JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIMICROBIAL ACTIVITY OF LACTIPLANTIBACILLUS AND LEVILACTOBACILLUS STRAINS ISOLATED FROM FERMENTED OLIVE AND THEIR APPLICATION IN MILK AND PASTILLA LEAF BIOPRESERVATION

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https://doi.org/10.55251/jmbfs.6021

ARTICLE INFO

Received 2. 4. 2022
Revised 20. 1. 2023
Accepted 23. 1. 2023
Published xx.xx.201x

Regular article

OPEN ACCESS

ABSTRACT

Biological preservatives are increasingly in demand as an alternative to chemicals, because of consumer's health and environmental concerns. Lactic acid bacteria isolated from fermented olives, considered as extreme environment, may encompass significant antimicrobial activity against microorganisms involved in foodborne diseases and food spoilage. Three LAB strains (*Levilactobacillus brevis* S27, *Lactiplantibacillus pentosus* S42, and *Lactiplantibacillus plantarum* S62), isolated from fermented olive, were studied for their antimicrobial activity against pathogenic bacteria (*Listeria innocua* and *E. coli*) and yeast (*Rhodotorula glutinis*), involved in foodborne diseases and food spoilage. The microbial culture, cell-free supernatant (CFS) of all LAB strains showed inhibition zone (IZ) values ranges of 26.33-28.83 mm, 14.16-16.33 mm and 10-12.16 mm against *R. glutinis*, *L. innocua* and *E. coli*, respectively. The IZ values ranges obtained with NCFS (neutralized CFS at pH 7) against *R. glutinis* and *L. innocua* were respectively of 17.83-20.50 mm and 13.16-14.50 mm, whereas no inhibition was obtained against *E. coli*. Their bioactive molecules of proteinaceous nature showed that among the four peptide bands obtained, two bands of approximately 42 and 56 kDa are common for the three LAB strains (S27, S42 and S62). Two additional bands of 46 and 89kD are observed in S42 and S27, respectively. The minimum inhibitory concentration of 10-fold concentrated CFS (10xCFS) obtained was 5% against *E. coli* and 4.5% against *L. innocua* and *R. glutinis*. The 10xCFS of *Lb. plantarum* S62, selected as the most efficient strain against pathogens, demonstrated its effectiveness in decontamination of milk at concentrations of 0.45% (*v*/*v*) and 0.5% (*v*/*v*) against *L. innocua* and *E. coli*. The CFS allowed, at 0.5 mL/piece of 5 cm diameter, a total inhibition of *R. glutinis*, without affecting the color of Pastilla leaf. The CFS of *Lb. plantarum* S62 can be used as a bio-preservative against pathogenic bacteria (*L. innocua* and *E. coli*) in milk, and against *R. glutinis* in Pastilla leaf.

Keywords: *Lactiplantibacillus*, *Levilactobacillus*, antimicrobial, food, biopreservation

INTRODUCTION

Spoilage of food products by bacteria and fungi (*E coli* O157:H7, *Salmonella enterica serovars Typhimurium*, *Listeria monocytogenes* and *Penicillium sp.*) is a worldwide problem. They can cause extensive damage to foods (i.e., unpleasant smell, taste, or appearance), as well as the formation of harmful substances for consumer's health. They are considered as a serious health problem, particularly for young, elderly and immuno-compromised patients **(Chakchouk-Mtibaa *et al.*, 2014)**.

Pastilla leaf, a traditional food product in North Africa made of wheat flour, is widely used in meals and patisserie preparations. In addition, there is an increase in consumption of half-cooked wheat-based products (i.e., Pastilla leaf, Pizza, Tacos, Burger…), which are usually packed and stored in refrigerator before consumption. During storage, these food products are subject to development of pathogenic and spoilage microorganisms (e.g., *Bacillus cereus*, *Clostridium botulinum*, *E. coli*, *Salmonella* and *Staphylococcus aureus*), which can lead to serious food-borne illnesses to the consumer **(Sabillón and Bianchini, 2016)**. These pathogens are mostly controlled with chemical preservatives **(Militello *et al.*, 2011)**. However, natural preservatives are increasingly demanded by consumers as alternative to chemicals, due to their negative effects on human health and environment **(Carocho *et al.*, 2015)**.

Lactic acid bacteria (LAB) are gaining interest of consumer in food biopreservation, due to their GRAS (generally recognized as safe) status and their antimicrobial activity **(DjadouniI and KihalII, 2012)**. They are able to produce various antimicrobial metabolites, including organic acids, hydrogen peroxide, diacetyl, reuterin, carbon dioxide, and bacteriocins **(Dortu and Thonart, 2009)**. They have proven to be effective in preserving foods and enhancing the nutritional quality of a variety of fermented food products **(Gad *et al.*, 2016; Dinev *et al.*, 2018)**. The use of selected probiotic LAB strains and/or their metabolites may ensure the inhibition of pathogenic and spoilage microorganisms, and also improve the functional properties of food products.

