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Probiotic properties of *Lactobacillus* strains isolated from table olive biofilms

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Running title: *L. pentosus* probiotic strains

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Abstract

In this work, sixteen strains with promising probiotic characteristics belonging to the *Lactobacillus pentosus* (13) and *Lactobacillus plantarum* (3) species and isolated from table olive biofilms, were tested for adherence to cell lines and to solvents, immunomodulatory and anti-proliferative properties on epithelial human cellular lines. Most *Lactobacillus* strains were able to regulate the production of cytokines by stimulating the production of pro-inflammatory (IL-6) and anti-inflammatory (IL-10) interleukins on macrophages, and by suppressing the secretion of IL-8 on HT-29 TNF- α induced model. *Lactobacillus* strains also showed anti-proliferative activity on the HT-29 cell line. No clear relation was found between adhesion to solvents and adhesion to HT-29 human cell line. *Lactobacillus pentosus* LPG1, which showed the best anti-inflammatory and immunomodulatory properties, was then tested in a dinitro-benzene sulfonic acid (DNBS)-induced chronic colitis murine model. As a measure of the inflammation, gut permeability and weight loss, as well as cytokine profiles, were determined. *L. pentosus* LPG1 improved mice health as observed by a significant reduction of weight loss, gut permeability, and beneficial cytokine modulation. Macroscopic scores and tissue damage was also lower in mice administered with LPG1 with respect to the DNBS-treated group. These results showed that *L. pentosus* LPG1 isolated from plant could have potential as probiotic for use as an anti-inflammatory and immunomodulatory agent for patients with inflammatory bowel disease.

Keywords: table olives, lactic acid bacteria, biofilms, animal models, probiotic

1. Introduction

Currently, there is a trend towards the search for functional foods which could act in two ways: providing prebiotic properties due to their inherent characteristics or acting as a carrier of beneficial microorganisms to consumers. In this sense, the role of fermented foods such as yogurt, cheese, kimchi, sauerkraut, among others, is well known [1]. However, at present, there is a demand for new alternatives to these recognized healthy foods, which in many cases are dairy products. Thus, new fermented foods with beneficial properties are emerging, as is the case of table olives [2-5].

Table olives are a major component of the Mediterranean diet and culture. Nowadays, they constitute one of the most important fermented vegetables in the world, especially in the Mediterranean basin, with a production that exceeds 2.5 million tons/year [6]. Among the diverse processing methods, alkali-treated green olives (Spanish style), ripe olives by alkaline oxidation (Californian style) and directly brined olives (natural olives) are the most common [7]. Lactic acid bacteria (LAB), mainly belonging to *Lactobacillus pentosus* and *Lactobacillus plantarum* species, are the most important bacteria in many table olive and other fermented vegetables [8,9]. LAB metabolize sugars producing lactic acid during olive fermentation, thus decreasing the pH. Moreover, they can produce bacteriocins which act against pathogen and spoilage microorganisms [10, 11]. In recent years, researchers have focused their efforts to isolate LAB strains from the fermentation of table olives with multifunctional characteristics (technological + probiotic) for use as starter cultures [12-15]. Also, many of these strains have demonstrated their ability to adhere to the surface of olives during fermentation forming biofilms, turning table olives as carriers of beneficial microorganisms to consumers [16-19].

Lactobacillus strains are widely studied for their probiotic potential. A beneficial effect of probiotic microorganisms is the regulation of the immune response. Thus, in last years, there is a trend for the search of new strains with immunomodulatory and anti-inflammatory properties that can act in the prevention and/or treatment of diseases related to abnormal immune functions, including allergic diseases [20] or intestinal inflammatory disorders such as Crohn's disease [21]. An adequate balance among pro and anti-inflammatory cytokines is needed to appropriately modulate response against infections [22]. For example, IL-10 has been considered as one of the most powerful anti-inflammatory cytokines, repressing the expression of inflammatory cytokines such as TNF- α , IL-6, and IL-1 by activated macrophages. IL-10 can also up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors [23].

Microorganisms play an important role in the proper functioning of the immune system. In this way, patients with inadequate and continuous activation of the mucosal immune system, driven by the intestinal microbiota from a genetically predisposed patient, appear to be prone to the development of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) or ulcerative colitis, characterized by a chronic and recurrent inflammation of the gastrointestinal tract [24]. In particular, it is suspected that in CD the intestinal microbiota

plays a role in the initiation and activation of the immune system, which leads to a characteristic inflammation [25].

