000 ***g*** for 5 min. Cell pellets were washed in 1 ml sterile PBS (same centrifugation conditions) and then resuspended in 375 μl sterile PBS. Three volumes of ice-cold 4% paraformaldehyde were added to the cell suspensions and mixed thoroughly; the suspensions were then incubated for 4 h at 4 °C. Fixed cells were washed twice in 1 ml of filtered ice-cold PBS (centrifugation as above) prior to resuspension in 150 μl of ice-cold PBS plus 150 μl of ice-cold 96% ethanol and stored at −20°C. FISH samples were shipped to the University of Reading on dry ice for hybridization and enumeration.

Hybridization and enumeration of bacteria

Probes (Table 1) were synthesized commercially (Sigma-Genosys; Haverhill, UK) and labelled with the fluorescent dye cyanine 3. FISH was performed as described by Martín-Peláez *et al.* (2008). Slides were viewed under a Nikon E400 Eclipse microscope (DM575 filter; Nikon, Surrey, UK). Cells were counted for 15 random fields of view, and the numbers of bacteria were determined by using the following equation:

$$DF \times ACC \times 6732{\cdot}42 \times 50 \times DF_{\text{sample}}$$

Where DF is the dilution factor [(300 μl/500 μl) × 10], ACC is the average cell count of 15 fields of view, and DFsample refers to the dilution of sample used with a particular probe or stain (e.g. 50× for Lab158 counts). The figure 6732·42 refers to the area of the well divided by the area of the field of view, and the factor 50 takes the cell count back to per millilitre of the sample.

DNA isolation and 16S rRNA gene amplification

Genomic DNA of *Lact. plantarum* CRL 1815 was isolated employing the PowerSoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instructions. Total DNA was extracted from 200 mg of faecal samples using a modification of the bead-beating method described by Godon *et al.* (1997), where samples were shaken at medium speed in two 2-min cycles, with 5 min ice incubation after each cycle. The same DNA extraction was modified for intestinal samples to increase its efficiency as described by Lepage *et al.* (2005). Prior to DNA extraction, intestinal samples were incubated overnight at 56 °C, 300 rev min^{-1} in a lysis buffer (Tissue lysis; Qiagen, Madrid, Spain) at a proportion of 1 : 10 (w/v). Homogenates were subsequently centrifuged at 19 000 ***g*** for 30 min and DNA extracted from the pellets.

The concentration and integrity of DNA were determined by electrophoresis on 0·8% agarose gel [1 × Tris-acetate-EDTA (TAE)] containing ethidium bromide,

Table 1 Details for fluorescence *in situ* hybridization probes used in this study

Probe name	Detects	Formamide (%) in hybridization buffer	Temperature (°C)		Reference
			Hybridization	Washing	
Ato291*	*Cryptobacterium curtum*, *Gordonibacter pamelaeae*, *Paraeggerthella hongkongensis*, all *Eggerthella*, *Collinsella*, *Olsenella* and *Atopobium* species	0	50	50	Harmsen *et al.* (2000)
Bif164*	Most *Bifidobacterium* species and *Parascardovia denticolens*	0	50	50	Langendijk *et al.* (1995)
Chis150	Most members of *Clostridium* cluster I, all members of *Clostridium* cluster II	0	50	50	Franks *et al.* (1998)
Clit135	Nine members of *Clostridium* cluster XI	0	50	50	Franks *et al.* (1998)
Ecyl387	*Clostridium innocuum*, *Eubacterium biforme*, *Eubacterium cylindroides*, *Eubacterium dolichum*, *Eubacterium tortuosum*, *Streptococcus pleomorphus*	20	50	50	Harmsen *et al.* (2002)
Erec482	Most members of *Clostridium* cluster XIVa	0	50	50	Franks *et al.* (1998)
Eub338I, Eub338II, Eub338III (Eub338 mix)*,†	Most Bacteria, *Planctomycetales, Verrucomicrobiales*	35	46	48	Daims *et al.* (1999)
Fpra655	*Faecalibacterium prausnitzii* and some *Subdoligranulum* species	0	58	58	Hold *et al.* (2003)
Lab158*	All *Oenococcus*, *Vagococcus*, *Melissococcus*, *Tetragenococcus*, *Enterococcus*, *Catellicoccus*, *Paralactobacillus*, *Pediococcus* and *Lactococcus* species, most *Lactobacillus*, *Weissella* and *Leuconostoc* species	0	50	50	Harmsen *et al.* (1999)
Prop853	Most members of *Clostridium* cluster IX	0	50	50	Walker *et al.* (2005)
Rbro730/Rfla729*‡	*Anaerotruncus colihominis*, *Ruminococcus bromii*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Desulfotomaculum alcoholivorax*	20	50	50	Harmsen *et al.* (2002)

