Global Journal of Biotechnology & Biochemistry 6 (3): 154-161, 2011
ISSN 2078-466X

Screening of Bacteriocin-Producing Lactic Acid Bacteria Isolated from West Algerian Goat's Milk

N. Saidi, M. Hadadji and B. Guessas

Laboratoire de Microbiologie Appliquée, Département de biologie Faculté des Sciences,
Université d'Oran BP 1524 El-menaouer, Es-Sénia, 31100, Oran, Algeria

Abstract: Fifty nine lactic acid bacteria isolated from Algerian goat's milk and previously selected according to their technological properties. They were screened for antimicrobial activity. From 3258 bacterial couples brought into the study, we observed 747 cases of inhibitions (22.93%). The results obtained showed that the lactobacilli have high spectrum of action. Thus, for the various species: *Lc. lactis* subsp. *lactis* (40.1%); *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (29.1%); *Leuconostoc* (25.8%); *Streptococcus* (56.9%); *Pediococcus* (36.5%) and *Lactobacillus* (13.5%). For the whole of the interactions brought into studies, the lactobacilli inhibited 392 strains of 1331 couples (29.5%); the other species have a more reduced spectrum of action: *Lactococcus* (18.76%); *Leuconostoc* (25.7%); *Streptococcus* (17.5%); and *Pediococcus* (5.34%). Six strains showed inhibitory activity in solid medium (well diffusion assay) when tested against the effects of organic acid and hydrogen peroxide were eliminated. These strains did not show inhibitory activity after treatment with proteinase K, trypsin, or α-chymotrypsin. *Lactococcus lactis* subsp. *lactis* LCL01 produced a heat stable substance with a proteinaceous nature and with bactericidal action, suggesting a bacteriocin-like. *Lactobacillus plantarum* LBP01 act positively on *Escherichia coli* by producing a bacteriocin.

Key words: Lactic Acid Bacteria · Bacteriocin · Milk · Bactericidal · *Lactococcus* · Interaction

INTRODUCTION

The isolation and characterization of news strains of lactic acid bacteria from various biotopes took a great interest these last decades [1-5].

Lactic acid bacteria are traditionally used as starters for food fermentations. Since they have a capacity to inhibit spoilage and pathogenic bacteria [6-10], they are important in food preservation and intestinal prophylaxis. Lactic acid bacteria are the most important groups for industrials purposes, since their fermentative activity involves a notable preservative capacity as a result of the drop in the pH and the antimicrobial activity of their metabolites such as lactic and acetic acid, diacetyl or bacteriocins.

Bacteriocins are antimicrobial peptides produced by many lactic acid bacteria (LAB), which are directed mainly to inhibit the growth of related species or species with the same nutritive requirements [11-15]. Some bacteriocins have been used to inhibit this pathogen in food, either through bacteriocin-producing cultures [16-17] or by the addition of pure or semi pure bacteriocin preparations [18]. Many lactic acid bacteria, including members of the genera *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus* and *Pediococcus*, are known to secrete bacteriocins [20], many of them inhibit *Listeria monocytogenes* [9,21]. Some bacteriocins have been used to inhibit this pathogen in food, either through bacteriocin-producing cultures [15] or by the addition of pure or semi pure bacteriocin preparations.

The aim of this study was the search for the bacteriocins produced by lactic acid bacteria isolated from Algerian goat's milk. The objectives of the present paper were as follow: (i) to determine the nature of lactic acid bacteria from raw goat's milk of West Algeria; (ii) to study the antibacterial potential of wild isolates of LAB; (iii) to characterize the main properties of this bacterial inhibitor in the crude extract and (iv) to determine the range of antimicrobial activity of LAB against a variety of microorganisms.

Corresponding Author: B. Guessas, Laboratoire de Microbiologie Appliquée, Département de biologie Faculté des Sciences, Université d'Oran BP 1524 El-menaouer , Es-Sénia, 31100, Oran, Algeria.

MATERIALS AND METHODS

Bacterial Strains: The following 59 strains of LAB tested for their antagonistic activity were isolated from the Algerian goat's milk: *Lc. lactis* subsp. *lactis* (eighteen strains), *Lc*. *lactis* subsp. *lactis* biovar. *diacetylactis* (four strains), *Ln. mesenteroides* subsp. *dextranicum* (five strains), *Sc. thermophilus* (five strains), *Pc. acidophilus* (three strains), *Lb*. *plantarum* (seven strains), *Lb*. *salivarus* (six strains), *Lb*. *brevis* (six strains) and *Lb*. *helveticus* (five strains). The procedures for isolating, identifying, technologically characterizing and selecting these strains were those described in earlier work [2].