The main objective of this work is to characterize the antimicrobial activity of some LAB strains (*Levilactobacillus brevis* S27, *Lactiplantibacillus pentosus* S42, and *Lactiplantibacillus plantarum* S62) isolated from traditional fermented olive and to evaluate their biopreservative effect on milk and Pastilla leaf against some spoilage microorganisms.

MATERIALS AND METHODS

LAB strains

Three LAB strains (*Levilactobacillus brevis* S27, *Lactiplantibacillus pentosus* S42, and *Lactiplantibacillus plantarum* S62), studied in this work, were previously isolated in the Laboratory of Bioresources, Biotechnology, Ethnopharmacology and Health (Mohammed Premier University, Oujda, Morocco) from traditional fermented olive brine. These strains were maintained in 20% glycerol (*v*/*v*) at -80ºC, and they were routinely reactivated in De Man Rogosa and Sharpe (MRS) broth (Biokar, France) at 37ºC for 18 hours before use.

Target strains

The microorganisms used as targets were bacteria (*L. innocua* and *E. coli*) and yeast (*Rhodotorula glutinis*), preserved at -80°C. The targets were cultured in Mueller-Hinton (MH) broth (Difco, France) for bacteria and yeast extract glucose (YEG) broth containing 10 g L^{-1} of yeast extract, 10 g L^{-1} of glucose for *Rhodotorula glutinis.* The cultures were adjusted to the absorbance of 0.5 McFarland standard at a wavelength of 625 nm before use.

Preparation of cell-free supernatants of LAB strains

The cell-free supernatant (CFS) of LAB strains was prepared from their culture obtained after 48 hours of incubation at 37°C in MRS broth. The culture of each strain was centrifuged at 4000 x g for 10 min at 4°C. The neutralized CFS (NCFS) of LAB strains was prepared by adding 4M NaOH to the CFS until pH 7. The CFS and NCFS obtained were filter-sterilized using 0.2 µm sterile filter (Sartorius, Germany) and stored at 4°C before use.

Effect of catalase and proteinase-K on the antimicrobial activity of LAB strains

The NCFS of the LAB strains was treated with catalase and proteinase-K. The catalase enzyme (4000 U mg^{-1}, Sigma-Aldrich) was sterilized by 0.2 µm sterile filter (Sartorius, Germany), then added to the NCFS at a final concentration of 1 mg mL^{-1}, and then incubated for 2 hours at 37°C, to eliminate the effect of hydrogen peroxide, resulting subsequently in catalase-treated NCFS. In order to verify the proteinaceous nature of the bioactive molecules of the CFS, the NCFS was treated with proteinase-K P-5056 (Sigma-Aldrich), diluted in a Tris-HC1 50 mM buffer (pH 7.5) at a final concentration of 2.5 µg mL^{-1}. The proteinase K-treated NCFS was sterilized by 0.2 µm sterile filter, and then incubated for 2 hours at 37°C. The remaining enzymatic activity of proteinase-K was inactivated by heating at 65°C for 10 min.

SDS-PAGE analysis of proteins precipitate from LAB strains

The CFS (50 mL) of each LAB strain, obtained by centrifugation at 4000 x g for 10 min at 4°C of LAB strains cultured in MRS broth at 37°C for 48 h, was supplemented with ammonium sulfate to 80% saturation under stirring conditions and maintained overnight at 4°C to precipitate the proteins. The precipitate was collected by centrifugation (9400 x g for 20 min at 4°C), and dissolved in 5 mL of phosphate-buffered saline (pH 7.4). The proteins precipitate obtained was analyzed by polyacrylamide gel (12%) electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and β-mercaptoethanol according to the method of **(Laemmli, 1970)**. The PAGE was carried out at a constant voltage of 200V for 45 min using Mini-PROTEAN® II Electrophoresis Cell. The gel was incubated in a staining solution of ethanol (40%, *v/v*), acetic acid (7%, *v/v*) and Coomassie G-250 (0.0025%, *w/v*), with gentle shaking for 30 minutes, to reveal the proteins. Finally, the molecular mass of the proteins that appeared, in the form of bands, is estimated using a molecular mass marker (BIOLINE, HyperPage II Prestained Protein Ladder, 10 to 250 kDa, Cat. No. BIO-33067).

Antimicrobial activity of LAB strains using overlay method

The antimicrobial activity of LAB strains (*Lb. brevis* S27, *Lb. pentosus* S42 and *Lb. plantarum* S62) was determined by the agar overlay method of **Aween *et al.* (2012)**. Briefly, overnight cultures of LAB strains were spot inoculated on MRS agar plates, and then incubated at 37°C for 48 hours. Subsequently, plates were overlaid with 15 mL of MH agar (0.75% agar) or 15 mL of YEG agar (0.75% agar), previously inoculated with 10^6 CFU mL^{-1} of bacteria (*L. innocua* and *E. coli*) or *R. glutinis*, respectively. After 24 hours of incubation at 37°C for bacteria and 25°C for *R. glutinis*, the inhibition zone (IZ) was measured (mm). All the tests were realized in triplicate.