*Lysozyme pretreatment performed as described by Martín-Peláez *et al.* (2008).
†All three probes used together at equimolar concentrations (50 ng μl^{-1}).
‡Both probes used together at equimolar concentrations (50 ng μl^{-1}).

prior to amplification of the V6 to V8 regions of 16S rRNA gene, using primers U968-GC-F and L1401-R (Zoetendal *et al.* 1998). PCR was performed using Hot Star *Taq* DNA polymerase (Qiagen, Spain). PCR mixtures (25 μL) contained the following: 1 × PCR buffer, 1·5 mmol l^{-1} Mg $(OAc)_2$, 0·1 mmol l^{-1} of each dNTP, 0·5 μmol l^{-1} primers and 2·5 U of Hot Star *Taq* polymerase. Several dilutions of template DNA were tested if the presence of PCR inhibitors was suspected after electrophoresis on agarose visualization (1 and 1·5 μl of crude extract or 1 μl of 1/10 dilution) and the highest PCR-positive dilutions used for further analysis. The DNA fragments were amplified using a GeneAmp PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the following programme: 95°C for 15 min; 30 cycles of 97°C for 1 min, 58°C for 1 min, and 72°C for 1 min 30 s; and 72°C for 15 min. Amplicons were analysed by electrophoresis on a 1·5% agarose gel containing ethidium bromide to check the correct size and estimate their concentration.

Temporal temperature gradient gel electrophoresis analysis

Temporal temperature gradient gel electrophoresis was performed using a Dcode Universal Mutation Detection System (Bio-Rad, Paris, France) for sequence-specific separation of PCR products. Electrophoresis was performed through a 1-mm-thick, 16 × 16 cm polyacrylamide gel [8% (w/v) acrylamide-bisacrylamide, 7 mol l^{-1} urea,

1·25 × TAE, 60 μl of TEMED and 600 μl of 10% ammonium persulfate] in 1·25 × TAE. For better resolution, the voltage was fixed at 20 V for 20 min at the beginning of electrophoresis; thereafter, electrophoresis was performed at a fixed voltage corresponding to 64 mA for 22 h with an initial temperature of 66°C and a ramp rate of 0·2°C per h. Gels were stained in a solution of SYBR Green I (Roche Diagnostics, Barcelona, Spain) and bands visualized under UV illumination using a gel image system (Vilber-Loumart Quantum ST4, Eberhardzell, Germany). Bands with a predominant profile, that is, appeared consistently across animals or with a high peak intensity, and bands that migrated to the same region of the gel as *Lact. plantarum* CRL 1815 were excised and treated for further analysis.

Band excision and sequence analyses

Bands were excised using sterile scalpel blades, and the gel fragments were washed once in 200 μl of sterile milliQ water prior to overnight incubation in 100 μl of sterile milliQ water at 4°C. Bacterial 16S rRNA gene sequences were then amplified by PCR (as above) from the band dialysates. The amplicons were purified using the Amicon Ultra-0.5, Ultracel-100 Membrane 100 kDa (Millipore, Billerica, MA, USA), and the size and concentration evaluated on 1·5% agarose gels containing ethidium bromide. PCR products were sequenced with an Applied Biosystems 373A DNA sequencer by the automated Sanger method (Sanger *et al.* 1977). Sequences were analysed with the software Chromas lite ver. 2.01 (Technelysium, South Brisbane, Australia), and their similarity with 16S rRNA gene sequences stored in GenBank and EMBL databases was searched with the Blast software (www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis and reading of temporal temperature gradient gel electrophoresis profiles

Unpaired Student's *t*-test was used to determine significant differences in the bacterial counts between experimental groups on the same day. Paired Student's *t*-test was used to determine significant differences in the bacterial counts within each group. Statistical significance was accepted at $P < 0{\cdot}05$.

Temporal temperature gradient gel electrophoresis profiles of a total of 15 gels were compared using Quantity One® ver. 4.6.3 software (Bio-Rad). The analysis took into account the number of bands, their position on the gel and their intensity. Only profiles with at least three bands were taken into account. An external reference standard or marker (AmpliSize™ Molecular Ruler (Bio-Rad) was used to align and normalize the profiles. Two markers and two *Lact. plantarum* DNA were added per gel. Statistical differences in the number of bands and their intensity within each group were analysed by Student's *t*-test. Cluster analysis and calculation of similarity between TTGE profiles were performed with Dice correlation coefficient, and a dendogram was constructed from this matrix by using neighbour-joining algorithm.

