# Partial characterization of bacteriocin-like peptide produced by *Lactobacillus acidophilus* DSM 20079

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**ABSTRACT:** Antagonistic effect of some strains of lactic acid bacteria and bifidobacteria were studied. *Lactobacillus acidophilus* DSM 20079 produced bacteriocin-like activity against different bacteria including some pathogenic and food-spoilage species. The strain produced antibacterial peptide after 2 hours, and the level of the peptide was at a maximum after 30 hr during fermentation. The peptide exhibited antibacterial activity against *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081, *B*acillus *subtilis, Bacillus series, Escherichia Coli, Enterococcus faecalis* and *Salmonella typhimurium* but not against *staphylococcus aureus, Lactobacillus acidophilus* DSM 9126, *Lactobacillus acidophilus* DSM 20242, *Lactobacillus delbrueckii ssp bulgaricus* DSM 20080 *Streptococcus thermophilus* DSM 20617, *Bifidobacterium infantis* DSM 20088 and *Bifidobacterium angulatum* DSM 20097. The bacteriocin-like peptide completely lost its activity by proteolytic hydrolysis with pepsin, trypsin and chemotrypsin. However, the antibacterial activity of the peptide was well maintained at pH between 2.0 and 9.0. The activity remained after heat treatment at 50-140$^{o}$C for 5-15 min. Most of bacteriocin-like peptide precipitated with 40-50% ammonium sulfate saturation. Growth rate of *Lactobacillus delbrueckii ssp bulgaricus* in MRS completely inhibited with addition of different levels of cell-free supernatant from *Lactobacillus acidophilus* DSM 20079 as measured at 620 nm. The molecular mass of the bacteriocin-like peptide was fount to be 33 kDa

## INTRODUCTION

Lactic acid bacteria and bifidobacteria are among the most frequently applied probiotic microorganisms. Lactic acid-producing bacteria include *Lactobacillus* ssp. are used widely throughout the fermented dairy, food, and meat processing industries (Cleveland et al., 2001). Most of the bacteriocins produced by this genus are active only against other lactic acid bacteria, but several display antibacterial activity against most phylogenetically divergent gram-positive bacteria and occasionally against gram-negative bacteria.

Many bacterial species produce peptide antibiotics, called bacteriocins, that often have an antimicrobial effect. These compounds have been extensively studied because of their potential applications in the food industry as natural biopreservatives and in pharmaceuticals as antimicrobials (Joerger and Klaenhammer, 1986; Bizani et al., 2005 and Bizani et al., 2008). Bacteriocins produced by lactic-acid bacteria have been the focus of many investigations because of their particular importance in the dairy industry (O'Sullivan et al., 2002). Based on their chemical structures, stability, and mode of action, bacteriocins have been classified as: (i) lantibiotics; (ii) small heat-stable peptides; (iii) large heat-labile proteins; and (iv) complex proteins that require carbohydrate or lipid moieties for activity (Klaenhammer**,** 1993). Bacteriocin-like inhibitory substances have been detected in several bacterial species, including *Streptococcus ssp* (Whitford et al., 2001 and Yonezawa and Kuramitsu, 2005) *Lactobacillus plantarum* C11 (Moll et al.,

1999), *Lactococcus lactis* subsp. *lactis* HV219 (Todorov et al., 2006), *Bacillus cereus* (Bizani et al., 2005) and *Bacillus licheniformis* (He et al., 2006).

Bacteriocin-like peptides exhibite antibacterial activity against Gram positive bacteria and few species of Gram negative bacteria. Brashears et al. (1998) reported that samples inoculated with at least $5.0 \times 10^7$ *L. lactis* per ml exhibited significant declines in numbers of *E. coli* after only three days of storage. Moreover, the viable cell numbers of *Lact. sake* decreased from approximately $4 \times 10^8$ to less than 10 cfu $ml^{-1}$ over a period of 4 h with addition of pentocin TV35b produced from *Lactobacillus pentosus* (Okkers et al., 1999). Most bacteriocins of lactic acid bacteria (LAB) act on sensitive cells by destabilization and permeabilization of the cytoplasmic membrane through the formation of transitoryporation complexes or ionic channels that allow the free flow of electrolytes, metabolites and water across the phospholipid bilayers and cause the reduction or dissipation of the proton motive force (PMF) which needed to make ATP by ATP synthase (Cintas et al., 2001 and Bizani et al., 2005**)**. Bacteriocin-like inhibitory substances are different in molecular mass, heat stability, optimum pH of the activity, antagonistic spectrum and susceptibility to hydrolize by proteolytic enzymes.