Antagonistic Substances Detection: Each set of master plates was replicated three times with a multi-inoculator on MRS or M17 agar and incubated at 30°C for 18 h. The replica plates were overlaid with molten agar seeded with other strain and incubated. Plates were then examined for zones of inhibition. We sought the zones of inhibition of growth which resulteds in clear rings around the strains sown into key. All strains demonstrating antagonism were transferred from the master plates, purified and stocked in 20% glycerol at-20°C.

Research of the Nature of the Inhibiting Agent: Inhibitions can be caused by several agents such as acidity, hydrogen peroxide, phages and bacteriocins. The research of the nature of the inhibiting agent was started in solid and liquid medium.

Acidity Production: The multiplication of LAB is accompanied by a production of acid causing the reduction in the intracellular and the extracellular pH [22]. To minimize the acid production, we used LBP medium containing 0.25% glucose and plugged with buffer phosphate.

Hydrogen Peroxide Production: To detect the production of H_2O_2, the cultures were carried out in the presence of catalase at a rate of 1mg/ml of medium. The enzyme and the indicator strain are mixed in the semi-solid medium (0.8% agar). After incubation, the reading of the results is done by comparison with the control without catalase.

Detection of Lytic Bacteriophage: To detect the presence of lytic bacteriophage, a portion of the clearing zone was cut from a spot deferred antagonism assay plate. The agar plug was added to 3 ml of broth and macerated with a sterile medium. The mixture was held at room temperature for 1 h. A 100µl amount of the suspension and 100µl of an indicator strain (grown overnight) were suspended in 8 ml of soft (0.8 %) agar. The soft-agar suspension was poured evenly over an agar plate and incubated overnight at 30°C. The formation of plaques indicate the phage activity.

Bacteriocins Production: The effect of the proteolytic enzymes on the inhibiting activity of the selected strains was carried out at the same time on liquid medium and solid medium. To ensure itself of the protein nature of the inhibiting substances, we used the proteolytic enzymes: pronase, α-chymotrypsin and trypsin. Each enzyme is dissolved in plug phosphates buffer (10 mM, pH 7.0) with a concentration of 10mg ml^{-1} and sterilized by filtration (0.45 µm). During the treatment by the pronase, the trypsin and the α-chymotrypsin, the filtrate containing these enzymes is incubated during 1hour with 37°C. The sensitivity of an antibacterial substance to a given enzyme is appreciated by determining the residual activity by measurement of the diameter of zone of inhibition.

Preparation of Culture Supernatants: Sterile cell-free culture of 18 h bacterial suspension was obtained by centrifugation (10000g for 15 mn at 4°C) and filtration through a 0.45 µm pore-size filter (Millipore). They were adjusted to pH 7.0, with NaOH 2 mol l^{-1}, to eliminate any effect of acidity. Inhibitory activity due to hydrogen peroxide was suppressed by the addition of catalase (3600 U ml^{-1}, Sigma). Filtrates were also treated with trypsin, α-chymotrypsin and protease (Sigma Chemical Co.). Enzymes were filter-sterilized in 50 mmol l^{-1} phosphate buffer, pH 7.0. Commercial protease preparations were used at a 1 mg/ml final concentration. Samples and blanks were incubated at 37°C for 1 hour and added to crude bacteriocin preparations at final concentration of 1 mg/ml. The supernatant of 500ml of two strains was concentrated 10-fold by using a rotavapor. The concentrated culture supernatant was used as a source of bacteriocin-like substance.

Kinetics of Growth: The antimicrobial effect of the supernatant was tested against the indicator strains in liquid medium M17 (20ml) and the filtrate was concentrated 10 fold or not concentrated (1%) with treatment or without was inoculated with 200µl from overnight culture of indicator strain. At interval, samples were removed for measurement of absorbance at 660 nm.

