Relationships between the genome and some phenotypical properties of Lactobacillus fermentum *CECT 5716, a probiotic strain isolated from human milk*

Nivia Cárdenas, Jonathan E. Laiño, Susana Delgado, Esther Jiménez, Marianela Juárez del Valle, Graciela Savoy de Giori, et al.

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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Relationships between the genome and some phenotypical properties of *Lactobacillus fermentum* CECT 5716, a probiotic strain isolated from human milk

Nivia Cárdenas · Jonathan E. Laiño · Susana Delgado · Esther Jiménez · Marianela Juárez del Valle · Graciela Savoy de Giori · Fernando Sesma · Baltasar Mayo · Leónides Fernández · Jean Guy LeBlanc · Juan M. Rodríguez

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Abstract *Lactobacillus fermentum* CECT 5716, isolated from human milk, has immunomodulatory, anti-inflammatory, and anti-infectious properties, as revealed by several in vitro and in vivo assays, which suggests a strong potential as a probiotic strain. In this work, some phenotypic properties of *L. fermentum* CECT 5716 were evaluated, and the genetic basis for the obtained results was searched for in the strain genome. *L. fermentum* CECT 5716 does not contain plasmids and showed neither bacteriocin nor biogenic amine biosynthesis ability but was able to produce organic acids, glutathione, riboflavin, and folates and to moderately stimulate the maturation of mouse dendritic cells. No prophages could be induced, and the strain was sensitive to all antibiotics proposed by European Food Safety Authority (EFSA) standards, while no transmissible genes potentially involved in antibiotic resistance were detected in its genome. Globally, there was an agreement between the phenotype properties of *L. fermentum* CECT 5716 and the genetic information contained in its genome.

Keywords *Lactobacillus fermentum* · Genome · Glutathione · Riboflavin · Folate · Antibiotic resistance · Dendritic cells · Human milk

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Introduction

It is well known that human milk is rich in many bioactive molecules, such as cytokines, immune cells, polyamines, and oligosaccharides; however, it has only recently been accepted that human milk also constitutes a source of commensal and probiotic bacteria that can colonize the infant gut and modulate its function (Fernández et al. 2013). Human milk bacteria seem to be uniquely adapted to reside in the human digestive tract and to interact with human hosts in symbiosis starting right after birth (Jeurink et al. 2013). Therefore, special interest has been focused on the selection of potentially probiotic lactobacilli or bifidobacterial strains from this biological fluid. Among these, *Lactobacillus fermentum* CECT 5716 has found commercial applications due to its repertoire of desirable properties, including its ability to inhibit the growth of a wide spectrum of pathogenic bacteria (Martín et al. 2005a; Olivares et al. 2006), its role in supporting maturation of the infant immune system by acting on both innate and acquired immunity through a variety of mechanisms (Díaz-Ropero

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et al. 2006; Olivares et al. 2007; Pérez-Cano et al. 2010), its anti-inflammatory activity (Mañé et al. 2009), its high rate of survival in conditions simulating those in the gastrointestinal tract (Martín et al. 2005a), prebiotic metabolism (Bañuelos et al. 2008), resistance against diet polyphenols (Cueva et al. 2010), its safety (Lara-Villoslada et al. 2009; Gil-Campos et al. 2012), and its in vivo efficacy to reduce the incidence of gastrointestinal and upper respiratory tract infections in infants (Maldonado et al. 2012).

L. fermentum, a heterofermentative species of the genus *Lactobacillus*, is a normal inhabitant of the human gastrointestinal tract, including that of breast-fed infants (Grover et al. 2013). Since the benefits of probiotic bacteria are recognized as being strain-specific, a better knowledge of the genetic basis for strain-specific traits in potentially probiotic strains is required. The genome of *L. fermentum* CECT 5716 was recently sequenced and consists of a circular chromosome of 2,100,449 bp, with a GC content of 51.49 % with no plasmids (Jiménez et al. 2010). The objective of this work was to evaluate the relationship between the genetic background of *L. fermentum* CECT 5716 and certain phenotypic traits, including antimicrobials, B group vitamin and glutathione biosynthesis, glycosidase activities, presence of phages, and occurrence of antibiotic resistance genes.

Material and methods

Bacterial strain and growth conditions

L. fermentum CECT 5716 (Martín et al. 2003) was routinely grown in de Man Rogosa and Sharpe (MRS) medium (Oxoid, Basingstoke, UK) at 32 °C under aerobic conditions. When required, it was grown in anaerobiosis using a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) with a gas mixture consisting of 10 % H_2, 10 % CO_2, and 80 % N_2.