Antimicrobial activity of LAB strains using well diffusion method

The antimicrobial activity of supernatants and the proteins precipitate of LAB strains against pathogens was determined using the well diffusion method of **Barefoot and Klaenhammer (1983)**. The targets (*L. innocua*, *E. coli*, and *R. glutinis*) were seeded with a concentration of 10^6 CFU mL^{-1} on MH agar for bacteria and YEG agar for *R. glutinis*. The wells performed in the medium were charged with 65 µL of supernatants (CFS, NCFS, catalase-treated NCFS or proteinase K-treated NCFS) and the proteins precipitate of LAB strains. Nisin (Sigma-Aldrich) (1 mg mL^{-1}) and cycloheximide (0.01 mg mL^{-1}) were used as positive controls for *L. innocua* and *R. glutinis*, respectively, in case of the proteins precipitate test. Then, the cultures were incubated at 37°C for 24 hours for bacteria, and at 25°C for 24 hours for *R. glutinis*. The IZ of the targets obtained around the wells were measured (mm). All the tests were realized in triplicate.

Minimum inhibitory concentration of concentrated CFS of LAB strains

The minimum inhibitory concentration (MIC) of 10-fold concentrated CFS (10xCFS), obtained from LAB strains cultured in MRS broth at 37°C for 48h, was determined in MH broth. The CFS obtained by centrifugation (4000 x g for 10 min at 4°C), was 10-fold concentrated at 30°C using a SpeedVac concentrator (Eppendorf ™ Concentrator Plus, with diaphragm vacuum pump). The concentrations of the 10xCF obtained (2%, 4%, 4.5%, 5%, 5.5%, 6%, 8% and 10% (*v/v*)), by dilution in distilled water, were filter sterilized (0.2 µm sterile filter) and added to MH broth (2 mL), and subsequently inoculated with a concentration of 10^6 CFU mL^{-1} of the target (*L. innocua*, *E. coli* or *R. glutinis*). The cultures were then incubated at 37°C for 48h for bacteria and 25°C for 48h for *R. glutinis*, and the lowest concentration of 10xCFS showing no visible microbial growth of the target was recorded as a MIC value. All the experiments were repeated three times.

Quantitative assay of antimicrobial activity of LAB strains

The quantitative assay of antimicrobial activity of *Lb. brevis* S27, *Lb. pentosus* S42, and *Lb. plantarum* S62 were carried out in 96-well microplate, according to the method of **Barman *et al.* (2018)**. Briefly, each well was charged with 175 µL of MH broth and 5 µL of overnight culture (0.5 McFarland/ 10^6 CFU mL^{-1}) of the target (*L. innocua*, *E. coli* or *R. glutinis*). The cultures were incubated at 37°C for bacteria and 25°C for *R. glutinis*. After 7 hours of incubation, a volume of 20 µL of MIC value (4.5%, *v/v*) of each type of supernatant of *Lb. plantarum* S62 was aseptically added to the culture. The supernatants used in the assay were 10xCFS, 10xNCFS (10-fold NCFS) and Protein precipitate. Gentamicin (0.1 mg mL^{-1}) and cycloheximide (0.01 mg mL^{-1}) (Sigma-Aldrich, USA) were used as positive controls against bacteria and yeast, respectively. During the incubation, the biomass of target strains was determined by measuring its absorbance at 600 nm (OD), after three hours of addition of the antimicrobial agent, using a microplate reader (Thermo Multiskan EX Microplate Photometer) controlled by a computer using the Ascent Software. The negative control contained the culture of targets with no antimicrobial addition. The biomass growth reduction of the target strains was calculated by the difference between the values of OD600nm obtained, after 10h of culture, in the absence (negative control) and in presence of LAB supernatant.

Biopreservation of milk

The 10xCFS of *Lb. plantarum* S62 was tested for its biopreservative effect against pathogens (*L. innocua* and *E. coli*) in ultra-heat-treated cow milk, purchased from local market (Oujda, Morocco). The assay was realized according to the method described by **Bajpai *et al.* (2016)**. Briefly**,** 8.9 mL of milk were inoculated with 10^6 CFU mL^{-1} of overnight culture of target strains (*L. innocua* or *E. coli*) and then added with 1 mL of MIC value of 10xCFS of *Lb. plantarum* S62, and then incubated at 37°C for 48h. The final concentrations of MIC values of 10xCFS in milk were 0.45% for *L. innocua* and 0.5% for *E. coli*. During the incubation, the biomass of the target strains was determined using colony forming unit method (CFU mL^{-1}). An assay without 10xCFS addition was also used as control. All assays were realized in triplicate.

