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Evaluating the Probiotic Potential of *Lactobacillus plantarum* Strains from Algerian Infant Feces: Towards the Design of Probiotic Starter Cultures Tailored for Developing Countries

Chahira Gheziel[1] · Pasquale Russo[2] · Mattia Pia Arena[2] · Giuseppe Spano[2] · Hadda-Imene Ouzari[3] · Omar Kheroua[1] · Djamel Saidi[1] · Daniela Fiocco[4] · Hanane Kaddouri[1] · Vittorio Capozzi[2]

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Abstract

Lactobacilli naturally present in the neonatal gut are believed to be beneficial for the human hosts and are investigated as potential probiotics. In this study, we aimed to characterize six *Lactobacillus plantarum* strains derived from the feces of a breast-fed infant, for the development of new probiotic cultures. Our attention was focused on *L. plantarum* in reason of the presence, within such species, of both pro-technological and probiotic strains, i.e., a combination of particular interest to design tailored probiotic starter cultures for developing countries. The bacterial isolates exhibiting lactobacilli-like phenotypic characteristics were identified as members of the *L. plantarum* group by 16S rRNA gene sequencing, and their diversity was evaluated by randomly amplified polymorphic DNA (RAPD) PCR patterns. The selected strains were screened for probiotic potential through in vitro tests. Firstly, bacterial survival was evaluated in an in vitro system simulating the human oro-gastrointestinal tract, using also milk as a carrier matrix. Besides, physiological traits such as antibiotic susceptibility, antimicrobial activity against selected enteric pathogens, and adhesion to abiotic surfaces and to gastric mucin were studied. Considering the resistance to simulated gastrointestinal digestion and the results from the biofilm and mucin adhesion tests, a strain-denominated *L. plantarum* LSC3 was selected for further evaluation of in vitro adhesion ability to intestinal mucosa and immunomodulatory activities. *L. plantarum* LSC3 was able to adhere efficiently to human enterocyte-like cells (Caco-2 cells), and decreased IL-8 transcription while increasing IL-10 mRNA level, as revealed by transcriptional analysis on LPS-stimulated human (THP-1) macrophages. Our results highlight that *L. plantarum* LSC3 fulfills major in vitro probiotic criteria as well as interesting immunostimulatory properties, and thus may be a promising candidate for further in vivo studies aiming at the development of novel probiotic starter cultures.

Keywords *Lactobacillus plantarum* · Probiotic · Infant feces isolates · Developing country

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✉ Daniela Fiocco
daniela.fiocco@unifg.it

[1] Laboratory of Physiology of the Nutrition and Food Security, Department of Biology, Faculty of Natural and Life Sciences, University of Oran, 1 Ahmed Ben Bella, 31000 Oran, Algeria

[2] Department of Agriculture, Food and Environment Sciences, University of Foggia, Via Napoli 25, 71122 Foggia, Italy

[3] Laboratoire de Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Université Tunis El Manar, 2092 Tunis, Tunisie

[4] Department of Clinical and Experimental Medicine, University of Foggia, Viale Pinto 1, 71122 Foggia, Italy

Introduction

Within lactobacilli, *Lactobacillus plantarum* is an important and versatile species of lactic acid bacteria (LAB) that is isolated from a variety of food- and nonfood-related environmental niches, such as fermented vegetables and the human gastrointestinal tract (GIT) [1]. The gut microbiota of healthy infants is normally colonized by *L. plantarum* strains that are delivered by breast-fed milk, which is an essential source of LAB susceptible to colonizing the gut mucosa, thereby interacting with the host immune system [2, 3]. The selection of LAB from fecal origin, as potential probiotic starter cultures for a range of bio-medical and technological applications, is considered a promising approach, since such bacterial group boasts a long-time association and adaptation to humans [4, 5]. Overall, given its safety attribute, its probiotic and

functional features, and its use as microbial food culture in different matrices [6–8], at present, *L. plantarum* represents an attractive biological agent with tremendous biotechnological potential [9]. As previously observed for other bacteria, the probiotic properties, including the ability to tolerate gut stress, seem to vary among the different strains of *L. plantarum*, due to genetic/phenotypic variations within the species [10, 11].

In order to screen and select microbial strains with probiotic abilities, FAO and WHO have established some basic criteria, such as the examination of tolerance to the oro-gastrointestinal (OGI) transit, production of antimicrobial substances and antibiotic susceptibility, adherence to human intestinal mucosa, and desired immunomodulation activity [12]. To evaluate probiotic survival and efficacy during the OGI transit, several in vitro assays, simulating the different parts of the human OGI tract, have been developed [13]. In such systems, candidate probiotics are often tested using different food preparations as delivery matrices, in order both to mimic probiotic food ingestion and to protect bacteria from the harsh gastrointestinal (GI) conditions, thus maximizing the number of live bacterial cells that can reach the target organ [14–16]. A good adhesion to the gut mucosa is another important feature, as it enhances probiotic intestinal colonization, while preventing enteropathogen attachment [17]. The adhesion capability of potential probiotics is usually assayed in vitro, e.g., using plate-immobilized mucus components and/or cultures of intestinal cell lines of human origin, such as Caco-2 cells, which were originally isolated from a human colon adenocarcinoma [18]. Some recent literature has demonstrated that dietary probiotics, in association with the intestinal microbiota, may induce immune and inflammatory responses in the intestine, e.g., by influencing the production of cytokines [19, 20]. Indeed, preliminary studies on potential probiotic microbes typically include the evaluation of their immune-modulatory effects during the interaction with host immune cells, for instance, taking advantage of feasible in vitro models, such as that provided by THP-1-derived human macrophages [21].