*Lactobacilli acidophilus* is widely used as fermenting agent in dairy products and our preliminary studies showed that *Lactobacillus acidophilus* DSM 20079 has antagonistic effect against *lactobacillus delbrueckii ssp bulgaricus* DSM 20081. In this study characteristics of bacteriocin-like peptide produced from *Lactobacillus acidophilus* DSM 20079 was investigated.

## MATERIALS AND METHODS

**Reagents and media.** Nutrient broth (NB), nutrient agar (NA), de Man-Rogosa-Shape (MRS), MRS agar and MacConkey agar were purchased from Oxiode (Oxide LTD Basingstoke, Hants, England) and Eosin Methylene Blue Agar (EMB-agar) from Difco (Detroit, Michigan, USA). Pepsin, trypsin and chemotrypsin were purchased from Sigma (St. Louis, MO, USA). All other chemical and reagents were of analytical grade.

**Bacterial cultures.** Indicator strains listed in Table 1 were obtained from the different sources presented in Table 1. The strains were kept frozen in 40% (v/v) glycerol at $-20^{o}$ C until needed and then were grown under the culture conditions indicated in the same table.

**Detection of antagonistic activity:** The well diffusion assay of Schillinger and Lucke (1989) was used to detect the inhibitory effect of supernatant of *Lactobacillus acidophilus* DSM 20079 against indicator bacteria. This involved seeding petri dishes with the test bacteria and introducing 0.05 ml (50µl) of *Lactobacillus acidophilus* DSM 20079 supernatant into holes bored with 3 mm cork borer. The plates were incubated aerobically at $37^{\circ}$C for 24 h after which they were examined for zones of inhibition. Bifidobacteria were incubated under anaerobic condition.

Table 1. Source of bacterial strains and growth conditions.

| Bacterial strain | Source | Media | Temperature |
|---|---|---|---|
| *Lactobacillus acidophilus* | DSM 9126 | MRS agar | 37°C |
| *Lactobacillus acidophilus* | DSM 20079 | MRS agar | 37°C |
| *Lactobacillus acidophilus* | DSM 20242 | MRS agar | 37°C |
| *Lactobacillus delbrueckii ssp bulgaricus* | DSM 20081 | MRS agar | 37°C |
| *Lactobacillus delbrueckii ssp bulgaricus* | DSM 20080 | MRS agar | 37°C |
| *Streptococcus thermophilus* | DSM 20617 | MRS agar | 40°C |
| *Bifidobacterium infantis* | DSM 20088 | MRS + Cystein | 37°C |
| *Bifidobacterium angulatum* | DSM 20098 | MRS + Cystein | 37°C |
| *Bacillus cereus* | ATCC 9634 | NB | 40°C |
| *Escherichia coli* | Food isolate | MacConkey | 37°C |
| *Enterococcus faecalis* | Food isolate | EMB agar | 37°C |
| *Salmonella typhimurium* | ATCC 14028 | NB | 37°C |
| *Staphylococcus aureus* | ATCC 25923 | NB | 37°C |
| *Bacillus subtilis* | ATCC 9372 | NB | 40°C |

DSM= Deutsche Sammlung Von Mikroorganismen, ATCC= American Type Culture Collection

**Effect of heat treatments**

Cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 was heated in screw capped test tubes at 50-140°C for 5-15 min in glycerol bath. After cooling to room temperature, the residual activities were measured using *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081 as indicator strain.

**Effect of proteolytic enzymes**

Cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 was mixed with pepsin, trypsin and chemotrypsin each alone at final concentration of 1 mg/ml and incubated at 37°C for 3h. After incubation, the residual activities were measured as indicated previously. The untreated supernatant served as the positive control.

**Effect of pH**

The pH of cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 was adjusted to values from 2-9 using 1N HCl or NaOH and kept at 4°C over night, then the residual activities were measured. In another experiment the pH of uninculated MRS broth was adjusted to 2-9 to clarify if the inhibition zones attributed to the changes in pH or to bacteriocin-like peptide.