Antimicrobial activity

To check for antimicrobial activity on solid medium, overnight cultures of *L. fermentum* CECT 5716 were spotted onto MRS-Cys agar plates, incubated at 30 °C for 18 h in anaerobiosis, and overlaid with 4.5 ml soft agar inoculated with ~10^5 colony forming units (cfu)/ml of the selected indicator strain. Plates were further incubated at 37 °C for 24 h and examined for clear halos of inhibition. Parallel bacteriocin activity in neutralized, cell-free *L. fermentum* CECT 5716 supernatants was assayed using the agar drop diffusion test as previously described (Langa et al. 2012). The following bacterial strains were used as indicators: *Lactobacillus acidophilus* NCDO 1748, *Lactobacillus brevis* LB9, *Lactobacillus casei* ATCC 334, *Lactobacillus coryniformis* Q8, *L. fermentum* ATCC 9338, *Lactobacillus hilgardii* LB76, *Lactobacillus jensenii Ov* 2967, *Lactobacillus paraplantarum* CNRZ 1885, *Lactobacillus pentosus* 128/2 and 55-1, *Lactobacillus plantarum* NC8 and ATCC 8014, *Lactobacillus reuteri* DSM 20016, *Lactobacillus salivarius* NCFB 2747, *Lactobacillus sakei* NCFB 2714, *Lactococcus lactis* MG 1363, *L. lactis* CNRZ 117, *L. lactis* IPLA 838, *L. lactis* IL 1403, *Streptococcus thermophilus* ST 112, *Leuconostoc citreum* IPLA 616, *Leuconostoc mesenteroides* INRA 33, *Pediococcus damnosus* NCDO 1832, *Pediococcus parvulus* P339, *Pediococcus pentosaceus* FBB63, *P. pentosaceus* PC1, *Enterococcus faecium* LP6T1a-20, *Enterococcus faecalis* EFI, *Listeria innocua* BL86/26, and *Staphylococcus carnosus* MC1. For this purpose, lactic acid bacteria were grown in MRS (Oxoid) medium, while the rest of indicator strains were grown in brain heart infusion (BHI, Oxoid) medium.

Hydrogen peroxide production by *L. fermentum* CECT 5716 was tested on MRS agar and broth following the procedure described by Song et al. (1999) and Yap and Gilliland (2000), respectively. The concentrations of L- and D-lactic acid and acetic acid in the broth supernatants were quantified using enzymatic kits (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions.

Antibiotic resistance/susceptibility

Initially, a search for antibiotic resistance genes was performed in the genome of *L. fermentum* CECT 5716. BLAST, BLASTX, and BLASTN programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to compare nucleotide and deduced amino acid sequences with those stored in the GenBank database. The minimal inhibitory concentration (MIC) of the 16 antibiotics included in this study (Table 1) was determined by a microdilution method using VetMIC plates for lactic acid bacteria (National Veterinary Institute of Sweden, Uppsala, Sweden), as described previously (Langa et al. 2012). The plates were incubated at 37 °C for 48 h, and the MIC was defined as the lowest concentration at which no growth was observed.

Fermentation of carbohydrates and enzymatic activities

L. fermentum CECT 5716 was grown on MRS-Cys prepared from the ingredients replacing glucose by an equivalent amount of cellobiose, lactose, maltose, or raffinose, and the bacterial growth was monitorized by measuring the optical density at 600 nm (OD_{600}).

A cell-free extract (CFE) of *L. fermentum* CECT 5716 was prepared to determine glycosidase, peptidase, and esterase activities using chromogenic substrates as described by Cárdenas et al. (2014). Glycosidase activities were assayed using the following *p*-nitrophenyl (*p*NP) derivatives as substrates: *p*NP-α-D-glucopyranoside (*p*NP-αGlu), *p*NP-β-D-

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Table 1 MIC values (μg/ml) of different antibiotics to *Lactobacillus fermentum* CECT 5715

Antibiotic	MIC (μg/ml)	Breakpoints (EFSA 2012)
Penicillin	0.25	–
Ampicillin	0.25	2
Ciprofloxacin	8	–
Gentamicin	0.5	16
Kanamycin	16	32
Streptomycin	8	64
Neomycin	1	–
Tetracycline	4	8
Erythromycin	0.12	1
Clindamycin	0.03	1
Chloramphenicol	4	4
Vancomycin	>128	Not required
Virginiamycin	0.25	–
Linezolid	1	–
Trimethoprim	1	–
Rifampicin	0.5	–

glucopyranoside (*p*NP-βGlu), *p*NP-α-D-galactopyranoside (*p*NP-αGal), and *p*NP-β-D-galactopyranoside (*p*NP-βGal) (Sigma), and the specific glycosidase activity was expressed as nanomole of *p*-nitrophenol released per milligram of protein in CFE and minute. Aminopeptidase activities were investigated using the chromogenic *p*-nitroanilide (*p*NA) derivatives of lysine (Lys-*p*NA), alanine (Ala-*p*NA), leucine (Leu-*p*NA), proline (Pro-*p*NA), and glycil-proline (Gly-Pro-*p*NA) (Sigma-Aldrich) and expressed as micromole of *p*NA released per milligram of protein in CFE and hour. Esterase activities were determined using α-naphthyl (α-NA) derivatives of acetic (C2), propionic (C3), butyric (C4), and caproic (C6) acids (Sigma-Aldrich) and expressed as micromole of α-naphthol released per milligram of protein in CFE and hour.

In addition, proteolytic activity was determined by the *o*-phthalaldehyde (OPA) spectrophotometric assay described by Church et al. (1983). This test is based on the reaction of free α-amino groups released by hydrolysis of casein, after a 24-h incubation period in milk at 30 °C and the precipitation of nonhydrolyzed protein using trichloroacetic acid (TCA), with OPA in the presence of β-mercaptoethanol to form a complex that strongly absorbs at an optical density at 340 nm (OD_{340}). An overnight bacterial culture in MRS was centrifuged and the pellet resuspended in the same volume of buffer sodium phosphate (0.32 mM, pH 7.2). The cell suspension was added (1 %, *v*/*v*) to 10 ml of heat-treated (121 °C for 5 min) reconstituted (10 %, *w*/*v*) skim milk (HT-RSM); uninoculated samples were used as control. Proteolytic activity was expressed as the increase in OD_{340} after reaction of free α-amino groups present in the TCA-soluble filtrate with OPA in comparison with an inoculated sample.