Recently, in the light of the increasing differences detected into the gut microbiome composition of different people from diverse geographical regions (as a function of the different diets and lifestyles), locally sourced probiotics have been proposed as new resources to improve the health of populations in developing countries [22, 23]. The characterization of probiotic properties of *L. plantarum* strains isolated from Algerian healthy infants remains very limited [24]. Yet, the presence within this species of both pro-technological and probiotic strains is of particular interest to design low-cost, probiotic starter cultures for developing countries, considering the opportunity to use the same strain as food starter cultures and as probiotic starter cultures.

In this study, six strains of the *L. plantarum* group, isolated from the feces of a healthy breast-fed Algerian infant, were identified by 16S rDNA sequencing and strain divergence among the isolates was evaluated by RAPD analysis. The isolated strains were tested in vitro for survival to conditions of the human gastrointestinal tract, antibiotic susceptibility, antipathogenic properties, and adherence to mucin and to abiotic surface. Based on findings from such preliminary analysis, one of the best-performing strains was chosen to be further studied in vitro for adhesion to Caco-2 monolayers and immune modulation of human macrophages.

Materials and Methods

Isolation Procedure, Bacterial Strains, and Growth Conditions

The lactobacilli used in this work were *L. plantarum* WCFS1 [25] and six *L. plantarum* strains isolated from human feces. Fecal samples were collected from a healthy infant feeding exclusively on breast-milk and aged less than 1 year, from the region of Oran, Algeria. Serially diluted fecal samples were streaked on de Man, Rogosa, and Sharpe (MRS, Liofilchem, Italy) agar (pH 6.2) supplemented with cysteine (0.05% [*w*/*v*], Sigma-Aldrich, St. Louis, MO) for the selective isolation of lactobacilli, and then incubated anaerobically in jars at 37 °C for 48 h. Isolated colonies were arbitrarily chosen and transferred to MRS agar plates incubated at 37 °C. The isolates were phenotypically examined by Gram staining, catalase production, and colony morphology. Based on such preliminary characterization, six isolates, putatively belonging to the *Lactobacillus* genus, were selected and propagated in MRS (pH 6.2) at 37 °C, while *L. plantarum* WCFS1 was grown at 30 °C in MRS.

The bacterial pathogens used in this study were *Listeria monocytogenes* CECT 4031, *Escherichia coli* O157:H7 CECT 4267, two methicillin-resistant strains of *Staphylococcus aureus*, i.e., strains MRSA1220 and MRSA1209, and two methicillin-susceptible strains of *S. aureus*, i.e., MSSA1208 and MSSA1070. *L. monocytogenes* and *E. coli* were grown in tryptone soya broth (TSB, Oxoid, Basingstoke, UK) at 37 °C. *S. aureus* strains were propagated in Brain Heart Infusion broth (BHI, Oxoid) at 37 °C.

Molecular Characterization

The genomic DNA of the isolated lactobacilli strains was purified using a genomic DNA extraction kit (Qiagen, Manchester, UK), following the manufacturer's instructions. The genomic DNA was used as a template to amplify and sequence 16S rDNA, resulting in species identification. Moreover, RAPD-PCR analysis was performed as previously described [26] in order to determine strain diversity.

BioNumerics software 7.6 version (Applied Maths, Belgium) was used for cluster analysis of RAPD-PCR.

Tolerance to a Simulated Oro-gastrointestinal Transit

Lactobacilli were grown until mid-exponential phase (OD_{600nm} 1), then centrifuged (2000×*g* for 10 min), and re-suspended (3×10^8 CFU mL^{-1}) into two different carrier matrices: (i) saline solution (NaCl 8.5 g L^{-1}) and (ii) commercial skim milk (0% fat milk, Candia, Oran Algeria). Commercial milk was subjected to thermal pre-treatment (90 °C for 10 min), and the absence of any contamination was checked by plating on MRS agar plates. The oro-gastrointestinal transit (OGT) tolerance assay was performed according to Bove et al. [11] with some modifications. Briefly, bacterial suspensions were incubated at 37 °C for 5 min, with 150 mg L^{-1} lysozyme at pH 6.0. Then, pepsin (3 g L^{-1}) was added and pH downshifted to a value of 2.0, in order to simulate gastric stress (30 min incubation at 37 °C). Finally, the intestinal stress was simulated by adjusting the pH to 6.5 and by adding bile salts (3 g L^{-1}) and pancreatin (1 g L^{-1}) (all from Sigma-Aldrich), over a 60-min incubation at 37 °C. The relative viability was calculated by comparing the colony-forming units (CFU) from control and OGT-stressed bacterial samples.

Antipathogenic Activity, Antibiotic Resistance, and Biofilm Formation Assays

The growth inhibitory activity of the lactobacilli strains was evaluated using the agar spot assay [27]. Briefly, 5 μL of each of the lactobacilli overnight cultures was spotted on MRS agar and grown for 24 h at 37 °C. Then, 150 μL from overnight cultures, of each pathogenic strain, was mixed with TSB soft agar (0.6% agar, *w*/*v*) and poured over MRS agar plates containing the developed lactobacilli. After 24-h incubation, the inhibition zones radius was measured.