**Precipitation of bacteriocin-like peptide**

Ammonium sulfate salt was added to cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 (100 ml) in saturation 20-90% with stirring according to He et al. (2006). The suspension was subjected to centrifugation at 12.000 g for 15 min at

4°C. The obtained pellet was dissolved in 5 ml 50 mM tris-HCl buffer at pH 7.0. The activities of dissolved precipitate were then measured against *Lactobacillus delbrueckii ssp bulgaricus* as indicator strain.

**Growth rate and production of bacteriocin-like peptide**

*Lactobacillus acidophilus* DSM 20079 was grown in MRS broth at 37°C for 30h. The obtained supernatant was mixed at different levels (0.25-1.5 ml/10 ml) to inoculated MRS with *Lactobacillus delbrueckii* ssp *bulgaricus* DSM 20081. The optical densities were measured (without dilution) according to Walker and Gilliland (1993) at 0, 2, 4, 6 and 24h spectro-photometrically at 620 nm (LKB Biochrom Ultrospec II, Campridge, England). Total plate count was also measured after incubation for 6h. The production of bacteriocin-like peptide was determined at 0, 2, 4, 6, 24, 30, 48 and 72h incubation period using *Lactobacillus delbrueckii* ssp *bulgaricus* as indicator.

**Determination of molecular weight of bacteriocin-like peptide**

**Size determination**

Samples collected from the 50% ammonium sulfate saturation were separated on SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). A molecular weight protein marker with sizes ranging from 94 to 14.4 kDa (Amersham International, UK) was used. The gels were fixed and stained with Coomassie Brilliant Blue R250 (Reactifs LBF, 92390 Villeneuve-La-Garenne, France).

## RESULTS AND DISCUSSION

**Spectrum of antagonistic activity**

The cell-free supernatants of *Lactobacillus acidophilus DSM 9126, Lactobacillus acidophilus DSM 20079, Lactobacillus acidophilus DSM 20242, Lactobacillus delbrueckii ssp bulgaricus DSM 20081, Lactobacillus delbrueckii ssp bulgaricus DSM 20080, Streptococcus thermophilus 20617, Bifidobacterium infantis DSM 20088* and *Bifidobacterium angulatum DSM 20098* were tested against each other. The results in Fig. 1 indicated that the supernatant of *Lactobacillus acidophilus DSM 20079* exhibited antagonistic effect against *Lactobacillus delbrueckii ssp bulgaricus DSM 20081*. Therefore, the cell-free supernatant of *Lactobacillus acidophilus DSM 20079* was used against some strains of Gram positive and Gram negative pathogenic and food-spoilage bacteria. Results in Table 2 showed that a broad spectrum of antagonistic activities against these bacteria was observed.

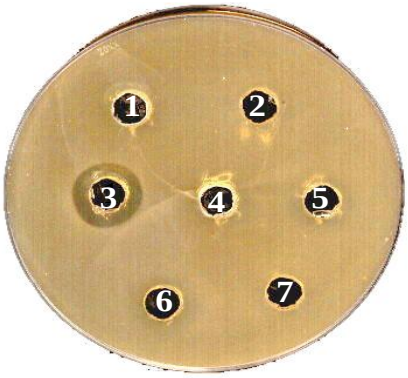


Figure 1. Antagonistic effect of some strains of lactic acid bacteria against *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081.
1=*Lactobacillus acidophilus* DSM 9126; 2= *Lactobacillus acidophilus* DSM 20242; 3= *Lactobacillus acidophilus* DSM 20079; 4= *Streptococcus thermophilus* DSM 20617; 5= *Lactobacillus delbrueckii ssp bulgaricus* DSM 20080; 6= *Bifidobacterium infantis* DSM 20088 and 7= *Bifidobacterium angulatum* DSM 20098.