Production of glutathione

Production of glutathione by *L. fermentum* CECT 5716 was assessed as described by Peran et al. (2006). Briefly, bacterial cells obtained in MRS broth cultures were washed with distilled water, suspended in 300 μl of TCA 7.5 % (*w*/*v*), and disrupted by stirring. The mixture was centrifuged (10,500×*g* for 2 min), and the supernatant (100 μl) was transferred to a new tube containing 300 μl of MilliQ water. A portion (20 μl) from this solution was mixed with 340 μl of 0.6 M phosphate buffer (pH 7.8) and 340 μl of 1.25 mM Tris (carboxyethyl) phosphine HCl (TCEP) in 20 mM HCl. The sample was placed in the dark for 15 min, and then 800 μl of 12 mM *ortho*-phthalaldehyde in 50 mM sodium acetate was added, and samples were placed at 4 °C for 15 min. Samples were analyzed by HPLC using a Spherisorb S3 ODS column at 0.8 ml/min in isocratic mode using 50 mM sodium acetate (pH 7.7)/acetonitrile (96:4) as mobile phase.

Production of vitamins B_2, B_6, and B_{12}

L. fermentum CECT 5716 bacterial cells from an overnight culture in MRS were washed three times with saline solution, resuspended in this solution at the original culture volume, and used to inoculate (4 %, *v*/*v*) either riboflavin-, folate-, or vitamin B_{12}-free culture media (Difco, USA). Then, the inoculated media were incubated at 37 °C for 18 h without agitation. After incubation, this washing-resuspension procedure was repeated, and the resulting cell solution was used to inoculate (2 %; *v*/*v*) the respective fresh vitamin-free media. This last step was repeated seven times and, after the last incubation, samples were taken to determine extra- and intracellular vitamin concentrations. For determination of folate concentration, a sample (500 μl) of bacterial grown vitamin-free medium was mixed with equal parts of a protecting buffer (0.1 M phosphate buffer, pH 6.8, containing ascorbic acid [1.5 %, *w*/*v*]) to prevent vitamin oxidation and degradation, while acetic acid (1 %, *v*/*v*) was added in the case of riboflavin. Immediately after the addition of either the protecting buffer or acetic acid, the mixes were centrifugated for 5 min at 5,000×*g*. Then, the supernatant was collected (extracellular sample) and boiled for 5 min, while the pellet was resuspended in 500 μl of protecting buffer, boiled for 5 min, and centrifuged for 6 min at 10,000×*g*, and the corresponding supernatant was also collected (intracellular samples). All supernatants were stored at −70 °C until used for vitamin quantification.

Folate concentrations were determined by a previously described microbiological assay using *Lactobacillus rhamnosus* NCIMB 10463 as the indicator organism (Laiño et al. 2012). Briefly, samples or different concentrations of HPLC-grade folic acid (Fluka BioChemika, Sigma-Aldrich, Switzerland) were placed with the indicator strain and incubated statically during 48 h at 37 °C in 96-well sterile microplates containing

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the folate-free medium (Deltalab, Argentina). The optical density was read at 580 nm (OD_{580}) using a microplate reader (VERSAmax tunable microplate reader, Molecular Devices, USA). The folate concentration of the samples was determined by comparing the OD with those obtained with the standard curve prepared using commercial folic acid. Riboflavin concentrations were determined in the same manner but using *L. rhamnosus* ATCC 7469 as the indicator strain grown in the riboflavin-free medium and confirmed by HPLC analysis as described previously (Juarez del Valle et al. 2014).

A reference method, the *Lactobacillus delbrueckii* B_{12} assay (Horwitz 2000), was used to prepare cells extracts and, then, to analyze production of cobalamin. A cyanocobalamin (Sigma) standard curve was used to quantify the cobalamin content in the intracellular extract. The protein concentration of the extracts was estimated as described above. *L. coryniformis* CECT 5711 (Martín et al. 2005), a cobalamin-producing strain, was used as a positive control.

Prophage induction

Prophage induction was carried as described previously (Langa et al. 2012). Exponential cultures of *L. fermentum* CECT 5716 (~OD_{600}=0.4) were treated with 0.25, 0.5 (MIC), and 1 μg/ml mitomycin C (final concentration), and incubation was continued for up to 5 h. Aliquots of the supernatants were placed on lawns of presumably susceptible *L. salivarius* strains growing in soft MRS (0.75 % agar) supplemented with 1 % hemoglobin, 10 mM $CaCl_2$, and 10 mM $MgSO_4$, placed on top of plates with the same medium (1.5 % agar). After incubation for 24 h, the generation of lysis plates was recorded.

Production of biogenic amines

The ability to form biogenic amines (tyramine, histamine, putrescine, and cadaverine) was assessed using the decarboxylase broth and the method described by Bover-Cid and Holzapfel (1999). The precursor amino acids (tyrosine, histidine, ornithine, and lysine, respectively) were purchased from Sigma. *L. fermentum* CECT 5716 was streaked onto the different decarboxylase medium plates and incubated for 4 days at 37 °C under aerobic and anaerobic conditions. A positive result was indicated by a change of the medium color to purple in response to the pH shift caused by the production of the more alkaline biogenic amine from the amino acid initially included in the medium.

