Effect of iron on the probiotic properties of the vaginal isolate *Lactobacillus jensenii* CECT 4306

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The vaginal microbiota of healthy, fertile women is dominated by lactobacilli. As a defence mechanism, these bacteria produce H$_2$O$_2$ to discourage colonization of the vagina by undesirable micro-organisms. In particular, *Lactobacillus jensenii* CECT 4306 is a strong producer of H$_2$O$_2$ and has been found to protect itself from the bactericidal effects of this compound through the activity of extracellular peroxidases. However, this peroxidase activity is dependent on the presence of Fe$^{3+}$, which is found in elevated concentrations in the vaginal mucosa as a consequence of the menstrual discharge. The aim of the present work was to evaluate whether Fe$^{3+}$ is able to modulate other potential probiotic properties of strain 4306. We found that Fe$^{3+}$ enhances the adhesion of *L. jensenii* CECT 4306 to mucin and to HT-29 and HT-29 MTX cells, and, in addition, improves the anti-inflammatory profile, as judged by an increase in the ratio of IL-10/IL-12p70 that were secreted by macrophages. A comparison of total, secreted and surface proteins produced in the presence and absence of Fe$^{3+}$ revealed significant differences in the concentration of the moonlighting protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In conclusion, Fe$^{3+}$ seems to improve the probiotic characteristics of *L. jensenii* CECT 4306, and future research of the interactions of this strain with its vaginal environment may reveal further information about different aspects of its probiotic potential.

INTRODUCTION

Since the first microbiological study of the human vagina, lactobacilli have been considered the dominant members of the microbiota of this organ (Doderlein, 1982), a finding that has been further confirmed by several recent metagenomic studies (Biagi *et al.*, 2009; Fettweis *et al.*, 2012; Martín *et al.*, 2014; Pavlova *et al.*, 2002; Song *et al.*, 1999; Verhelst *et al.*, 2004; Vitali *et al.*, 2007; Wilks *et al.*, 2004; Zhou *et al.*, 2004). In total, around 20 different species of lactobacilli have been consistently isolated from the vagina, although only one or two species predominate at a time in a given individual. Dominant strains vary as a function of race and lifestyle, but strains of *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii* and *Lactobacillus gasseri* are the most frequent (Biagi *et al.*, 2009; Pavlova *et al.*, 2002; Song *et al.*, 1999; Verhelst *et al.*, 2004; Vitali *et al.*, 2007; Wilks *et al.*, 2004; Zhou *et al.*, 2004).

The presence of lactobacilli discourage the establishment of undesirable micro-organisms, mainly enteric bacteria, and the overgrowth of indigenous microbes, such as *Candida albicans* and *Gardnerella vaginalis*, whose excessive proliferation causes vaginitis, vaginosis and other pathologies (Sobel, 2000; Thorsen *et al.*, 1998). These beneficial effects result from two mechanisms: (i) exclusion, as lactobacilli form biofilms that mask the epithelial cell receptors to urogenital pathogens, and (ii) growth inhibition due to the generation of antimicrobial compounds such as organic acids and hydrogen peroxide (H$_2$O$_2$) (Martín & Suárez, 2010). The production of organic acids is a consequence of the strictly fermentative sugar metabolism of lactobacilli and generates the low pH (between 4 and 4.5) found in the healthy vagina, which inhibits the growth of most pathogens (Boskey *et al.*, 2001). Instead, H$_2$O$_2$ exerts its bactericidal...
Effect through the generation of oxidizing metabolites that disrupt enzyme functions and introduce breaks in cell DNA in the presence of myeloperoxidase and halides (Martin et al., 2008).

The vaginal isolate L. jensenii CECT 4306 has been found to produce high levels of H$_2$O$_2$ (Martin & Suárez, 2010). This strain may protect itself from the bactericidal effects of H$_2$O$_2$ through extracellular peroxidases, which are active in the presence of haemin, haemoglobin and Fe$^{3+}$ (Martin & Suárez, 2010); these cofactors are abundant in the vagina due to the bloody menstrual discharge.

