Agronomy Research **13**(4), 907–918, 2015

Detection of bacteriocin-producing lactic acid bacteria from milk in various farms in north-east Algeria by a new procedure

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Abstract. Twelve samples of bacteriocin-producing lactic acid bacteria were isolated from raw milk. The screening procedure has the advantage of differentiating directly on agar plates active colonies among thenatural microbial population without subsequent culture. Five of milk isolates had effective inhibitory activity against *Staphylococcus*, *Bacillus* and all *Listeria monocytogenes* strains tested. In addition, two bacteriocinogenic isolates were effective against Gram-negative bacteria including *Pseudomonas aeruginosa* and *Escherichia coli*. The action of the bacteriocins was eliminated by a proteolytic enzyme. Simulation tests in liquid medium showed a 3 log reduction of *Listeria* growth in presence of bacteriocin during a period stockage of 14 days at 4°C.

Key words: bacteriocin, lactic acid bacteria, screening procedure, *Listeria monocytogenes*.

INTRODUCTION

Lactic acid bacteria (LAB) have long been used in the processes of fermentation (milk, meat, vegetables) by participating in organoleptic properties and by ensuring a better conservation of the products. This conservation is mainly due to the production of organic acids by these bacteria (lactic acid, acetic acid) with concomitant decrease of pH that inhibits undesirable contamination flora. Other LAB metabolites such as: hydrogen peroxyde, diacetyl, some enzymes, antibiotics and reuterin can also contribute towards the overall preservative potential of these products. In addition, the LABs can synthesize and excrete antimicrobial compounds of a proteinaceous nature known as bacteriocins (Gibbs, 1987; Axelsson et al., 1993, O'Sullivan et al., 2002).

Research on bacteriocins has been the object of particular attention because of their potential advantages for applying them as natural food biopreservatives (Jeppesen & Huss, 1993; Deegan et al., 2006). Combination of these bacterial metabolites and traditional methods of conservation (heat and chemical treatments) led a higher inhibitory action than that of the hurdles applied separately, with the inherent improvement of nutritional and organoleptic quality of foods sanitized by combined treatments (Aymerich et al*,* 1998).

Some bacteriocins are inhibitory towards a broad spectrum of bacteria which include spoilage microorganisms responsible for modifications of food texture and food-borne pathogens such as *L. monocytogenes* and *Clostridium botulinum* (Blom et al., 1999). Since the bacteriocins may be hydrolysed in the human digestive tract and are active at low pH, these properties render them useful as substitutes of some antibiotics

used for pharmaceutical purposes (Piard & Desmazeaud, 1992). Also, the emergence of bacterial resistance has lead researchers to combine efforts towards developing novel antimicrobial alternatives (Parisien et al., 2007)

The present report describes the development of an original and appropriate methodology for the detection of antimicrobial producing LAB in local fresh and fermented milks, for the purpose of selecting those with inhibitory effects against *L. moncocytogeneses* and/or other pathogenic/food borne microorganisms. This work also describes the simulation of the bacteriocin extracts activity in liquid medium and identification of producer LAB.

MATERIALS AND METHODS

Strains and culture conditions

Lactic acid bacteria (LAB) strains tested for antimicrobial activity were isolated from fresh and fermented milks (curdled milk and buttermilk). These milks came from various farms situated in north-east Algeria (Bazer, Mezloug, Bousselam). A total of 90 samples were collected in 3 farms at a rate of 30 samples per farm corresponding to 10 samples for each type of milk. Samples were collected aseptically in sterile flasks and then rapidly forwarded to the laboratory. The indicator strains used for screening bacteriocin detection are shown in Table 1. *Listeria* strains were cultured at 30°C in tryptone soya yeast (TSY), MRS or M17 for LAB at 30°C and in nutrient medium for the other types of organisms at an incubation temperature of 37°C.

Screening for bacteriocinogenic lactic acid bacteria (LAB) by the mixture method

The technique of detecting cells active against on indicator organisms was based on a novel approach which consisted of adding 0.5 ml of appropriate serial decimal dilutions of the milk to 2 ml of an overnight culture of *L. monocytogenes* CLIP74910 diluted first one hundred timesin tryptone soya yeast (TSY) broth. Milk flora was appropriately diluted in order to obtain well isolated colonies. The surface of Petri dishes containing MRS or M17 agar was inoculated by spreading 0.1 ml of this mixture from every dilution. Milk and indicator organism were cultured at the same time contrary to the traditional procedure where the indicator organism is inoculated into soft agar that is then poured over the plate onto which the milk flora has been grown. Incubation was carried out at 30°C over periods of few days to one week in the search for inhibition zones.