Biopreservation of Pastilla leaf

The biopreservative effect of CFS of LAB strains (*Lb. brevis* S27, *Lb. pentosus* S42, and *Lb. plantarum* S62), against *R. glutinis*, was tested on Pastilla leaf, purchased from local market (Oujda, Morocco). For this, pieces of Pastilla leaf (5 cm diameter) were aseptically cut and introduced in sterile petri dishes, then sterilized by ultraviolet light for 30 min in microbiological safety cabinet (PSM-II). After sterilization, 0.5 mL of CFS, 10xCFS, NCFS or 10xNCFS was totally sprayed on each Pastilla leaf sample. Pastilla leaf samples added with 0.5 mL of cycloheximide (0.01 %, w/v) or distilled water (0.5 mL) were used as controls. Subsequently, the Pastilla leaf samples were inoculated in the middle with 0.1 mL of overnight culture (10^6 CFU mL^{-1}) of *R. glutinis*, and then incubated at 25°C for 72 h, and the growth zone diameter of *R. glutinis* on Pastilla leaf was measured (mm). All assays were realized in triplicate.

Statistical Analysis

The results obtained were presented as the means ± standard deviation. The student-Newman-Keuls (S-N-K) comparison test was used to identify the group of means by the one-way analysis of variance (ANOVA), followed by means comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Antimicrobial activity of cultures of LAB strains

Results of antimicrobial activity of LAB strains (*Lb. brevis* S27, *Lb. pentosus* S42, and *Lb. plantarum* S62) against bacteria (*L. innocua* and *E. coli*) and yeast (*R. glutinis*) are reported in (Tab 1). The inhibition zone (IZ) values ranged from 27.33

to 29.83 mm, from 18.33 to 19.83 mm, and from 14.33 to 15.66 mm against *R. glutinis, L. innocua* and *E. coli*, respectively. The IZ values obtained against *R. glutinis* were higher than those obtained against *L. innocua* and *E. coli*. Among the LAB strains, *Lb. pentosus* S42, showed the significantly (P<0.05) lowest value against *R. glutinis;* while the other LAB strains (*Lb. brevis* S27, and *Lb. plantarum* S62) exhibited higher, but not significantly (P<0.05) different, IZ values. No significant difference (P<0.05) was observed between the three LAB strains in IZ values obtained against each target of bacteria.

Table 1 Inhibition zone values (in mm) of LAB strains obtained against *R. glutinis, E. coli*, and *L. innocua* with overlay method.

LAB strains	Inhibition zone (mm)		
	R. glutinis	***E. coli***	***L. innocua***
***Lb. brevis* S27**	29.16^{a}±0.57	14.33^{a}±1.04	18.66^{a}±2.56
***Lb. pentosus* S42**	27.33^{b}±1.25	15.66^{a}±0.28	18.33^{a}±1.89
***Lb. plantarum* S62**	29.83^{a}±2.25	15.16^{a}±0.57	19.83^{a}±2.25

Values are mean ± standard error of triplicates
[a-b] Means in the same column of each target strain with different lower-case letters differed significantly (P<0.05).

Antimicrobial activity of supernatants of LAB strains

The IZ values of supernatants of LAB strains, obtained against the targets, are reported in (Tab 2). The crude CFS (pH 4.2) demonstrated the same trend in IZ values against the targets as that obtained with the cultures of LAB strains (Tab 1), but with lower values. Hence, the CFS of all LAB strains showed IZ values ranges of 26.33-28.83 mm, 14.16-16.33 mm and 10-12.16 mm against *R. glutinis*, *L. innocua* and *E. coli*, respectively. The IZ values ranges obtained with NCFS (neutralized CFS at pH 7) against *L. innocua* and *R. glutinis* were respectively of 13.16-14.50 mm and 17.83-20.50 mm, whereas no inhibition was obtained against *E. coli*. The same trend was observed in results of NCFS of LAB strains treated with catalase. However, no inhibition was detected in CFS of the three LAB strains treated with proteinase K, indicating the proteinaceous nature of bioactive molecules involved in inhibition of *L. innocua* and *R. glutinis*.
Lb. plantarum S62 showed a significant highest IZ values (P<0.05) with its NCFS (pH7) against *R. glutinis* and its CFS (pH4.2) against *L. innocua*; While, no significant difference (P<0.05) was observed between the three LAB strains in terms of IZ values obtained with the other supernatants (catalase-treated NCFS and proteinase K-treated NCFS).

Table 2 Inhibition zone values (mm) of supernatants (CFS, NCFS, catalase-treated NCFS and proteinase K-treated NCFS) of LAB strains obtained against *R. glutinis, E. coli* and *L. innocua*

LAB strains	Inhibition zone (mm)											
	R. glutinis				***E. coli***				***L. innocua***			
	pH4.2	**pH7**	**Cat.**	**Prot-K**	**pH4.2**	**pH7**	**Cat.**	**Prot-K**	**pH4.2**	**pH7**	**Cat.**	**Prot-K**
Lb. brevis S27	26.33^{a}± 1.04	17.83^{b}± 2.25	18.33^{a}± 0.76	ND	10.00^{a}± 2.50	ND	ND	ND	14.16^{c}± 1.25	13.16^{a}± 2.25	13.00^{a}± 1.00	ND
Lb. pentosus S42	26.66^{a}± 1.75	18.16^{b}± 1.75	18.83^{a}± 0.57	ND	12.16^{a}± 2.08	ND	ND	ND	15.33^{b}± 1.25	13.66^{a}± 1.15	13.66^{a}± 1.04	ND
Lb. plantarum S62	28.83^{a}± 1.52	20.50^{a}± 2.78	20.50^{a}± 2.00	ND	11.83^{a}± 1.60	ND	ND	ND	16.33^{a}± 0.76	14.50^{a}± 1.50	14.50^{a}± 0.50	ND

