RESEARCH ARTICLE

ANTIFUNGAL ACTIVITY OF NEWLY ISOLATES OF LACTIC ACID BACTERIA

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Abstract

A total of 54 strains of lactic acid bacteria isolated from silage, camel milk and carrot were screened for antifungal activity with overlay and confrontation assay. Six of them, belonging to the genera *Lactobacillus*, *Lactobacillus plantarum* and *farciminis* strains produce antifungal compounds active against *Aspergillus* spp., *Fusarium roseum*, *Trichoderma* spp., *Penicillium* spp. and *Stemphylium* spp. Approximately 16.66% of the isolates showed inhibitory activity against *Aspergillus* spp. The antifungal compounds were originated from organic acids, the maximum antifungal activity was observed at pH 2.0, but it decreased at pH 3.0÷7.0. Inhibitory activity did not change after treatment with proteolytic enzymes, heating, refrigeration and freezing. No inhibitory activity could be detected against the mould *Aspergillus* spp. with non-concentrated cell free supernatant and volatile substance.

Introduction

Biopreservation is defined as "extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products" (Stiles, 1996). Lactic acid bacteria, as mentioned earlier, have a major potential to be used in biopreservation methods because they are safe to consume and they naturally occurring microbiota of many foods: fermented dairy products, sausages and meat, fermentation of olives and vegetables, sourdough baked products and silage, and they have been known as a positive influence in the gastrointestinal tract of humans and other mammals as probiotics they are afforded GRAS status (Generally Recognized as Safe) in the United States which suggests the non toxic nature of metabolites produced by these organisms (Stiles, 1996; Marco, 2011; Magnusson *et al*., 2001). The preservative action of lactic acid bacteria is due to several antimicrobial metabolites, including organic acids (lactic acid, acetic acid), bacteriocins, hydrogen peroxide and others (Dalié *et al*., 2010; Magnusson *et al.,* 2001; Muhialdin *et al.,* 2011).

Fungal growth is a frequent cause of spoilage in food and feed that can cause considerable

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economic loss. Research on the antifungal activity of lactic acid bacteria have started in the late 50s and early 60s, with Guillo (1958) who developed an active product against *Candida albicans* by *Lactobacillus acidophilus*, and Marth and Hussong (1963) who tested the filtrate of *Leuconostoc citrovorum* against four yeast species *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, *Torula glutinis* and *Mycotorula lipolytica*. Collins and Hardt (1980) found that the sterilized filtrate of a culture of *Lactobacillus acidophilus* could slightly delay the growth of *Candida albicans* compared to freshly prepared broth. Wiseman and Marth (1981) demonstrated the inhibition of *Aspergillus parasiticus* by *Streptococcus lactis* C10 strain, without identification of the inhibitor agent. Currently, several research have identified the antifungal substances produced by lactic acid bacteria (Gourama, 1997; Niku-Paavola *et al.,* 1999; Magnusson and Schnürer, 2001; Falguni *et al.,* 2010; Ndagano *et al.*, 2011), and the synergistic effect of sodium acetate content in the culture medium with organic acids produced by these bacteria (Stiles *et al.,* 2002). In addition to antifungal metabolites, nutrient competition has also been proven as an antifungal obstacle (Bayrock and Ingledew, 2004).

The aim of our work was the isolation of newly *Lactobacillus* strains from natural and traditional fermented product and evaluation their antimicrobial activity on the conidia and mycelia growth of some spoilage fungus strains, especially against *Aspergillus* spp. strains.

Materials and methods

Sampling isolation and identification of lactic acid bacteria

Lactic acid bacteria were isolated from carrot, silage and camel milk. The carrot was cut into small pieces (1g) and suspended into 9 ml MRS broth (De Man *et al.,* 1960) and incubated at $37^{o}C$ under anaerobic conditions in jar with gas pak (BBL^{TM} GasPak^{TM} Catalyst, Becton Dickinson) for 24h. Then, 1ml of the appropriate dilutions was plated out (duplicates) using MRS agar medium with $CaCO_3$ (5 g/l). The plates were incubated at

$37^{o}C$ for 48h under anaerobic conditions. Plates containing separated colonies, acid formers (were identified by the presence of clear zones around the colonies), were selected for isolation. Purification of the isolates was done by repeated pour plating technique using the same agar medium until pure cultures were obtained. Only Gram positive, rod and catalase-negative bacteria were kept and maintained on MRS agar in refrigerator, and on MRS broth or skim milk with 30 % glycerol in freezing until used for antifungal activity assay.