The goal of this work was to confirm the probiotic potential of diverse LAB strains previously isolated from the surface of table olives, through the measure of their *in vitro* immunomodulatory properties, adhesion capacity to cell line, hydrophobicity, anti-proliferative activity in cancer colon cell lines, and validate their protective effect in an *in vivo* dinitro-benzene sulfonic acid (DNBS)-induced colitis mouse model.

2. Materials and methods

Microorganisms assayed

Thirteen *Lactobacillus pentosus* (Lp1, Lp2, Lp3, Lp4, Lp5, Lp6, Lp7, Lp8, LPG1, Lp10, Lp11, Lp13, Lp14) and three *Lactobacillus plantarum* (Lpl12, Lpl15, Lpl16) strains, all of them previously isolated from olive biofilms in different industries which process natural and lye treated green olives, were used in the present study. All these microorganisms belong to the Table Olive Microorganisms Collection (TOMC) of Instituto de la Grasa (CSIC, Seville, Spain) and they were identified by molecular methods [26]. Two commercial probiotic strains, *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG, were also used as probiotic control. These strains have been previously subjected to diverse *in vitro* preliminary probiotic tests as cholesterol removal, survival to gastric and pancreatic digestions, co-aggregation, phytase, α -glucosidase and β -galactosidase activities, inhibition of pathogens, hemolytic activity, presence of bile salt hydrolase genes and antibiotic resistance, with promising results [26]. Strains were stored at -80°C in 20% glycerol (v/v) until further analysis. Prior its use, they were grown at 37°C in microaerophilic conditions in the Man Rogosa Sharp (MRS) broth (Difco, France) for 12 h and sub-cultured once before each experiment.

Bacterial adhesion to the cell monolayer

The human colon adenocarcinoma cell line HT-29-MTX (mucus producer) was used to determine the adhesion ability of LAB strains. The procedure was developed according to the protocol described by Turpin et al. [27] with slight modifications. The bacterial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland) supplemented with 1% L-cysteine, 1% L-glutamine, streptomycin (100 mg/ml), penicillin (100 U/ml), and 10% heat-inactivated fetal bovine serum, and incubate at 37° C with 10% CO₂. The medium was changed every two days. Once the confluence was obtained, the cells were trypsinized and seeded in in DMEM 24-well culture plates at a concentration of 1×10^5 cells/well. The DMEM medium was changed daily. Cell differentiation was reached after 21 days incubation. Two days before the adhesion test, antibiotics were removed from the media.

The bacterial cells were harvested from overnight cultures by centrifugation for 5 min at 4500 x g, the pellet was washed twice and re-suspended in phosphate-buffered saline (PBS) to 5×10^7 CFU/mL, and 50 μ l of each bacterial suspension was added to the appropriate well. After 2 h of incubation at 37°C in a 10 % CO₂ air atmosphere, the cell monolayer was washed twice with PBS to remove the non-attached bacteria [27]. Attached bacteria were then separated by adding 200 μ l of a 15 % (v/v) solution of trypsin-EDTA (Gibco Life Technologies) in PBS, and incubated at room temperature until cell detachment [28]. Then, 300 μ l of DMEM was added to stop the trypsin reaction, and appropriate dilutions were plated

on MRS agar medium and incubated at 37°C during 48 h to elucidate the number of viable adherent bacteria. Adhesion was expressed as the percentage of adhered cells with respect to the number of input cells.

Bacterial adhesion to hydrocarbons

Microbial adhesion to solvent (chloroform, ethyl acetic or o-xylene) was developed following the protocol described by Vinderola and Reinhimer [29] with slight modifications. Briefly, overnight cultures were centrifuged at 4,500 x g and washed twice with PBS 1X. Then, the cell suspension was adjusted to an $A_{600\text{nm}}$ value of 1.0 in the same buffer. Afterward, 3 ml of this suspension was mixed with 0.6 ml of chloroform, ethyl acetic or o-xylene (Sigma, St. Luis, USA) and vortexed for 120 s. The phases were allowed to separate for 1 h at 37 °C. After that, the aqueous phase (lower) was carefully removed, and the $A_{600\text{nm}}$ was measured. The experiment was developed by duplicate. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface adhesion, which was calculated with the formula $\% \text{ Adhesion} = [(A_o - A)/A_o] \times 100$, where A_o and A are the absorbances before and after extraction with chloroform, ethyl acetate or o-xylene.