The antibiotic susceptibility of lactobacilli strains was determined by the disc diffusion assay according to Tulini et al. [28]. Antibiotics, i.e., ampicillin (10 μg), penicillin G (10 units), oxacilline (1 μg), amikacin (30 μg), gentamicin (30 μg), tetracycline (25 μg), chloramphenicol (25 μg), and clindamycin (2 μg), were placed on the surface of plates and incubated at 37 °C for 24 h. The inhibition zone diameters were measured, and susceptibility was expressed in terms of resistant (R) and susceptible (S).

The production of biofilms on glass and plastic surface, i.e., glass tubes and 96-well polystyrene microtiter plates, was assayed as previously described [29]. Lactobacilli were grown in MRS broth for 48 h at 37 °C. Biofilm rings were washed twice with distilled water, and then stained with crystal violet (0.5%, *w*/*v*). The biofilms were solubilized with acetic acid (30%, *v*/*v*), and optical density (OD) was measured at 570 nm.

In Vitro Adhesion to Intestinal Mucin

Porcine gastric mucin (type III, Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) at pH 7.0, and bound to 96-well PolySorp microplates (Nunc Immuno plates, Nunc, Roskilde, Denmark), according to a previously described technique [30], with minor modifications. Briefly, plates were covered with 100 μL of mucin (0.2 mg mL^{-1}), and incubated at 4 °C overnight. After PBS washing, wells were blocked with 200 μL of PBS-2% bovine serum albumin (BSA) for 2 h, at room temperature. Then, wells were washed twice with PBS. Bacterial suspensions (100 μL; 3×10^8 CFU mL^{-1}) of each strain were added, and plates were incubated for 2 h at 37 °C. After removing nonadhered cells by PBS washes, well-adherent bacteria were stained with 0.1% crystal violet (95 μL per well) for 45 min. After washing, the plates were dried and the stain was released with 30% (*v*/*v*) acetic acid (100 μL per well), and the OD_{520nm} was determined in an EON Microplate Spectrophotometer (Biotek, VT, USA). PBS alone was included in all experiments as blank wells. The averages of six absorbance values were calculated. Strains were classified as strongly adherent ($OD_{520nm} > 0.3$), weakly adherent ($0.1 < OD_{520nm} < 0.3$), or nonadherent ($OD_{520nm} < 0.1$).

Caco-2 Adhesion Assay

Caco-2 cells were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin, at 37 °C with 5% CO_2. Caco-2 cells were seeded in 96-well cell culture plates (2×10^5 cells per well) and cultivated for 3 weeks in order to obtain steady monolayers. The medium was changed every 2 days. Microbial adhesion assays were performed as previously described [31]. In brief, 24 h prior to the adhesion assay, the complete growth medium was replaced with absolute DMEM. *L. plantarum* LSC3 was grown to mid-exponential phase (OD_{600nm} 1), centrifuged, resuspended in DMEM, and then incubated with Caco-2 cells (0.1 mL per well) for 1 h, at 37 °C, with 5% CO_2 (ratio 1000:1, bacteria to Caco-2 cells). Wells were PBS-washed, then Caco-2 cells and adherent bacteria were detached by addition of trypsin and resuspended in PBS. The number of cell-attached bacteria was determined by plating serial dilutions onto MRS agar. CFU from washed wells (cell-bound bacteria only) were compared with those from control unwashed wells (unbound and bound bacteria), in order to calculate the adhesion percentages. The adhesion capacity of the investigated strain was compared to that of the recognized probiotic strain *L. plantarum* WCFS1. Adhesion assays were conducted in three independent experiments, with duplicate determinations.

Stimulation of THP-1 Macrophages

Human THP-1 cells were grown in RPMI (Gibco, Carlsbad, CA); supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin; and incubated at 37 °C in humidified atmosphere with 5% CO_2. THP-1 cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA), as described elsewhere [21]. Macrophages were incubated with 100 ng mL^{-1} lipopolysaccharides (LPS) from *E. coli* O127:B8 (Sigma-Aldrich), with or without addition of *L. plantarum* LSC3 cells (ratio of 1000:1, bacteria to macrophages) from mid-exponential cultures. Negative and positive controls were represented by untreated macrophages and macrophages treated with LPS only, respectively. After 1 and 4 h incubation, total RNA was extracted from macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). The transcriptional analysis of immune-related genes, i.e., coding for interleukins IL-8 and IL-10, was performed by quantitative RT-PCR (qRT-PCR) in a real-time instrument (ABI 7300, Applied Biosystems, Foster City, CA, USA), as previously described [32].

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Significant differences were assessed using one-way analysis of variance (ANOVA) and Student's *t* test, with $p < 0.05$ as the minimal level of significance. StatPlus Pro software (Version 5.9.8; Analysis software, USA) was used for analyses.

Gene Accession Number

L. plantarum 16S rDNA sequences were deposited in the GenBank data library under accession numbers MG717919 (LSC3), MG717920 (LSC4), MG717921 (LSC11), MG717922 (LSC22), MG717923 (LSC2), and MG717924 (LSC).