Table 2. Antagonistic spectrum of cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 against some bacterial strains.

| Bacterial strain | Source | Antagonistic activity |
|---|---|---|
| *Lactobacillus acidophilus* | DSM 9126 | - |
| *Lactobacillus acidophilus* | DSM 20242 | - |
| *Lactobacillus delbrueckii ssp bulgaricus* | DSM 20081 | +++ |
| *Lactobacillus delbrueckii ssp bulgaricus* | DSM 20080 | - |
| *Streptococcus thermophilus* | DSM 20617 | - |
| *Bifidobacterium infantis* | DSM 20088 | - |
| *Bifidobacterium angulatum* | DSM 20098 | - |
| *Bacillus series* | ATCC 9634 | ++ |
| *Escherichia coli* | Food isolate | ++ |
| *Enterococcus faecalis* | Food isolate | ++ |
| *Salmonella typhimurium* | ATCC 14028 | ++ |
| *Staphylococcus aureus* | ATCC 25923 | - |
| *bacillus subtilis* | ATCC 9372 | +++ |

(-) No antagonistic activity; (++) medium inhibitory activity; (+++) high inhibitory activity.

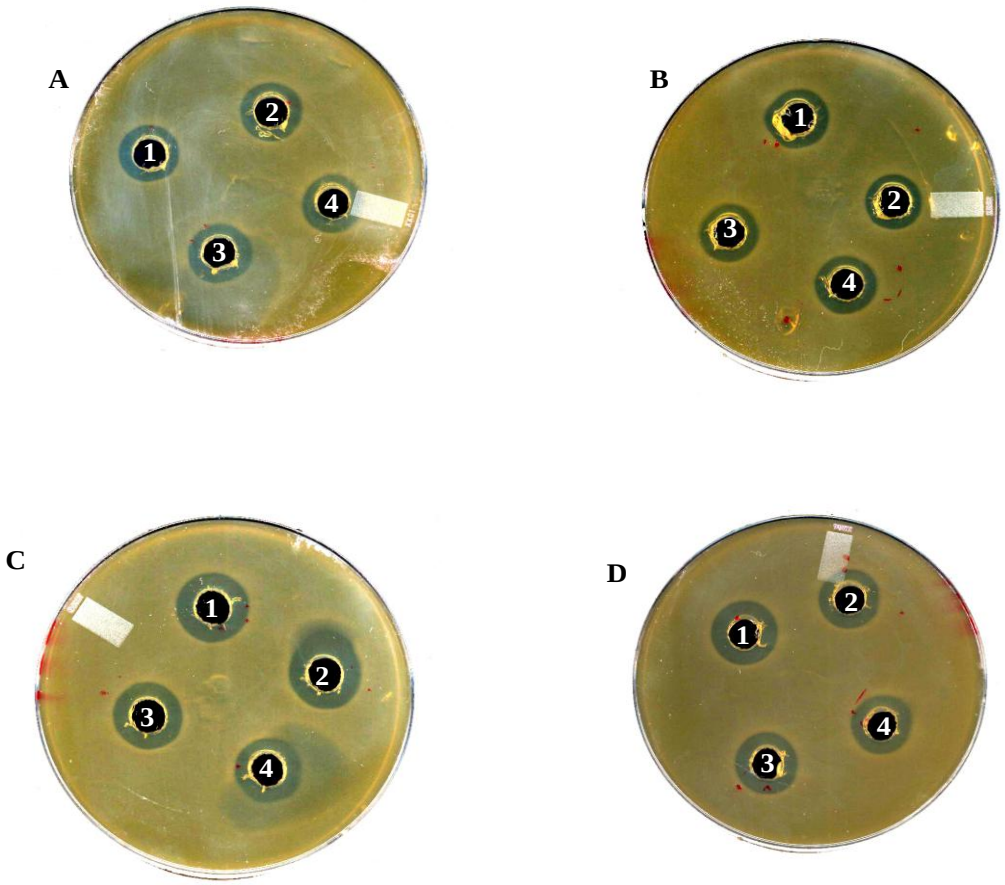


Figure 2. Effect of heat treatment on the stability of bacteriocin-like peptide produced from *Lactobacillus acidophilus* DSM 20079. (A, heating at 110°C. B, heating at 120°C. C, heating at 130°C and D heating at 140°C. 1, 2, 3, and 4 are heating times for 0, 5, 10 and 15 min., respectively).

**Effect of heat treatment**

The antagonistic activity of cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 was measured after heat treatment in the range 50 – 140°C. Results in Fig. 2 indicated that the cell-free supernatant was very heat stable during heating at indicated temperature for 5-15 min.