Each whitish colony presenting an inhibition zone was isolated and inoculated into MRS or M17 broth. After incubation from 1 to 2 days at 30°C, a fraction of the culture was streaked onto MRS agar to verify the purity of the isolated cultures. After a second subculture in MRS broth, cultures were preserved at -15°C in cryotubes containing MRS or M17 broth supplemented with 15% of glycerin until use.

Detection of antagonistic activity of isolated cultures

Supernatant fluids were obtained by growing thenhibitory producer strains overnight in MRS or M17 broth. After incubation at 30°C over 18 to 20 h, the cultures were centrifuged and the cell-free supernatant recovered and divided into aliquots that were untreated (crude extract), lyophilized, precipitated with ammonium sulfate subjected to adsorption-desorption method as described by Yang, R. et al. (1992).

Table 1. Indicator strains and origin

Strains	References	Origin
Listeria monocytogenes	CLIP 74910	Pasteur Institute, Paris, France
Listeria monocytogenes	CLIP 74904	
Listeria monocytogenes	CLIP 74903	
Listeria monocytogenes	CLIP 74902	
Listeria ivanovii	CLIP 12229	
Lactobacillus casei subsp. *rhamnosus*	B445	Process Ingineering Laboratory, Nancy University, France
Escherichia coli	CIP 7424	Department of Biology, Setif University, Algeria
Staphylococcus aureus	CIP 7625	
Pseudomonas aeruginosa	76110	
Bacillus subtilis	CIP 5862	
Escherichia coli	K12	
Pseudomonas syringae	11	
Bacillus megaterium	12	
Escherichia coli	18	Parasitology Laboratory, Setif Hospital, Algeria
Escherichia coli	21	
Escherichia coli	101	
Escherichia coli	153	
Escherichia coli	931	
Escherichia coli	ATCC 122	
Escherichia coli	120	
Pseudomonas aeruginosa	254	
Pseudomonas aeruginosa	152	
Staphylococcus aureus	290	
Klebsiella pneumoniae	766	
Staphylococcus aureus	15	
Bacillus sp.	19	
Proteus mirabilis	198	Parasitology Laboratory, El Eulma Hospital, Algeria
Staphylcoccus aureus	71	
Staphylococcus aureus	76	
Bacillus sp.	105	

For the first method, 50 ml aliquots of cell-free cultures were lyophilized (freezing step at -15°C during 24 h; sublimation step for 24 h) and suspended in 5 ml of distilled water (LS supernatant). The ammonium sulfate precipitation of cell-free supernatants was performed as follow: a volume of 50 ml of culture supernatant were made up to 60% saturation by addition of ammonium sulfate and kept overnight at 4°C with gentle stirring. After centrifugation (10,000 × g, 20 min, 4°C), the sedimented pellet was recovered and suspended in 3 ml of 0.1 M potassium phosphate buffer at pH6 (ASPS supernatant). For adsorption-desorption method, a 100 ml of supernatant culture was

used and its pH adjusted at 6.5 to allow adsorption of the bacteriocin to the wall of the producer cell. Then, a temperature of 70°C for 30 min was applied to the culture to kill cells and to inactivate proteolytic enzymes. Cells were then removed by centrifugation at 10,000 × g (20 min, 4°C), and washed twice with 5 mM sodium phosphate buffer at pH 6.5. Cell precipitates were suspended in 5 ml of 100 mM NaCl solution adjusted to pH 2 for allowing desorption of the bacteriocin. Stirring was applied for 2 hour at 4°C and the supernatant (ADS) was recovered after centrifugation at 18,000 × g (30 min, 4°C).

To exclude inhibitory effects of hydrogen peroxide or organic acids, the cell-free extract solutions were dialyzed overnight at 4°C by a dialysis membrane with a 3.5 kDal cutoff against 1.0 liter of distilled water with two changes of distilled water. After dialysis, the solution in the dialysis bag was filter-sterilized (0.2 µm pore-size filter) or heated (70°C, 20 min). Samples were stored at -15°C until use.