Immunomodulatory properties

The anti-inflammatory properties of the LAB strains were evaluated by measuring their ability to suppress the secretion of IL-8 on HT-29 cells stimulated with TNF- α . First, HT-29 differentiated cells were grown in a 24-well plate at a concentration of 1×10^5 cells/well in DMEM (Lonza, Switzerland), supplemented with 1% L-glutamine (200 mM), 0.1% penicillin/streptomycin, and 10 % heat-inactivated fetal bovine serum (FBS). Plates were incubated for 7 days at 37°C in 10 % CO₂ air atmosphere until confluence ($\sim 1.83 \times 10^6$ cells/well). The medium was changed daily. Twenty four hours before bacterial co-culture, the concentration of serum in the medium was changed to 5%. To stimulate the IL-8 production, on the co-culture day, the HT-29 cells medium was replaced with fresh medium supplemented with 10 ng/mL of recombinant human TNF- α (Peprotech, USA).

The RAW 264.7 murine macrophage-like cell line model was also used to investigate the IL-6 and IL-10 modulation capacity of the olive *Lactobacillus* strains. RAW 264.7 cells were grown in DMEM media, supplemented with 2% L-glutamine, streptomycin (100 mg/mL), penicillin (100 U/ml), and 10% FBS. Cells were seeded in a 24-well plate at a density of 5×10^5 cells/mL and then incubated for 24 h at 37°C in a 10 % CO₂ air atmosphere.

Overnight culture of the strains suspensions were treated as described above. For IL-8 determination, the activity of UV-inactivated cells was also evaluated. First, cells were killed by UV treatment (20 min). Bacteria viability was verified by plating on MRS agar. Live or inactive bacteria were added at a multiplicity of infection of 1:40. Plates were then incubated during 6 h at 37°C in 10 % CO₂ air atmosphere. Afterward, supernatants were collected and frozen at -80°C until further analysis. Interleukins (IL) concentrations were determined by Enzyme-linked immunosorbent assay analysis (ELISA) [30]. For IL-6 and IL-10 evaluation, plates were incubated during 24 h. IL concentration in supernatants was determined by the ELISA analysis following MABTECH protocol (Sweden).

Cell viability

The evaluation of the anti-proliferative activity of the *Lactobacillus* strains to cell lines was developed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay, which is based on the reduction of MTS by actively growing cells or the neutralized and non-neutralized cell-free supernatants (Promega, Madison, Wisconsin, USA). First screening with viable olive *Lactobacillus* strains was conducted in the human colon cancer HT-29. The cell-free supernatants of those with the best anti-proliferative activities in the previous test were also tested in HCT-116 and Caco-2 cells lines.

Human colon cancer cell lines (Caco-2, HTC-116, and HT-29) were cultured in DMEM medium (Lonza, Switzerland) containing 1% L-glutamine (200 mM), streptomycin (100 mg/mL), penicillin (100 U/ml), and 10% fetal bovine serum. For the Caco-2 cell lines, 1% of non-essential amino acids were added. Cell lines were incubated at 37°C in a humidified atmosphere with 10% CO₂. Trypsin-EDTA (PAA, Austria) was used for cells detachment after confluence. Each cancer cell line was seeded in a 96-well plate (1.0×10^4 cells/ well) in DMEM medium and incubated during 24 h at 37°C in a 10% CO₂ atmosphere. Overnight cultures of bacterial suspensions were centrifuged for 5 min at 4500 x g. The pellet was washed twice with PBS 1X. Then, 20 µl of the bacterial suspension was added at a multiplicity of infection (MOI) of 1:100 and plates were incubated for 24 and 48 h at the same conditions described above. 5-Fluorouracil (Roche, Italy) was used as a positive control at 1mM final concentration. In the case of the supernatant, the suspensions were treated as described above, the sediments were discarded, and 20 µl of filtered suspensions were added.

The determination of the number of viable cells was determined according to the Promega protocol. Briefly, after removing the medium from the plates and washing with PBS 1X, 120 µl of DMEM medium containing 200 µl/mL of CellTiter 96® Aqueous One Solution Reagent medium was added. Plates were incubated for 2 h and A_{490nm} was determined. The percentage of viable cells was calculated as follows: cell proliferation (%) = (OD of experimental group/OD of the control group) × 100.

Murine model

C57BL male mice (6-8 weeks) were kept in the animal care facilities of the National Institute of Agricultural Research (IERP, INRA, Jouy-en-Josas, France) under specific pathogen-free conditions, for a minimum of 1 week before experimentation. All the experiments were previously approved by the corresponding local committee and performed in accordance with the European Community standards for the care of animals.

