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Original Research Article

Antimicrobial activity of lactic acid bacteria and bacteriocins isolated from a traditional brine table olives against pathogenic bacteria

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A B S T R A C T

Among 65 LAB were screened for exhibition of antagonistic activities using agar well diffusion method. It was found that 40 isolates exhibited antibacterial activity against *Listeria monocytogenes,* including 35 strains displayed large inhibition spectra results in excellent inhibition zones diameters larger than 15 mm. Of these, 8 strains (LBA01, LBA07, LBA09, LBB08, LBC02, LBC04, LBD02, and LBD05) were also active against Gram-negative bacterium *Escherichia coli* O157 and were identified as *Enterococcus faecium*. Results showed that the LAB strains retained activity in the supernatants after effects of organic acid and hydrogen peroxide were eliminated. It was determined that the bacteriocins produced by both strains *Enterococcus faecium* LBB08 and *Enterococcus faecium* LBC02 that have been purified by the method of Yang were active towards *Listeria monocytogenes* and *Escherichia coli* O157. This result suggests the possibility to use the strains studied as a "natural" substance for microbiological control in a variety of industrial applications.

Introduction

Faced with the anxiety of consumers towards certain foods and growing craze for organic products, it was necessary to seek to improve the processes of biological conservation. Although the food industry has many conservation techniques, fermented foods are still very important, and represent an important part of our diet (Yamamoto *et al.*, 2003). The interest of the consumer for these products is attributed to the organoleptic quality (McKay and Baldwin, 1990), and microbiological stability accorded by the presence of lactic acid bacteria (LAB) (Caplice and Fitzgerald, 1999).

However, as a result of extensive research the food industry has appealed these selected microbial strains for a well controlled fermentation, and widely used as starters for the elaboration of an unlimited number of foods in which they contribute to eliminate any possibility of contamination by undesirable microorganisms (Deegan *et al.*,

2006). This family of bacteria is responsible for the conservation by the production of a wide range of antimicrobial metabolite comprising organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds and bacteriocins which are considered as natural food ingredients and they have been consumed for millennia by mankind as LAB (Alfonzo *et al.,* 2013) The lactic bacteria and bacteriocins which are defined as proteins or peptides biologically active possess bactericidal activity against the pathogenic bacteria and which are defined as proteins or peptides biologically active possess bactericidal activity against the pathogenic bacteria and/or alteration of food (Tagg and McGiven, 1971), are used extensively for the preparation of an unlimited number of foods for several potential applications as functional starter cultures in the food area (Yamamoto *et al.,* 2003).

Also contribute to the pleasant sensory profile of the final product by the improvement of the nutritional value of different foods such as vegetables, fish, meats, dairy and sausages and prolong their shelf life why not in control of mold growth by the action of the antifungal properties of BAL (Crowley *et al.,* 2013; El-Ghaish *et al.,* 2011; Ghrairi *et al.,* 2008; Lindgren and Dobrogosz, 1990).

Very little work has been based on the research of LAB produced from natural vegetables. Traditional fermentation olives with the greatest qualities of fermentation is a very practical replied to Morocco and represent the heart of Moroccan cuisine and possessing an undeniable attraction because is considered to be more organoleptically superior to similar canned olives.

In the present work we isolated strains of lactic acid bacteria from the brine table olives, test their bactericidal, and undertaken to bridge the gap in knowledge the existence of bacteriocinogenic activities produced by bacteriocins, with the aim of evaluating their potential use as a biopreservatives.

Materials and Methods

Bacterial strains and growth conditions:

The samples of traditional brine table green olives were collected randomly from local markets (souk). 65 LAB considered in this study were isolated from this collection were characterized using Gram stain, cell morphology and catalase reaction tests. These strains those described in our previous work were suffered has different technical isolation and identification.

LAB strains isolated from brine table olives Gram-positive and catalase-negative isolates were grown in MRS broth (Biokar Diagnostics, France) at 37 °C for 24 h and subcultured twice in MRS broth supplemented with 15% glycerol and kept frozen at −20 °C.

The indicator strains chosen for their pathogenic effect were *Listeria monocytogenes* (CECT 4031), *Staphylococcus aureus* (CECT 239) and *Pseudomonas aeruginosa* (CECT 116) were obtained from Spanish Collection of Type Cultures CECT and *Escherichia coli* O157: H7 from strain collection.