The cell-free extracts were tested for bacteriocin activity against indicator bacteria by using agar diffusion methods (agar spot test or agar well test). The agar spot technique was performed as follows: a fraction of 0.1 ml of an overnight culture of indicator bacteria was poured onto an appropriate medium agar plate. Then, one drop of each supernatant fluid with antibacterial activity was spotted on the plate. After incubation for 24h at temperatures optimal for the indicator bacteria, inhibition was indicated by a clear zone around spots (Yang et al., 1992; Cintas et al., 1998). Concerning the agar well test, a quantity of 20 ml of TSY agar was poured onto a sterile Petri dish. Then, the plate was recovered with a 0.3 ml of molten agar (0.7% agar) inoculated with indicator organisms. Wells of uniform diameter (6 mm) were bored in the agar. Aliquots (150 µl) of the tested cell-free supernatants or of positive and negative controls were dispensed in wells, and plates were incubated overnight at optimal temperature during 24 h. Inhibition of growth was determined by an area of inhibition surrounding each agar well (Herranz et al., 2001).

Sensitivity to heat and pronase

Cell-free extracts previously concentrated by the adsorption-desorption method were subjected to heating (63 and 70°C for 30 min; 80; 90 and 100°C for 10 min) and protease treatment (100 µl of pronase E at 2 mg ml^{-1} added to 100 µl of bacteriocin solution and incubated at 37°C for 1 h). The residual activity was measured by the agar spot test against a sensitive indicator lawn. An untreated preparation of bacteriocin served as the control.

Identification of bacteriocinogenic cultures

Cultural, morphological and physiological characteristics of selected isolates and their behavior in certain physico-chemical conditions were determined. Identification tests enclosed Gram coloration, mannitol mobility, catalase, cytochrome-oxydase, peroxydase, sugar fermentation profiles and fermentative type. The fermentation of sugars included glucose, lactose, galactose, fructose, saccharose and glycerin. Development of strains was verified under a wide range of temperature, pH and NaCl concentrations.

Proteins quantification of bacteriocins extracts

Considering that bacteriocins are of protein nature, their quantity was estimated according to Macart et al. (1986) method for quantification of protein by using the following reagent: 0.004% (P per V) of blue of Coomassie G 250; 4% (vol vol^{-1}) of ethanol (96%); 0.003% (wt vol^{-1}) SDS and 10% (vol vol^{-1}) of phosphoric acid (85%). This product was found to be stable for over 3 months. Serum bovine albumin (BSA) dissolved in distilled water at 2 mg ml^{-1} served as a standard. Fractions of 100 µl of standard solutions and bacteriocin samples at appropriate dilutions were added to 2 ml of reagent and mixed. After 10 minutes, the optical density (OD) of the mixture was measured at 595 nm by using a spectrophotometer (Genesys).

Bacteriocin sensitivity measurement

The growth rate of *L. monocytogenes* CLIP74910 on TSY in presence or absence of bacteriocin were estimated according to the method of Huang et al. (1994). The tubes containing 10 ml of TSY broth were inoculated with 100 µl of overnight *Listeria* cultures corresponding approximatively to a final population of 10^6 UFC ml^{-1} and, to which we added 0.2 ml of bacteriocin preparation. A tube without addition bacteriocin extract served as a negative control. Because *Listeria* is a psychrophilic bacterium, tubes were placed in refrigeration at 4°C, and the OD was measured at 600 nm at time 0 and at 2 days intervals until 14 incubation days. Cultures at 37°C were also performed and samples were removed at different time intervals for OD measurements at 600 nm and for viable counts. Measurements of plating counts and OD were carried out at 37°C for 0, 4 h, 8 h, 24 h, 30 h, and 48 h on *Listeria* cultures in order to establish a standard curve.

RESULTS AND DISCUSSION

The detection of antibacterial-producing strains in milk and fermented milks was performed directly on MRS and M17 agar by inoculating these media with a combination of an appropriate dilution of the product under study and an overnight indicator culture of *L. monocytogenes*. The colonies showing halos after incubation of plates at 30°C for 2–7 days were selected and, their visualization is reported in Fig. 1. A total of 40 and 10 strains grown on MRS and M17 agar respectively, exhibited inhibitory activity against *L. monocytogenes* strain (results not shown). Only 30 isolates were found by the agar spot assay to secrete antibacterial substances into the growth medium that could be detected without concentration of the supernatant fluids. Results also shows that twelve of these isolates secreted antibacterial compounds that preserved their inhibitory effect after dialysis. This fact and their sensitivity to pronase and the positive correlation between inhibitory activity and protein concentration suggest that the 12 supernatants are bacteriocins.