Stimulation of immature dendritic cells

Mouse immature dendritic cells (DCs) were isolated from the spleen of female C57BL/6 mice (6–10-week age) and characterized as described previously (Lu et al. 1995). For propagation of isolated DC, they were routinely cultured at 37 °C in a humidified 5 % CO_2 atmosphere in Iscove's modified Dulbecco's medium (IMDM) with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 20 pM β-mercaptoethanol (complete IMDM; all the components were from Sigma) and supplemented with 0.4 ng/ml mouse granulocyte-macrophage colony-stimulating factor (rGM-CSF) (R&D Systems, Minneapolis, USA). The culture medium was changed every 4 days, and granulocytes and mature DCs were removed by gentle washes, after which the cultures were replenished with new medium containing fresh rGM-CSF (Lu et al. 1995).

The monoclonal anti-mouse antibodies IA/Ed (2G9) and CD86/B7.2 (GL1), which specifically recognize mouse major histocompatibility complex (MHC) II and B7.2, respectively, were purchased from PharMingen (San Diego, USA). Phycoerythrin and streptavidin were obtained from Sigma.

MRS-Cys overnight cultures of *L. fermentum* CECT 5716 were recovered by centrifugation at 6,000×*g* for 5 min and washed twice with PBS. Then, 2×10^7 cfu was distributed in 100-μl aliquots of IMDM devoid of antibiotics and added to 10 ml fresh DC cultures containing 2×10^6 cells. The cocultures were incubated for 90 min at 37 °C, and non-inoculated DC cultures were included as negative controls. After the incubation period, cells were washed with PBS and 2 mM EDTA and maintained for 18 h at 37 °C in complete IMDM supplemented with gentamicin (250 μg/ml) and tetracycline (10 μg/ml) to kill the remaining bacteria. Then, cells were washed twice with PBS and stained with the anti-MHC class II and anti-B7.2 antibodies in order to detect both DC and the potential activation of the surface markers. Staining was performed according to standard immunofluorescence techniques, while labeling of the antibodies with phycoerythrin was carried out following the manufacturer instructions (Sigma). Finally, flow cytometry analysis was performed with a FACS scan (Becton Dickinson, San Jose, CA), and the resulting data were analyzed with the WinMDI 2.8 software. A total of 10,000 cells were analyzed through a viable cell gate determined by forward and right angle light scatter parameters to exclude subcellular particles.

Parallel, the genome of *L. fermentum* CECT 5716 was searched for the presence of open reading frames (ORFs) encoding putative proteins related to DC functionality, such as DC-SIGN1 or DC-SIGN2, or potentially involved in human molecular mimetism. For this purpose, the Words In Proteins (WIP) algorithm developed by Era7 Information Technologies (Granada, Spain) was used.

Bioinformatic analysis

BioCyc Database Collection (http://biocyc.org/; BioCyc 18.1) was used to search for genes involved in selected metabolic

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activities (glycosyl hydrolase, proteolytic and lipolytic activities, sugar, peptide, and amino acid transport, pyruvate fermentation, and glutathione synthesis) and their orthologs in other *L. fermentum* strains in order to compare them. KEGG Pathway database was employed to search for folate and riboflavin metabolic pathways (http://www.kegg.jp/kegg/pathway.html). Homology of genes involved in glutathione, folate, and riboflavin biosynthesis with those of other *L. fermentum* strains was performed with BLASTN 2.2.30 (Zhang et al. 2000). Information about whole genome comparison of available sequence data of ten *L. fermentum* strains (CECT 5716, IFO 3956, F-6, ATCC 14931, 28-3-CHN, 3-CHN, FTDC8312, NB-22, 3872, Lf1, and MTCC 8711) was taken from the National Center for Biotechnology (NCBI, http://www.ncbi.nlm.nih.gov/genome/genomes/711).

Results

Analysis of antimicrobial activity

L. fermentum CECT 5716 cells displayed clear antimicrobial activity against 26 strains of Gram-positive bacteria, including a variety of lactobacilli, lactococci, leuconostocs, pediococci, listeria, enterococci, streptococci, and staphylococci. High concentrations of both L- and D-lactic acid enantiomers (mean±SD, 4.92±0.29 and 3.94±0.27 mg/ml, respectively) were detected in the supernatants obtained from MRS cultures of this strain. Acetic acid could be also detected and quantified in its culture supernatants (1.73 mg/ml±0.24).

In contrast, this strain did not produce hydrogen peroxide in the assayed conditions, while its neutralized cell-free supernatants did not show bacteriocin-like activity.

Antibiotic resistance/susceptibility

MIC values were lower or equal to the microbiological breakpoints established by the European Food Safety Authority (EFSA 2012) (Table 1). RAST server (http://rast.nmpdr.org/) was used for the search for specific antibiotic resistance genes in the genome of *L. fermentum* CECT 5716. Several predicted coding sequences were classified as putative related with non-transmissible antibiotic resistance, including three multidrug efflux pumps, two β-lactamases, four determinants of resistance to fluoroquinolones, and four ribosome protection proteins related with tetracycline resistance (Table 2).