Screening of LAB for their antimicrobial activity

For detection of antimicrobial activity, the agar well diffusion method (AWDA) as described by (Casla *et al.,* 1996; Sumathi, 2012). MRS agar plates (1.5% agar), in which wells (5 mm in diameter and of 180 µl in capacity) were formed by placing the

oxford cylinders stainless steel, were overlaid with 25 ml of molten BHI agar (Biokar Diagnostics, France) (0.75% agar) inoculated with an overnight culture of pathogenic bacteria (about 10^8 cfu/ml). After solidification of the overlayer, the molds were carefully removed and were filled with 60 µl of approximately 10^8 cfu/ml of the production strain.

Finally the plates were incubated aerobically at the proper temperature for the growth of the target strain for 24 h at 37 °C. Antagonist effect resulting in the appearance of the clear zone around the wells of the growth of the putative inhibitor strain was inspected.

Nature of antibacterial compounds from LAB

The elimination of the antimicrobial effect of organic acids (Hwanhlem *et al.,* 2011), the strains of LAB were grown for 24 h at 37°C in MRS broth and then centrifuged at 14,000g for 5 min. The supernatant fluid was filtered through a syringe filter with a pore size of 0.22 µm (Millipore, France) and adjusted to pH 6.5 with NaOH (1 N) to rule out acid inhibition. the inhibitory action of hydrogen peroxide (Merck, Germany) was eliminated by the addition of a sterile solution of catalase (Fluka, Germany) (300 U/ml) at 25°C for 30 min (Ammor *et al.*, 2006), The antagonistic activity of the samples was detected using the well diffusion agar method.

Extract large amounts of bacteriocins from lactic acid bacteria

For extraction and purification of bacteriocins, the method of Yang *et al.* (Yang *et al.*, 1992) was applied for only two lactic strains (LB08 and LC02) were selected for their great stability antagonizes, each producing strain was cultured in 1 liter of MRS broth adjusted to pH 6.5 to early stationary phase about (10^8 cfu/ ml) for 18 h at 37°C. The culture was then heated at 70 °C for 25 min to inactivate the proteases which may degrade the bacteriocins, after cooling the cells are recovered by centrifugation at 15,000 x g for 15 min.

The pellets were washed with 5 mM sodium phosphate (Solvachim, Casablanca) (pH 6.5) and they were resuspended in 50 ml of 100 mM NaCl (Fluka, Germany) at pH 2.0. After stirring for one hour at 4 °C, the cells were centrifuged at 29,000 x g for 20 min. The cells were resuspended in 5 mM sodium phosphate pH 6.5 and the supernatants were dialyzed in dialysis bags Cellu-Sep T2/Nominal MWCO: 6,000 - 8,000 (Membrane Filtration Products, INC, USA) against dH_2O at 4°C for 24 h, the whole is stirred to prevent any formation around the dialysis tubing and the liquid against dialysis must be renewed frequently and then freeze-dried.

To test the activity of these supernatants, AWDA the well diffusion method is implemented as described above. A volume 60 µl of supernatants is located in each well and the plates were incubated at 37 °C for 24 h. The activity expressed in arbitrary units (AU) ml^{-1}. One arbitrary unit (AU) was defined as the highest dilution that produced a halo of inhibition of the indicator strain 2 mm in diameter (Champagne, 2007).

Results and Discussion

Isolation of LAB

A total, 65 LAB strains were isolated from the traditional brine table green olives, were collected randomly from local markets. The strains were isolated in culture medium MRS and identified as LAB using the criteria of being Gram-positive and catalase

negative. Of these 65 isolates, only 8 isolates were showed inhibition zones diameters after excluding inhibition due to organic acids and hydrogen peroxide, were subjected to a different biochemical tests.

Detection of antagonistic activity

The agar well diffusion assays were used to study the antibacterial activity of the 65 strains of lactic acid bacteria isolated from the traditional brine table green olives were shown to produce inhibition zones against some indicator microorganisms. The sensitivity of the indicator strains was estimated based on the diameter (mm) of the inhibition zones. Inhibitory spectra of these isolates are presented in Table 1.