**Effect of proteolytic enzymes**

The antagonistic activity was completely abolished with addition of proteolytic enzymes (pepsin, Trypsin and Chymotrypsin in ratio of 1mg/ml) to cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 and incubation for 3h at 37°C (Fig. 3). These results are in good agreement with those obtained by He et al. (2006) and Stern et al.

(2006) who reported that the activity of cell-free supernatant of *Bacillus licheniformis* and *Lactobacillus salivarius* NRRL B-30514 was completely lost with addition of proteinase K, Trypsin, beta-chymotrypsin and papain.

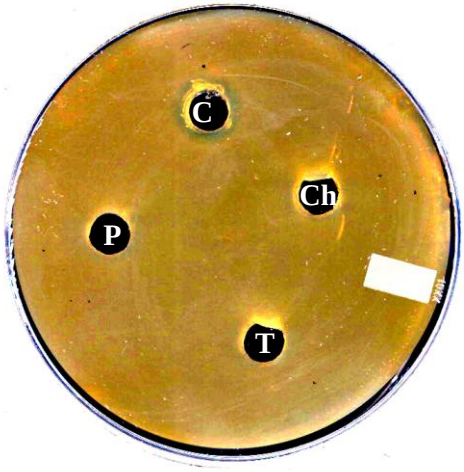


Figure 3. Effect of proteolytic enzymes on the activity of bacteriocin-like peptide produced from *Lactobacillus acidophilus* DSM 20079 during incubation at 37°C for 3hrs.(C is control, Ch is chemotrypsin, T is trypsin and P is pepsin)

**Effect of pH**

The pH of cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 was adjusted in the range 2.0 – 9.0 and then the antagonistic activity was measured against *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081 as indicator bacterium. Results in Fig. 4 showed that the cell-free supernatant was stable and maintained its activity at the indicated pH values. In another experiment the pH of uninculated MRS was adjusted to pH 2.0-9.0 and the effect of pH was tested to clearfy if this inhibitory effect is attributed to pH or the bacteriocin-like peptide. Results in Fig. 4 clearly indicated that the inhibitory effect is attributed to the bacteriocin-like peptide.

