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Production of Antimicrobial Agent against *Listeria monocytogenes* ATCC 15313 by a Lactococcus Strain No. 1−74 from Algerian Cheese El−Klila 1

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Abstract From a traditional cheese, El-Klila, produced in Algeria, 198 strains of lactic acid bacteria were isolated. Twenty strains among them produced the antimicrobial agent against *Listeria monocytogenes* ATCC 15313. The strain (No. 1−74) with the highest productivity (60k.eq. μg/ml) was coccus in the form. A high quantity of the agent was produced when it was cultured at 25−35°C, pH 3.2−5.7, in the presence of metal ions (1mM) such as Ca^{2+}, Ni^{2+} and Ba^{2+} for 36h, under stationary culture conditions. Heavy metal ions such as Ag^{+}, Cu^{2+}, Co^{2+}, and Zn^{2+}, excess oxygen inhibited the growth and the productivity of the agent.

Key words: antimicrobial agent, El-Klila, lactic acid bacteria, *Listeria monocytogenes*,

INTRODUCTION

We reported in the previous paper (BOUBEKRI and OHTA, 1995), that many strains of *Enterococcus*, *Leuconostoc*, and *Pediococcus* were isolated from a traditional cheese, El-Klila, produced from cow-milk in Setif and Betna cities, Algeria. Also, these strains have been characterized morphologically and physiologically. Furthermore, we found that these lactic acid bacteria had a binding activity to carcinogens such as heterocyclic amines (BOUBEKRI and OHTA, 1994), which are produced during cooking of foods.

El-Klila cheese is produced from raw cow-milk using a traditional method under poor hygienic conditions. However, it is consumed by many people in the area mentioned above without any problems. But, during production, the cheese is exposed to contamination by pathogenic microorganisms such as *Listeria monocytogenes*. This is a Gram-positive, facultative anaerobic pathogen that has been involved in numerous food poisoning outbreaks and food product recalls (FARBER and PETERKIN, 1991). The strain is also responsible for causing listeriosis in humans and animals (FARBER and PETERKIN, 1991).

Many lactic acid bacteria are known to produce bacteriocin with antimicrobial activity against food spoilage and pathogenic bacteria (KLAENHAMMER, 1988; DAESCHEL, 1990). For examples, the bacteriocin 'nisin' produced by strains of *Lactobacillus lactis* sub. sp. *lactis*, has been used in processed cheese product (Food and Drug Administration, 1988). Lactococci also are known to produce bacteriocins (KLAENHAMMER, 1988). These bacteriocins have advantages as food preservatives, since many have antimicrobial activity against *L. monocy-*

togenes and *Clostridium botulinum* and many inhibit these pathogens in actual foods (BERRY *et al.*, 1990, BERRY *et al.*, 1991, OKEREKE and MONTRILLE, 1991; PUCCI *et al.*, 1988).

In this study, we tried to re-isolate many strains of lactic acid bacteria from El-Klila to find out the strains with the antimicrobial activity against *L. monocytogenes*. Furthermore, the preferable culture conditions were investigated with the selected lactococcus strain 1−74 for the production of the antimicrobial agents.