Fermentation of carbohydrates and enzymatic activities

L. fermentum CECT 5716 was able to grow in maltose, lactose, and raffinose (Fig. 1), as expected by the presence of putative-specific glycoside hydrolases in the genome such as α-glucosidase (EC 3.2.1.20; LC40_0498), α-galactosidase (EC 3.2.1.22; LC40_1129), and β-galactosidase (EC 3.2.1.23; LC40_0186 and LC40_0187) (Supplemental Table S1). These results related to its fermentation ability were phenotypically confirmed with the determination of glycosyl hydrolase activities in CFE of the bacterium using chromogenic substrates (Table 3). The highest glycosidase activities

Table 2 Putative antibiotic resistance genes found in the genome of *Lactobacillus fermentum* CECT 5716 using the RAST server

Subsystem	Role	NCBI annotation/protein ID
Tetracycline resistance, ribosome protection type	Translation elongation factor G	Elongation factor G (EF-G)/protein_id = "ADJ41530.1"
Resistance to fluoroquinolones	DNA gyrase subunit B	DNA gyrase subunit B/protein_id = "ADJ40687.1"
Resistance to fluoroquinolones	DNA gyrase subunit A	DNA gyrase subunit A/protein_id = "ADJ40688.1"
Resistance to fluoroquinolones	Topoisomerase IV subunit B	ATP-hydrolyzing DNA topoisomerase/ protein_id = "ADJ41204.1"
Resistance to fluoroquinolones	Topoisomerase IV subunit A	Topoisomerase IV subunit A/protein_id = "ADJ41205.1"
Beta-lactamase	Beta-lactamase class C and other penicillin-binding proteins	Cardiolipin synthase and penicillin-binding protein/protein_id = "ADJ40943.1" and protein_id = "ADJ41256.1
Tetracycline resistance, ribosome protection type, too	Ribosome protection-type tetracycline resistance related proteins, group 2	Elongation factor G/protein_id = "ADJ41369.1"
Multidrug resistance efflux pumps	Multi-antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family, of MDR efflux pumps	Na^+-driven multidrug efflux pump/ protein_id = "ADJ41424.1"
Multidrug resistance efflux pumps	Multidrug efflux transporter, major facilitator superfamily (MFS)	Multidrug transport protein/protein_id = "ADJ41342.1"

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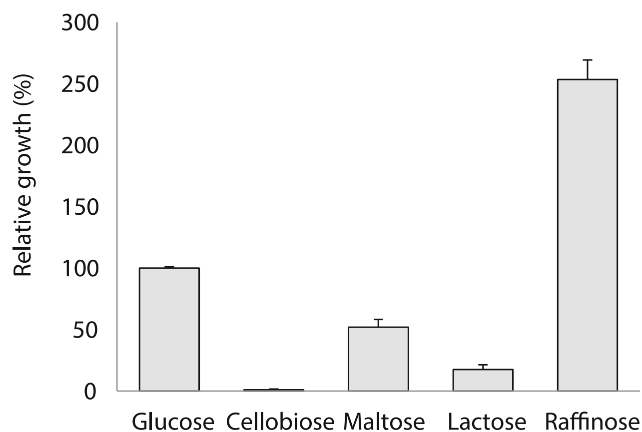

Fig. 1 Relative growth of *L. fermentum* CECT 5716 in MRS-Cys containing selected saccharides as the carbon source after 24 h at 32 °C under aerobic conditions. $OD_{600\ nm}$ of *L. fermentum* CECT 5716 grown in broth containing glucose was 2.050

were found for α-glucosidase and α-galactosidase substrates (7.86±0.14 and 7.46±0.20 nmol *p*NP mg^{-1} protein h^{-1}, respectively), while β-glucosidase activity was almost undetectable (0.05±0.03 nmol *p*NP mg^{-1} protein h^{-1}) in the same conditions. Genome searching revealed the existence of other putative glycolytic hydrolase genes such as maltose phosphorylase (LC40_0017; pseudo-gene), xylosidase (LC40_0286), muramidase (LC40_0905), and sucrose-6-phosphate hydrolase (LC40_1000, preceded by a phosphoenolpyruvate-dependent sugar phosphotransferase system: LC40_0998) (Supplemental Table S1). The strain neither grew when cellobiose was the only carbon source present in the broth (Fig. 1) nor showed β-glucuronidase activity and, correspondingly, no

Table 3 Glycosidase, peptidase, and esterase activities in cell-free extracts of *Lactobacillus fermentum* CECT 5716

Substrates for	Enzyme activity
Glycosidase activity (nmol *p*NP mg^{-1} protein h^{-1})	
*p*NP-α-D-glucopyranoside	7.86±0.14
*p*NP-β-D-glucopyranoside	0.05±0.03
*p*NP-α-D-galactopyranoside	7.46±0.20
pNP-β-D-galactopyranoside	3.87±0.92
Peptidase activity (μmol *p*NA mg^{-1} protein h^{-1})	
Ala-*p*NA	0.58±0.07
Leu-*p*NA	0.69±0.05
Lys-*p*NA	0.97±0.14
Pro-*p*NA	0.86±0.00
Gly Pro-*p*NA	0.94±0.01
Esterase activity (μmol α-naphthol mg^{-1} protein h^{-1})	
α-NA acetate (C2)	0.34±0.33
α-NA propionate (C3)	0.16±0.13
α-NA butyrate (C4)	0.78±0.50
α-NA caproate (C6)	0.16±0.14

genes involved in such activity could be detected in its genome, although a putative PTS system cellobiose-specific transporter subunit IIC (LC40_0732) was identified (Supplemental Table S1).