The aim of this work was to evaluate whether Fe$^{3+}$ might influence other potential mutualistic effects of L. jensenii CECT 4306 as a further step in its evaluation for use as a probiotic organism. Specifically, we tested the effects of iron on adhesion of L. jensenii to mucin and epithelial cells as well as on its immunomodulatory properties.

**METHODS**

**Bacterial strains, cell lines and growth conditions.** L. jensenii CECT 4306 and Lactobacillus rhamnosus GG (ATCC 53103) were obtained from the Colección Española de Cultivos Tipo and the American type Culture Collection, respectively. The lactobacilli were grown in MRS broth (Difco). When necessary, 1.5 % (w/v) agar (Difco) was added to the liquid medium. Solid-medium cultures were incubated in anaerobic jars using the AnaeroGen Compact system (Oxoid). All the strains were grown at 37 ºC in an atmosphere of 5% CO$_2$. Culture medium was changed daily until 100% confluence was achieved.

**Protein manipulations.** Secreted, surface and total proteins of L. jensenii CECT 4306 were obtained as described by Martín et al. (2012). Samples were analysed by SDS-PAGE using 12.5% (w/v) polyacrylamide gels (Laemmli, 1970).

2D electrophoresis was performed with CyDye difference gel electrophoresis (DIGE) at the Proteomic Service of the Centro Nacional de Biotecnología (CNB-CSIC, Madrid, Spain). For this, 60 µg of protein was used for the reference map gels (Cy2) and 50 µg for those on which the samples were labelled with fluorescent dyes (Cy3 and Cy5). The effect of Fe$^{3+}$ on protein synthesis was analysed by spot matching across the gels. Experiments were conducted in triplicate. A threshold of ±1.2-fold was selected to identify the proteins of interest.

**Determination of immunomodulatory effects.** Anti-inflammatory assays were performed following the procedure described by Kechoua et al. (2013). Briefly, 50,000 HeLa or HT-29 cells per well were seeded in 24-well culture plates (Nunc). Twenty-four hours before bacterial challenge, the culture medium was changed to one containing 5% FBS. Experiments were initiated on day 7 after seeding, when cells had reached confluence (~1.83 x 10$^5$ cells per well). On the day of the coculture, bacteria were added at a m.o.i. of 40 in 50 µl DMEM; the volume was then brought up to 500 µl with DMEM medium. Cells were stimulated simultaneously with recombinant human TNF-z (5 ng ml$^{-1}$; Peprotech) for 6 h at 37 ºC in 10% CO$_2$. Following coculture, cell supernatants were collected and frozen at −80 ºC until the production of IL-8 could be quantified by an ELISA (Biologend). The total protein concentration was determined using the Bradford reagent (Sigma) according to the manufacturer’s instructions. All samples were analysed in triplicate.

For experiments on RAW 263.7 macrophages, 1 x 10$^5$ cells per well were seeded in 24-well culture plates (Nunc). Co-culture experiments were initiated the day after seeding via the addition of bacteria at an m.o.i. of 40 in 50 µl DMEM in a total volume of 500 µl. Culture supernatants were collected, mixed with complete EDTA-free protease inhibitor (Roche Applied Bioscience) following the instructions of the manufacturer, and stored at −80 ºC until IL-10 and IL-12 quantification by ELISA (Mabtech).