Gram positive, rod and catalase negative bacteria were identified by physiological and biochemical test: growth at $45^{o}C$, CO_2 production from glucose, arginine degradation, carbohydrates fermentation (glucose, mannitol, melibiose, rhamnose, arabinose, sucrose, xylose, inositol, lactose, trehalose, sorbitol, amygdalin, fructose, maltose and galactose) were determined by using sterile multiwell plates (Gusils *et al.,* 2004). Esculin fermentation was determined by the hydrolyzed of esculin in the medium to esculetin and dextrose. The esculetin reacts with ferric chloride in the media to form a black-brown color.

Indicator fungal strains and inoculum preparation

The indicator fungal strains, *Aspergillus* spp., *Fusarium roseum*, *Trichoderma* spp., *Penicillium* spp. and *Stemphylium* spp., used were mainly provided by the laboratory of parasitology and mycology of the hospital of Sidi-Bel-Abess, the laboratory of microbiology of the University of Mascara and the Laboratory of phytopathology of the University of Oran Es-Senia in Algeria.

The mould was grown on malt extract agar medium at $30^{o}C$ for 5 days. The spores were collected after vigorously shaking of plates with 10 ml of sterile water, and adjusted suspension to 10^3 spores/ml by haemocytometer (Mgnusson *et al.,* 2003).

Screening assay for antifungal activity

The antifungal activity of LAB was determined on solid media by overlay method described by Magnusson *et al.,* 2003 and confrontation assay described by Brunner *et al.,* 2005.

Overlay assay

LAB cells were streaked in two lines on MRS agar plates and allowed to grow at 30°C for 48h. Then 10 ml of soft malt extract (0,7 % agar) containing 0,1 ml of a spore suspension of mould (10^3 spores/ml) was then poured onto the agar plates and incubated at 30°C. After 72h, the zone of inhibition was measured. The degree of inhibition was calculated as the area of inhibited growth in relation to the total area of the Petri dish and the scale was the following: - = no visible inhibition, + = no fungal growth on 0,1–3 % of plate area, ++ = no fungal growth on 3–8 % of plate area, +++ = no fungal growth on >8 % of plate area.

Confrontation assay

LAB cells were streaked on MRS agar plates, plates were incubated for 48 h at 30°C, and then the indicator mould (5 mm of disc) was inoculated in the same plates and incubated at 30°C for 72h, after incubation growth diameter of fungus was measured.

Antifungal activity of LAB cell-free supernatant

Lactic acid bacteria strains were grown on MRS broth at 30°C, after 24h of incubation cells were centrifuged at 8000 g for 10 min. The filtration of the supernatant by filter Millipore (0,22 μ) was necessary to prevent further growth of bacterial cells. Then 100 μl of cell free supernatant was spotted onto the well in the surface of MRS agar plates overlaid with 10 ml of soft malt extract (0,7 % agar) which had been inoculated with 0,1 ml of a mould spore suspension (10^3 spores/ml). After incubation of the plates for three days at 30°C, the zones of fungal inhibition were examined.

Antifungal properties evaluation

Effect of proteolytic enzymes

The effect of proteolytic enzymes was determined by the method of Hirsch (1979), 10 μl of the enzymes chymotrypsin and pepsin (Sigma Aldrich) (1 mg/ml, prepared in 50 mM Tris-HCL; pH 8.0) was applied around the producer colonies after chloroform treatment. The plates were incubated at 37°C for a further 4h, then the surface of MRS agar plates overlaid with 10 ml of soft malt extract (0,7 % agar) which had been inoculated with 0,1

ml of a spore suspension (10^3 spores/ml). After incubation of the plates for three days at 30°C, they were examined for zones of inhibition.