Agar Well Diffusion Method: Twenty strains were screened for the antibacterial activities of LAB including *Enterococcus* (ten strains), *E. coli* (one strains), *Bacillus substilus* (one strain) and *Staphylococcus aureus* (two strains). LAB cultures were screened for antagonistic substances detection by the agar well-diffusion method [11]. Wells were cut with a sterile tube (8 mm in diameter) in agar media plates seeded with an indicator culture. The culture supernatant (50 µl) obtained previously was placed into the wells with or without treatment. After diffusion of the supernatant into the agar (4 h at 4°C), the agar plats were incubated overnight at the appropriate temperature. The assays were performed at a final concentration of 1mg/ml for all enzymes. Samples with and without treatment were held at appropriate temperature for 1hour. The remaining activity in both samples after enzymes digestion was detected by the agar well-diffusion method, against sensitive indicator. To test for heat activity, culture supernatant was heated at 121°C for 15 min. The purpose of the study achieved is to see whether the antibacterial substance produced by *Lactococcus lactis* subsp. *lactis* (Lc01) has indeed a bactericidal effect or a bacteriostatic effect. Stability of the concentration in viable bacteria showeds a bacteriostatic effect; whereas, a reduction in this concentration indicateds a bactericidal effect [23].

RESULTS

Screening for Bacteriocinogenic LAB: The whole results obtained are gathered in table 1 and figure 1 shows the action of our strains on indicator strains (*Lb. salivarius* LBS01 and *Lc. lactis* subsp. *lactis* LCL09). Out of 3258 bacterial couples brought into experiments, we observed 747 cases of inhibitions (22.93%). It was noted that *Lc. lactis* subsp. *lactis* was the strongest inhibiting species among the Lactococci; it is responsible for 107 inhibitions from 121 (88.43%). *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* is responsible for only 14 inhibitions (11.57%). For the whole of the interactions brought into experiment, the lactobacilli inhibited 392 strains from 1331 (29.5%); the other species have a more reduced spectrum of action: *Lactococcus* (18.76%); *Leuconostoc* (25.7%); *Streptococcus* (17.5%); and *Pediococcus* (5.34%).

Nature of Inhibitory Agent

Acidity and Hydrogen Peroxide Production: The acid production is responsible for six cases of inhibition (40%). We noted four types of responses (Table 2): (i) the inhibition is lost with LCL05, LCL13, LNM04, LBP01; and LBH01; (ii) the activity was decreased with LCL18 and LCN04; (iii) an increase of inhibition with LCL10 and (iv) the inhibition is maintained with LCL01, LCL10, LCL18, SCT05, LBP02 and LBH03.

Table 1: Interaction between LAB isolated from Algerian goat milk and the tested strains

		Strains inhibited					
Strains inhibiting		*Lc.*	*Ln.*	*Sc.*	*Pc.*	*Lb.*	Total
Lactococcus	NC	484	110	110	66	429	1199
	NI	121	21	32	12	39	225
	%	25	19.1	29.1	18.2	9.09	18.77
Leuconostoc	NC	110	25	25	15	105	280
	NI	40	2	5	0	25	72
	%	36.4	8	20	0	23.8	25.71
Streptococcus	NC	110	25	25	15	105	280
	NI	22	5	6	0	16	49
	%	20	20	24	0	15.2	17.50
Pediococcus	NC	66	15	15	9	63	168
	NI	9	0	0	0	0	9
	%	13.6	0	0	0	0	5.35
Lactobacillus	NC	514	124	123	74	496	1331
	NI	196	32	70	27	67	392
	%	38.1	25.8	56.9	36.5	13.5	29.45
Total	NC	1284	299	298	179	1198	3258
	NI	388	60	113	39	147	747
	%	30.2	20.1	37.9	21.7	12.3	22.93

NC: number of couples; NI: number of inhibitions; %: percentage of inhibitions

Table 2: Nature of inhibitory agent (indicator strain: *Lc. lactis* subsp. *lactis* LCL09)

				Medium added with 1mg/ml of			
Strains	Codes	M	MT	Ca	C	P	T
Lactococcus lactis subsp. *lactis*	LCL01	5	5	5	0	0	0
	LCL05	4	0	3	4	0	0
	LCL10	6	9	5	0	4	0
	LCL13	6	0	5	7	5	0
	LCL14	7	6	0	0	0	4
	LCL18	9	5	3	0	0	0
biovar. *diacetylactis*	LCN04	6	3	0	5	0	4
Ln. mesenteroides subsp. *dextranicum*	LNM01	4	5	0	4	4	5
	LNM04	4	0	0	5	5	4
Sc. thermophilus	SCT05	5	5	6	0	0	0
Lactobacillus plantarum	LBP01	6	0	0	5	5	5
	LBP02	6	6	5	7	0	7
	LBP03	6	0	0	0	7	4
Lactobacillus helveticus	LBH01	5	0	1	5	0	0
	LBH03	6	6	7	0	0	0