Moderate colitis was induced to mice by using dinitro-benzene sulfonic acid (DNBS), applying the protocol described by Martín et al. [31]. Briefly, mice were first anesthetized with enflurane (Abbott, Abbott Park, USA). To induce colitis, 200 mg/kg of DNBS solution (ICN, Biomedical Inc.) in 30% ethanol (EtOH) was injected intrarectally via, using a 10-cm-long piece of PE90 tubing (ClayAdam, USA) attached to a tuberculin syringe which was inserted 3.5 cm into the colon. Control mice (non-colitis group) were only supplied with EtOH. In order to prevent dehydration (DNBS period) mice were supplied with 6 % sucrose in drinking water for the first 3 days after DNBS injection. Ten days after DNBS period, mice were intragastrically administered with a suspension of 200 µL containing either 1×10^9 CFU of the LPG1 strain or 200 µL of PBS, daily for a total of 10 days (gavage period). The study groups were established as follows: control non-colitis group (PBS, n=8), control colitis group

(DNBS, n=8) and *Lactobacillus pentosus* LPG1 (DNBS-LPG1, n=8). Twenty-one days after the first DNBS injection, colitis was reactivated with a second injection of 100 mg/kg of DNBS solution (recovery period). As a measure of the DNBS effect, the bodyweight of the mice was evaluated prior to killing them. Mice were sacrificed 3 days after the second DNBS challenge (day 24 after the beginning of the experiment).

Effect of DNBS in the inflammation intensity is normally visible at a macroscopic level. Thus mucosal damage in stool consistency and colon tissue, as an indicator of diarrhea, was measured to determine this effect. Macroscopic scores were measured according to the system for DNBS colitis described by Qiu et al. [32]. The next parameters were determined to evaluate the mucosal damage: adhesions between the colon and other intra-abdominal organs, ulcerations, thickening of the colon wall and presence of hyperemia. For this purpose, mice were sacrificed by cervical dislocation, and the abdominal cavity was opened. The small intestine and colon were removed and opened longitudinally. Macroscopic damage was assessed immediately and scored from 0-9.

Intestinal permeability was measured as previously described by Martín et al. [33]. Briefly, 0.6 mg/g body weight of fluorescein-conjugated dextran (FITC-dextran 3000-5000 Da, Sigma-Aldrich) was administered by oral gavage to mice on the sacrifice day. Blood samples were obtained from the retro-orbital venous plexus 3.5 h later. Then, plasma FITC levels were determined by the use of a fluorescence spectrophotometry microplate reader (Tecan, Lyon, France). As standard, FITC-dextran was serially diluted.

To determine the colonic ILs, the colon was mechanically dissociated with a GentleMax disruptor (Miltenyi Biotech, Paris, France) followed by Ficoll-plaque density gradient centrifugation GE Healthcare, Vélizy-Villacoublay, France). Cells were then homogenized in PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzethonium chloride, 0.05 % Tween-20, 10 mM EDTA, and 20 KIU Aprotinin A using a tissue homogenizer (100 mg tissue/mL buffer). Afterward, suspensions were centrifuged at 4°C at $12,000 \times g$ for 20 min, and the supernatants were transferred to microtubes and stored at -80°C until further analysis. Concentrations of IL-4, IL-5, IL-13, IL-22, INF γ , and TGF- α were determined by ELISA.

Statistical analysis

Statistical analysis of data was done by using Prisma 5.0 software (GraphPad Software Inc., La Jolla, CA, United States). One-way analysis of the variance (ANOVA) and Dunnett's test as *post hoc* comparison analysis was developed, and $p < 0.05$ was considered as statistically significant level.

3. Results

Bacterial adhesion to HT29-MTX cell line

This analysis revealed an adhesion capacity to HT29-MTX cells by all the *Lactobacillus* strains that ranged from 0.17 (*L. plantarum* Lp12) to 2.25 % (*L. pentosus* Lp14) with an average percentage of $0.73(\pm 0.37)$ % (Figure 1). ANOVA test showed that only *L. pentosus* Lp14 and *L. rhamnosus* GG had significant differences ($p > 0.05$), showing the highest adhesion capacity.