Effect of temperature

Lactic acid bacteria were streaked in two lines on MRS agar plates and allowed to grow at 30°C for 48h. Then, the plates were exposed to chloroform for 30 min to kill the bacteria. Plates only with antifungal compounds were incubated at 45°C, 70°C, 100°C and120°C for 20 min, stored at 8°C and -18°C for 15 days. 10 ml of soft malt extract (0,7 % agar) containing 0,1 ml of inoculum of mould (10^3 spores/ml) was then poured onto the agar plates treated with temperature (heating, refrigeration, freezing). After incubation of the plates for three days at 30°C, the inhibition zones were examined.

Effect of pH

Overlay assay described above was used, the pH values of malt extract agar medium was changed at 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0.

Detection of volatile substance

The detection of volatile antifungal compounds was evaluated by sealed plates method (Fiddaman et Rossall, 1993). Lactic acid bacteria were inoculated on MRS agar plates sealed with parafilm and allowed to grow at 30°C for 24h, in parallel agar disc of mould was inoculated on other Petri dish and allowed to grow at 30°C for 24h, and then the two plates were sealed together with parafilm and incubated at 30°C for 72h. The growth of a fungus was determined by the measurement of colony diameter compared to control, to evaluate the inhibition by volatile substance

Fungicide or fungistatic effect

Fungicide or fungistatic effect was determined by the method of Kerr (1999) on the conidia germination by transferring a disc agar from a clearing zone of inhibition into novel medium of Malt extract agar medium and incubated at 30°C for 72h, and on the mycelium growth by agar disc broth, the mould of *Aspergillus* was incorporated in an agar disc which was incubated in MRS broth inoculated with *Lactobacillus* spp. strains, after seven days of incubation at 30°C, the agar disc was

transferred into novel medium of Malt extract agar medium and incubated at 30°C for 72h.

Results and discussion

Screening of lactic acid bacteria strains for antifungal activity

A total of 54 lactic acid bacteria (LAB) isolates that showed clear zone on the MRS agar with CaCO$_3$ were screened for antifungal activity towards *Aspergillus* spp. by streaking out the bacteria in two parallel lines onto MRS plates. Results showed that approximately 16,66 % of the isolates had antifungal activity, but only 11,11 % can be characterized as lactobacilli as they are Gram positive, rod, catalase negative and fermented different carbohydrates (Table 1).

Table 1. Phenotypic characterization of antifungal lactobacilli isolates

Test	LAB strains					
	LB54	LB52	LB53	LB51	LB24	LB20
Source of isolation	Silage	Silage	Silage	Silage	Carrot	Camel milk
Growth at 45°C	-	-	-	-	-	-
CO$_2$ production from glucose	-	-	-	-	-	-
Arginine degradation	-	-	+	-	-	-
Gelatin degradation	-	-	-	-	-	-
Carbohydrates fermentation						
Glucose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Esculin	+	-	+	+	+	-
Melibiose	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Xylose	-	+	-	-	-	-
Inositol	-	+	+	+	-	+
Lactose	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Maltose	+	+	+	+	+	-
Galactose	+	+	+	-	+	+
	Lb[a].plantarum	*Lb.plantarum*	*Lb.farciminis*	*Lb.plantarum*	*Lb.plantarum*	*Lb.plantarum*

[a] *Lactobacillus* spp.

In this study the antifungal properties of five strains of *Lactobacillus plantarum* and one strain of *Lactobacillus farciminis* were evaluated.

The antifungal activity of *Lactobacillus plantarum* has also been reported by other authors: Laitila *et al.* (2002); Sjogren *et al.* (2003); Strom *et al.* (2005); Sathe *et al.* (2007); Delavenne *et al.* (2012); but no publication on the antifungal activity of *Lactobacillus farciminis*.