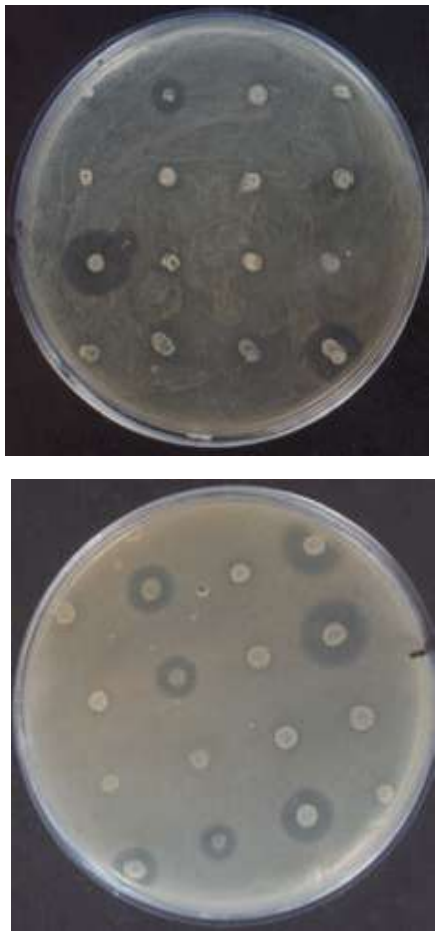
M: medium M17 or MRS without treatment, MT: medium plugged at pH7, Ca: medium added with catalase, C: medium added with α-chymotrypsin, P: medium added with protease, T: medium added with trypsin.

Seven cases of inhibition (46.7%) were observed when the catalase is added to M17 or MRS (hydrogen peroxide production).

Bacteriocin Production: We had different responses to the action of proteolytic enzymes (Table 2). The inhibitory agent is: (i) sensitive for the three enzymes for LCL01, LCL18, SCT05 and LBH03; (ii) sensitive only to the protease that belong LCN04 and LBP02 and (iii) resistant only to the protease of LCL10. Table (2) also shows that the antimicrobial substance was also proteinic in nature and resistant only to α-chymotrypsin with LCL05 and LBH01 and; sensitive only to the trypsin with LCL13. We noted that for three strains (LNM01, LBP01 and LBP03), the acidity and hydrogen peroxyde production were the main inhibitory agents.

In Liquid Medium: Only one filtrate concentrated 10 times gave us conclusive results (Figure 2). Inhibition by *Lc. lactis* subsp *lactis* (LCL01) is maintained with supernatant at pH 7, added with catalase or protease or α-chymotrypsin. The inhibition observed in solid medium added with trypsin is loss in liquid medium. It is that, in liquid medium, the bacteriocins are in a chemical configuration which makes the action of the proteolytic enzymes.

The inhibitory activities of culture supernatants were not modified after a treatment for 60 min at 120°C. Inhibitory activity was fully observed for supernatants which adjusted pH values 7 and with catalase. Inhibitory activity was totally lost by proteinase K, α-chymotrypsin



(a)

(b)

Fig. 1: Interaction between LAB and different indicator strains: (a) *Lb. salivarus* LBS01; (b) *Lc. lactis* subsp. *lactis* LCL09).

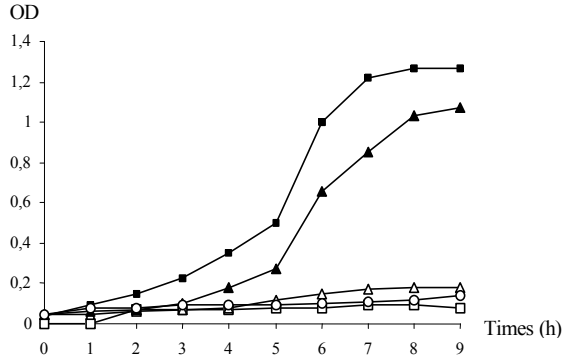


Fig. 2: Kinetics of growth of *Lc. lactis* subsp. *lactis* (LCL09) with the filtrate of *Lc. lactis* subsp. *lactis* LCL01. ■: M17, Δ: M17 added with filtrate at pH 7 and catalase, ▲: M17 added with filtrate at pH 7 and α-chymotrypsin, ○: M17 added with filtrate at pH 7 and trypsin, □: M17 added with filtrate at pH 7 and protease.