Anti-inflammatory properties (IL-8 variation)

We tested the anti-inflammatory properties of the *Lactobacillus* strains based on their ability to reduce the production of pro-inflammatory IL-8 by HT-29 cells stimulated with TNF- α to produce the ILs. Figure 2 shows the IL-8 variation after co-cultivation with live cells and UV-inactivated bacteria strains. *Lactobacillus* strains display a reduction of IL-8 levels in comparison to the group PBS (untreated cells, negative control). The values ranged from 0.0 to 56.6% of blocking secretion. Thus, the statistical analysis revealed that live *L. pentosus* Lp2, Lp7, Lp8, LPG1, Lp11, Lp13, Lp14, and *L. plantarum* Lp112 and Lp115, as well as the probiotic strains, were able to significantly reduce IL-8 levels, highlighting *L. pentosus* LPG1 with the higher value of blocking secretion. The second approach with UV-inactivated cells showed effect in the IL-8 secretion on HT-29 cells stimulated with TNF- α . The values ranged from 0.0 to 37.8% of blocking secretion, highlighting *L. pentosus* Lp7.

Immunomodulation of IL-6 and IL-10 in cell line murine macrophage

The capacity of the *Lactobacillus* strains to regulate the production of both ILs by RAW 264.7 murine cell line was also determined. The values obtained for this analysis ranged from 250.3 pg/ml (strain Lp115) to 1557.7 pg/ml (strain Lp8) for IL-6, and from 5180.67 pg/ml (strain Lp115) to 24069.24 pg/ml (strain LPG1) for IL-10. As Figure 3 shows, *L. plantarum* Lp115, and Lp116 evidenced the lowest stimulatory activity compared with the other *Lactobacillus* strains for both, IL-6 and IL-10. Results were compared with the obtained for the probiotic strain *L. casei* Shirota. ANOVA test revealed that *L. pentosus* Lp3, Lp8, and LPG1 showed a significantly higher capacity to stimulate the IL-6 production, while Lp7, Lp115 and Lp116 strains significant lower stimulation capacity. In the case of IL-10 modulation, mostly *Lactobacillus* strains isolated from table olive showed higher activities than the observed in the commercial probiotic strains. Thus Lp1, Lp2, Lp3, Lp5, Lp8, LPG1, Lp10, Lp11, Lp112, and Lp13 strains were able to stimulate significantly the production of IL-10. Nevertheless, the best balance in the stimulation of IL-6/IL-10 was obtained after incubation with LPG1 strain.

Microbial adhesion to solvents (MATS)

MATS test was developed to study the properties of the bacterial surface. Result of this analysis is shown in Table 1. The adhesion percentage to ethyl acetate, which determine the electron acceptor properties of the bacterial cell surface varied from 17.60 % (± 0.61) for Lp7 to 48.46 % (± 3.06) for Lp116, which was the only strain with a significant higher affinity to ethyl acetate ($P < 0.05$) than reference strains *L. casei* Shirota (36.19%). The rest of the *Lactobacillus* strains showed similarly or lowers values of affinity to this solvent.

Affinity values to chloroform and o-xylene were generally higher, indicating the donor nature of the strains and hydrophobic properties, respectively. An average affinity of 70.95% was obtained for chloroform and 54.23% for o-xylene. However, values higher to 80.0% were obtained for some *Lactobacillus* strains for both solvents. Thus Lp2, Lp4, Lp112, Lp115, and Lp116 showed significant high affinities to chloroform than *L. casei* Shirota ($P < 0.05$) and Lp4, Lp6 Lp115 and Lp116 to o-xylene. Low hydrophobicity was found in the probiotic strains *L. casei* Shirota and *L. rhamnosus* GG (Table 1).

Microbial effect on cell viability

The MTS method is commonly used to scrutinize the cytotoxicity and viability of living cells. To simulate the interaction between the cellular mucosa and bacteria, the

metabolic activity of the cell lines was measured after two different co-incubation times (24 and 48 h). Figure 4 illustrates the antiproliferative potential of the *Lactobacillus* strains in HT-29 cells. After 24 h of incubation a decreasing metabolic activity in HT-29 was not observed, indicating that all *Lactobacillus* strains are likely to leave the cellular viability unchanged or even improved after this incubation time. However, MTS data at 48 h incubation indicated that *Lactobacillus* strains Lp3, Lp6, LPG1, Lp112, Lp115 and the probiotic strain *L. rhamnosus* GG had a statistically significant cytotoxic effect on HT-29 with respect to the non-inoculated control group. The free-cell supernatant of the strains could also decrease the viability of each cell line (data not shown). Nevertheless, no effect was observed when the pH was neutralized, suggesting that the cytotoxic effect could be due to the lactic acid present in the supernatant.