Nucleotide sequence accession numbers

The sequences of the bands amplified with universal primers in this study have been assigned GenBank accession numbers from JN674462 to JN674499 and JN674501.

Results

For the evaluation of the impact of microencapsulated *Lact. plantarum* administration on the rat faecal microbiota, both the composition and biodiversity of this microbiota were analysed by employing FISH and TTGE approaches.

All animals completed the feeding study, and there were no signs of dietary intolerance or health problems.

Composition of the faecal microbiota

The overall microbial load (Eub338 counts) remained essentially stable in all animal groups throughout the study (Fig. 1). The administration of *Lact. plantarum* CRL 1815 encapsulated in xanthan : gellan gum significantly ($P < 0{\cdot}05$) reduced Bif164, Chis150 and Ecyl387 counts over time, while Clit135 levels increased significantly ($P < 0{\cdot}05$). An increment on Clit135 and a decrease on Bif164 levels took place as well with nonloaded xanthan : gellan gum capsules. Increased Lab158 counts compared to pretreatment levels were seen on day 3 in the free *Lact. plantarum* CRL 1815 group and on days 7 and 10 in both jamilan : gellan gum groups (with and without probiotic).

Significantly ($P < 0{\cdot}05$) higher levels of Lab158 counts were only seen in animals receiving *Lact. plantarum* CRL 1815 contained in jamilan : gellan gum capsules (compared to jamilan : gellan gum capsules alone and *Lact. plantarum* group) (Fig. 1). While Bif164 counts decreased over time for both xanthan : gellan gum capsule groups and the control group, its levels remained relatively constant for animals in both jamilan : gellan gum capsule groups (with or without bacteria).

On average, the ten group-specific probes detected ~50% of the rat faecal microbiota at day 1 (range, 34–75% of Eub338 counts). Greater variation was observed between the proportions each probe accounted for after treatments than was seen for probe counts (data