Values are mean ± standard error of triplicates
[a-c] Means in the same column of each parameter with different lower-case letters differed significantly (P<0.05).
pH4.2, cell free supernatant (CFS); pH7, neutralized CFS (NCFS); Cat., NCFS treated with catalase; Prot-K, NCFS treated with catalase and proteinase K; ND: not detected

Antimicrobial activity of the proteins precipitate from LAB strains

The proteins precipitate of the three LAB strains showed IZ values ranges of 11.83-13.16 mm and 18.33-20.66 mm against *L. innocua* and *R. glutinis*, respectively; While, IZ values obtained with the positive controls were 12 mm with nisin against *L. innocua* and 22.3 mm with cycloheximide against *R. glutinis* (Tab 3). No significant difference was observed between the IZ values obtained with the three LAB strains against *L. innocua*. However, *Lb. plantarum* S62 showed the significantly (P<0.05) highest IZ value against *R. glutinis*, which is not significantly (P<0.05) different from that obtained with cycloheximide (0.01 mg mL^{-1}), used as positive control.

Table 3 Inhibition zone values of proteins precipitate of LAB strains obtained against *L. innocua* and *R. glutinis*

LAB strains	Inhibition zone (mm)	
	L. innocua	***R. glutinis***
***Lb. brevis* S27**	13.16^{a}±1.25	18.50^{b}±1.00
***Lb. pentosus* S42**	11.83^{a}±1.25	18.33^{b}±1.25
***Lb. plantarum* S62**	12.33^{a}±1.04	20.66^{ab}±1.25
Nisin (1 mg mL^{-1})	12.00^{a}±0.86	NT
Cycloheximide (0.01 mg mL^{-1})	NT	22.33^{a}±0.28

Values are mean ± standard error of triplicates
[a-b] Means in the same column of each target strains with different lower-case letters differed significantly (P<0.05).
NT: not tested.

SDS-PAGE analysis of the proteins precipitate

The SDS-PAGE of the proteins precipitate of the CFS of LAB strains cultures (*Lb. brevis*, *Lb. pentosus* S42 and *Lb. plantarum* S62) is reported in figure. 1. The results showed that among the four peptide bands obtained, two bands of approximately 42 and 56 kDa are common for the three LAB strains (S27, S42 and S62). Two additional bands of 46 and 89kDa are observed in S42 and S27, respectively.

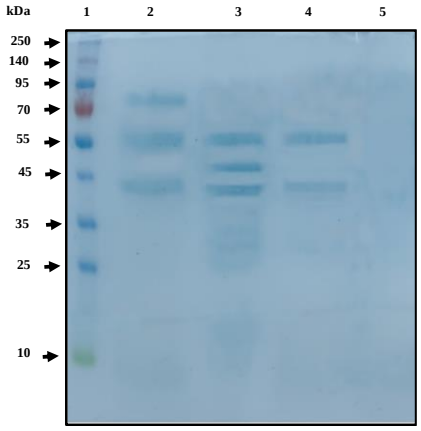


Figure 1 SDS-PAGE analysis of proteins precipitate obtained from LAB strains. Lane 1: Molecular mass markers; Lanes 2, 3 and 4: proteins precipitate from *Lb. brevis* S27, *Lb. pentosus* S42 and *Lb. plantarum* S62, respectively; Lane 5: proteins precipitate from sterile MRS broth. The gel was stained with Coomassie Brilliant Blue R250 to reveal proteins.

MIC of concentrated CFS

The MIC values of the 10xCFS obtained for the three LAB strains (*Lb. brevis* S27, *Lb. pentosus* S42, *Lb. plantarum* S62) were shown to be 4.5% (*v*/*v*) against *L. innocua* and *R. glutinis*, and 5% (*v*/*v*) against *E. coli*.

Antimicrobial activity of LAB strains in liquid medium

The effect of the supernatants (10xCFS and 10xNCFS) and protein precipitate from the three LAB strains on the growth of targets microorganisms (*L. innocua, E. coli* and *R. glutinis*) was evaluated in 96-well microplate for 10 hours, and the results are reported on Figure 2. The concentrations of the supernatants (10xCFS, 10xNCFS) and proteins precipitate tested were 0.45% (*v*/*v*) against *R. glutinis* and *L. innocua*, and 0.5% (*v*/*v*) against *E. coli*. Gentamicin (0.1 mg mL^{-1}) and cycloheximide (0.01 mg mL^{-1}) were used as positive controls against bacteria (*L. innocua* and *E. coli*) and *R. glutinis*, respectively. The supernatants (10xCFS, 10xNCFS) and proteins precipitate and the positive controls (gentamicin and cycloheximide) were added to the cultures of targets after 7 hours of incubation, and the biomass growth was measured after three hours of addition of the

antimicrobial agents. The negative control contained the culture of targets with no antimicrobial's addition.