Their activity was stable under heating (60°C for 30 min or 100°C for 10 min). Such temperature stability is very convenient to use the bacteriocin extracts as a food preservative since many processing procedures involve a heating treatment. Many authors have demonstrated bacteriocin resistance to pasteurization and high temperature (121°C for 30 min) (Deraz et al. 2005). Thermoresistance is a characteristic of lactic acid bacteria bacteriocins having low-molecular-weight and a simple structure (Desmazeaud, 1994).

A wide range of Gram-positive and Gram-negative bacteria were used to check the inhibition spectrum of these 12 supernatants. Results are shown in table 2. Inhibition activity was demonstrated by test strains against practically all tested and 40Z had the advantage of being effective against Gram-negative bacteria so inhibiting some strains of *Pseudomonas*, *Klebsiella* and *E.coli.* Most of the bacteriocins produced by LAB are active only against LAB and other gram-positive bacteria (Line et al., 2008; Ray, 1996; Bhunia et al., 1991). None of the tested strains displayed inhibitory activity against *Lactobacillus casei* subsp. *rhamnosus* and *Proteus mirabilisListeria, Staphylococcus* and *Bacillus* strains. In addition, two bacteriocin extracts from isolates 28Z.

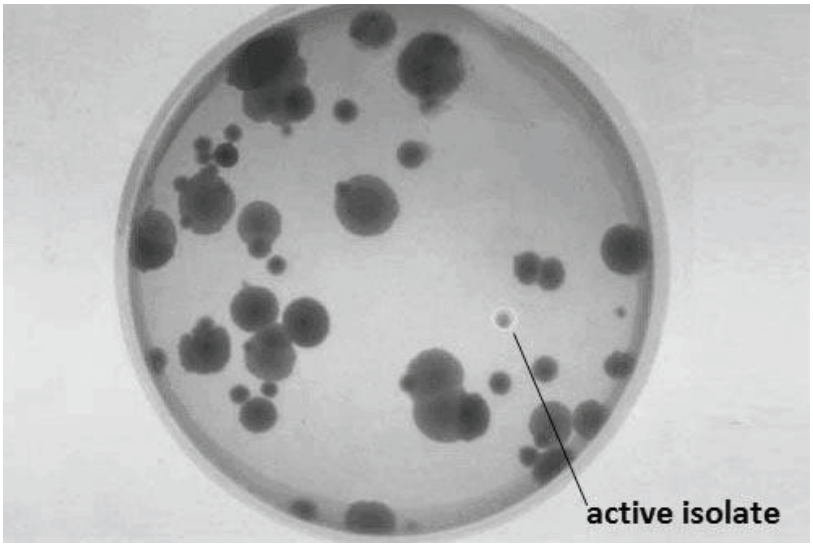

Figure 1. Screening of bacteriocinogenic strains from milk by mixture method.

The resistance of Gram-negative bacteria to bacteriocins seems to be due to the complexity of their cellular wall in comparison to Gram-positive bacteria, containing lipopolysaccharides (LPS) which are absent in Gram-positive bacteria. The results of the present work suggest that the 28Z and 40Z antibacterial extracts could destabilize the LPS layer of Gram-negative bacteria (Kalchayanand et al., 1992; Motta et al., 2008) and, correspond qualitatively to those described by several authors. Rodriguez et al. (2005) described a slight inhibitory effect of bacteriocins of LAB in cheeses on the survival of *E. coli*, which cannot be related to differences in pH values. The authors attributed the effect to a higher sensitivity of injured cells of Gram-negative bacteria to bacteriocins, an injury due to a prolonged acid exposition at the low pH values of cheese (Kalchayanand et al, 1992). Tan et al. (2000) described inhibition of both an ampicillin resistant *E. coli* and a *Salmonella typhii* strains by a bacteriocin produced by *Enterococcus faecalis VRE* 1492. Ponce et al. (2008) described similar inhibition activity on Gram-negative foodborne pathogens for some LAB strains from organic vegetables. In recent study, LAB isolated from olives had developed antimicrobial activity against *Listeria, E. coli and Enterococcus* strains (Gaamouche et al., 2014).