MATERIALS AND METHODS

Listeria monocytogenes ATCC 15313 was used as a test strain throughout this experiment. It was cultured with brain/heart infusion medium (B.H.I. medium, Difco Co., U.S.A.). The lactic acid bacteria were isolated from the Algerian traditional cheese, El-Klila 1 and 3. These cheese are traditionally made from cow milk in Algeria. Samples K1 and K3 were collected in Setif city and Batna, respectively, Algeria. The cheese sample (hard and solid material) was ground to a fine powder in a mortar with a pestle. Ten grams of the ground sample were put into 100ml of autoclaved (121°C, 15min) 10% (w/v) skim milk (Meiji Milk Co., Tokyo) solution and incubated at 30°C, for 48h. Then the culture was shaken to homogenize the coagulated material in the culture, and was diluted with sterile saline solution (0.9% (w/v) NaCl). An aliquot (0.1ml) of the diluted culture was spread on the B.C.P agar medium (a selective medium for lactic acid bacteria, Nissui Co., Tokyo), and incubated at 30°C, for 48h. Individual colonies that made the medium yellowish, were picked up and were streaked onto the B.C.P. agar plate medium. The incubation was carried out under the same conditions as above. This one colony-isolation procedure was repeated twice. The purified isolates of lactic acid bacteria were inoculated onto the slope medium of B.C.P. agar supplemented with 1% (w/v) solid $CaCO_3$. After incubation at 30°C, for 48h, they were preserved at 4°C until used. The anaerobic cultivation was carried out in an anaerobic jar (5L) with an anaerobic system (Becton Dickinson Co., U.S.A.). The shaking cultivation was carried out on a test tube shaker (TC 300, Takasaki Co., Saitama) at 240 r.p.m. The pH was measured with a pH meter (Model 240, Corning Co., U.S.A.). Growth amount was determined by measuring optical density (O.D.) at 660nm with a spectrophometer (U-2000, Hitachi Co., Tokyo). The growth amount was expressed with OD_{660}. The production of antimicrobial agent was determined as follows: One loopful of lactic acid bacteria grown on the slope agar medium was inoculated into 10ml of MRS liquid medium (pH 5.7, Merck Co., Germany) in a test tube (I.D. ϕ, 16mm × 160mm high). The medium composition of MRS (pH 5.7, g/l): peptone, 10.0; meat extract, 8.0; yeast extract, 4.0; D-glucose, 2.0; potassium hydrogen phosphate, 2.0; Tween 80, 1.0; di-ammonium hydrogen citrate 2.0; sodium acetate, 5.0; magnesium sulfate, 0.2; manganese sulfate, 0.04. This liquid medium was used as a basic medium throughout the experiment. The incubation was carried out at 30°C, for 48h. The cultured broth was centrifuged at 17,000 × *g*, at 4°C, for 5min. The supernatant fluid was taken and filtered through a membrane filter (pore size, 0.45 μm, Advantec, Tokyo). The filtrate was subjected to a determination experiment with an antimicrobial agent. One loopful of *L. monocytogenes* ATCC 15313 grown on the B.H.I. agar slope medium was inoculated into 7ml of B.H.I. liquid medium in a test tube and incubated at 30°C, overnight. An aliquot (0.1ml) of the liquid culture was inoculated into

12ml of B.H.I. agar medium (agar concentration, 0.8% (w/v)) and kept at 48°C. The inoculated agar medium was poured into a Petri dish (I.D. ϕ, 90mm). The surface of the solidified agar plate medium was dried for about 20min. in a clean bench (MG3, Hitachi, Tokyo). Five penicillin cups (stainless steel, I.D. ϕ, 5mm; O.D. ϕ, 7mm; height, 10mm) were put on the surface of the agar medium to keep the same distance from each cup. One hundred micro liters of the filtrate prepared above was poured in a cup with a micropipet. The plate was kept at a room temperature (about 25°C) for 2h in order to allow the agent to be absorbed into the agar medium. Then the plates were incubated at 30°C, overnight. After the cups in the Petri dishes were taken off, the clear diameter of the growth inhibition zone was measured with a ruler in milli meters. The correlation standard curve between the concentration of the antimicrobial agent and the growth inhibition zone was determined with kanamycin (Meiji Seika Co., Tokyo) as a standard material. Referring to this standard curve, the productivity of the antimicrobial agents was expressed as the amount equivalent to kanamycin per culture broth (k.eq. μg/ml).

Cells of lactic acid bacteria were harvested by centrifugation at 17,000 $\times$ g, for 5min. The cells were washed and resuspended into the same volume of phosphate buffer (pH 7.0, 20mM) as the amount from which they were harvested. The cell suspension was subjected to ultrasonication with a sonicator (201M, Kubota Co., Tokyo) at 20°C, for 10min. The disrupted cell suspension was then centrifuged to obtain the supernatant. Total sugar content was determined with a phenol-sulfuric acid method. All other chemicals with 1st grade were commercially obtained.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria with productivity of antimicrobial agent against *L. monocytogenes*: The individual colonies which colored the B.C.P. agar medium yellowish, were picked up as lactic acid bacteria colonies and streaked onto the same medium. After repeating isolation procedure by streaking, 198 total strains of lactic acid bacteria were isolated: 121 strains from El-Klila 1, and 77 strains from El-Klila 3.