Murine model

Based mainly on the *in vitro* anti-inflammatory property described above, *L. pentosus* LPG1 was selected for evaluating the protective effects in a chronic colitis murine model induced by the DNBS.

As shown in Figure 5, DNBS administration affected mouse weight, resulting in an average weight loss of about 3% at the end of the experiment, in contrast to a gain of 4% in the control mice without DNBS (PBS control group). Interestingly, the administration of LPG1 significantly ($P<0.05$) limited the weight loss, even increasing body weight by 5%. Gut permeability to FITC-dextran was also evaluated in DNBS-treated mice to assess barrier function. As Figure 5 shows, the permeability of mice treated with DNBS increased significantly, confirming an alteration in the barrier function in terms of permeability. However, the administration of LPG1 strain decreased significantly the intestinal permeability concerning the DNBS mice. Those data could also be explained by a significant lower macroscopic score in the mice administered with LPG1 with respect to the DNBS-treated mice (Figure 5).

The production of the colonic cytokines IL-4, IL-5, IL-13, IL-22, IFN- γ , and TNF- α was analyzed by ELISA. As Figure 6 shows, levels of IFN- γ , IL-5, IL-22 (cytokines reported as increased in active IBD patients) were higher in samples from DNBS-treated mice than in the healthy one. Values of those cytokines after administration of LPG1 decreased significantly ($P<0.05$) with respect to the DNBS-treated group, being even lower than the healthy control in the case of IL-22 and IFN- γ . Significant differences were also observed in IL-4, IL-13 levels, although a reduction was observed in TNF- α in the LPG1 treatment, it was not statistically significant.

4. Discussion

Most of the studies carried out until now to prove the potential probiotic of table olive related microorganisms were executed exclusively using *in vitro* models [1,2,5,14,26]. Recently, Guantario et al. [34] evaluated *in vivo* the probiotic potential of lactobacilli isolated from Nocellara del Belice table olives in a *Caenorhabditis elegans* model, revealing that one strain of *L. pentosus* species significantly induced longevity of the nematode population and its protection from pathogen-mediated infection. In addition, Grounta et al. [35] studied the effect of *L. pentosus* B281 and *L. plantarum* B282, isolated from table olives on the survival of *Listeria monocytogenes* and *Staphylococcus aureus* using as a model *Galleria mellonella* larvae. However, no studies using superior animal models have been performed until now.

This work uses a murine model to validate results obtained preliminary by diverse *in vitro* tests.

The ability to adhere to the intestinal mucosa is recognized as one of the main selection criteria for probiotics [36]. Our study did not show high values of adhesion to HT29-MTX compared to the probiotic strains used as control. This way, no differences were found between the *Lactobacillus* isolated from olive biofilms and the probiotic *L. casei* Shirota. Results obtained in this work are similar to previously described for other *Lactobacillus* strains [27]. In the case of adhesion to organic solvents, the affinity of the strains to chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) describe the electron donor and electron acceptor properties of the *Lactobacillus* cell surface, respectively. On the contrary, the hydrophobic characteristic of the bacteria is determined by the adhesion to an apolar solvent such as xylene [37], which are attributed to carboxylic groups and Lewis acid-base interactions [38,39]. Probiotic microorganisms are considered to attach easily to cell lines due to their high hydrophobicity. However, in this study, we did not found a clear relation between MATS and adhesion to the cell lines. Thus attachment to surfaces depends not only on the hydrophobicity of cells; factors responsible for this process include also Brownian movement, Van der Waals attraction, gravitational forces and surface electrostatic charges [40]. It has also been reported that microorganisms can switch between hydrophobic and hydrophilic phenotypes in response to changes in environmental conditions (temperature, the composition of nutrients, etc.) and growth phases [41,42].

Previous studies have shown that the administration of certain probiotics inhibits the progression of cancer cells *in vitro* and in animal models [43,44]. Other authors have identified some molecules involved in this process [45]. The MTS compound (Owen's reagent) is bio-reduced by cells into a colored formazan product that is soluble in a tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The metabolites secretion of some of the *Lactobacillus* strains could have decreased the viability of the cells and the activity of this reaction. Our findings revealed a decrease in the viability of HT-29 after 48 h of incubation with most of the *Lactobacillus* assayed. Supernatants could also inhibit the growth of the cells, however, when they were neutralized, no effect was observed, suggesting that lactic acid produced by microorganisms was largely responsible for this inhibition.