Concerning antimicrobial substances lost after dialysis for 38 remainder supernatants, it is probable that are formed by organic acids, hydrogen peroxide (Muriana & Luchansky, 1993) or non-proteinaceous low molecular mass compounds (LMMC) (Niku-Paavola et al., 1999) capable to cross the 3.5 kDa cutoff dialysis membrane used.

Table 2. Inhibitory spectrum of the antibacterial substances produced by selected isolates.

	Indicator strains	1Z	7Z	9Z	12Z	15Z	23Z	28Z	32Z	36Z	40Z	44Z	50Z
Gram negative	*Pseud. aeruginosa 7625*			-	-		-		-	-		-	-
	Pseud. aeruginosa 254	-	-	-	-	-	-	-	-	-	-	-	-
	Pseud. aeruginosa 152	-	-	-	-	-	-	+	-	-	+	-	-
	Pseud. syringae 11	-	-	-	-	-	-	+	-	-	±	-	-
	Kleb.. pneumoniae 766	-	-	-	-	-	-	+	-	-	+	-	-
	Prot. mirabilis 198	-	-	-	-	-	-	-	-	-	-	-	-
	Esch. coli CIP 7424	-	-	-	-	-	-	+	-	-	+	-	-
	Esch. coli 18	-	-	-	-	-	-	+	-	-	-	-	-
	Esch. coli 21	-	-	-	-	-	-	-	-	-	-	-	-
	Esch. coli 101	-	-	-	-	-	-	+	-	-	-	-	-
	Esch. coli 153	-	-	-	-	-	-	-	-	-	-	-	-
	Esch. coli 931	-	-	-	-	-	-	-	-	-	-	-	-
	Esch. coli ATTC 122	-	-	-	-	-	-	-	-	-	-	-	-
	Esch. coli 120	-	-	-	-	-	-	+	-	-	-	-	-
	Esch. coli k12	-	-	-	-	-	-	+	-	-	±	-	-
Gram positive	*L. monocyt. CLIP74910*	+	+	±	±	+	±	+	±	±	+	±	±
	L. monocyt. CLIP74904	+	+	±	±	+	±	+	±	±	+	±	±
	L. monocyt. CLIP74903	+	+	+	+	+	+	+	+	+	+	+	+
	L. monocyt. CLIP74902	+	+	±	±	+	±	+	±	±	+	±	±
	L. ivanovii CLIP 12229	+	+	+	+	+	+	+	+	+	+	+	+
	S. aureus CIP 7625	+	+	+	+	+	+	+	+	+	+	+	+
	S. aureus 290	+	+	±	-	+	-	+	-	±	+	-	+
	S. aureus 71	+	+	-	-	+	±	+	+	±	+	+	-
	S. aureus 76	-	-	+	+	+	+	+	±	±	+	±	+
	B. subtilis CIP 5862	+	+	+	+	+	+	+	+	+	+	+	+
	Bacillus sp. 19	+	+	±	±	+	-	+	-	-	+	-	±
	Bacillus sp. 105	+	-	-	-	-	-	±	+	+	±	±	-
	B. megaterium 12	+	+	+	+	+	+	+	+	+	+	+	+
	L. casei sp. rhamnosus B445	-	-	-	-	-	-	-	-	-	-	-	-

+ inhibition of indicator strain;
- absence of inhibition of indicator strain;
± weak inhibition.

Fig. 2 shows the inhibitory activity of 5 isolates exhibiting the strongest activity against *Listeria* when the agar well diffusion test was used. According to physiological assays, morphological characteristics and carbohydrate fermentation of these isolates (data not shown), all of them produce whitish, bulging and mucous colonies similar to those of lactic acid bacteria on MRS or M17 agar plate, are Gram-positive, catalase-

negative, oxydase-negative, benzidine-negative and non motile. On the basis of their biochemical and morphological characteristics, strains 1Z and 28Z seem to be, respectively, identical to *Pediococcus acidilactici and P. pentosaceus,* the strain 7Z to *Streptococcus thermophilus*, the strain 15Z to *Lactococcus plantarum* and the strain 40Z to *Lactobacillus* sp.