All strains were tested for production of the antimicrobial agent. As summarized in Table 1, 9 strains produced about 60k.eq. μg/ml, 11 strains, between 10 and 50k.eq. μg/ml of antimicrobial agents. Other strains had no ability to produce the agent. Consequently,

Table 1. Effect of Culture Methods

Methods		Growth (OD_{660})	Antimicrobial agent (k.eq. μg/ml)	Final pH
Shaking	a*	3.08	25	4.23
	b*	4.10	40	4.68
Anaerobic	a	3.74	28	4.28
	b	4.30	40	4.63
Stationary	a	5.13	32	3.89
	b	5.46	40	4.65

* supplement without (a) or with (b) solid $CaCO_3$ (1% (w/v)).

about 12% of the isolates were found to produce the antimicrobial agents against *L. monocytogenes* ATCC 15313.

The morphology of these 11 strains with higher productivity were tested under a microscope (BH-12, Orympus Co., Tokyo). They were all found to be in the form of coccus.

The best strain (No. 1−74) with productivity and growth was selected for the further experimentation. This strain was Gram positive and coccus. This was the strain that was isolated from the El-Klila 1 sample. The El-Klila cheese is traditionally made from raw cow-milk under poor hygienic conditions. *L. monocytogenes* is a pathogenic bacteria, that often contaminates dairy products. A high population of lactic acid bacteria with the capacity to produce the antimicrobial agents against this pathogen is thereby preferred for the traditional production of the cheese under such environmental conditions.

The following experiments were carried out for establishment of suitable cultural conditions for the production of the antimicrobial agents by this strain.

a) Influence of culture temperature: The strain No. 1−74 was inoculated in to 10ml of MRS medium in a test tube (I.D. ϕ, 16mm × 160mm high) supplemented with or without 1% (w/v) solid $CaCO_3$. The incubation was carried out at 20, 25, 30, 35, 40, and 45°C, for 48h. As shown in Fig. 1, the maximal growth temperature was found to be between 25 and 35°C, for 48h-cultivation. The temperatures for two-thirds of the maximal growth was observed at 20 and 40°C; and the temperature for half of the maximum was 45°C. The antimicrobial agent was produced at constant level (60k.eq. μg/ml) between 20 and 35°C in the medium with solid $CaCO_3$. An almost similar amount of the agent (60k.eq. μg/ml) was produced at

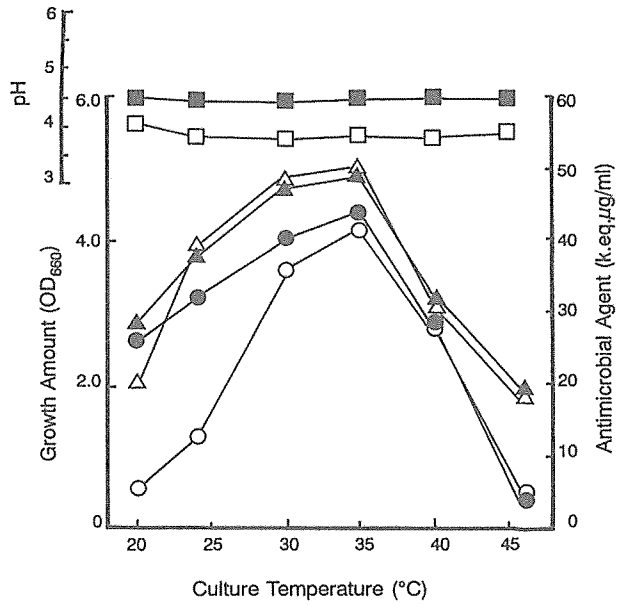

Fig. 1. Effect of temperature
Symbols: (closed symbols, added with $CaCO_3$ in medium; open symbols, without $CaCO_3$)
□, ■, pH; △, ▲, growth; ○, ●, antimicrobial agent.

Table 2. Effect of Productivity Enhancing Substances

Substances	Grow (OD_{660})		Antimicrobial agent (k.eq. μg/ml)	Final pH
Skim milk	a[2)]	5.18	33	4.27
	b[2)]	5.72	40	4.65
L.m.[1)] cells	a	5.23	27	4.23
	b	5.60	40	4.67

[1)] 2mg dead and dried cells of *Listeria monocytogenes* ATCC 15313.
[2)] see the legend for Table 1

35°C in the medium with solid $CaCO_3$. Below 30°C and above 40°C, however, the productivity decreased. The production of agent was poor at 50°C (about 6k.eq. μg/ml). It is possible that the presence of solid $CaCO_3$ bufferized the medium for the production of the agent from the low pHs. The solid $CaCO_3$ neutralized medium resulting in the stabilization of the agent. The solid $CaCO_3$ had influenced the production of the agent. The final pH of the medium with $CaCO_3$ was around 4.40, while it was about 3.82 without $CaCO_3$.