**Adhesion assays.** For adhesion assays, 2500 cells per well were seeded in 12-well culture plates (Nunc) and cultivated at 37 ºC in a 5% (v/v) CO$_2$ atmosphere. Culture medium was changed daily until confluence for Caco-2, HT-29 and HeLa cells (Tallon et al., 2007) and for 21 days for HT-29 MTX cells to allow proper mucus production. Adhesion of L. jensenii to porcine gastric mucin (type III; Sigma-Aldrich) and to Caco-2, HT-29, HT-29 MTX and HeLa monolayers was tested following the procedure described by Tallon et al. (2007). Approximately 10$^{10}$ lactobacilli (as determined by plate count) were used to test adhesion to mucin and a 50:1 ratio of bacteria/eukaryotic cells to test adhesion to epithelial cells. Overnight bacterial cultures and confluent eukaryotic cell cultures were used in all cases. For mucin adhesion tests, the data were normalized using the adhesion values of L. rhamnosus GG because of the reported strength of its adhesion to this compound (Guéimonde et al., 2004); adhesion of L. rhamnosus GG to mucin was given the arbitrary value of 1. Assays were performed at least in triplicate and the data are expressed as the mean ± SD.

**Fig. 1.** Adhesion to mucin. Data were normalized using the adhesion values of L. rhamnosus GG (ATCC 53103) because of the strength of its adhesion to this compound (Guéimonde et al., 2004); these values were arbitrarily set as 1. Results are expressed as the mean ± SD of at least three independent experiments. *P<0.05.
The bands of interest were excised from the gels and digested with porcine trypsin. The resulting peptides were analysed by MALDI-TOF MS at the Proteomic Service of the CNB-CSIC, and matched against the non-redundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism Software version 5.00 for Windows. The groups were compared using a non-parametric Kruskal–Wallis test followed by Dunn’s multiple-comparison post-hoc analysis. For 2D analysis, differences between spots were analysed statistically with Student’s t-test. A P-value of less than 0.05 was considered significant.

**RESULTS**

**Influence of Fe^{3+} on the adherence of L. jensenii CECT 4306 to epithelial cell cultures**

To test for the effect of iron on the adhesion of *L. jensenii* CECT 4306 to epithelial cell cultures, assays were performed that used cultures grown overnight with or without Fe^{3+}. First, we characterized the strain’s adhesion to mucin (Fig. 1). When strains were incubated in the absence of Fe^{3+}, *L. jensenii* was around 20 times more adherent to mucin compared with *L. rhamnosus* GG, while this value increased to 50-fold for strains of *L. jensenii* that had been incubated in the presence of Fe^{3+} (P<0.05) (Fig. 1).

*L. jensenii* CECT 4306 adhered better to HT-29 cells and to the mucus produced by the HT-29 MTX intestinal cell lines than it did to Caco-2 cells (Fig. 2b, d). For all three of these cellular models, adhesion significantly increased (P<0.05) when the lactobacilli had been incubated in the presence of Fe^{3+} (Fig. 2b, d). Conversely, while *L. jensenii* adhered well to HeLa cells, no differences were observed as a function of incubation with Fe^{3+} (Fig. 2a).

**Immunomodulatory properties of L. jensenii CECT 4306: influence of Fe^{3+}**

*L. jensenii* CECT 4306 lowered secretion of the inflammatory mediator IL-8 by TNF-α-induced HT-29 cells (Fig. 3a)

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**Fig. 2.** Adhesion to epithelial cell cultures. Adhesion of *L. jensenii* CECT 4306 grown with or without Fe^{3+} to HeLa (a), Caco-2 (b), HT-29 (c) and HT-29 MTX cells (d). Results are expressed as mean c.f.u. per eukaryotic cell (±sd) of at least three independent experiments. *P<0.05.
and this effect was slightly larger (but non-significant) when the bacteria had been pre-incubated overnight in media containing 2 mM Fe$^{3+}$. The same was observed with TNF-$\alpha$-stimulated HeLa cells (Fig. 3b). $L. jensenii$ did not affect IL-8 secretion by HT-29 or HeLa cells in the absence of TNF-$\alpha$ stimulation (data not shown).

Similarly, $L. jensenii$ CECT 4306 induced a non-inflammatory secretion profile of RAW 263.7 macrophages, as demonstrated by an increase of the IL-10/IL-12p70 ratio (Fig. 3c). Moreover, prior overnight incubation of the lactobacilli in media containing Fe$^{3+}$ further increased the induced anti-inflammatory profile of the macrophages ($P<0.05$) (Fig. 3c).