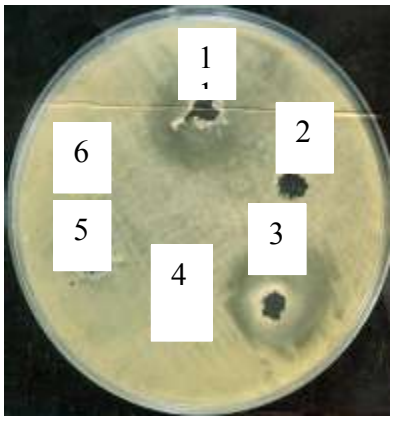


Fig. 3: Inhibition of *Lc. lactis* subsp. *lactis* LCL09 by LCL01 culture supernatant by the agar well diffusion assay. Wells contained cell-free supernatant was treated:
well 1: at pH 7, well 2: heated for 60 min at 120°C, well 3: adding catalase (1 mg/ml), well 4: adding trypsin (1 mg/ml), well 5: adding α-chymotrypsin (1 mg/ml) and well 6: adding protease (1 mg/ml).

and trypsin. This result suggests that a heat-stable proteinaceous compound was responsible for the inhibitory activity of the culture supernatant of *Lc. lactis* subsp. *lactis* LCL01.

Agar Well Diffusion Method: The results obtained confirmed that the inhibitory factor is a substance of a proteinaceous nature. No inhibition zone was detected after treatment with the enzymes (Figure 3).

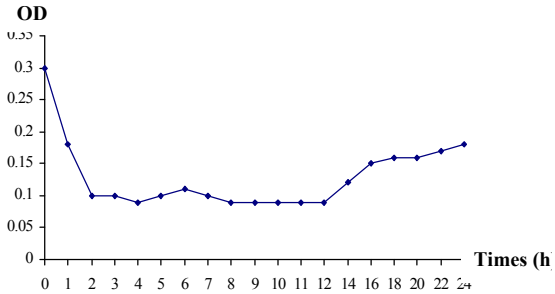


Fig. 4: Mode of action of antibacterial substance produced by *Lc. lactis* subsp. *lactis* (LCL01) against *Lc. lactis* subsp. *lactis* LCL 09

Fig. 5: Spectrum of action of LAB against *E. coli*. (Only one strain inhibit *E. coli*, *is Lactobacillus plantarum* LBP01).

Mode of Action: The viability of the bacterial indicator *Lc. lactis* subsp. *lactis* LCL11 in the supernatant incubated at 30°C with *Lc. lactis* subsp. *lactis* LC01 was measured. After only one hour of incubation, a reduction of growth was observed (initially the OD was 0.18) and After became 0,3 after two hour of incubation (Figure 4), it decreases to 0.1. The antibacterial substance produced by LCL01 thus presenteds a mode of action of the bactericidal type and this bactericidal effect was relatively fast. The speed of the bactericidal effect is a characteristic of the majority of the bacteriocins.

Spectrum of Activity: Our results showed that the bacteriocins didn't inhibit the growth of the tested gram-negative bacteria. On the other hand, *E. coli* was the only one which was affected by the inhibiting substance produced by *Lb. plantarum* LBP01 which has a characteristic of the bacteriocin-like (Figure 5).

DISCUSSION

The objective of the present study was to make an extensive screening program of lactic acid bacteria isolated from Algerian goat's milk, in order to demonstrated the antagonistic activity [2,25]. The knowledge of the interactions between lactic acid bacteria remains a significant criterion for strains selection used in industrial fermentations. From our study, we obtained 747 cases of inhibitions (22.93 %); this percentage is relative because it depends on the culture conditions and also on the indicating strain used [26]. Barefoot and Klaenhammer [27] and Schillinger and Lücke [28] recorded lower (15.5%) and higher (81%) results. Rammelsberg and Radler [29] found that out of 79 *Lactobacillus* only 12 had an inhibiting activity (15%). El-Shafei *et al.* [30] reported that 36 out of 100 isolates from traditionally fermented products produce a bacteriocin such as the nisin [30] and Navarro *et al.* [31] stated that 9 out of 42 strains of lactic acid bacteria produce bacteriocins.