Among 65 LAB were screened for exhibition of antagonistic activities, it was found that 40 isolates exhibited antibacterial activity against *L. monocytogenes* (Todorov *et al.,* 2013) including 35 strains displayed large inhibition spectra results in excellent inhibition zones diameters larger than 15 mm. Of these, 8 strains were also active against *Escherichia coli* O157, and it was noticed that antagonistic activity toward *E. coli O157* of all 8 LAB strains was lower than this capability against *L. monocytogenes*.

Gram positive bacteria are generally more sensitive to the bactericidal effect of lactic acid bacteria (Mataragas *et al.,* 2003; Vignolo *et al.,* 1996), which can be explained by the presence in Gram-negative bacteria an additional outer membrane separated from the cell wall by the periplasmic space and which contains lipids may protect the cytoplasmic membrane from the action of the LAB and these antimicrobial compounds (Gao *et al.,* 1999)**.** However, the strains of selected LAB could not inhibit *S. aureus* (CECT 239) and *Pseudomonas aeruginosa* (CECT 116) were resistant, moreover similar results were reported by some papers (Tantillo *et al.,* 2002).

Nature of antibacterial compounds from LAB

The antimicrobial activity of these lactic acid bacteria may be due to various antimicrobial compounds such as organic acids by decreased pH levels, hydrogen peroxide, or presence of bacteriocins (Luo *et al.,* 2011).

In order to confirm the proteinaceous character of the bacteriocin substance, cell-free extracts for Only 8 strains showed significant inhibition against *L. monocytogenes* and *E. coli O157* were subjected to neutralization, and addition of catalase to eliminate the activity of organic acids and hydrogen peroxide, as shown in Table 2.

The results obtained showing that all 8 strains of LAB still exert antibacterial effect against pathogenic microorganisms confirming that these inhibitory compounds are of proteinaceous nature of the bacteriocins compounds (De Vuyst *et al.,* 1996; Liserre *et al.,* 2002).

Table.1 Inhibitory spectra of LAB isolate exhibiting antibacterial activity

Isolated N°	Inhibition zone (mm)	
	L. monocytogenes	*E. coli*
LBA01	20.4 ± 5.5	10.5 ± 0.7
LBA02	16.4 ± 7.9	ND
LBA03	16.8 ± 9.2	ND
LBA04	15.6 ± 7.7	ND
LBA05	15.2 ± 7.7	ND
LBA06	17.4 ± 7.3	ND
LBA07	22.8 ± 10.4	11.5 ± 0.7
LBA08	18.0 ± 11.0	ND
LBA09	20.0 ± 0.0	10.5 ± 0.7
LBA10	10.3 ± 0.6	ND
LBB01	13.1 ± 1.4	ND
LBB02	16.8 ± 4.4	ND
LBB03	14.0 ± 5.4	ND
LBB04	17.3 ± 5.7	ND
LBB05	19.8 ± 13.6	ND
LBB06	17.0 ± 0.0	ND
LBB07	13.7 ± 2.9	ND
LBB08	26.8 ± 4.0	12.5 ± 0.7
LBB09	16.4 ± 4.2	ND
LBB10	16.7 ± 6.0	ND
LBC01	14.7 ± 4.0	ND
LBC02	20.0 ± 0.0	13.0 ± 1.4
LBC03	19.7 ± 1.2	ND
LBC04	19.3 ± 1.5	12.5 ± 0.7
LBC05	17.7 ± 2.1	ND
LBC06	19.0 ± 0.8	ND
LBC07	17.0 ± 4.0	ND
LBC08	17.0 ± 4.0	ND
LBC09	16.0 ± 1.7	ND
LBC10	17.0 ± 1.0	ND
LBD01	17.5 ± 1.5	ND
LBD02	21.3 ± 2.6	13.5 ± 2.1
LBD03	21.0 ± 1.0	ND
LBD04	19.9 ± 6.9	ND
LBD05	22.4 ± 1.3	10.5 ± 0.7
LBD06	18.6 ± 6.0	ND
LBD07	15.0 ± 0.0	ND
LBD08	17.2 ± 5.2	ND
LBD09	18.0 ± 0.0	ND
LBD10	17.3 ± 4.0	ND

ND: not determined.