Results

Molecular Identification of the Lactobacilli Strains

Fifteen bacterial isolates were obtained from the fecal samples of an Algerian healthy breast-fed infant. In order to select possible lactobacilli strains, a preliminary identification was performed by analyzing Gram staining, catalase production, and colony morphology. Such phenotypic characterization suggested that 12 of the isolated strains were LAB, all being Gram-positive, catalase-negative, and with rod or spherical shape. Of these, only six isolates displayed a rod-shaped morphology, so their 16S rDNA gene was sequenced and BLAST analysis revealed a high similarity to *L. plantarum* sequenced genomes (99% similarity to GenBank sequences). 16S rDNA is a highly conserved genomic region whose sequencing cannot differentiate closely related species, as those within the so-called *L. plantarum* group, which comprises six species, i.e., *L. plantarum*, *L. pentosus*, *L. paraplantarum*, *L. fabifermentans*, *L. xiangfangensis*, and *L. mudanjiangensis* [33]. Therefore, the six isolates were identified as members of this group. For reasons of simplicity, they were referred to as *L. plantarum* and accordingly named as *L. plantarum* LSC3, *L. plantarum* LSC4, *L. plantarum* LSC2, *L. plantarum* LSC22, *L. plantarum* LSC11, and *L. plantarum* LSC. The six isolates assigned to the *L. plantarum* group were analyzed by RAPD-PCR in order to assess inter species diversity. Cluster analysis of RAPD-PCR profiles (Fig. S1) managed to differentiate the six isolates to at least five different strains, highlighting the genetic distances among the different biotypes and pointing to a high genetic similarity between strains LSC2 and LSC4.

Survival of *L. plantarum* Strains under Simulated Oro-gastrointestinal Transit

The tolerance of the six *L. plantarum* strains to progressive oral, gastric, and intestinal stress was investigated using an in vitro model (i.e., OGT assay) that mimics the human digestive process [11]. The survival of the *L. plantarum* strains was evaluated using either saline solution or milk as carrier matrices (Fig. 1). The data obtained from the first step of simulated GI conditions indicated that the bacterial viability was not affected by oral stress in either matrices. Conversely, the survival was significantly reduced under gastric conditions (pH 2.0). Indeed, under acid conditions, viability decreased by about 3 log units, in the presence of saline solution, with no significant differences between the tested strains, whereas, in the presence of milk, at the same OGT step, a lower reduction of bacterial survival was observed in a range of 1–2 log units, and with significant differences between bacterial strains (Fig. 1). On the other hand, under intestinal stress, a tendency to retain viability was observed for all bacterial samples in both matrices. In saline solution, the percentage of survival after intestinal stress decreased by 2–3 log units, and there were no major differences between bacterial strains, whereas in milk, the survival percentage was reduced by 1–2 log units.

Overall, our results indicated that in the presence of saline solution, there was no significant inter-strain differences in the survival rate, through the entire digestive process. By contrast, the viability of strains LSC2, LSC3, and LSC22 was significantly improved by the presence of milk, compared to the other strains ($p < 0.05$), throughout the OGT.

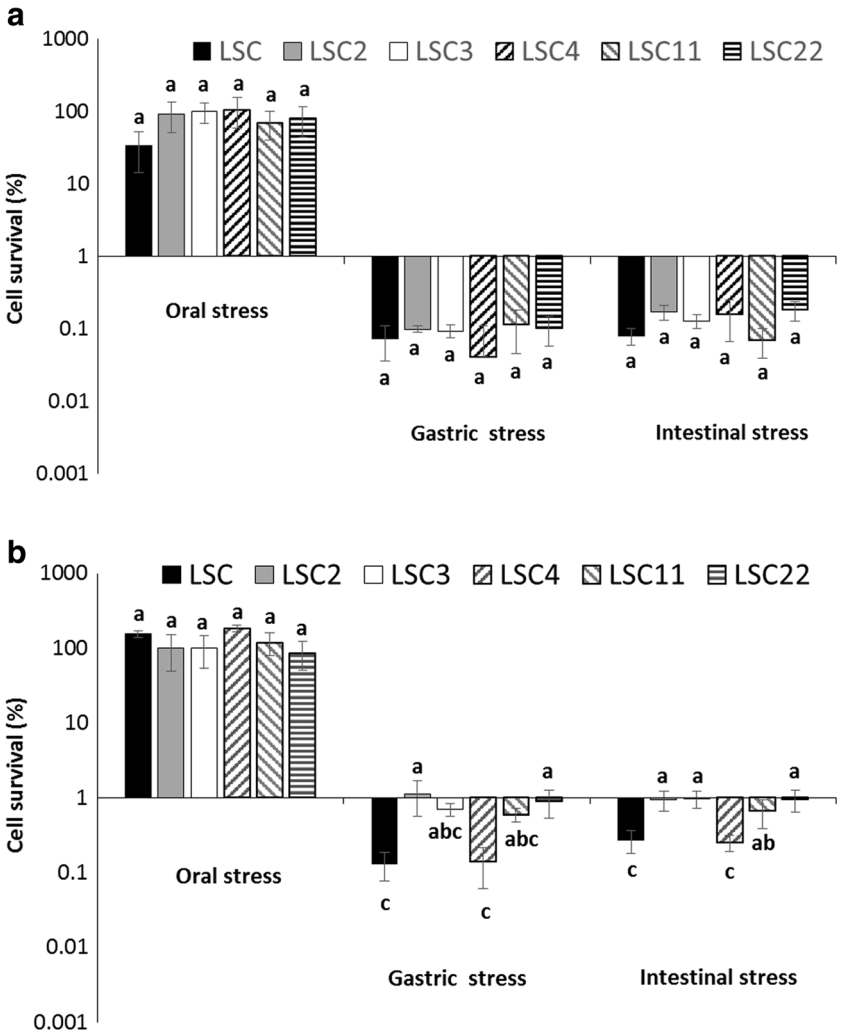

Fig. 1 Tolerance of the investigated *L. plantarum* strains to an in vitro simulated oro-gastrointestinal transit (OGT), using saline (**a**) or milk (**b**) as vehicle matrices. The bacterial survival is expressed as a percentage relative to untreated control samples and is reported after each stage of the simulated OGT (see the experimental procedure for details). The viability was determined by CFU count analysis. Values represent mean ± SD of two independent experiments. Different superscript letters indicate significant differences ($p < 0.05$) in survival rate between the *L. plantarum* strains, according to ANOVA test. *L. plantarum* strains LSC3, LSC4, LSC2, LSC22, LSC11, and LSC