L. fermentum CECT 5716 did not show remarkable proteolytic activity (ΔOD_{340}=0.07) as determined by the OPA method, while discrete peptidase activities were exhibited by the CFE of the strain (Table 3). More than 50 genes encoding for proteins related to proteolysis (~25) and peptide and amino acid transport (~30) were found in the genome sequence of *L. fermentum* CECT 5716 (Supplemental Tables S2 and 3). Apart from proteases involved in physiological processes, several proteolytic enzymes that would allow hydrolysis of milk protein to obtain essential amino acids were identified including four endopeptidases (serine-type LC40_0013 and LC40_0417 and metalloendopeptidases LC40_0482 and LC40_1149), one oligoendopeptidase (LC40_1124, similar to PepF), six dipeptidases (LC40_0361, LC40_0652, LC40_0730, LC40_0819, LC40_0857, and LC40_1116), and five aminopeptidases (LC40_0125, LC40_0190, LC40_0568, LC40_1057, and LC40_1058) (Supplemental Table S3). Significantly, genes encoding several peptidases specific for proline-containing peptides were recognized: X-prolyl dipeptidyl aminopeptidase (PepX; LC40_1057) that liberates Xaa-Pro dipeptides from the N-terminus of peptides, two prolidases or Xaa-Pro dipeptidases (PepQ; LC40_0361 and LC40_0819), and proline aminopeptidase (PepI; LC40_0417) that releases N-terminal proline residues from a peptide.

Regarding the esterase activity of *L. fermentum* CECT 5716, the preferred chromogenic substrate was the butyrate derivative (Table 3). In contrast to proteolytic enzymes, only six genomic sequences related to esterase activity were inferred by computational analysis (LC40_0189, LC40_0973, LC40_0208, LC40_0642, and LC40_0987), and three of them encoded proteins of the esterase/lipase superfamily containing the GXSXG active site and the characteristic alpha/beta hydrolase fold of hydrolases (Supplemental Table S4).

Furthermore, the genes comprising the whole pyruvate fermentation pathway to diacetyl, acetoin, and 2,3-butanediol, considered as important flavor compounds in dairy products, were found distributed across the chromosome/acetolactate synthase (EC 2.2.1.6; LC40_0670), acetolactate decarboxylase (EC 4.1.1.5; LC40_0293, acetoin dehydrogenase (EC 1.1.1.303; LC40_0950), and butanediol dehydrogenase (EC 1.1.1.4; LC40_0618).

Production of glutathione and vitamins B_2, B_6, and B_{12}

L. fermentum CECT 5716 produced relatively high amounts of glutathione (1.54±0.29 mM) in culture media. The synthesis of glutathione from its three amino acids precursors (L-glutamate, L-cysteine, and glycine) is usually carried out in

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two steps: the first step consists in the condensation of L-glutamate and L-cysteine by the enzyme glutamate-cysteine ligase or gamma-glutamylcysteine synthetase (EC 6.3.2.2), while the second and final steps, where the glycine is added to the C-terminal end of γ-glutamylcysteine, are catalyzed by the enzyme glutathione synthetase (EC 6.3.2.3) (Supplemental Fig. S1). A total of four genes encoding proteins with glutamate-cysteine ligase activity were identified in the *L. fermentum* CECT 5716 genome (LC40_0421, LC40_0664, LC40_0686, and LC40_0924). Putative proteins encoded by all four genes (ADJ41044.1, 456 amino acids; ADJ41274.1, 298 amino acids; ADJ41296.1, 501 amino acids; and ADJ41495.1, 512 amino acids) displayed the glutamate-cysteine ligase family 2 (GCS2) motif and were related to the glutamate-cysteine ligase activity; the product of LC40_0686 was the only putative protein that was also associated to glutathione synthetase activity (Supplemental Fig. 1).

In addition, this strain was able to grow in absence and produce both vitamins B_2 and B_6. The concentrations (mean ±SD) of intracellular and extracellular riboflavin were 290±30 and 333±50 ng/ml, respectively (total concentration 623 ng/ml). In the case of folate, the intracellular and extracellular concentrations were 42±5 and 106±8 ng/ml, respectively (total concentration, 148 ng/ml). The gene clusters responsible for the production of both vitamins (riboflavin: LAF_0736, LAF_0773, LAF_0774, LAF_0775, and LAF_0776; folates: LAF_0498; LAF_0888, LAF_1066, LAF_1080, LAF_1081, LAF_1082, LAF_1336, LAF_1338, LAF_1339, LAF_1340, and LAF_1341) were identified in the genome of *L. fermentum* CECT 5716 (Supplemental Figs. S2 and 3). This strain did not produce cyanocobalamin, and no gene involved in the biosynthesis of this vitamin could be found in its genome.

Prophage induction

L. fermentum CECT 5716 supernatants did not generate typical phage-related inhibition halos or plaques of lysis on lawns of any of 11 *L. fermentum* strains tested. Genes encoding a complete prophage were not detected in the genome of *L. fermentum*; however, a few phage-related genes were found (e.g., LC40_0445 phage antirepressor, LC40_0324 phage helicase, LC40_0325 phage primase, LC40_0327 phage terminase, LC40_0328 phage portal protein, and LC40_0329 phage capsid protein).

Production of biogenic amines

Production of biogenic amines (tyramine, histamine, putrescine, or cadaverine) by *L. fermentum* CECT 5716 could not be detected and, in correspondence, no gene involved in the biosynthesis of such compounds could be detected in its genome.