Comparison between the secreted, surface-associated and total proteomes of $L. jensenii$ CECT 4306 grown in the presence or absence of Fe$^{3+}$

$L. jensenii$ was grown in MRS broth with or without the addition of 2 mM Fe$^{3+}$, and the crude extracts of secreted (Fig. 4a) and cell-surface-associated proteins (Fig. 4b) from these cultures were compared following separation by SDS-PAGE. The most intense bands were excised manually and identified by MS (Table 1). Among the extracellular proteins detected, cell-wall hydrolases (R2, R5, R51) and a surface antigen (R52) were identified. In addition, a moonlighting protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (R4, R53) was also found. Unfortunately, we were unable to identify R3, R51, R54, R57 and R58, as no matches were found in the databases. Growth in the presence of Fe$^{3+}$ appeared to increase the abundance of secreted proteins (Fig. 4a) while decreasing the number of surface proteins present (Fig. 4b).

The total proteomes of $L. jensenii$ CECT 4306 grown in the presence or absence of Fe$^{3+}$ were resolved by SDS-PAGE. The bands that demonstrated the greatest changes in intensity were excised manually and identified by MS (Fig. 4c, bands 1–3; Table 1) as GAPDH.

Differences in the total proteomes were also revealed by 2D DIGE (Fig. 5, Table 2). When cultured in the presence of

![Fig. 3. Changes induced by $L. jensenii$ CECT 4306 in the secretion of immunomodulation factors. IL-8 secretion (measured in pg ml$^{-1}$ and corrected by the protein concentration of the extracts in mg ml$^{-1}$; final units pg ml$^{-1}$) by TNF-$\alpha$-induced HT-29 (a) and HeLa cells (b) in the absence or presence of $L. jensenii$ grown in media with or without added Fe$^{3+}$. (c) Change in the IL-10/IL-12 ratio produced by RAW 263.7 macrophages following incubation with $L. jensenii$, which was pre-incubated in the presence or absence of Fe$^{3+}$. The results are expressed as the mean ± SD of at least three independent experiments. *$P<0.05$, **$P<0.001$.](http://mic.sgmjournals.org/711)
Fe$^{3+}$, *L. jensenii* overproduced six proteins and underproduced 14 (over/underproduction defined as more than a twofold change in production) when compared with strains grown in the absence of Fe$^{3+}$ (Table 2). The most upregulated proteins were identified as GADPH isoforms (spots 136, 1177, 1187, 1189, 1236 and 1252), ATP synthase β subunit (spot 656) and glutamyl-tRNA glutamine amidotransferase β subunit (spot 767).

**DISCUSSION**

The vaginal exudate is a complex mixture of proteins, including immunoglobulins and enzymes, polysaccharides and monosaccharides, and aliphatic acids (Geshnizgani & Onderdonk, 1992). This rich vaginal environment is protected against the invasion of undesirable microorganisms by some species of *Lactobacillus*, such as *L. crispatus*, *L. iners* and *L. jensenii*; these species are so well adapted to the vaginal environment that they are seldom found outside the vagina (Macklaim *et al.*, 2011).

One of the unique characteristics of the vaginal ecosystem is the periodic change triggered by the menstrual cycle, which temporarily increases the pH of this organ and provides trace elements such as iron, which may influence the growth of the autochthonous microbiota (Cohen *et al.*, 1984). *L. jensenii* CECT 4306 is a strong H$_2$O$_2$ producer that depends on Fe ions to synthesize an extracellular peroxidase, which plays an important role in protecting the bacterium from its own antimicrobial activity (Martin & Suárez, 2010). Here, we aimed to study the effect of Fe$^{3+}$ on other mutualistic characteristics of *L. jensenii* CECT 4306, to evaluate this strain’s potential to be used as a commercial probiotic.