Antipathogenic Activity, Antibiotic Resistance, and Biofilm Formation

The antibacterial activity of the isolated *L. plantarum* strains was studied against common food-borne pathogens such as *L. monocytogenes*, enterohemorrhagic *E. coli*, and *S. aureus*. Table 1 reports the antibacterial activity as a halo of growth inhibition produced on agar plates against these pathogens. No inhibition against *L. monocytogenes* CECT 4031 was observed, whereas all *L. plantarum* strains showed a strong (zone of inhibition > 6 mm) antibacterial activity against *E. coli* O157:H7 CECT 4267 and against all the tested *S. aureus* strains.

The *L. plantarum* isolates were also assayed for antibiotic resistance (data not shown). All the strains were sensitive to all the tested antibiotics, except to penicillin G and oxacilline. Moreover, the isolates were investigated for adhesion and biofilm formation on two types of abiotic surfaces, i.e., glass and plastic. Strains LSC3 and LSC22 were the best biofilm producers ($OD_{590} > 0.2$) on plastic and glass surfaces, respectively (Table 1). *L. plantarum* LSC was a mild biofilm producer, whereas strains LSC4, LSC11, and LSC2 showed no ability to adhere on either abiotic surfaces ($OD_{590} < 0.1$).

In Vitro Adhesion to Intestinal Mucin

Immobilized porcine mucin was bound by all the six tested strains. However, inter-strain differences in the mucin-binding affinity were apparent (Fig. 2). Adhesion to mucin was significantly higher in LSC22 and LSC3 ($p < 0.05$) than in the other strains. No significant differences in mucin binding were observed between strains LSC, LSC2, and LSC4. The adhesion level of LSC11 was the lowest, though not significantly different from that of LSC2 and LSC4 (Fig. 2). Based on these results, by using the criteria given in the "Materials and Methods" section, LSC22 and LSC3 were classified as strongly adherent, LSC, LSC4, and LSC2 as weakly adherent, and LSC11 as a nonadherent strain.

Table 1 Antimicrobial and biofilm-forming activities of the examined *L. plantarum* strains

L. plantarum strain	Antimicrobial activity[a]			Biofilm formation[b]	
	L. monocytogenes	*E. coli* O157:H7	*S. aureus*[c]	Glass	Plastic
LSC3	–	+++	+++	+	++
LSC4	–	+++	+++	–	–
LSC11	–	+++	+++	–	–
LSC22	–	+++	+++	++	–
LSC2	–	+++	+++	–	–
LSC	–	+++	+++	+	+

− inhibition zone ≤ 1 mm, + inhibition zone ≤ 2 mm, ++ 2 mm ≤ inhibition zone ≤ 5 mm, +++ inhibition zone ≥ 6 mm

[a] The size of clear zonal inhibition of the growth was measured

[b] Optical density measured at 590 nm to quantify the biofilm formation on abiotic surface (glass and plastic). The examined *L. plantarum* were classified as follows: −, nonbiofilm producer ($OD_{590} < 0.1$); +, mild biofilm producer ($0.1 < OD_{590} < 0.2$); and ++, strong biofilm producer ($OD_{590} > 0.2$)

[c] The results of inhibition on the four tested *S. aureus* strains (i.e., MRSA1220, MRSA1209, MSSA1208, and MSSA1070) were the same, so they have been presented in a single column

Adhesion to Enterocyte-like Cells by *L. plantarum* LSC3

The preliminary evaluation of some of the probiotic properties of the fecal isolates revealed *L. plantarum* LSC3 as one of the best-performing strains, indeed exhibiting a relevant tolerance to the OGI transit in the presence of milk, the strongest ability to produce biofilms on plastic surface, and a high level of adhesion to immobilized mucin. Therefore, this strain was selected to be further investigated for probiotic abilities, including its potential to bind to the intestinal mucosal surfaces. The adhesion capacities of the *L. plantarum* LSC3 strain was evaluated in vitro on Caco-2 cell monolayers, and *L. plantarum* WCFS1 was used as a positive control as it was previously shown to exhibit good adherence compared to other commercial probiotic lactobacilli [34]. The adherence was expressed as percentage, and values of 7.4 ± 1.5 and 11.2 ± 0.5% were observed for *L. plantarum* LSC3 and *L. plantarum* WCFS1, respectively (Fig. 3).