Table.2 Inhibitory activity of selected LAB strains against bacterial indicator strains after eliminating effect of organic acid and hydrogen peroxide

Producer strain		Detection of inhibitory activity	
		L. monocytogenes	*E. coli*
LBA01	*E. faecium*	++	+
LBA07	*E. faecium*	++	+
LBA09	*E. faecium*	++	+
LBB08	*E. faecium*	++	+
LBC02	*E. faecium*	++	+
LBC04	*E. faecium*	++	+
LBD02	*E. faecium*	++	+
LBD05	*E. faecium*	++	+

(+ +), inhibition zone of 15 to 20 mm, (+) small inhibition zone.

Eight LAB strains (LBA01, LBA07, LBA09, LBB08, LBC02, LBC04, LBD02, and LBD05) which exhibited widest zones of inhibition toward tested pathogenic bacteria were identified as *Enterococcus faecium* based on their morphological properties and biochemical tests. The presence of *Enterococci* play an important role fermented food products (Franz *et al.,* 1999; Hugas *et al.,* 2003), such as olives (Omar *et al.,* 2004; Randazzo *et al.,* 2010), in which *Enterococcus faecium* have been isolated from the brines of olives (Asehraou *et al.,* 1992; Lavermicocca *et al.,* 1998). As alternative the antimicrobial activity of *Enterococcus faecium* (Barbosa *et al.,* 2014; Ferreira *et al.,* 2007; Jin *et al.,* 2000) or their bacteriocins has attracted a great deal of attention as a novel approach to contribute to potential applications as functional starter cultures in the food preservation (Settanni and Corsetti, 2008).

Extraction and purification of bacteriocin

From these strains two strains were selected for this study, based on their super stability of antibacterial activity and broad spectrum of inhibition.

The extraction and purification of bacteriocins produced by *E. faecium* LB08 and *E. faecium* LC02 was performed by the purification technique described by Yang *et al.* (Yang *et al.,* 1992) which seems more economical faster and reproducible. This method is based on the influence of pH on the adsorption and release of each bacteriocin from the cell surface.

To carry evidence of the production of bacteriocin by the LAB strains, the antagonist activity was detected by the AWDA method and shows that bacteriocins were manifested by the appearance of zones of inhibition and were also reported to inhibit the growth of *L. monocytogenes* and *E. coli O157,* as shown in Table 3.

Bacteriocins produced showed the same results against indicator strains, gave the highest level of activity against *L. monocytogenes* (Ibarguren *et al.,* 2010; Vera Pingitore *et al.,* 2012), which exhibited inhibitory efficiency by targeting the cytoplasmic membrane (Delves-Broughton *et al.,* 1996; Gálvez *et al.,* 2007).

Table.3 Inhibitory spectra of bacteriocins produced by LAB

Bactriocin strain	Detection of inhibitory activity	
	L. monocytogenes	*E. coli*
Enterocin LBB08	17.0 ± 1.9	13.2 ± 1.5
Enterocin LBC02	16.2 ± 2.4	12.0 ± 1.0

The results showed that the bacteriocins were able to inhibit *E.coli O157* Gram-negative, which are generally recognized insensitive to bacteriocinogenic activity of *E. faecium* (Belgacem *et al.,* 2010) for the same reason that lactic acid bacteria (Bhunia *et al.,* 1991). The antagonist activity was unaffected by heating at 70 °C for 25 min and shows super thermal stability for both enterocins (Belgacem *et al.,* 2010), and the two bacteriocins exhibited activities of 800 AU/ml for the inhibition of *L. monocytogenes*.

As well as other favorable properties have demonstrated for LAB and bacteriocins produced by LAB isolated from the traditional brine table green olive may hold them to explore their potential application for microbiological control and used to improve the quality of the sensory properties of food products as a "natural" substance in a variety of industrial applications (Balciunas *et al.,* 2013; O'sullivan *et al.,* 2002).

In Conclusion, Lactic acid bacteria isolated had good antimicrobial activity against foodborne pathogens (*L. monocytogenes* and *E. coli* O157). The bacteriocins produced by the *E. faecium* strains showed a large inhibitory spectrum and strong inhibitory activity. These studies highlight the possibility that these bacteriocins and LAB will be notified of great interest in terms of security especially in combination with other antimicrobial compounds in further research.

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