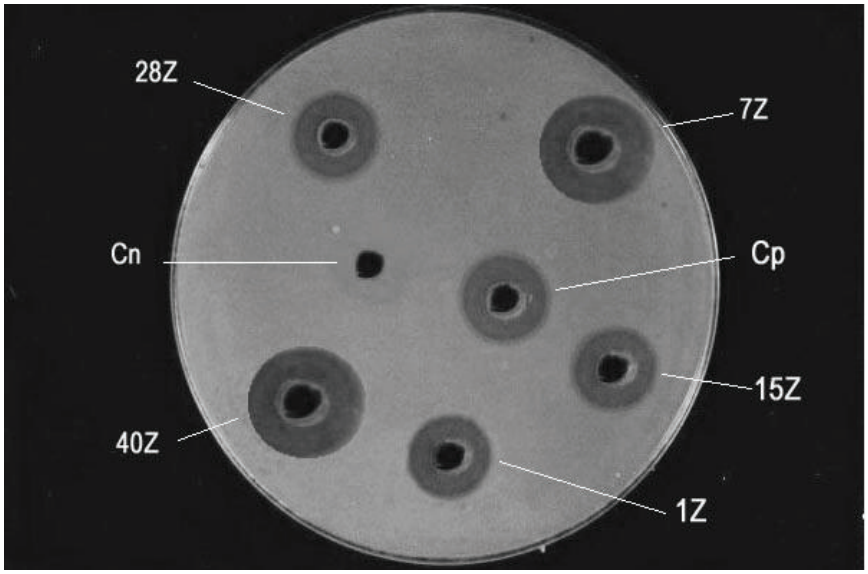

Figure 2. Activity of bacteriocin extracts against *Listeria monocyogens* CLIP 74910 using agar well diffusion assay. Bacteriocin extracts are adsorbed/desorbed, dialyzed and filter-sterilized before testing against indicator lawn. Cn – negative control (sterile MRS broth); Cp – positive control (filter-sterilized Nisaplin solution at 10%).

The growth patterns of four strains of *L. monocytogenes* and one strain of *L. ivanovii* in TSY broth subjected to antibacterial compounds derived from supernatant broths of the active strains B1Z, B7Z, B15Z, B28Z, B40Z were also determined. The results are shown in Fig. 3. All *Listeria* strains grew in TSY with/without the antibacterial substances produced by either isolates. However, The OD measured in TSY supplemented with antibacterial substances produced by either isolates were significantly lower than O.D. detected in the control TSY at the initiation of the experiment and at the end of incubation period. Analysis was done within 24 h old-cultures indicating that the anti-*Listeria* effects of either supernatant manifested themselves rapidly. After this initial reduction, the population of *Listeria* remained essentially constant throughout the first 3 days. Thereafter, OD values increased progressively to 14 days with values fluctuating approximately from 0.02 to 0.15. By way of contrast, in the absence of bacteriocin, values of OD are much higher and vary from 0.02 to 0.55 with an acceleration of growth rate after 5 at 7 days incubation depending on *Listeria* strain tested. This acceleration is seen from an inflection on the growth curve. OD values of controls are about 3.5 to 5 times higher than OD of strains in presence of bacteriocins at the same incubation period.