Immunomodulatory Effect of *L. plantarum* LSC3

L. plantarum LSC3 was evaluated in vitro for its capacity to influence the production of two cytokines with pro- and anti-inflammatory characters, i.e., IL-8 and IL-10, respectively. The transcriptional level of these cytokine-encoding genes was investigated by qRT-PCR, in LPS-stimulated human THP-1 macrophages. As expected, challenging THP-1 macrophages with *E. coli* LPS led to a significant upregulation of the pro-inflammatory IL-8 gene, relative to non-LPS-stimulated cells (Fig. 4). The IL-8 gene transcriptional level was significantly enhanced after 4 h of LPS treatment (19.7 ± 0.1 relative gene expression). The upregulation was time-dependent as the transcriptional level was found to increase

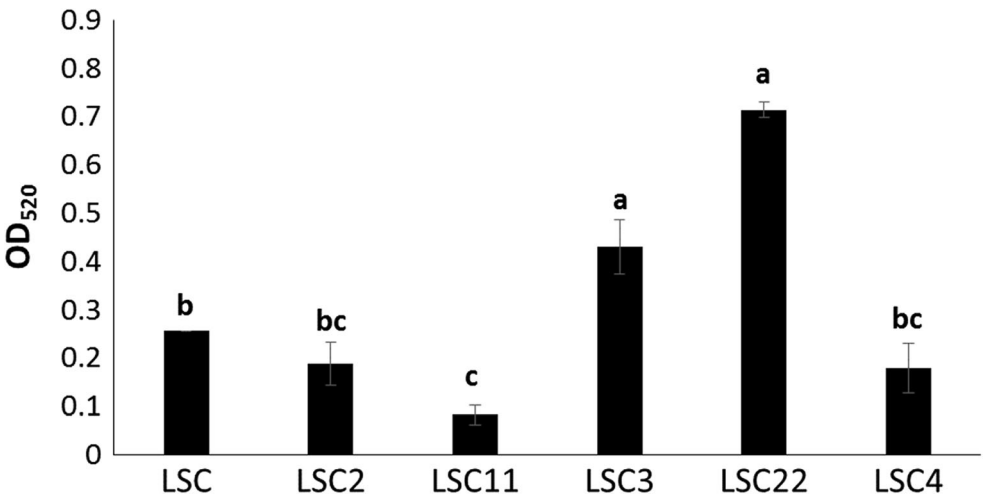

Fig. 2 Ability of the tested lactobacilli to adhere to immobilized porcine mucin. The data are expressed as optical density measured at 520 nm. Values represent mean ± SD of six absorbance measurements. Different superscript letters indicate significant differences ($p < 0.05$) in mucin adhesion between the different *L. plantarum* strains, according to ANOVA test. *L. plantarum* strains LSC3, LSC4, LSC2, LSC22, LSC11, and LSC

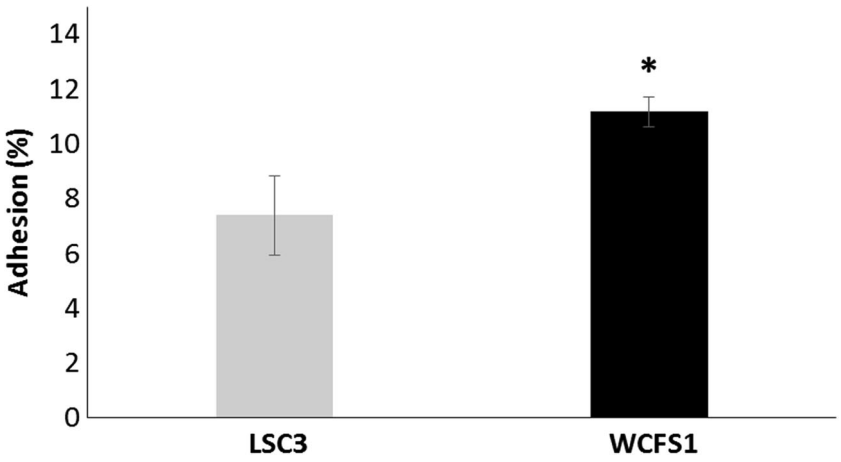

Fig. 3 Bacterial adhesion to Caco-2 cells. The adhesion ability of *L. plantarum* LSC3 and *L. plantarum* WCFS1 was expressed as the percentage of adhesion. Values represent mean ± SD of three different experiments. Statistical analyses were carried out by ANOVA test, and significant differences are relative to the control strain (*$p < 0.05$)

from 1 to 4 h, following LPS simulation. Conversely, the relative expression of the gene encoding the anti-inflammatory IL-10 cytokine decreased to 0.2 and 0.6, after 1 and 4 h, respectively (Fig. 4). On the other hand, when THP-1 macrophages were co-incubated with LPS and *L. plantarum* LSC3 cells, the transcriptional upregulation of the IL-8 gene was significantly reduced to the extent of 5.9 ± 1.0, as compared with the positive control ($p < 0.05$), after 4 h of incubation (Fig. 4). Moreover, under simultaneous treatment with LPS and *L. plantarum* LSC3, the relative expression of IL-10 was 0.3 ± 0.1 and 1.0 ± 0.1, after 1 and 4 h, respectively, therefore indicating a higher, though not significantly different, expression compared to the treatment with LPS only.