The results showed that, compared to the negative control, 0.45% of the 10xCFS of the three LAB strains allowed a reduction of around 50% of biomass growth of *L. innocua*, which is almost similar to that obtained with the positive control (gentamicin, 0.1 mg mL^{-1}). While, a reduction of around 40% of biomass growth inhibition was observed with 10xNCFS and proteins precipitate (Figure 2.: a1, a2 and a3). Against *E. coli*, 0.5% of the 10xCFS preparation reduced the biomass growth of around 50%, However, no significant inhibition was observed with 0.5% of 10xNCFS and proteins precipitate, when compared to the negative control (Figure 2: b1, b2 and b3). Against *R. glutinis*, 0.45% of the 10xCFS led to 50% of reduction in biomass growth when compared to the negative control. However, in presence of 0.45% of 10xNCFS and proteins precipitate, *R. glutinis* showed a continuous biomass growth, but at lower level than that observed in the negative control (Figure 2.: c1, c2 and c3).

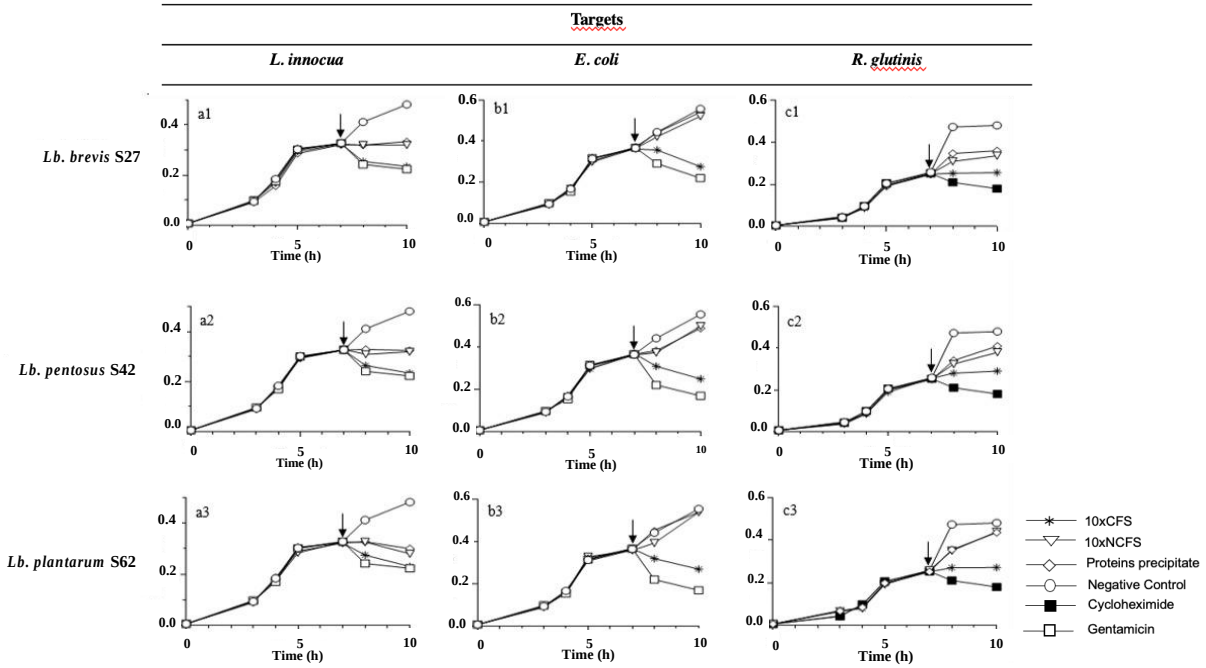


Figure 2 Quantitative assay of antimicrobial activity of LAB strains. a1: *Lb. brevis* S27 against *L. innocua*. a2: *Lb. pentosus* 42 against *L. innocua*. a3 *Lb. plantarum* S62 against *L. innocua*. b1: *Lb. brevis* S27 against *E. coli*. b2: *Lb. pentosus* 42 against *E. coli*. b3: *L. plantarum* S62 against *E. coli.* c1: *Lb. brevis* S27 against *R. glutinis.* c2: *Lb. pentosus* 42 against *R. glutinis.* c3: *Lb. plantarum* S62 against *R. glutinis.* The arrow indicates the addition of MIC

Biopreservation of milk

The inhibitory effect of 0.45% and 0.5% of 10xCFS of *Lb. plantarum* S62 on the biomass growth of respectively of *L. innocua* and *E. coli*, was evaluated in UHT-milk, and the results are reported in Figure 3. The results showed important reduction of biomass growth of *L. innocua* and *E. coli* in milk, with final reduction values respectively of 5 Log CFU mL^{-1} and 4 Log CFU mL^{-1}, respectively, when compared to the controls.