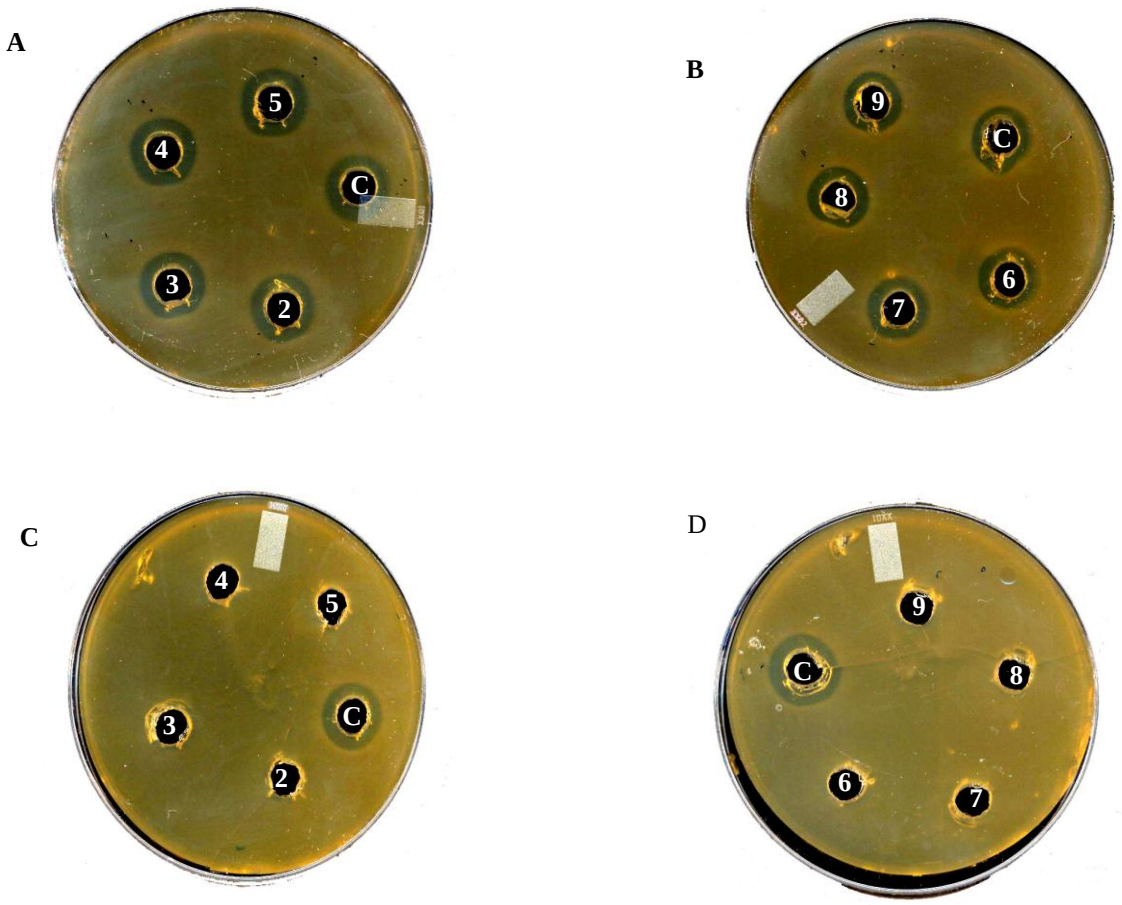


Figure 4. Effect of pH values on the activity of bacteriocin-like peptide produced from *Lactobacillus acidophilus* DSM 20079. A is pH values from 2-5; B is pH values from 6-9; C is pH values of uninculated MRS broth from 2-5 and D is pH values of uninculated MRS broth from 6-9.

**Growth rate and production of bacteriocin-like peptide**

*Lactobacillus acidophilus* DSM 20079 was grown in MRS for 24h at 37°C. Growth rate at 620 nm and the antagonistic activity were measured after incubation at 0, 2, 6, 24, 30, 48 and 72h. Results in Fig. 5 showed that the antagonistic effect was detected after 2h and reached to the maximum after 30h of incubation. Growth rate of control samples measured at 620 nm increased significantly after incubation for 4h and substantially increased until the end of incubation period. However, the optical densities of treated samples remained almost constant on incubation for 6h and increased significantly with increasing the incubation period for 24h (Fig. 6). It seems therefore, that the baceriocin-like peptide produced by *Lactobacillus acidophilus* DSM 20079 has bacteriostatic effect against *Lactobacillus debrueckii ssp bulgaricus*. This conclusion was confirmed by measuring total count after 6h of incubation. The total count of control samples was

$6.49\times10^{8}$ while it was $4.78\times10^{4}$ for treated sample with 0.5ml supernatant of *Lactobacillus acidophilus* DSM 20079.

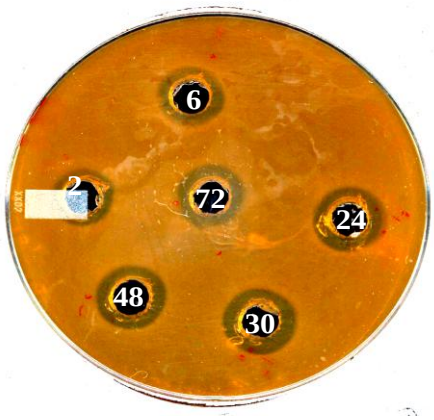


Figure 5. Production of bacteriocin-like peptide from *Lactobacillus acidophilus* DSM 20079 at 2, 6, 24, 30, 48 and 72h incubation at 37°C.