Lactobacillus spp. strains were able to inhibit the conidia germination, and the mycelia growth. The conidia were more affected than the mycelia. This result is similar to those obtained by Muhialdin and Hassan (2011).

In tested confrontation assay mycelia growth was inhibited and no conidia forming was observed from the survival mycelia in the first two days of incubation, the vertical and horizontal diameters of

the mycelium inhibited by lactobacilli was 3 cm, except with *Lactobacillus plantarum* LB20 strain was equal to 4,5 and 3,7 cm respectively.

However, no growth of the mycelium was observed when the bacteria were streaked in all the surface of plates, due to the high concentration of antifungal compound present on the medium (Table 2, Figure 1).

Table 2. Antifungal activity of LAB and cell-free supernatant tested by overlay and confrontation assays

LAB strains	Overlay method[a]	Supernatant	Confrontation method[b]		Spectrum			
			Two line	All surface	*Trichoderma spp.*	*Penicillium spp.*	*Fusarium roseum*	*Stemphylium spp.*
LB54	+++	-	++	++	+++	+++	+++	+++
LB52	++	-	+	++	+++	+++	+++	+++
LB53	++	-	+	++	+++	+++	+++	+++
LB51	+++	-	+	++	+++	+++	+++	+++
LB20	+++	-	+	+++	+++	+++	+++	+++
LB24	+++	-	++	+++	+++	+++	+++	+++

[a] - = no visible inhibition; + = no fungal growth on 0,1–3 % of plate area; ++ = no fungal growth on 3–8 % of plate area; +++ = no fungal growth on > 8 % of plate area.

[b] - = no inhibition; + = condia formation; ++ = no conidia formation; +++ = no conidia and mycelia growth.
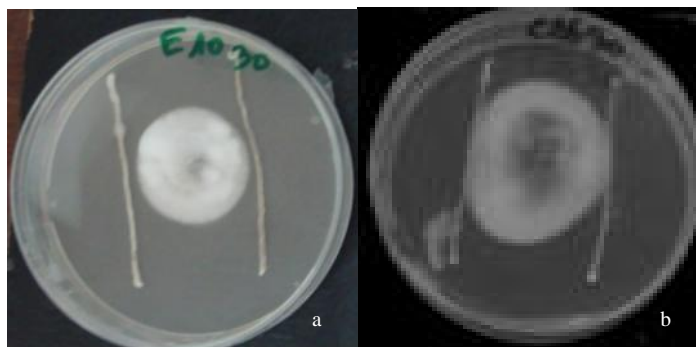

Figure 1. *Antifungal activity of selected LAB strains assayed by confrontation assay.*

(a) Lactobacillus plantarum LB20; (b) Lactobacillus plantarum LB54

Lctobacillus plantarum and *Lactobacillus farciminis* were reported to inhibit the growth of the mycelia of many spoilage fungi including: *Penicillium* spp., *Fusarium roseum*, *Stemphylium* spp. and *Trichoderma* spp. rather than *Aspergillus* spp. (Table 2).

No antifungal activity was detected by the non concentrated cell-free supernatants of all the strains (Table 2). Miescher Schwenninger and Meile (2004), Lan *et al.* (2012) reported that no inhibitory activity could be observed with non-

concentrated cell-free supernatant of strains *Propionibacterium* spp; *Weissella cibaria* 861006 and *Weissella paramesenteroides* 860509.

Antifungal properties characterization

Effect of proteolytic enzymes

The antifungal substances treated with protelotytic enzymes (pepsine, chymotrypsin) showed an inhibitory activity (Table 3), suggesting that the antifungal activity could be not due to protein. Gerez *et al.* (2009) also reported that proteinase K

did not affect the inhibitory activity of the cell-free supernatant. Niku-Paavola *et al.* (1999) working with *Lb. plantarum* showed that proteolytic enzymes did not affect the antifungal activity of the culture filtrate. Ndagano *et al.* (2011) reported that the treatment of supernatant by protelotytic enzymes (pronase, pepsine, Proteinase K and chymotrypsin) showed an inhibitory activity.