Among the requirements for probiotics, as established by FAO/WHO, adhesion to the mucosal surface is considered to be crucial (FAO/WHO, 2006). At the time of this study, we had no access to a convenient vaginal cell model; we therefore utilized throughout our study two well-established and well-characterized epithelial cell line models for adhesion, Caco-2 and HT-29, as well as the HeLa cell line, which is of cervical origin. Here, we show that *L. jensenii* CECT 4306 adhered strongly to mucin, HT-29 and HT-29 MTX cell cultures following both growth in standard media and growth in the presence of a high concentration of Fe$^{3+}$, but with significant increases under the latter conditions. Overall, this suggests that the cation induced the synthesis and/or surface presentation of new adhesins. However, the changes observed in the secreted and surface proteomes were too complex to allow identification of a single protein that might be responsible for the phenotypic change. Among the proteins whose expression changed as a result of the presence of Fe$^{3+}$, several were identified as cell-wall hydrolases. Recently, the peptidoglycan hydrolase Acm2 of *Lactobacillus plantarum* has been found to recognize mucin and has been hypothesized to serve as a generic mechanism for guiding cell adhesion (Beaussart *et al.*, 2013). The involvement of surface-associated GAPDH in lactobacilli adhesion to mucin and eukaryotic cells has also been reported (Kinoshita *et al.*, 2008; Martín *et al.*, 2012; Sánchez *et al.*, 2008). Although we did not find an increase in the concentration of obvious adhesin

![Fig. 4. Representative protein patterns obtained following SDS-PAGE of the secreted (a), surface (b) and total (c) proteomes of *L. jensenii* CECT 4306 grown with or without Fe$^{3+}$. M, molecular mass standards.](image_url)
Table 1. Proteins that showed a significant change in abundance, as identified by MS, as a function of the incubation of *L. jensenii* CECT 4306 in the presence or absence of Fe$^{3+}$.

| Band | Putative function | Micro-organism | Accession number | MM† | pI‡ | Match§ | MSMS|| | MOWSE¶ |
|------|-------------------|----------------|------------------|-----|-----|--------|------|------|-------|
| R2   | Cell-wall hydrolase | *Lactobacillus casei* ATCC 334 | gil116493849 | 49.4 | 4.9 | -      | 2    | 66   |
| R4   | GAPDH | *Lactobacillus casei* ATCC 334 | gil116494473 | 36.7 | 5.7 | -      | 4    | 111  |
| R5   | Cell-wall-associated hydrolase | *Lactobacillus casei* BL23 | gil191637071 | 23  | 4.9 | -      | 5    | 259  |
| R51  | Cell-wall-associated hydrolase | *Lactobacillus casei* BL23 | gil116493849 | 49.4 | 4.9 | 7      | 138  |
| R52  | Surface antigen | *Lactobacillus casei* ATCC 334 | gil116493849 | 49.4 | 4.9 | 7      | 138  |
| R53  | Surface antigen | *Lactobacillus casei* ATCC 334 | gil116493849 | 49.4 | 4.9 | 7      | 138  |
| 1    | GAPDH | *Lactobacillus bulgaricus* ATCC 11842 | gil104773773 | 36.7 | 5.5 | 4      | 105  |
| 2    | GAPDH | *Lactobacillus bulgaricus* ATCC 11842 | gil104773773 | 36.7 | 5.5 | 6      | 155  |
| 3    | GAPDH | *Lactobacillus acidophilus* NCFM | gil58337019 | 36.7 | 5.9 | 12     | -    | 355  |

*Spot labels as shown in Fig. 4.
†Theoretical molecular mass.
‡Theoretical isoelectric point.
§Number of tryptic peptides matching the corresponding theoretical sequence, which allowed protein identification.
||Fragmented peptides that allowed protein identification.
¶Molecular mass search score.
proteins when cultures were grown in the presence of Fe$^{3+}$, it is possible that exposure to this cation affected other elements that are proposed to play a role in adhesion, such as polysaccharides (Andreu et al., 1995; Boris et al., 1997, 1998; Vélez et al., 2007), or changes in the superficial hydrophobicity of the cell (Courtney et al., 2009). Alternatively, it is possible that we did not detect an increase in the production of these proteins because their synthesis is only upregulated in the presence of mammalian cells. This is an area that certainly deserves further research.