b) Influence of culture methods: Generally, lactic acid bacteria are one group of facultative anaerobic bacteria. The strain No. 1−74 was inoculated in 6 test tubes (I.D. ϕ, 16mm × 160mm high) containing 10ml of MRS liquid medium with or without 1% (w/v) solid $CaCO_3$. The first two tubes were incubated aerobically by setting them on a test tube shaker. The second two tubes were incubated anaerobically in an anaerobic jar. The last two ones were incubated by standing them in a test tube rack. In all cases, the incubation was carried out at 30°C, for 48h. As shown in Table 2, the maximal growth amount was observed for the stationary culture in the medium with $CaCO_3$. The final pH was around 4.60. Higher growth amount was obtained in the medium with $CaCO_3$ under all culture conditions, than in the medium without $CaCO_3$. Antimicrobial agent was produced consistantly in the media with solid $CaCO_3$ under all different culture conditions. In contrast, 28k.eq. μg./ml of the agent was produced in the medium without $CaCO_3$ under anaerobic and aerobic conditions. The solid $CaCO_3$ probably enhanced the production of the agent under the last two culture conditions by adjusting the pH of the medium. The micro aerobic (stationary) culture condition was the preferred condition for the production of the agent by this strain.

c) Influence of productivity enhancing substances: L. monocytogenes is found to contaminate dairy products. It grows well in the milk products. Further, the productivity of antimicrobial agents is enhanced by the presence of inducers like the corresponding bacterial cells, or their debris. Therefore, the MRS liquid medium with or without (1%, w/v) solid $CaCO_3$, was supplemented with skim milk (1%, w/v) or 2mg of dry dead cells of *L. monocytogenes*. The stationary incubation was carried out at 30°C, for 48h. As shown in Table 3, no effect of the addition of the substances was found on the production of the agent in the medium with or without $CaCO_3$. The agent might be produced constitutively.

d) Influence of various kinds of sugars: Various kinds of sugar shown in Table 4 were added at a concentration of 2% (w/v) in the liquid medium with the same composition at that of

Table 3. Effect of Growth Stimulants

Substances	Growth (OD_{660})	Antimicrobial agent (k.eq. μg/ml)	Final pH
Yeast extract	5.22	35	4.19
Soy bean meal	5.61	35	4.28
Waste mollasse	5.10	35	4.16
C.S.L.[1)]	5.26	35	4.11
Meat extract	5.23	35	4.10
Control	5.43	35	4.17

[1)] C.S.L.: Corn steep liquor.
Solid $CaCO_3$ (1% w/v) was supplemented in the medium.

Table 4. Effect of Sugar Forms

Sugars	Growth (OD_{660})	Antimicrobial agent (k.eq. μg/ml)	Final pH
Arabinose	1.75	7	4.83
Xylose	1.62	7	5.46
Fructose	4.23	35	4.22
Galactose	4.06	35	4.28
Glucose	4.16	40	4.12
Lactose	4.00	35	4.28
Maltose	4.12	30	4.24
Saccharose	3.95	35	4.43
Dextrin	2.21	8	5.16
Sol. Starch[1)]	1.83	8	5.37
None (control)	1.62	10	4.17

[1)] Soluble Starch:
Two percent (w/v) was added.

MRS, except glucose. This medium was prepared by combining the ingredients of MRS. The cultivation was carried out in 10ml liquid medium in a test tube under stationary culture condition in the presence of solid $CaCO_3$. As shown in Table 4, the strain grew abundantly on glucose, lactose, galactose and sucrose. It produced 35−40k.eq. μg/ml of antimicrobial agent in the medium containing these sugars. The final pH of the medium was about 4.26. The strain did not utilize arabinose, xylose, dextrin, and starch. A small amount of the agent was produced in the absence of sugars (control experiment).

e) Influence of growth stimulants: Yeast extract, defatted soy bean meal (Yoshihara Oil Co., Osaka), corn steep liquor (Wako Co., Osaka) or meat extract (Kyokuto Co., Tokyo) was added at a concentration of 0.05% (w/v) to the MRS liquid medium with $CaCO_3$. Incubation was carried out at 30°C, for 48h. As shown in Table 5, no stimulants for growth or the production of the agent were found among these materials. Further additional minor nutrients might not be required in this experiment.