However, Rouse *et al.* (2008) in their works the concentrated supernatant of *Lb. plantarum* treated with proteinase K essentially eliminated their antifungal activity. Mauch *et al.* (2010); Guo *et al.* (2011) showed that the proteolytic treatment reduced the antifungal activity of *Lb. brevis* and *Lb. reuteri* R2.

Table 3. Sensitivity of antifungal compounds to proteolytic enzymes and thermic treatments

LAB selected strains	Control	Proteolytic enzymes		Heating	Refrigeration	Freezing
		Pepsin	Chymotrypsin			
LB54	+ [a]	+	+	+	+	+
LB52	+	+	+	+	+	+
LB53	+	+	+	+	+	+
LB51	+	+	+	+	+	+
LB20	+	+	+	+	+	+
LB24	+	+	+	+	+	+

[a] presence of inhibition zone

Effect of temperature

No loss of antifungal activity was observed after heating, refrigeration and freezing treatment (Table 3). Similar results have been reported by Niku-Paavola *et al.* (1999) after heating the culture filtrate of *Lb. plantarum* for up to 15 min at 120°C and by Rouse *et al.* (2007) when heating the concentrated supernatants of *Lb. plantarum*, to either 80°C for 1h or 121°C for 15 min. Gerez *et al.* (2009) reported that heat treatment (100°C for 10 min) of the supernatant of *Lb. plantarum* don't affect the antifungal activity. Miescher Schwenninger *et al.* (2005) reported also the heating of the supernatant of *Lactobacillus paracasei subsp. paracasei* SM20, SM29, and SM63 for 10 min at 100°C don't affect the antifungal activity. However, Gourama (1997) showed the loss of inhibitory activity at 100°C for a period of 10 min. Magnusson and Schnu¨rer (2001) suggested that the loss of activity after storage at -28°C for only 2 days might be due to an irreversible precipitation-denaturation process. Falguni *et al.* (2010) observed that the antifungal activity of the supernatant was lost during prolonged storage. The activity was stable at both refrigerator (7–10°C) and deep freezer (-20°C) temperatures until the sixth day, but there was

rapid reduction of activity under both storage conditions after the sixth day. After the 10th day, in the refrigerated sample the activity was not completely lost, whereas the sample stored in the deep freezer exhibited a complete loss of activity after the 10th day.

Effect of pH

A total inhibition growth of *Aspergillus* spp. by lactic acid bacteria at the pH 2.0 was observed. But the use of malt extract agar medium with pH values at 3.0, 4.0 and 5.0, decreased the antifungal activity of all strains (Fig. 2), therefore most probably to the secretion of organic acids which depends strongly on the pH, since they are active in the undissociated form. In this form, their lipophilic condition permits them to penetrate across the membrane. At a higher intracellular pH, the acid dissociates to release protons and conjugate bases, which disrupt the membrane proton motive force (Cabo *et al.,* 2002).

Since lactic acid bacteria produce organic acids, these might also activate other antifungal compounds such as peptides by lowering the pH. These compounds are therefore also eliminated by neutralization. For example, the antifungal substance described by Magnusson and Schnurer

This paper is available on line at http://www.bioaliment.ugal.ro/ejournal.htm

(2001) is such a low pH activated compound. The activity of this peptide was stable at pH values between 3.0 and 4.5 but rapidly decreased between 4.5 and 6.0.
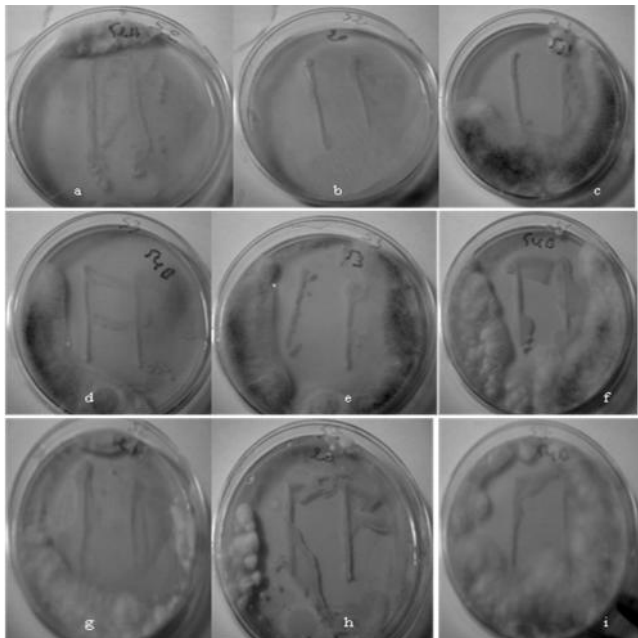