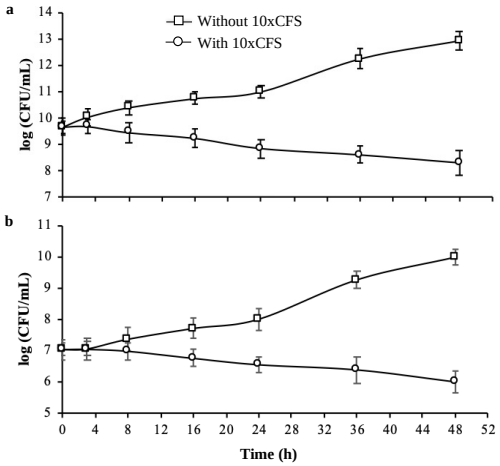


Figure 3 Biomass growth (CFU mL^{-1}) of *L. innocua* (a) and *E. coli* (b) in milk during 48 hours of incubation at 37°C in the absence and presence of 10xCFS of *Lb. plantarum* S62.

Biopreservation of Pastilla leaf

The antifungal effect of supernatants (CFS, 10xCFS, NCFS and 10xNCFS) of *Lb. plantarum* S62 was evaluated on Pastilla leaf contaminated with *R. glutinis* and maintained at ambient temperature, and the results are reported in Tab 4 and Figure 4. The results showed total inhibition (100%) of *R. glutinis* in Pastilla leaf samples treated with the CFS, 10x-CFS, 10xNCFS and cycloheximide (positive control). However, in presence of NCFS a biomass growth of *R. glutinis* was observed in samples, with growth zone diameter of 7.5 mm, against 10 mm obtained with distilled water (negative control) after 3 days of incubation. However, the concentrated supernatants (10xCFS and 10xNCFS) led to the browning of Pastilla leaf samples (Figure 4).

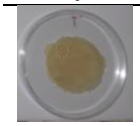
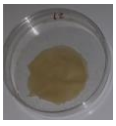
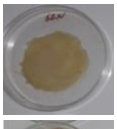
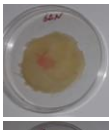
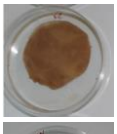
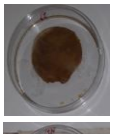
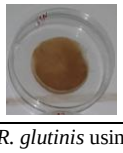
Treatment	Observation	
	Day 0	Day 3
Inoculation with *R. glutinis* and addition of distilled water		
Inoculation with *R. glutinis* and addition of cycloheximide		
Inoculation with *R. glutinis* and addition of CFS		
Inoculation with *R. glutinis* and addition of NCFS		
Inoculation with *R. glutinis* and addition of 10×CFS		
Inoculation with *R. glutinis* and addition of 10×NCFS		

Figure 4 Biopreservation of Pastilla leaf against *R. glutinis* using CFS, NCFS, 10xCFS and 10xNCFS of *Lb. plantarum* S62.

Table 4 Growth zone of *R. glutinis* on traditional Pastilla leaf in presence of different supernatants of *Lb. plantarum* S62.

	CFS	10xCFS	NCFS	10xNCFS	H_2O	CH
Growth Zone diameter (mm)	ND	ND	7.50±0.00	ND	10±0.50	ND

Values are mean ± standard error of triplicates.
ND: no growth detected, CFS: cell-free supernatant, 10xCFS: 10-fold concentrated CFS, NCFS: neutralized CFS, 10xNCFS: 10xfold concentrated neutralized CFS, H_2O: distilled water, CH: Cycloheximide.