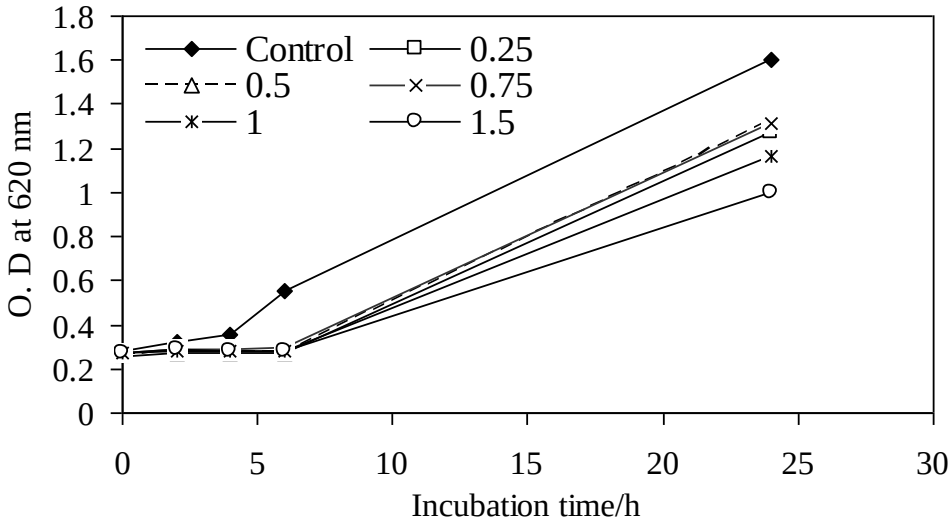


Fig. 6. Effect of cell-free supernatant from Lactobacillus acidophilus DSM 20079 on growth rate of Lactobacillus delbrueckii ssp bulgaricus DSM 20081.

**Quantification of antagonistic activity**

MRS broth was inoculated with three-subcultured *Lactobacillus acidophilus* DSM 20079 and incubated at 37°C for 24h. The obtained cell-free supernatant was diluted with sterilized water. The antagonistic effect of diluted supernatant (50 µL) was measured against *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081 as indicator bacterium. Results showed that the highest dilution of the cell-free supernatant was about 25 fold. Therefore, it was estimated that the antagonistic activity of the cell-free supernatant of *Lactobacillus acidophilus* DSM 20079 could reach about 500 arbitrary unit/ml. (Fig. 7).

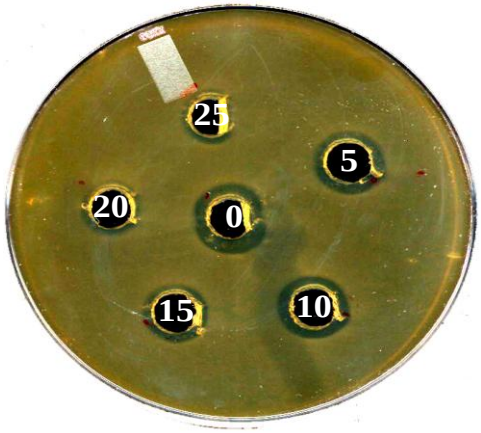


Figure 7. The antagonistic effect of diluted supernatant (50 µL) of *Lactobacillus delbrueckii ssp bulgaricus* DSM 20079 against *Lactobacillus delbrueckii ssp bulgaricus* DSM 20081.

**Precipitation of bactriocin-like peptide with different ammonium saturation.**

Ammonium sulfate was added to cell-free supernatant in concentration 20-90% saturation. The obtained pellet was dissolved in tris buffer (50 mM) and antagonistic activity was determined. The maximum antagonistic activity was found at 40 and 50% saturation (Fig. 8). He et al (2006) reported that the maximum antagonistic activity of cell-free supernatant of *Bacillus licheniformis* was at 60% saturation.

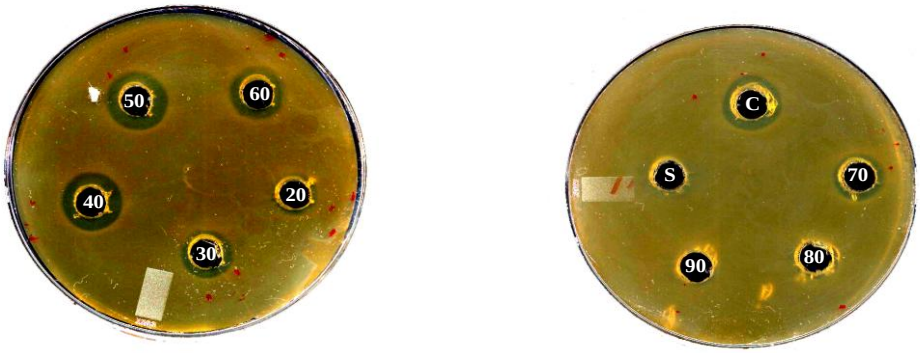


Figure 8. Antagonistic activity of bacteriocin-like peptide precipitated by different saturation of ammonium sulfate. C is control (cell-free supernatant), S is supernatant remains after 90% saturation, 20-90% is ammonium sulfate saturation.