f) Influence of initial pH: Five milli liters of the double concentrate MRS liquid medium in test tubes was sterilized at 121°C, for 15min. The pHs of 2.3 to 9.2 was adjusted to the desired pHs with sterile 1N NaOH and 1N HCl solutions aseptically. The amount of the

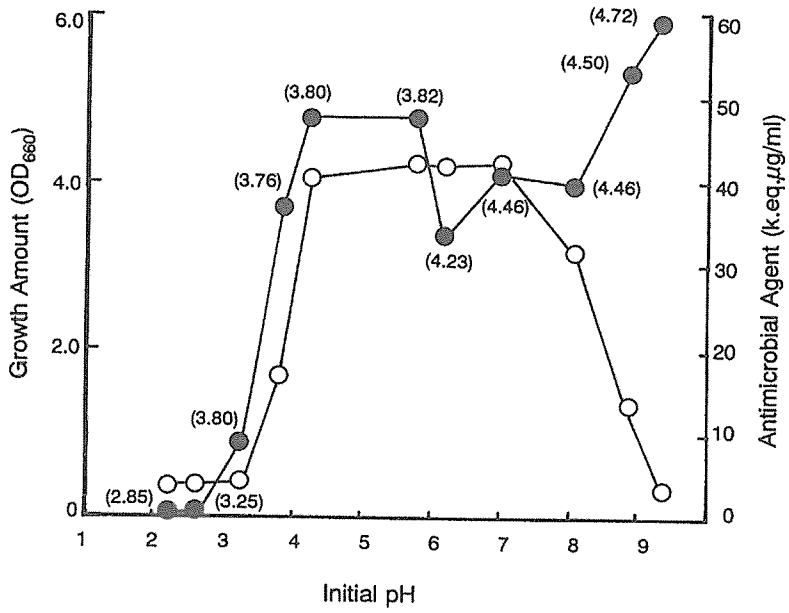

Fig. 2. Effect of initial pH
Symbols: ●, growth; ○, antimicrobial agent.
The number in bracket shows the final pH.

solutions to be added had been determined in a preliminary experiment. Sterile distilled water was added to the pH adjusted media to make up a total volume of 10ml. Then, one loopful of the slope culture of the strain was inoculated into the liquid medium, and incubated at 30°C, for 48h. As shown in Fig. 2, the strain grew profusely at the initial pHs between 3.8 and 5.7, and between 8.2 and 9.2. But, the growth rate decreased below pH 3.2, and above pH 10. This strain grew at alkaline pHs, because it produced an acidic substance (lactic acid) which decreased the pH to a level conductive to growth. This was verified experimentally when the final pH was measured at around 4.2. However, this strain barely grew below pH 3.2. The antimicrobial agent was produced optimally between pH 3.8 and 8.2, but at the lower and higher pH than the range shown above, the agent was produced at lower concentrations. During the 48h-incubation, it is possible that the agent is produced after the cells grow. Thus, prolonged cultivation may be required for the production of the agent. The nutrient components in the medium were consumed by the cells at high pHs. For this reason, little agent was produced. Anyway, the further experimentation would be required.

g) Influence of metal ions: Various kinds of metal ions were added to the MRS medium at 1mM final concentration. The metal ions were all in the form of chloride. As shown in Table 5, the divalent ions such as Ca^{2+}, Ni^{2+} and Ba^{2+} enhanced the growth by 20% and the production by 25%, compared with the control experiment (without addition of metal ion). On the other hand, Ag^{+}, Cu^{2+}, Co^{2+}, and Zn^{2+} inhibited cell growth and agent productivity. Other metal ions had no influence on the growth and the productivity of the agent at this concentration.

Table 5. Effect of Metal Ions

Metal ions	Growth (OD_{660})	Antimicrobial agent (k.eq. μg/ml)	Final pH
KCl	4.65	35	4.03
NaCl	4.70	35	3.96
AgCl	4.32	8	3.88
$BaCl_2$	5.21	40	3.89
$CaCl_2$	5.15	50	3.96
$CuCl_2$	2.34	20	4.36
$CoCl_2$	4.62	20	3.90
$FeCl_3$	4.34	40	3.94
$LiCl_2$	3.89	40	3.92
$MnCl_2$	4.52	50	3.93
$MgCl_2$	4.68	35	3.93
$NiCl_2$	5.06	50	3.94
$RbCl_2$	4.10	35	3.91
$SnCl_2$	4.52	35	3.87
$ZnCl_2$	4.89	25	3.94
None (control)	4.45	35	3.90

One mM metal ion was added.