The immunomodulatory ability of bacteria is other of the vital criteria for the assessment of a probiotic strain [46]. The acute kinetics of plasma levels of pro and anti-inflammatory molecules has been thoroughly studied. Groeneveld et al. [22] reported that mediators with a pro-inflammatory effect predominate in a first phase, while anti-inflammatory phenomena appear in later phases. It is known that IL-6 concentration increases after trauma and chronic diseases. This cytokine has a role as a pro-inflammatory mediator and also as an anti-inflammation regulator, which stimulates potent anti-inflammation cytokines such as IL-10 [47]. In this study, most strains were able to stimulate the production of the pro-inflammatory cytokine IL-6 and the anti-inflammatory IL-10. However, the ability of *L. pentosus* LPG1 to regulate the immune response was remarkable, presenting an effective balance in the stimulation of both types of cytokines. Thus, the high stimulation in the pro-inflammatory cytokine IL-6 was also regulated by a high stimulation of the production of the anti-inflammatory cytokine IL-10. Specific strains of *Lactobacillus* have been shown to induce pro-inflammatory (IL-1, IL-6, IL-12, TNF- α , etc.) and anti-inflammatory cytokines such as IL-10 and TGF- β [48-50].

Among the main IL, IL-8 is especially known to be a potent promoter of the adhesion of monocytes and neutrophils that are found in the blood circulation to the endothelial cells that form the blood vessels, which helps them to pass from the blood to the inflamed tissue facilitating its action [51]. However, the high level of this cytokine has also been related to different pathologies, for example participating in the inflammatory processes of some diseases, such as rheumatoid arthritis or ulcerative colitis. Nine of the *Lactobacillus* strains, isolated from the surface of the olive, could significantly reduce IL-8. However, the LPG1 activity stood out with respect to the others. Similar results were also found in previous works for other *Lactobacillus* strains [28].

The anti-inflammatory properties of the *L. pentosus* LPG1 were also validated in a murine DNBS-induced acute colitis model. We report that this strain significantly improved the symptoms of chronic colitis. Treatment with LPG1 not only prevented the weight loss of mice but also decreased gut permeability. The use of probiotic microorganisms has been determined to be a substantial factor in the reduction of intestinal permeability, increasing the barrier function, and thus, preventing the passage of intestinal microorganisms and other antigens in patients with IBD [52]. This effect is produced through the communication with epithelial cells and different sets of cells implicated in both innate and acquired immune response via pattern-recognition receptors [53]. For example, several strains of *Lactobacilli* can increase mucus production by intestinal goblet cells through the up-regulation of MUC3 gene expression, which results in increased mucus production by intestinal goblet cells [54,55]. Macroscopic score and tissue damage were also significantly lower in mice administered with our strain. The onset, evolution, and ultimately, the resolution of inflammatory processes in inflammatory bowel diseases are governed by the responses of the cytokines involved in this process [56]. Our results showed that *L. pentosus* LPG1 tended to block the secretion of cytokines known to be involved in IBD pathogenesis, and it is especially remarkable the inhibitory effect on IL-22 since it is a cytokine that promotes pro-inflammatory gene expression in patients suffering IBD.

5. Conclusion

We show, for the first time, the protective effect of an *L. pentosus* strain isolated from plant origin in a murine chronic colitis model. These results provide evidence that *L. pentosus* LPG1 is an anti-inflammatory bacterium with therapeutic potential for patients with inflammatory bowel disease. Because of this strain was isolated from olive fermentations, it could also be used as a starter culture for producing probiotic olives. However, deeper studies are necessary to validate these properties in human clinical studies.

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Compliance with ethical standards

This article contains studies with animals performed according to their welfare. The authors declare that they have no conflict of interest.

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Table 1. Cell-surface characteristics of the *Lactobacillus* strains isolated from table olive biofilms, measured by the MATS test. Results are expressed as a percentage (%) of the average of duplicates for ethyl acetic, chloroform and o-xylene treatments. The parenthesis shows standard deviations between duplicates. The asterisk indicates significant differences ($P<0.05$) when compared with *L. casei Shirota*.