Stimulation of immature dendritic cells

The WIP algorithm showed that the genome of *L. fermentum* CECT 5716 does not encode proteins related to DC functionality, such as DC-SIGN1 or DC-SIGN2, or potentially involved in human molecular mimetism. The ability of *L. fermentum* CECT 5716 to stimulate the in vitro maturation of immature DCs was assessed. The strain enhanced presentation of the coestimulatory molecule B7.2 (CD86) and MHC class II on the surfaces of DC. These markers were detected in 39.2 and 42.0 %, respectively, of the co-cultured DC. In contrast, the values corresponding to DC that were not exposed to the bacterial strains were significantly lower (11.9 and 10.1 %, respectively).

Global comparison between *L. fermentum* CECT 5716 and other *L. fermentum* strains

The complete genome of *L. fermentum* CECT 5716 consists of a circular chromosome which contains 1,051 predicted protein-encoding genes, 54 tRNA-encoding genes, and 20 rRNA-encoding genes. The comparison of the CECT 5716 and other *L. fermentum* genomes revealed that it was highly similar to that of IFO 3956 (Supplemental Fig. S4).

Discussion

The relationship between the genotype and phenotype of *L. fermentum* CECT 5716 regarding some specific traits was evaluated in this study. Lactobacilli have been long considered to a part of the microbiological barrier to infection by intestinal pathogens. The production of inhibitory substances, such as organic acids, bacteriocins, or hydrogen peroxide, may affect undesirable or pathogenic bacteria. In this study, *L. fermentum* CECT 5716 was able to produce lactic acid and acetic acid, but production of bacteriocin-like substances or hydrogen peroxide could not be detected. This is in agreement with its genome since it did not contain genes or gene clusters involved in the biosynthesis of antimicrobial substances other than organic acids. Thus, production of such acids and/or exclusive competition processes may be responsible for the antimicrobial activity of this strain. In fact, *L. fermentum* CECT 5716 has the ability to inhibit the growth or the adhesion to mucins of a wide spectrum of pathogenic bacteria (Martín et al. 2005a; Olivares et al. 2006) and increases the survival of mice infected with this pathogen (Olivares et al. 2006). This strain exerts a protective role even in a in vivo murine septic shock model (Arribas et al. 2009), while a trial in 6-month-old infants led to a reduction in the incidence of intestinal and respiratory infections (Maldonado et al. 2012).

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Obligately heterofermentative lactobacilli, such as *L. fermentum*, produce CO_2, ethanol, acetate, and lactate from metabolism of glucose, a fact that is clearly reflected in the genome of *L. fermentum* CECT 5716. Although the primary catabolic pathway for glucose metabolism in *Lactobacillus* is assumed to be the glycolytic pathway, the *pfk* and the *fba* genes encoding 6-phosphofructokinase and fructose-bisphosphate aldolase, respectively, are absent from the *L. fermentum* CECT 5716 genomes. The *pfk* and *fba* genes are essential for the glycolytic pathway, and it has been suggested that the lack of such genes may be a specific feature of heterofermentative lactobacilli (Morita et al. 2008). On the other hand, *L. fermentum* CECT 5716 has the complete gene set for the pentose phosphate pathway, which indicate that this strain uses the pentose phosphate pathway to metabolize glucose, a fact previously observed in the genomes of *L. reuteri* JCM 1112 and *L. fermentum* IFO 3956 (Morita et al. 2008).

L. fermentum CECT 5716 showed a particularly high α-galactosidase activity. α-Galactooligosaccharides found in legumes, such as soybeans, can cause gastrointestinal disorders since mammals lack this enzyme in their small intestines, which is necessary for their hydrolysis. In a previous study, LeBlanc et al. (2005) found that *L. fermentum* CRL 722 was able to deliver active α-galactosidase activity in the small intestine of rats. Later, it was observed that this strain was able to overcome host α-galactosidase deficiency, as evidenced by reduction of hydrogen excretion in rats consuming soya alpha-galactooligosaccharides (LeBlanc et al. 2008). This observation offers interesting perspectives in lactic acid bacteria (LAB) application as vectors for delivery of digestive enzymes.

This study confirmed that *L. fermentum* CECT 5716 is able to produce glutathione, a fact previously described (Peran et al. 2006) but uncommon among lactobacilli, which was related to the presence of genes involved in the biosynthesis of this antioxidant compound. Glutathione is a sulfhydryl-derived compound that actively participates in the antioxidant mechanisms of the intestinal mucosa, preserving it from oxidant-induced tissue damage. Several antioxidant compounds, such as flavonoids or vitamin E, have been reported to exert anti-inflammatory activity through the restoration of glutathione colonic mucosal levels in experimental models of rat colitis (Gonzalez et al. 2001; Camuesco et al. 2004). Different studies have reported diminished glutathione content in inflammatory bowel diseases (Miralles-Barrachina et al. 1999) and that glutathione supplementation results in beneficial effects in experimental colitis (Ardite et al. 2000; Loguercio et al. 2003). *L. fermentum* CECT 5716 has already shown a strong anti-inflammatory effect in a 2,4,6-trinitrobenzenesulfonic acid (TNBS) model of rat colitis (Peran et al. 2006). These authors suggested that the inhibitory effect on leucocyte infiltration was the result of the preventative effect exerted by the probiotic against the free radical derived oxidative injury that takes place after TNBS instillation in the colonic tissue, since the intestinal anti-inflammatory effect was associated with a restoration of the colonic glutathione levels in comparison with non-treated colitic rats. Therefore, they concluded that the free radical scavenger properties attributed to glutathione produced by this probiotic was crucial in its anti-inflammatory effect. In addition, other *L. fermentum* strains (E-3 and E-18) have also been reported to possess strong anti-oxidative properties (Kullisaar et al. 2002).