Lactobacilli have been shown to modulate innate defence responses as a means of preventing pathogen colonization and of blocking inflammatory cytokine secretion in a strain-specific (Karlsson & Jass, 2012; Rose et al., 2012) and challenge-dependent manner (Zhao et al., 2012). *L. jensenii* CECT 4306 reduced the inflammatory response induced by TNF-α in HT-29 cell cultures, as demonstrated by its suppression of IL-8 secretion. However, this effect appears to be Fe-independent as IL-8 secretion was only slightly, and non-significantly, affected when the bacteria were prior incubated in media containing Fe$^{3+}$. It also generated an anti-inflammatory shift of the ratio of IL-10/IL-12p70 produced by macrophages, although in this case the anti-inflammatory effect was enhanced by the bacteria’s prior growth in the presence of Fe$^{3+}$.

The data presented above indicate that Fe$^{3+}$ influences the behaviour of *L. jensenii* and its relationship with the surrounding environment. This is of particular relevance because strain CECT 4306 is indigenous to the vagina, an environment in which the concentration of Fe$^{3+}$ fluctuates with the menstrual cycle. For this reason, we decided to investigate the changes in the total proteome that were induced by incubation in media containing a high concentration of Fe$^{3+}$ with respect to the proteomes of cultures grown under standard laboratory conditions. Most of the proteins showing significant concentration changes were enzymes involved in the metabolism of carbohydrates and, to a lesser extent, amino acids. In many cases, these proteins were isoforms which demonstrated different migration patterns in 2D gels as a result of exposure to Fe$^{3+}$. This could suggest that Fe$^{3+}$ might act as a cofactor for these enzymes, and that its presence at high concentrations would increase the relative amount of the metal-charged isoform while lowering the relative abundance of the uncharged version. Interestingly, GAPDH isoforms demonstrated the largest variation in concentration as a result of the presence or absence of Fe$^{3+}$ in the *L. jensenii* propagation media. Apart from being a component of the glycolytic pathway, GAPDH is constitutively associated with the cell wall of certain lactic bacteria strains (Sánchez et al., 2009). It has been implicated in the adhesion of lactobacilli to eukaryotic cells and was involved in resistance to H$_2$O$_2$ in spontaneous mutants of *Lactococcus lactis*, due to its oxidation of Cys-containing proteins (Rochat et al., 2012). Furthermore, the presence of GAPDH on the bacterial surface could be explained by possible changes in membrane permeability, perhaps due to the presence of iron, as has been observed in *L. plantarum* 299v (Saad et al., 2009). Conspicuous variations in protein concentration were also detected for an ATP synthase and a glutamyl-tRNA-Gln amidotransferase; however, the significance of these changes remains obscure.

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**Fig. 5.** 2D gel electrophoresis of total proteomes of *L. jensenii* CECT 4306 obtained from cultures grown in MRS broth supplemented with (a) or lacking (b) Fe$^{3+}$. Proteins that displayed a significant difference in concentration are identified by a number in a yellow box. Proteins with the largest changes in concentration (at least twofold) are framed in red.
Table 2. Proteins of *L. jensenii* CECT 4306 that demonstrated a change in concentration of more than 1.2-fold between cultures incubated in the presence or absence of Fe$^{3+}$. Proteins were separated by 2D gel electrophoresis and identified by MS or MS/MS.