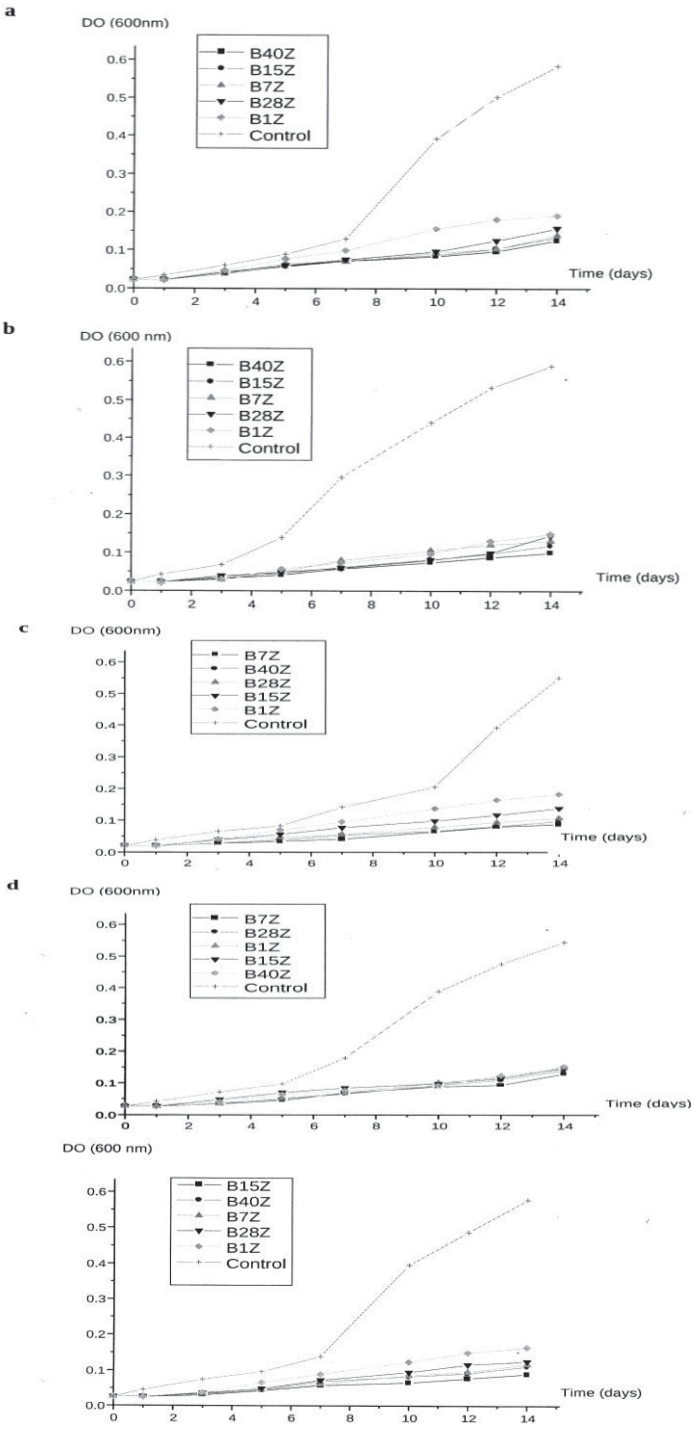

Figure 3. Populations of *Listeria monocytogenes* strains 74903 (a); 74910 (b); 743904 (c); 74902 (d) and *Listeria ivanovi* 12229 (last graph) incubated at 4°C for up to 14 days in TSY supplemented with bacteriocin preparations derived from 5 selected isolates.

In this study, bacteriocin extracts inhibited markedly the growth of *L. monocytogenes* strains tested in liquid media. The reduction of *Listeria* populations in presence of bacteriocins is about of 3 log as according to the standard curve obtained (data not shown). Similar results were obtained by Huang and al. (1994) having used milk which was experimentally contaminated with *Listeria* cultures in presence and in absence of pediocin 5. In another study, tests were made on raw milk contaminated artificially by *L. monocytogenes* in both the presence and in the absence of a bacteriocin produced by *Carnobacterium piscicola JG 126*. Under these conditions, piscicolin 126 reduced the number of *Listeria* from 4 to 5 log during the first day of storage (Wan et al., 1997). However, the *Listeria* growth appeared again after an incubation period of 24 h. This phenomenon was also observed for the nisin with apparition of mutants resistant to this bioactive component (Wan et al., 1995). As reported by Altuntas et al. (2012), all *L. monocytogenes* isolated from foods were sensitive to the cell-free supernatant of a bacteriocin-producing strain *P. acidilactici* while some *Listeria* strains were resistant to antibiotics. The results suggest the possibility to use the bacteriocinogenic strains or their bacteriocins as supplements to food in order to reduce unwanted contamination.

CONCLUSION

The used method allowed direct detection of isolates with antagonistic activity onto agar plate by facilitating the distinction of active colonies among the microbial population. This screening procedure is easy to execute and could be used as an alternative method to traditional antagonism tests. Further research is needed to identify compounds produced by the selected LABs, their purification and sequencing. This type of work is in progress in our laboratory. Bacteriocins active against pathogens and food spoiling microorganisms are presented as an interesting alternative to chemical preservatives in a variety of industrial applications.

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