Traditionally, LAB have been considered consumers of group B vitamins. However, several studies have reported the strain-specific ability of some LAB to synthesize folate (Sybesma et al. 2003; LeBlanc et al. 2007; Santos et al. 2008), riboflavin (Martín et al. 2010; Jayashree et al. 2011) and/or cobalamin (Taranto et al. 2003; Martín et al. 2005). In this study, *L. fermentum* CECT 5716 produced riboflavin and folate but not cobalamin. The contribution of the microbiota to the folate requirements of the high cell turnover intestinal epithelium is unknown, but a mechanism for luminal folate absorption by cells in the human colon has been reported (Dudeja et al. 2001), which suggests that folate produced in situ by the colonic microbiota may be utilized by cells in the colonic epithelium. In fact, it has recently been shown that bacterially synthesized folate is absorbed across the large intestine and incorporated into the liver and kidneys of piglets (Asrar and O'Connor 2005). *L. fermentum* CECT 5716 was originally isolated from breast milk, and it is interesting to note that oral administration of human milk to rats increased plasma folate concentration and total cecal material folate content by 42 and 48 %, respectively (Krause et al. 1996). These authors suggested that the improved folate status of rats fed human milk-containing diets was due, at least in part, to increased folate synthesis by folate-synthesizing microbes in the cecum and colon. Furthermore, it has been suggested with the expanding availability of genome sequences that it is not only possible to identify potential vitamin-producing probiotic strains but also to understand the intertwined mechanisms for their biosynthesis, all of which will be exploited to increase the vitamin-producing capacities in the gastrointestinal tract of humans (LeBlanc et al. 2013).

Cobalamin biosynthesis is limited to a few representatives of bacteria and archaea and, among LAB, *L. reuteri* was the first one reported to be able to produce this vitamin (Taranto et al. 2003). Recently, the comparison of the genomes of *L. reuteri* JCM 1112 and *L. fermentum* IFO 3956 showed that JCM 1112 possesses the gene sets (*cbi*, *cob*, and *hem*) for cobalamin biosynthesis (Morita et al. 2008). A comparison of this genetic locus with the corresponding locus in *L. fermentum* IFO 3956 revealed that this gene cluster is inserted into a locus that is common to both bacteria (and also to *L. fermentum* CECT 5716), suggesting that such a cluster is a genomic island that has been acquired through lateral gene transfer. Such an island is also absent in *L. fermentum* CECT 5716 genome.

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Antibiotic MIC values were lower or equal to the microbiological breakpoints established by the EFSA (2012), and were within the susceptible ranges reported elsewhere (Egervarn et al. 2007). The RAST server (http://rast.nmpdr.org/) was used for the search for specific antibiotic resistance genes in the genome of *L. fermentum* CECT 5716. Several predicted coding sequences were classified as putatively related with antibiotic resistance (Table 2). Among them, three multidrug efflux pumps, two β-lactamases, four determinants of resistance to fluoroquinolones, and four assigned to ribosome protection proteins related with tetracycline resistance were detected. However, none of these genes correlated with a clear antibiotic resistance phenotype, and this fact may account for the susceptibility of the strain to tetracycline and other antibiotics. The presence of genes displaying homology to tetracycline resistance genes is not surprising, as elongation-like factors involved in translation are related to ribosome protection-type tetracycline resistance proteins (Connell et al. 2003).

RAST annotation assigned a role in resistance to fluoroquinolones to four ORFs: *par*E, *par*C, *gyr*A, and *gyr*B. However, these genes are actually thought to be necessary for DNA replication. High and moderate MICs to ciprofloxacin, an inhibitor of DNA synthesis, have been previously reported in lactobacilli (Katla et al. 2001; Danielsen and Wind 2003).

Multidrug efflux transporters are involved in many detoxifying activities in the cell (Piddock 2006) and are widely spread among many LAB species. Therefore, they are anticipated to have a physiological role in other cellular processes rather than be associated with antibiotic resistance. Finally, ORFs encoding β-lactamase class C proteins and other penicillin-binding proteins in *L. fermentum* CECT 5716 are probably involved in peptidoglycan biosynthesis, having no relationship with β-lactam resistance. Globally, no transmissible antibiotic resistance gene could be detected in the *L. fermentum* CECT 5716 genome and, therefore, this strain can be considered as safe from this point of view.

In this work, the cell surface expression of B7-2 and MHC class II determinants by mouse DC was moderately stimulated by *L. fermentum* CECT 5716. Using the same model, better results were reported for *L. salivarius* CECT 5713 but the genome of such strain contained genes involved in DC-mediated molecular mimetism that have not been found in that of *L. fermentum* CECT 5716 (Langa et al. 2012).

Globally, the results of this work provide some clues on the relationship between some phenotypic properties of *L. fermentum* CECT 5716 and their subjacent molecular basis. In silico analysis has shown to be effective in predicting specific beneficial properties of this probiotic bacterium since all of the evaluated phenotypes were correlated with the presence of the genes or gene clusters involved. Functional genomics and comparative genomics such as those performed partially in this study pave the way for the evaluation and search for novel probiotic strains or to understand the mechanisms by which these beneficial microorganisms provide benefits to their host.

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