Discussion

In recent years, the gastrointestinal flora has been receiving attention as a potential source of helpful microbial strains, including lactobacilli liable to be isolated and selected for their probiotic and pro-technological characteristics, thereby providing starter cultures for the production of health-promoting

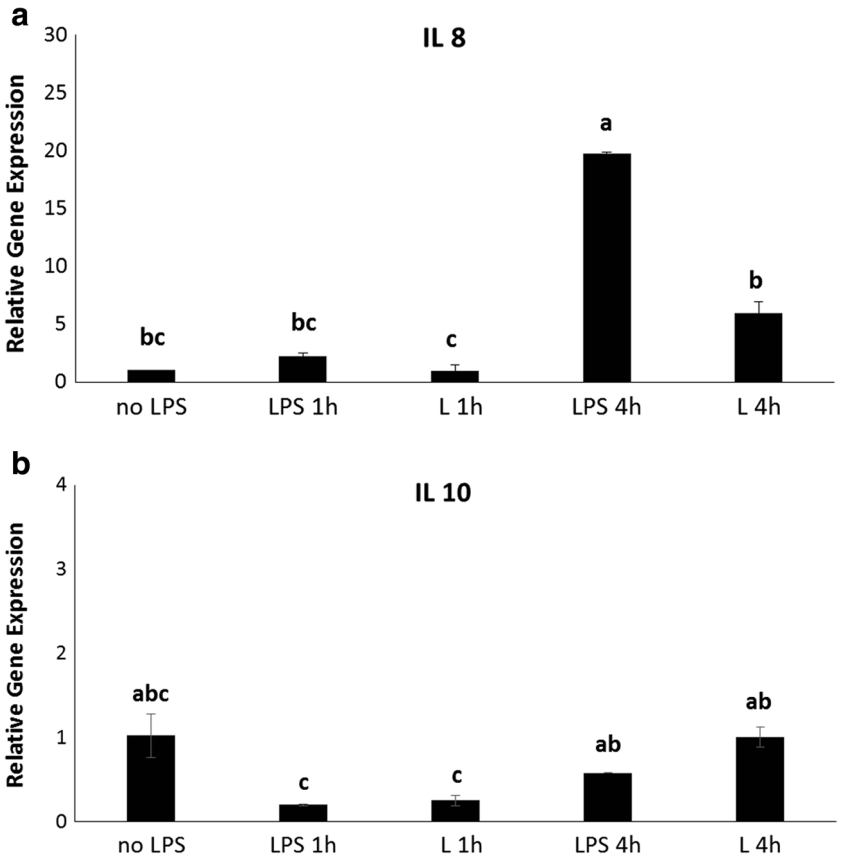

Fig. 4 Relative transcriptional level of IL-8 and IL-10 genes in LPS-stimulated THP-1 macrophages with or without co-incubation with *L. plantarum* LSC3. Relative gene expression values were obtained by qRT-PCR after 1- and 4-h treatments. Values represent the mean ± SD of at least two different experiments. Statistical analyses were carried out by ANOVA test. Superscripts of different letters indicate significant differences ($p < 0.05$). Unstimulated THP-1 cells, no LPS; LPS-stimulated THP-1 cells, LPS (positive control); LPS-stimulated THP-1 cells co-incubated with *L. plantarum* LSC3, L

functional food. An increasing awareness of the different composition of the gut microbiome in people from diverse world regions has been prompting the screening of locally sourced probiotics as opportunities to improve the health of populations, especially in developing countries, where the cost-effectiveness of any health-care strategy is pivotal [23]. With this regard, in this work, we focused our attention on *L. plantarum* as a species with a dual significance in food biotechnology, being used in the implementation of both food and probiotic starter cultures. Indeed, such a combination is of particular interest to conceive low-cost, tailored, probiotic starter cultures for developing countries.

Among the members of the gut microbiota, bifidobacteria and lactobacilli are acknowledged as beneficial to the host, and they dominate the intestinal microflora of healthy infants feeding exclusively on breast milk [35]. In the present study, six lactobacilli, isolated from the feces of a breast-fed infant and identified as members of the *L. plantarum* group, were analyzed for genetic diversity and evaluated in vitro for their potential probiotic properties.

In order to benefit the human host, probiotics should withstand the unfavorable conditions of the gastrointestinal tract and reach the intestine in a viable status [11]. Therefore, as recommended by the FAO-WHO [12], the capacity to resist to gastrointestinal stress is an important criterion to select potential probiotic strains. The incorporation of probiotics into a food matrix can protect microbial cells from GI challenges, thus enhancing their survival and colonization ability. In this regard, milk exhibits good buffering properties and is often used as a carrier matrix [36]. In the present work, the isolated *L. plantarum* strains were assayed for survival to an in vitro OGI system, using either saline solution or milk as a carrier matrix. In agreement with previous reports [13, 37], the viability of the tested *L. plantarum* strains, in either matrices, was not significantly affected by exposure to oral stress, yet it dropped drastically under gastric conditions. Similar results have been previously reported [34, 38], and highlight the impact of low pH on the survival of lactobacilli, indicating an extremely acidic environment as one of the most detrimental factors affecting the growth and viability of probiotics. Overall, the recovery in viability, observed under intestinal conditions, suggests that the analyzed *L. plantarum* strains could reach the intestine with significant concentrations of live cells, even after exposure to acid challenge. Our findings confirm that milk can protect bacterial cells, as it contributed to a significant increase in the survival rate of the tested *L. plantarum* strains throughout the gastrointestinal transit, as compared with saline solution. Furthermore, the presence of milk highlighted the differential survival aptitude of the investigated strains, resulting in a substantially improved performance of specific strains, i.e., LSC2, LSC3, and LSC22. As previously indicated [39], milk and/or its components can enhance the tolerance of probiotics to simulated GI transit. Based on our data, such protective effect could be strain-specific.