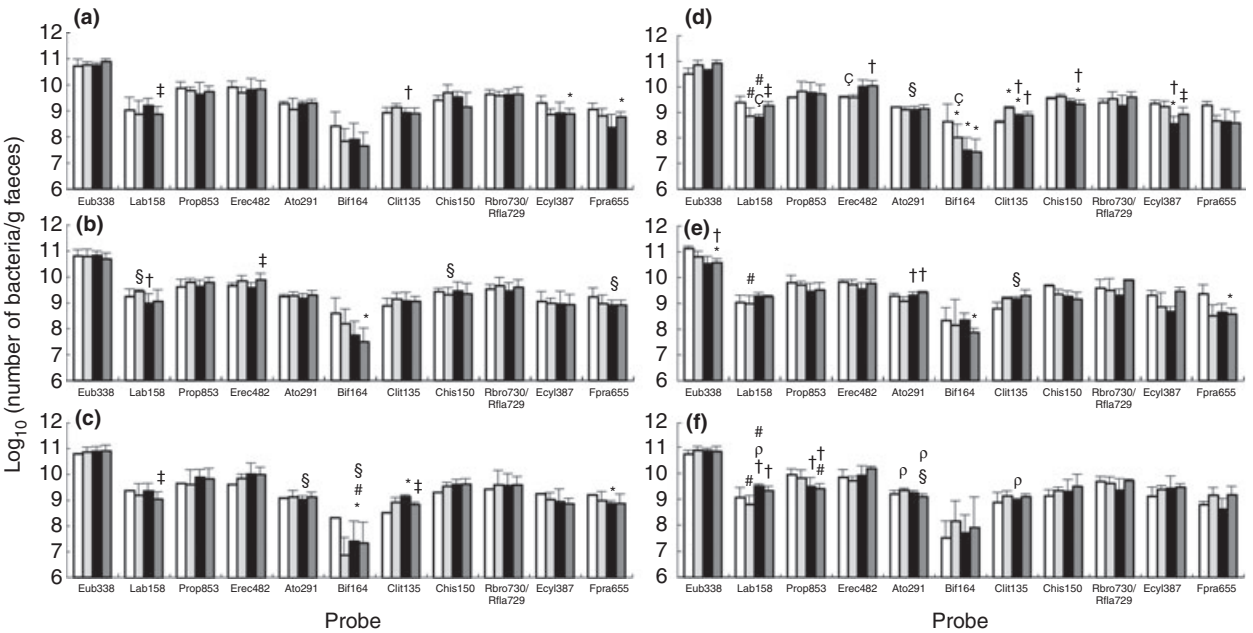

Figure 1 Investigation into the effects of encapsulation of *Lactobacillus plantarum* CRL 1815 on the faecal microbial population of rats during a feeding study: day 1, white bars; day 3, light-grey bars; day 7, black bars; day 10, dark-grey bars. Error bars represent standard deviation. (a) Control, $n = 8$; (b) free *Lact. plantarum* CRL 1815, $n = 8$; (c) xanthan : gellan gum (1% : 0·75%) capsules, $n = 4$; (d) xanthan : gellan gum (1% : 0·75%)– *Lact. plantarum* capsules, $n = 4$; (e) jamilan : gellan gum (1% : 1%) capsules, $n = 4$; (f) jamilan : gellan gum (1% : 1%)– *Lact. plantarum* capsules, $n = 4$. Intragroup variations: *Significantly different to day 1 ($P < 0·05$); †Significantly different to day 3 ($P < 0·05$); ‡Significantly different to day 7 ($P < 0·05$). Intergroup variations at same time point: §Significantly different from (a) ($P < 0·05$); #Significantly different from (b) ($P < 0·05$); çSignificantly different from (c) ($P < 0·05$); ρ,Significantly different from (e) ($P < 0·05$).

not included). The most notable effect was seen following administration of empty capsules (independent of type), with greater coverage of the microbiota seen (32·30 and 55·48% for xanthan : gellan gum group and 25·53 and 77·87% for jamilan : gellan gum group, days 1 and 10, respectively). Erec482 and Rbro730/Rfla729 proportions registered the main increases, averaging 6% at day 1 and 17% at day 10. After administration of xanthan : gellan gum capsules, an increase in proportions of Prop853 (6·91 to 13·86%) and Chis150 (3·13 to 7·60%) was detected, while jamilan : gellan gum capsules administration showed an increase in proportions of Ato291 (1·5 to 7·5%), Ecyl387 (1·5 to 7·5%) and Lab158 (0·89 to 4·99%). These effects on the microbiota proportions were not seen following administration of capsules containing *Lact. plantarum* CRL 1815, with the exception of an increase in proportions of Erec482 after jamilan : gellan gum-*Lact. plantarum* CRL 1815 beads (14·09 to 23·34%).

Biodiversity of the faecal microbiota

Molecular analysis of the faecal and intestinal samples was performed by TTGE for all the samples recovered (Fig. 2 and 3). The band number of the TTGE profiles varied markedly between rats (3–21 bands), although no significant differences were seen in relation to the treatment administered. Unique faecal and mucosa-associated microbiotas were seen for each rat, although some common bands were evident.

The banding profiles of the faecal and intestinal samples were analysed using the neighbour-joining algorithm, and the dendograms obtained did not demonstrate any obvious clustering according to the treatment. However, clustering was observed in relation to the sample day (i.e. length of treatment) and the sample site (small, large intestine) (Fig. 2 and 3). This suggests that the overall structure of the gut microbiome was not modified by encapsulated probiotic administration.

Bands that appeared consistently across animals or with high peak intensity, as well as those bands migrating to the same region of the gel as *Lact. plantarum* CRL 1815 band, were excised and sequenced. Analysis of the sequences using BLAST allowed the identification of these sequences with >80% similarity to sequences in the database (Tables 2 and 3). This identification should be considered tentative, because we have only amplified a 400-bp fragment. Band CJ1-1 (*Eubacterium eligens* ATCC 27750) appeared to be a constituent of the faecal