The presence of inhibition ring does not mean production of bacteriocin inevitably. From the tests, it was necessary to know the exact nature of the inhibiting agent. It may be that inhibition is due to the production of organic acids, hydrogen peroxide, phages and/ or bacteriocin [11,32].

The isolated bacterial strains were not lysogenic, certain authors announced that the ranges of lyses are not always detectable. Indeed in certain cases, the lysogenic phage exists but does not give ranges of lysis [33]. When the interaction between the lactic acid bacteria is not due to the bacteriophages, it is caused by the release of molecules as the hydrogen peroxide [34,35]; organic acids or the bacteriocin [11,32]. We showed that the inhibition caused by *Ln. mesenteroides* subsp. *dextranicum* (LNM04) and *Lb*. *plantarum* (LPB01) was due to the acid and hydrogen peroxide production. For the 15 strains tested the acid and/or hydrogen peroxide production is responsible for approximately 40% of inhibitions.

In the current work, the following strains *Lc. lactis* subsp *lactis* LCL05, LCL13 and LCL14, *Lc. lactis* subsp *lactis* biovar *diacetylactis* LCD04, *Lb*. *plantarum* LBP02 and LBP03 synthesized a bacteriocin-like.substance. Gilliland and Speck [36] showed that the addition of catalase in the culture media reduced the inhibition but does not eliminate the production of H_2O_2 with *Lb*. *acidophilus*. According to those authors, the hydrogen peroxide is partially responsible for antagonism. The antibacterial action produced by *Lb*. *acidophilus* is probably due to a combination of factors including acidity, hydrogen peroxide and other inhibiting substances. The inhibitory agents produced by the isolated lactic acid bacteria examined in this study could be characterized as bacteriocins-like, since inhibition due to acid, hydrogen peroxide and bacteriophages have been excluded. Also, the proteinaceous nature of the inhibitory substances produced by the strains was confirmed by their protease sensitivity. Some inhibiting substances were characterized as being antimicrobial proteins called bacteriocins. They should be sensitive to the action of the proteolytic enzymes [11,12,37,38]. Some strains produce only proteomic substance which would act like bacteriocin.

The response of the culture (solid medium or liquid medium) to the action of proteolytic enzymes was not the same. It is possible that this antagonist agent containeds only one substance made up of protein nature. In the literature, the recovered bacteriocins have various reactions with the proteolytic enzymes action. The inhibiting substance produced by *Ln. gelidum* UAL187 was sensitive to the treatment of pronase and trypsin [39]. *Lb. brevis* produces brevicin 37 whose action was inactivated by the pronase E and trypsin just as the casicin 80 synhesized by *Lb. casei* is sensitive to the protease E and α-chimotrypsin [29]. The action of the proteolytic enzyme didn't raise the inhibition completely, The antimicrobial activity of *Lb. plantarum* J-51 is lost after treatment with protease [41]. Gasserin, a bacteriocin produces by *Lb. gasseri* is sensitive to the action of proteolytic enzymes and resistant to heat [40] like leucocin BC2 and lactocin G13 produces respectively by *Leuconostoc mesenteroides* and *Lactococcus lactis* [41].

Several authors raised the difference between the results of inhibition on solid medium and liquid medium. In the majority of the cases, inhibition is lost in liquid medium. This can be due to several factors; the activity can be lost by filtration through the membrane of 0.2µm case of the bacteriocin of *Pediococcus damnosus* B69 [29]. Schillinger and Lücke [28] noted the same thing; out of 19 strains only 6 presented an activity in liquid medium. The absence of inhibiting activity of the filtrates can be due either to weak concentration of the inhibiting substance [43], or with the loss of the activity after filtration.

The resistance of Gram-negative bacteria is attributed to the particular nature of their cellular envelope and the mechanisms of action described for the bacteriocins utilizing an adsorption of these molecules to the sensitive cells. According to Bhunia and al.,[24], the pediocin

AcH produced by *Pc. acidilactici* H interacts with the lipotechoïc acids, absent in Gram-negative bacteria. These molecules would play the role of a nonspecific reception site necessary to produce the bactericidal effect. Bhunia *et al.,* [24] assigned the resistance of the Gram-negative bacteria to the pediocin AcH to the barrier which their external membrane would represent. The incapacity of the bacteriocins to cross this barrier is due to their molecular weight and/or their hydrophobic properties [43,44]. The external membrane is made permeable, either by a physical treatment [45], or by a chemical treatment [46,44] the Gram-negative bacteria will become sensitive to the bacteriocins.

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