Strain	Solvent		
	Ethyl acetic	Chloroform	o-xylene
<i>L. pentosus</i> Lp1	9.47 (± 13.14)*	91.27 (± 3.47)	52.83 (± 2.72)*
<i>L. pentosus</i> Lp2	18.56 (± 0.29)*	94.19 (± 0.15)*	36.79 (± 0.70)
<i>L. pentosus</i> Lp3	30.61 (± 5.04)	41.94 (± 3.47)*	32.83 (± 0.42)
<i>L. pentosus</i> Lp4	24.28 (± 0.75)*	94.90 (± 1.69)*	96.42 (± 1.17)*
<i>L. pentosus</i> Lp5	36.75 (± 3.82)	65.86 (± 0.21)	53.54 (± 1.76)*
<i>L. pentosus</i> Lp6	33.76 (± 1.13)	72.46 (± 3.96)	83.05 (± 1.95)*
<i>L. pentosus</i> Lp7	17.60 (± 0.61)*	75.80 (± 7.10)	21.54 (± 1.88)*
<i>L. pentosus</i> Lp8	36.82 (± 2.64)	63.51 (± 8.13)*	47.86 (± 0.54)*
<i>L. pentosus</i> LPG1	36.79 (± 6.71)	36.28 (± 0.57)*	52.21 (± 0.65)*
<i>L. pentosus</i> Lp10	19.94 (± 0.54)*	23.72 (± 2.75)*	19.74 (± 2.08)*
<i>L. pentosus</i> Lp11	19.73 (± 2.78)*	60.32 (± 2.40)	39.23 (± 2.92)
<i>L. plantarum</i> Lpl12	21.35 (± 1.88)*	85.86 (± 8.74)	56.42 (± 2.48)*
<i>L. pentosus</i> Lp13	27.45 (± 1.77)	66.48 (± 2.77)*	58.12 (± 1.15)*
<i>L. pentosus</i> Lp14	29.82 (± 10.86)	64.60 (± 0.61)	38.64 (± 1.33)
<i>L. plantarum</i> Lpl15	28.56 (± 0.22)	90.44 (± 3.12)	79.52 (± 0.92)*
<i>L. plantarum</i> Lpl16	48.46 (± 3.06)*	97.40 (± 1.13)*	96.29 (± 1.05)*
<i>L. casei</i> Shirota	36.19 (± 0.64)	78.95 (± 5.95)	35.36 (± 1.33)
<i>L. rhamnosus</i> GG	35.85 (± 2.50)	73.06 (± 0.87)	38.77 (± 2.61)

Figure legends

Figure 1. Percentage of adhesion of the *Lactobacillus* strains isolated from table olive biofilms to HT-29-MTX cells. Results are expressed as mean from two independent experiments performed by triplicate. The asterisk indicates significant differences ($P < 0.05$) as compared to the probiotic strain *L. casei Shirota*.

Figure 2. Effect of live and UV-inactivated (20 min) *Lactobacillus* strains isolated from table olive biofilms on interleukin 8 (IL-8) production by HT-29 cells stimulated with TNF- α . Results are expressed as a percentage of IL-8 production in the presence of IL-8 alone (co-culture HT-29 cells without bacteria). The asterisk indicates significant differences as compared to the PBS control group ($P < 0.05$).

Figure 3. Effect of the *Lactobacillus* strains isolated from table olive biofilms on the production of the pro-inflammatory interleukin IL-6 and the anti-inflammatory interleukin IL-10 by RAW 264.7 cells. The asterisk indicates significant differences in the stimulation of IL-6 with respect to the probiotic strain *L. casei Shirota*. The guide indicates significant differences ($P < 0.05$) in IL-10 stimulation.

Figure 4. Effect of the *Lactobacillus* strains isolated from table olive biofilms on the viability of HT-29 cancer cells after 24 and 48 h incubation. Data are expressed as mean viability (%). Asterisks denote statistically significant differences ($P < 0.05$) as compared to the probiotic strain *L. casei Shirota*.

Figure 5. Effects of *L. pentosus LPG1* isolated from the table olive epidermis in a dinitro-benzene sulfonic acid (DNBS) chronic colitis model. Body weight variation of mice, gut permeability, macroscopic score and tissue damage. Results are presented as means $\pm$ SEM. The asterisk indicates significant differences as compared to the DNBS group ($P < 0.05$).

Figure 6. Colonic interleukin (IL) levels in a dinitro-benzene sulfonic acid (DNBS) colitis model. Control non-inflamed group (PBS), control inflamed group (DNBS), and *L. pentosus* LPG1 group (DNBS-LPG1). Results are presented as means $\pm$ SEM. The asterisk indicates significant differences as compared to the DNBS group ($P < 0.05$).

HT-29