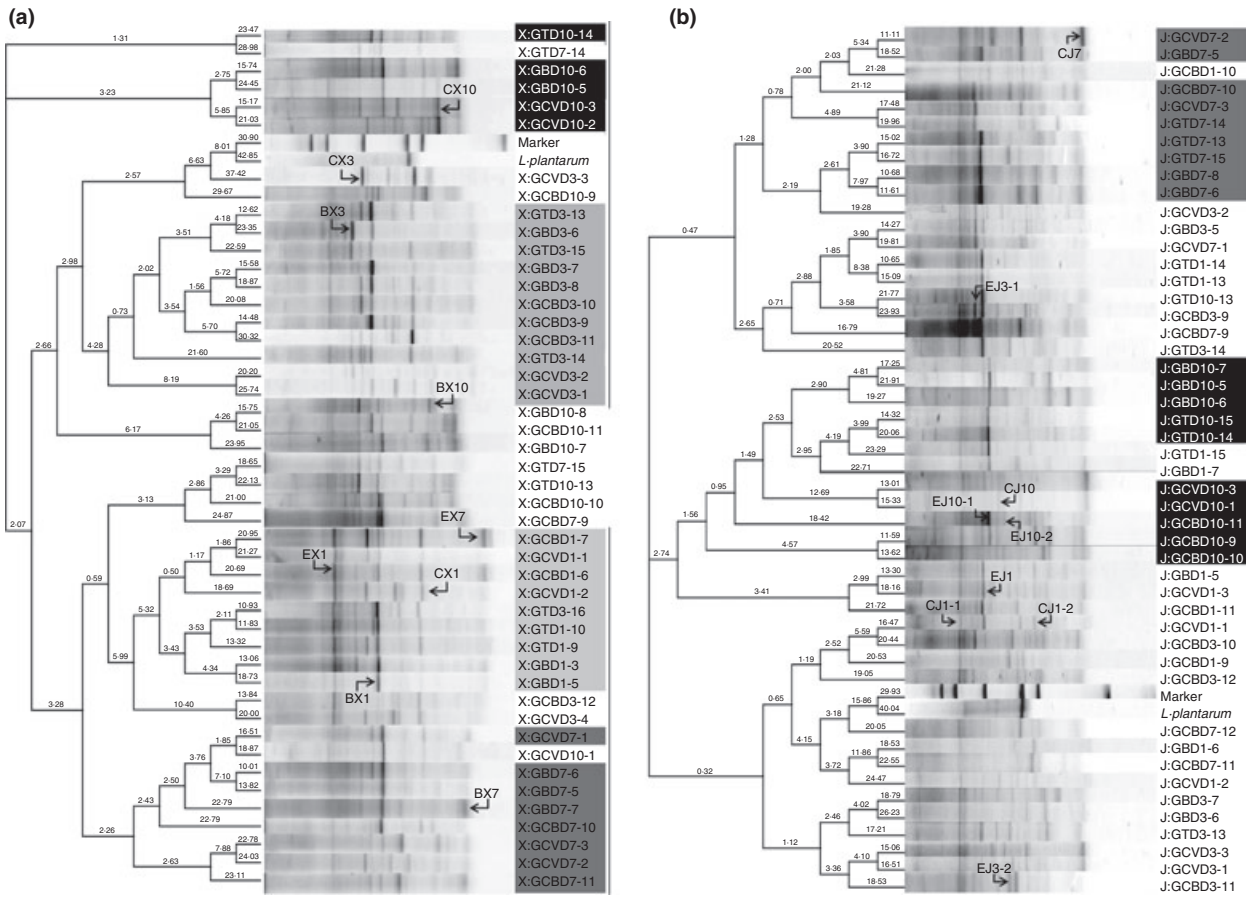

Figure 2 Cluster analysis of Temporal temperature gradient gel electrophoresis profiles of the V6 to V8 regions of 16s rDNA gene from the rat faecal microbiota performed using Quantity One® ver. 4.6.3 software. (a) Xanthan : gellan gum study; (b) jamilan : gellan gum study. Dice coefficient and neighbour-joining were applied. Percentage of similarity is detailed on tree branches. T, control group; B, free *Lactobacillus plantarum*; CV, capsules; CB, encapsulated *Lact. plantarum*. D numbering indicates sampling day. Ending hyphenated number refers to sample number within one sampling day. Numerated arrows in gels A and B point to the bands subjected to re-amplification and sequencing and correspond to the amplicon identifications of the Table 2. Relevant sample clustering: day 1-control samples, light-grey boxes; day 3, medium-grey boxes; day 7, dark-grey boxes; day 10, black boxes.

microbiota of rats, present in the profiles of all animals during the jamilan : gellan gum experiment (Fig. 2). Band EX1 (*Coprococcus eutactus* ATCC 27759) was common for profiles from animals during the xanthan : gellan gum experiment. Bands most closely related to *Clostridium mayombei, Robinsoniella peoriensis* and *Akkermansia muciniphila* were present in both faecal and mucosa-associated profiles (Tables 2 and 3). This diversity identified for faecal and intestinal samples included mainly members of the *Verrucomicrobiaceae*, *Clostridiaceae* and *Eubacteriaceae* families as well as typical enterobacterial strains such as *Escherichia coli*, as was expected according to previously published data on the rat intestinal microbiota (Salzman *et al.* 2002).