The application of LAB in the field of bio-preservation is mainly due to their aptitude to release antimicrobial molecules, such as bacteriocines and organic acids [40]. The antimicrobial activity is also desirable in probiotics, as it can confer antagonism against potentially harmful microbes, thus contributing to prevention of their colonization on the host mucosa. All the investigated *L. plantarum* isolates exhibited antagonistic activity against pathogenic strains of *E. coli* and *S. aureus*, and, according to a previously proposed classification [41], can be defined as very strong inhibitors of pathogen growth. In order to select a new isolate as a safe probiotic for human consumption, the evaluation of its antibiotic susceptibility is pivotal. In this regard, all the investigated *L. plantarum* strains were found to be resistant to penicillin G and oxacilline, which agrees with previous studies reporting a similar antibiotic resistance in LAB [42, 43]. On the other hand, the tested strains exhibited sensitivity to a wide range of antibiotics (i.e., chloramphenicol, ampicillin, clindamycin, amikacin, gentamicin, and tetracycline), which are commonly used to treat bacterial infections. Similar patterns of antibiotic sensitivity were reported by [28, 44], whereas other studies reported the occurrence of chloramphenicol and tetracycline resistance in some strains of *L. plantarum* and *L. pentosus* [5, 45], inferring that antibiotic susceptibility is quite variable and depends upon specific LAB strains.

Another desirable characteristic of probiotics is the ability to form biofilms, i.e., complex, multi-strain communities tightly associated to a surface. Indeed, when such a close association is established on the host mucosal surfaces, this can promote the colonization and long-term permanence of beneficial microbes in the intestinal habitat [46, 47]. For this reason, the screening of potential probiotic strains usually includes the evaluation of their biofilm-forming capacity. Since a mucus layer protects the host mucosae, this is the first site of host-microbe interaction. Consequently, possessing mucin-binding abilities improves the gut colonization by probiotic lactobacilli, while preventing enteric infections by competitive exclusion of pathogenic microbes [48]. Here, analyses of the adhesion capacity on abiotic surfaces and on porcine mucin revealed a certain variability in the biofilm-forming aptitude, with some of the tested *L. plantarum* strains exhibiting good adhesion potential, especially on mucin. In agreement with a previous work, which found a remarkable variability in the mucus-binding phenotype exhibited by *L. reuteri* [49], our results confirm that the binding properties of lactobacilli to host intestinal mucus are strain-specific.

Based on the preliminary evaluation of the probiotic attributes of the fecal isolates, strain LSC3 was identified as one of the best-performing strains, and thus it was selected to be further examined for probiotic features, including its potential adhesion to intestinal cells and immunomodulatory activities.

The adhesion level of *L. plantarum* LSC3 to Caco-2 cell monolayers was at least identical or higher than other probiotic lactobacilli previously tested [50]. This is a promising finding, since a good level of adhesion to human enterocytes may reinforce the intestinal barrier and prolong the permanence of probiotics in the intestine, thereby increasing the time span of their health benefits [51].

Imbalances in the composition of the gut microbiota and an overproduction of pro-inflammatory cytokines can underline chronic intestinal inflammations [52]. Because probiotics may modulate the intestinal microbiota as well as the production of cytokines, they can serve as therapeutic adjuvants in the treatment of inflammatory diseases [53, 54]. In our study, *L. plantarum* strain LSC3 was found to counteract in vitro the effect of a pro-inflammatory stimulation in human macrophages, by inducing a decreased upregulation of IL-8 and by attenuating the downregulation of IL-10, upon LPS stimulation. Our results are consistent with several in vitro studies, which found a significantly decreased secretion of IL-8, upon treating host cells with different probiotic strains, suggesting an immunosuppressing/anti-inflammatory ability [55, 56]. The observed upregulation of IL-10 is consistent with earlier experiments [21, 32], which used analogous in vitro models and diverse lactobacilli, including *L. plantarum* strains. However, several other studies reported that *Lactobacillus* species had no such effect on IL-10 production [57, 58], thereby entailing that probiotic immunomodulation is strain-specific. The increased IL-10 expression, which we observed in human macrophages upon exposure to *L. plantarum* LSC3, is an intriguing effect. Indeed, probiotic bacteria with such properties might contribute to a counter-balance in the production of intestinal pro-inflammatory cytokine and maintain gut homeostasis, thus protecting the host from inflammation-associated diseases [59]. Our findings are in accordance with previous studies documenting how probiotic lactobacilli contribute to the intestinal immune homeostasis by influencing the expression of pro-inflammatory genes [60].

In summary, the in vitro evaluation of six *L. plantarum* strains, isolated from the fecal sample of a breast-fed infant, pointed to strain LSC3 as the most promising one. This strain survived to simulate the OGI transit, particularly when using milk as a carrier matrix; moreover, it showed high antagonistic activity against two potential food-borne pathogens, and sensitivity to most tested antibiotics. Besides, it adhered efficiently to mucin and enterocytes, and transcriptional analysis in immunostimulated macrophages highlighted its anti-inflammatory potential. Overall, *L. plantarum* LSC3 exhibits in vitro some major requirements and properties of an efficient probiotic. However, in vivo characterization is necessary and further functional properties, such as riboflavin-producing capacity and amylolytic and antioxidant activity, could be investigated, which might contribute to the application of this strain for the development of health-promoting products.

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This paper is dedicated to the memory of our friend and colleague Prof. Chekroun Abdallah.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Statement This article does not contain any studies with human or animal participants performed by any of the authors.

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