DISCUSSION

E. coli and *L. monocytogenes* are the main bacteria involved in foodborne illnesses **(Yang *et al.*, 2018)**. *Rhodotorula spp*. are saprophytic yeasts involved in food spoilage **(Albertyn *et al.*, 2014)**, and they are considered as emerging opportunistic pathogens for susceptible patients **(Wirth and Goldani, 2012)**.
Human listeriosis outbreaks are mostly linked to contaminated dairy products **(Matto *et al.*, 2018)**, due to the tolerance of *L. monocytogenes* to their processing and storage conditions **(Bucur *et al.*, 2018)**. **Quinto *et al.* (2020)** demonstrated the survival capacity of *E. coli* in milk and dairy products for several weeks. Wheat flour, from different countries, was reported to be contaminated by various pathogenic and spoilage microorganisms, including bacteria (i.e. *E. coli*, *Salmonella*, *Bacillus cereus*, *Staphylococcus aureus*), yeasts and molds (*Aspergillus*, *Fusarium*, *Penicillium*) **(Magallanes López and Simsek, 2021)**, which can lead to the loss of quality and safety of processed wheat-based products **(Myoda *et al.*, 2019)**.
The three LAB strains (*Levilactobacillus brevis* S27, *Lactiplantibacillus pentosus* S42, and *Lactiplantibacillus. plantarum* S62), were studied for their antimicrobial activities. The high antifungal activity obtained in these LAB strains may be due to their initial isolation as potential antifungal strains against *Candida pelliculosa* **(Abouloifa *et al.*, 2020a)**, and they demonstrated important antifungal activity against pathogenic and spoilage yeasts (*Candida pelliculosa* and *R. glutinis*) and molds *(Aspergillus niger*, *Penicillium digitatum*, *Fusarium oxysporum*, and *Rhizopus sp*.) **(Abouloifa *et al.*, 2021)**. The important antibacterial and antifungal activities, obtained with the three probiotic LAB strains, indicate their possible use in food processing as food biopreservative.
All the supernatants prepared from the three LAB strains demonstrated antifungal activity higher than their antibacterial activity. Among these strains, *Lb. plantarum* S62 showed the highest inhibitory effect against *R. glutinis* and *L. innocua*; and the highest IZ values were obtained with its crude CFS, followed by that obtained with neutralized CFS (NCFS), and finally by that obtained with catalase treated-NCFS. It is worth noticing that no antibacterial nor antifungal activity was obtained with proteinase K-treated NCFS. These findings indicate the diversity of biomolecules involved in antimicrobial activity of *Lb. plantarum* S62, which include organic acids, hydrogen peroxide and proteinaceous compounds. These results are in agreement and confirm those obtained by **Abouloifa *et al.*, 2021**.
The proteinaceous compounds obtained from the three LAB strains were analyzed by SDS-PAGE, and the results showed the presence of four peptide bands of 42, 46, 56 and 89 kDa, which can be involved in the inhibition of *L. innocua* and *R. glutinis*. Their relatively high molecular weights (>30 kDa) indicate that these peptides can be compared to bacteriocins of the classes III and IV **(Zacharof and Lovitt, 2012).** Peptides with molecular weight of 45kDa, 55 kDa and 122 kDa, obtained from LAB strains, which are close to that obtained in this work, were actives against *L. monocytogenes* and *L. innocua* **(Lash *et al.*, 2005 ; Muhammad *et al.*, 2019 ; Heidari *et al.*, 2022).** The antibacterial activity was demonstrated in peptides (extracellular protease) of 89 kDa, obtained from an Algicolous Fungus *Xylaria psidii* KT30 **(Indarmawan *et al.*, 2016)**; While the antifungal activity was obtained with peptides of molecular weight of 45 kDa and 56 kDa **(Atanassova *et al.*, 2003 ; Di Biase *et al.*, 2014)** and with peptide of 42 kDa identified as chitinase from *Tricotherma atroviride* **(Harighi *et al.*, 2007).**
The MIC values obtained with the 10xCFS indicate the high inhibitory effect of the three LABs against *R. glutinis* (yeast) and *L. innocua* (Gram-positive bacteria) than *E. coli* (Gram negative bacteria). The highest inhibition of biomass growth of these pathogens was obtained with 10xCFS, followed by that obtained with 10xNCFS, and finally by that obtained with proteinase K-treated NCFS. The high inhibition obtained with 10xCFS can be due to the increase of concentration of the antimicrobial compounds produced by the LAB strains. The capacity of supernatants (10xCFS, 10xNCFS and proteinase K-treated NCFS) of *Lb. plantarum* S62 in reduction of biomass growth of *L. innocua* and *R. glutinis*, is of great interest in food processing, as they can be used as natural protective and curative agent against these pathogens.
The CFS, 10xCFS and 10xNCFS of *Lb. plantarum* S62, have been shown to be effective in protecting the Pastilla leaf from growth of *R. glutinis*. Previous works reported the inhibitory effects of LAB against pathogenic yeasts **(Afzali *et al.*, 2020)**. In this work, *Lb. plantarum* S62 demonstrated its effectiveness as bio-protective agent against *R. glutinis*, considered as emerging opportunistic pathogen **(Wirth and Goldani, 2012)**.

CONCLUSION

In this work, three LAB strains (*Lb. plantarum* S62, *Lb. pentosus* S42, or *Lb. brevis* S27), obtained from traditional fermented Moroccan green olives, were studied for their antimicrobial activities in food systems (in milk and Pastilla leaf). LAB strains demonstrated significant antifungal activity against *R. glutinis* and antibacterial effect against *L. innocua* and *E. coli*. Among the studied LAB strains, *Lb. plantarum* S62 and its 10xCFS exhibited the highest antibacterial and antifungal activity and demonstrated important protective effects against pathogens and spoilage microorganisms in milk and Pastilla leaf, respectively. These results indicate the possibility of using *Lb. plantarum* S62, to develop a food bio-preservative.

Acknowledgments: This work was financially supported by the CNRST (PPR/19/2015) and the "PHC-Maghreb" program of the French Ministry of Foreign Affairs and Ministry of Higher Education, Research and Innovation "code Campus France: 43791TM, code PHC: 01MAG20".

Conflicts of Interest Statement: The authors declare no conflicts of interest.

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