Bands migrating to the same region of the gel as the probiotic were only identified as *Lact. plantarum* in two profiles from animals treated with jamilan : gellan gum-encapsulated probiotic out of 92 profiles where *Lact. plantarum* was administered. *Lact. plantarum* was identified as one of the closest relatives of the excised bands EXI1 and EJI3 (Table 3), and both clones came from bands excised from the profiles of ileum samples.

Discussion

Microencapsulation of probiotics has been widely studied for its effective protection against adverse conditions encountered both in dairy products and the upper GIT, which actively contribute to reduce probiotic viability and, therefore, their concomitant effect on host health. This protection has been proved *in vitro*; however, there are few published *in vivo* studies in this area of

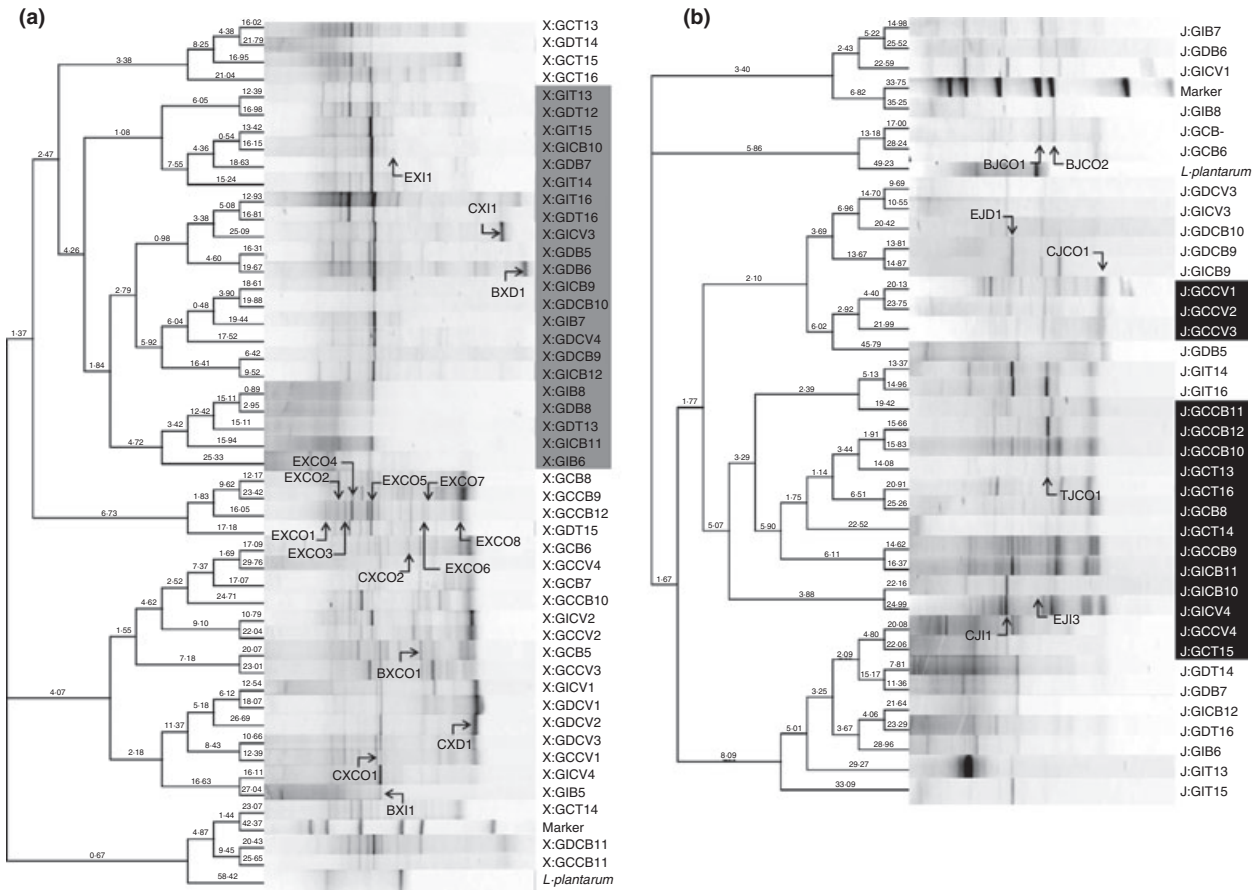

Figure 3 Cluster analysis of Temporal temperature gradient gel electrophoresis profiles of the V6 to V8 regions of 16s rDNA from the rat ileo-colonic mucosa-associated microbiota performed using Quantity One® ver. 4.6.3 software. (a) Xanthan : gellan gum study; (b) jamilan : gellan gum study. Dice coefficient and neighbour-joining were applied. Percentage of similarity is detailed on tree branches. T, control group; B, Free *Lactobacillus plantarum*; CV, capsules; CB, encapsulated *Lact. plantarum*. D, duodenum; I, ileum; C, colon. Number refers to the animal's sample. Numerated arrows point to the bands subjected to re-amplification and sequencing and correspond to the amplicon identifications of the Table 3. Relevant sample clustering: small intestine samples, dark-grey boxes; colon samples, black boxes.

microencapsulation. Furthermore, given the diversity of encapsulating material employed and the wide use of polysaccharidic polymers, it is reasonable to think that the capsules themselves may alter the intestinal microbiota.

Previous studies in our laboratory demonstrated that xanthan : gellan gum (1%:0·75%) and jamilan : gellan gum (1%:1%) are suitable materials for probiotic micro-encapsulation (Jiménez-Pranteda *et al.* 2012); they were, therefore, considered for an *in vivo* investigation. As was already demonstrated for Eub338, Erec482 and Lab158 (Salzman *et al.* 2002), the probes used to study the human faecal microbiota, and selected for this study, successfully worked for murine faecal microbiota. However, Bac303 probe (Manz *et al.