Figure 2. *Effect of pH on the antifungal compounds produced by lactobacilli. pH 2.0 (a) Lb. plantarum LB52; (b) Lb. plantarum LB20;pH 3.0 (c) Lb. plantarum LB51; (d) Lb. plantarum LB54. pH 6.0 (e) Lb. farciminis LB53; (f) Lb. plantarum LB54; (g) Lb. plantarum LB52; pH 7.0; (h) Lb. plantarum LB20;(i) Lb. plantarum LB54*

De Muynck *et al.* (2004) suggested that the neutralization of the supernatant of *Lb. acidophilus* LMG 9433, *Lb. amylovorus* DSM 20532, *Lb. brevis* LMG 6906 and *Lb. coryniformis subsp. coryniformis* LMG 9196 strains to pH values of 5.0, 5.5 and 6.0 indeed retired the antifungal activity.

In our study, at pH 6.0 and 7.0 the antifungal activity of *Lactobacillus plantarum* LB52 and *Lactobacillus plantarum* LB20 strains remained, therefore the antifungal activity not only due to undissociate organic acids, but also by a dissociate organic acids.

Eklun (1983), Skirdal and Eklund (1993) suggested that the inhibition of *Candida albicans, Penicillium chrysogenum, Cladosporium cladosporioides* and *Ulocladium* spp. by sorbic acid was shown to be due to both undissociated and dissociated acid. The dissociated acid caused more than 50 % of the growth inhibition of *Candida albicans* at pH 6.0 (Eklun, 1983).

Volatile compounds effect

No inhibition of mould was observed on any of the dishes sealed with the test strains.

Fungicide or fungistatic effect

Lactobacillus plantarum LB20 and *Lactobacillus plantarum* LB24 strains showed a fungicide effect (Table 4) on the growth of the mycelium, when the mycelium was transferred from a liquid medium to solid one. However, the other strains showed a fungistatic effect.

All tested LAB strains showed a fungistatic effect on the conidia germination. The difference between the fungistatic and fungicide effect on the conidia and the mycelium growth may be due to the state of water: bound water and free water.

No growth of the mycelium was observed in co-culture with LAB in liquid medium even after seven days.

Table 4. *Effect of lactobacilli strains on conidia germination and mycelia growth*

LAB strains	Agar disc-broth	Fungistatic and fungicide effect	
		Mycelium	**Conidia**
LB54	- [a]	+ [b]	+
LB52	-	+	+
LB53	-	+	+
LB51	-	+	+
LB20	-	++ [c]	+
LB24	-	++	+

[a] no mycelium growth;   [b] fungistatic effect; [c] fungicide effect

These data suggested that the antifungal compounds could be not a bacteriocin because this compound was rapidly adsorbed onto the producer cells, or formed spontaneous aggregates, or degraded by proteolytic enzymes, therefore the antifungal effect was eliminated during this phenomenon (Dalié *et al*., 2010; Falguni *et al.,* 2010).

Conclusions

This study shows that LAB isolated from natural product were found to have an antifungal activity against *Aspergillus* spp., produced heat stable compounds, active in acidic pH, and their antifungal activity did not change after treatment with proteolytic enzymes, refrigeration and freezing.

These antifungal compounds have potential to be used as food biopreservation to inhibit conidia germination and mycelia growth of spoilage fungi depending on food type, and pH of food especially in heat, and cold processed foods.

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