**Determination of molecular weight**

Gradient 8-18% SDS polyacylamide gel (Amersham, Biosciences) was used. Fig. 9 shows the electrophoretic patterns of the pellet obtained from the supernatant of *Lactobacillus acidophilus* DSM 20079 after precipitation with 50% ammonium sulphate saturation and redissolved in 50 mM tris buffer (The solution was dialyzed against water for 48h with stirring under cooling to remove ammonium sulphate salts before injection). The SDS-PAGE of the pellet resulted in a single band with estimated molecular mass of 33 kDa. (Fig. 10).

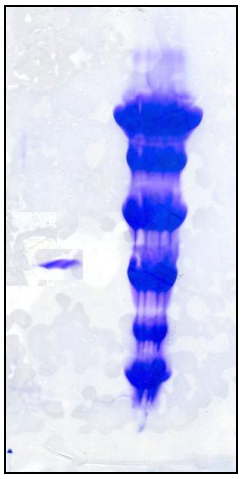

Figure 9. The SDS-PAGE of supernatant of *Lactobacillus acidophilus* DSM 20079 after precipitation with 50% ammonium sulphate saturation and redissolved in 50 mM tris buffer (lane 1 ) and molecular weight (94 – 14.4 kdalton) standard proteins (lane 2).

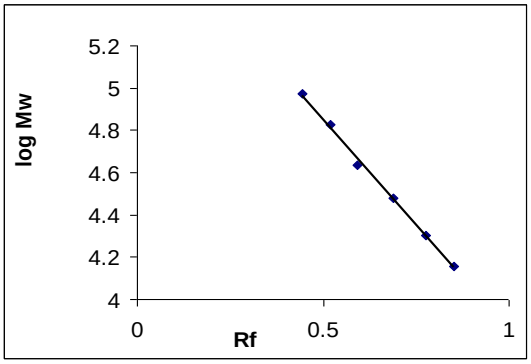


Figure 10. The Rf of protein marker against log of molecular weight

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# التوصيف الجزئي لبكتريوسين شبيه بالبتيد بواسطة Lactobacillus acidophilus DSM 20079

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الرياض، المملكة العربية السعودية

**الملخص:** تم دراسة التأثير المضاد لبعض سلالات البكتريا المنتجة لحمض اللبن والبفيدوبكتريا. أنتجت بكتريا *Lactobacillus acidophilus DSM 20079* نشاط شبيه للبكتريوسين ضد بكتريا مختلفة بما فيها أنواع من البكتريا الممرضة وتلك المسببة لفساد الأغذية. أنتجت السلالة ببتيد مضاد للبكتريا بعد ساعتين وكان مستوى الببتيد عند الحد الأقصى بعد 30 ساعة من التخمر. أظهر الببتيد نشاط للبكتريا ضد *Lactobacillus delbrueckii ssp bulgaricus DSM 20081,* و *Bacillus series* ATCC 9634 و *Escherichia coli* و *Enterococcus faecali* و *Salmonella typhimurium ATCC14028* و *bacillus subtilis* ATCC9372 ولكن ليس ضد *Lactobacillus acidophilus DSM 9126,* و *Lactobacillus acidophilus DSM 20242* و *Lactobacillus delbrueckii ssp bulgaricus DSM 20080,* و *Streptococcus thermophilus 20617, Bifidobacterium infantis DSM 20088 , Bifidobacterium angulatum DSM 20098.*

فقد البكتريوسين الشبيه بالببتيد نشاطه كلية بالتحلل البروتينى باستخدام الببسين والتربسين والكيموتربسين، إلا أن النشاط المضاد للبكتريا للببتيد ظل محتفظاً بنشاطه عند أس هيدروجيني من 2 إلى 9، كما احتفظ الببتيد بنشاطه المضاد للبكتريا بعد معاملته حرارياً على 50–140°م لمدة 5–15 دقيقة.

تم ترسيب البكتريوسين الشبيه بالببتيد باستخدام 40–50% كبريتات الأمونيوم. ثبط الببتيد معدل نمو بكتريا *Lactobacillus delbrueckii ssp bulgaricus DSM 20081* كلية بإضافة مستويات مختلفة من عائم *Lactobacillus acidophilus DSM 20079* الخالي من الخلايا وتم قياس ذلك عند طول موجي 620 نانوميتر. كما بلغ الوزن الجزيئي للببتيد المعزول 33 كيلو دالتون.