* 1996), commonly used to target *Bacteroides* and *Prevotella* species in humans and animals, failed to distinguish hybridized cells in our rat faecal samples. This problem was already previously detected for canine faecal samples because of background fluorescence (Jia *et al.* 2010). Nevertheless, with the probes employed, around 75% of the faecal population of certain samples was detected. What is more, facultative anaerobes and obligate anaerobes groups that constitute murine intestinal microbiota were widely covered with the probes employed in this study.

Throughout the study, interindividual variations were detected in the composition of the rat GIT microbiota, using both FISH and TTGE. This is in agreement with previously reported data for rat caecal (Montesi *et al.* 2005), canine faecal (Jia *et al.* 2010) and human mucosa-associated microbiotas (Zoetendal *et al.* 2002; Lepage *et al.* 2005). Despite this, the administration of nonprobiotic capsules consistently increased the coverage of the microbiota detected by the probes employed (FISH data). This may reflect the potential of these capsules as a

Table 2 Identification by sequencing of the bands amplified with 16S rRNA gene universal primers from faecal samples of the four groups of rats

Amplicon IDs*	Closest relative (Accession number)	% Similarity
BX1	*Clostridium mayombei* DSM 6539T (FR733682.1)	99
BX3, EJ3-1	*Clostridiaceae* bacterium (AB298768.2)	89, 89
BX7, CJ7	*Akkermansia muciniphila* ATCC BAA-835 (CP001071.1)	95, 100
BX10	*Ruminococcus* sp. (EU728791.1)	93
CJ1-1	*Eubacterium eligens* ATCC 27750 (CP001104.1)	83
CJ10	*Lachnospiraceae* bacterium (EU728729.1)	87
CX1, CJ1-2	*Robinsoniella peoriensis* (GU322806.1)	93, 93
CX3	*Eubacterium fissicatena* DSM 3598T (FR749935.1)	93
CX10, EJ10-2	*Clostridium citroniae* DSM 19261 (HM245936.1)	94,95
EJ1	Rumen bacterium (GU324393.1)	88
EJ3-2	*Ruminococcus obeum* (X85101.1)	91
EJ10-1	*Butyrivibrio crossotus* DSM 2876T (FR733670.1)	96
EX1	*Coprococcus eutactus* ATCC 27759 (EF031543.1)	94

*E, encapsulated *Lact. plantarum* CRL 1815; B, free *Lact. plantarum* CRL 1815; C, capsules; X, xanthan–gellan gum; J, jamilan–gellan gum. Number: sample day. Hyphenated number refers to band number when more of one band was excised from different samples of the same experimental group.