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<tr>
<th>Spot*</th>
<th>Putative function</th>
<th>Functional category</th>
<th>Gene†</th>
<th>MW‡</th>
<th>pI§</th>
<th>Match¢</th>
<th>MOWSE¶</th>
<th>−FE vs +FE#</th>
<th>P (t-test)</th>
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Table 2. cont.

| Spot* | Putative function                  | Functional category             | Gene† | MW‡ | pI§ | Match|| | MOWSE§ | −FE vs +FE# | P (t-test) |
|-------|-----------------------------------|---------------------------------|-------|-----|-----|--------|--------|--------|------------|------------|
| 1229  | GAPDH * Lactobacillus delbrueckii  | GAP Glycolysis gluconeogenesis  | gap   | 36564.38 | 5.51 | 10 | 256 | 3 | 4.8e-007 |
|       | subsp. bulgaricus ATCC 11842       |                                 |       |       |     |      |     |     |            |            |
| 1236  | GAPDH * Lactobacillus delbrueckii  | Glycolysis                      | gap   | 36564.38 | 5.51 | 8  | 280 | −2.45 | 1.3e-006 |
|       | subsp. bulgaricus ATCC 11842       | Glycolysis gluconeogenesis      |       |       |     |      |     |     |            |            |
| 1252  | GAPDH * Lactobacillus delbrueckii  | Glycolysis                      | Gap   | 36564.38 | 5.51 | 11 | 309 | −2.53 | 4.5e-006 |
|       | subsp. bulgaricus ATCC 11842       | Gluconeogenesis                  |       |       |     |      |     |     |            |            |
| 1399  | None identified                    |                                |       |       |     |      |     |     | 1.97      | 0.00017   |
| 1480  | Citrate lyase beta-chain           | Energy production and conversion|       | 33620.26 | 4.96 | 29 | 94  | 1.97 | 0.00017   |
|       | *Lactobacillus helveticus CNRZ32   |                                |       |       |     |      |     |     |            |            |
| 1677  | None identified                    |                                |       |       |     |      |     |     | 1.97      | 0.04      |
| 1834  | Xanthosine triphosphate pyrophosphatase * Lactobacillus delbrueckii ATCC 11842 | Folate biosynthesis Starch and sucrose metabolism | GeneID: 4085216 | 22294.19 | 5.42 | 1 (MSMS) | 44 | 1.54 | 0.00040   |
| 1886  | None identified                    |                                |       |       |     |      |     |     | 1.5       | 0.00045   |
| 1936  | GAPDH * Lactobacillus crispatus    | Glycolysis gluconeogenesis      |       | 36539.29 | 5.38 | 7  | 148 | 1.62 | 0.00081   |
| 1948  | Ribosomal subunit interface protein * Enterooccus faecalis V583 | Ribosome                         | GeneID: 1200654 | 21158.09 | 5.89 | 2 (MSMS) | 43 | 1.61 | 0.00016   |
| 2178  | None identified                    |                                |       |       |     |      |     |     | 1.56      | 0.022     |

*Spot labels as shown in Fig. 5.
†Gene identity according to the GenBank database.
‡Theoretical molecular mass.
§Theoretical isoelectric point.
||Number of tryptic peptides matching the corresponding theoretical sequence or fragmented peptides that allowed protein identification.
||Molecular mass search score.
#Mean ratio, in fold-change, of increase/decrease in spot volumes in L. jensenii CECT 4306 grown without versus with Fe³⁺ (ratio <0: protein upregulated with Fe³⁺; ratio >0: protein downregulated with Fe³⁺).
In conclusion, we found that growth in the presence/absence of Fe³⁺ affected the adhesion of \textit{L. jensenii} CECT 4306. In addition, the immunomodulatory properties of this strain were also modified in the presence/absence of Fe³⁺, although this effect was of a lesser magnitude and was dependent on the human cell line used. This was the case even though Fe³⁺ was not actually present during the interactions of the bacteria with the human cells. These results prompt us to highlight the importance of growth conditions on the characterization of potential probiotic strains and the potential future use of \textit{L. jensenii} CECT 4306 as a probiotic strain.

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