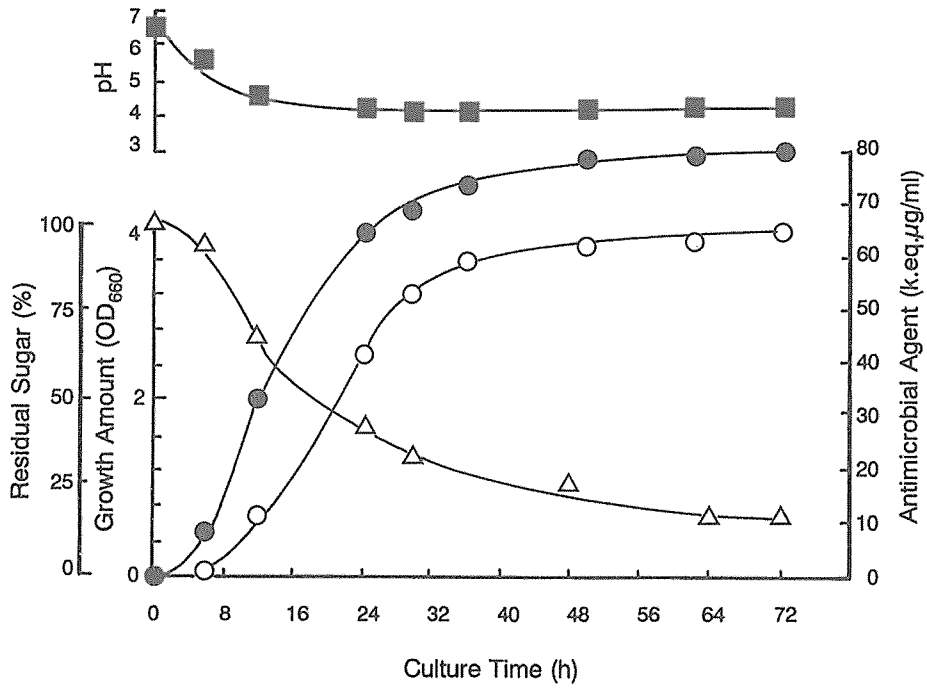

Fig. 3. Time course of production of antimicrobial agent
Symbols: ■, pH; ●, growth; △, residual sugar; ○, antimicrobial agent.
* kanamycin equivalent

h) Time course of production of antimicrobial agent: Overnight culture of the strain 1−74 grown in MRS medium was used as a seed culture. An aliquot (0.1ml) of the seed culture was inoculated into 10ml of MRS liquid medium supplemented with 1% (w/v) solid $CaCO_3$ in a test tube. Twenty test tubes were used as one set for this experiment. Incubation was performed at 30°C under stationary culture conditions. Every two test tubes was withdrawn at the timed intervals plotted in Fig. 3, and subjected to the chemical analysis. As shown in Fig. 3, the growth reached its maximum after a 30h-incubation period. The pH of the culture decreased from 6.5 to 4.3. The total of amount sugar in the medium decreased with progress of cell growth. The antimicrobial agent was produced after 12h-incubation, and reached the maximum (60k.eq. μg/ml) after 52h-incubation. The production of agent followed about 6h behind the growth. The agent could be the second metabolite of the strain.

i) Localization of the agent: Cells grown on MRS liquid medium at 30°C, for 48h were collected and disintegrated with a sonicator. The supernatant fluid was tested for the antimicrobial activity. As a result, no activity was detected in the fluid. The agent was found to be produced extracellularly.

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アルジェリアのチーズ「エルークリラ1」から分離した乳酸球菌 No. 1−74 株による *Listeria monocytogenes* ATCC15313 に対する抗菌物質の生産

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アルジェリアの伝統的チーズ「エルークリラ１」から乳酸菌を198株分離した。その内20株が，*Listeric monocytogenes* ATCC15313 に対する抗菌物質を産生した。最も多量に抗菌物質を産生した菌株 No. 1−74 は球菌であった。この抗菌物質は，pH 3.7～5.7, 25～35°C で，1mM の Ca^{2+}，Ni^{2+} や Ba^{2+} の存在下で，36時間静置培養した場合に本抗菌物質を良好に産生した。Ag^{+}, Cu^{2+}, Co^{2+} や Zn^{2+} などの重金属は本菌株の生育及び抗菌性物質の産生を抑制した。

キーワード：エルークリラ，抗菌物質，*Listeria monocytogenes*，乳酸菌