Table 3 Identification by sequencing of the bands amplified with 16S rRNA gene universal primers from intestinal samples of the four groups of rats

Amplicon IDs*	Closest relative (Accession number)	% Similarity
BJCO2	*Escherichia coli* (HQ012019.1)	99
BXCO1, TJCO1, BJCO1	*Robinsoniella peoriensis* (AF445283.2)	96, 94,94
BXD1	*Bifidobacterium animalis subsp. animalis* (HQ851037.1)	100
BXI1	*Ruminococcus gnavus* (NR_036800.1)	94
CJCO1	*Akkermansia muciniphila* ATCC BAA-835 (CP001071.1)	99
CXCO1	*Jeotgalicoccus halotolerans* (NR_025643.1)	95
CXCO2	*Escherichia* sp. (HE583396.1)	85
CXD1, CXI1	*Bacillaceae* bacterium (GU111727.1)	100,99
EJD1	*Clostridium* sp. (AB610575.1)	99
EXCO1	*Staphylococcus* sp. (GQ222244.1)	91
EXCO2	*Clostridium litorale* (NR_029270.1)	85
EXCO3, EXCO4, EXCO6	Rumen bacterium (GU324393.1)	88, 89, 86
EXCO5, CJI1	*Clostridium mayombei* DSM 6539T (FR733682.1)	97, 95
EXCO7	*Paenibacillus* sp. (FJ449653.1)	92
EXI1	*Lactobacillus letivazi* (AJ417738.1)-*Lactobacillus plantarum* (JN128733.1)	93/92
EJI3	*Lactobacillus plantarum* (HQ328838.1)	96

*E, encapsulated *Lact. plantarum* CRL 1815; B, free *Lact. plantarum* CRL 1815; C, capsules; T, control; X, xanthan–gellan gum; J, jamilan–gellan gum. CO, colon; I, ileum; D, duodenum. Number indicates number of band when more of one band was excised from different samples of the same experimental group and gastrointestinal tract location.

substrate for fermentation by the indigenous intestinal microbiota, especially since the major increases were seen for polysaccharide-degrading bacteria such as *Eubacterium rectale* group (detected by probe Erec482) and *Ruminococcus* sp. (detected by probe Rbro730/Rfla729) (Flint *et al.* 2008). This may allow degradation of the capsules, which could play an important role on the targeted delivery of the bacteria encapsulated. Previously, Kosaraju (2005) described natural polysaccharides as an appropriate material for colon-targeted delivery by virtue of their property to undergo degradation by the enzymes produced by the colonic microbiota. This primary carbohydrate degradation makes these substrates readily available to those bacterial groups that are unable to degrade polymerized carbohydrates directly and, therefore, contributes to the overall increases detected (Gibson and Roberfroid 1995). This degradation hypothesis is reinforced by the fact that capsules were not recovered in the faeces in any experimental group. Capsules initially presented a size between 1 and 2 mm, and if present in faeces should have been easily detected.

Of the two polymeric capsules used in this study, jamilan : gellan gum appeared to be a better choice for delivering probiotics, because administration of

xanthan : gellan gum capsules (with or without probiotic) was shown to reduce the *Bifidobacterium* population (Saulnier *et al.* 2009) and increase Chis150 levels (*Clostridium* clusters I and II, which include potentially pathogenic species) (Duerden and Brazier 2009). This was further supported as the only increases in faecal Lab158 counts compared to pretreatment and *Lact. plantarum* group levels were detected following administration of *Lact. plantarum* encapsulated in jamilan : gellan gum capsules. Basal Lab158 counts were similar to probiotic dosage, which may have reduced the chances of observing a clearer numerical impact of probiotic administration on this population. Similar results were obtained by TTGE analysis, with bands migrating to the same region of the gel as the probiotic only seen in profiles from animals treated with jamilan : gellan gum-encapsulated probiotic. So, even though previous *in vitro* studies of the protective effect of the polymers employed in this work against simulated gastrointestinal conditions showed better results with xanthan : gellan gum (Jiménez-Pranteda *et al.* 2012), when applied on *in vivo* conditions, the biocompatibility of this polymers reverted this results. It is very important and highly recommended then to test the effect of the encapsulating materials on the faecal microbiota when targeting the gut as, depending on the individual characteristics of each particular polysaccharide, they may act negatively on the gut microbiota and defeat the purpose of a probiotic administration.

In conclusion, we have shown that probiotic encapsulation with EPS is an interesting approach for colon-targeted delivery of probiotics. Jamilan : gellan gum (1%:1%) capsules were shown to be better than xanthan : gellan gum (1%:0·75%) capsules for this approach, as their effect on the indigenous microbiota was innocuous and some evidence of *Lact. plantarum* CRL 1815 delivery to the target site was seen in TTGE profiles. Future work should develop and employ more directed monitoring systems for the probiotic strain to afford conclusive investigation into the effects of encapsulation on protective effect and delivery of probiotic (including viability).

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