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Investigation of Bacteriocin Production Capability of Lactic Acid Bacteria Isolated From Foods [1] [2]

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Summary

The aim of this study was to isolate bacteriocin-producing lactic acid bacteria (LAB) from foods. A total of 12700 LAB were isolated from foods and screened for their bacteriocin production. Among them, 601 isolate showed a clear antimicrobial activity against indicator strains. After neutralization 35 of the cultures retained active. The antimicrobial activities of strains were lost after treatment with proteases. The antimicrobial metabolites were heat stable and were active pH range of 2-12. The bacteriocin which produced by *Lactococcus lactis ssp. lactis* isolated from kashar samples was partially purified by ammonium sulphate precipitation, dialysis, following ion-exchange chromatography. It was also found that this bacteriocin was cationic. The apparent molecular mass of partially purified bacteriocin, as indicated by activity detection after Tricine-SDS-PAGE, was 2.4 kDa, 4 kDa or 15 kDa. In this study antimicrobial substance of LAB from food showed strong antibacterial activity against Gram-negative and Gram-positive indicator microorganisms. Those were also found that these bacteriocins were stable at temperatures used in food manufacturing and storage. Bacteriosinogenic LAB or bacteriocins might be useful as a natural preservative for inhibiting food-borne pathogens in particular *Listeria monocytogenes* in foods.

Keywords: *Animals food, Lactic acid bacteria, Bacteriocin, Antimicrobial activity*

Gıdalardan İzole Edilen Laktik Asit Bakterilerinin Bakteriyosin Üretme Yeteneklerinin Araştırılması

Özet

Bu çalışmada, gıdalardan bakteriyosinojenik laktik asit bakterilerinin (LAB) izolasyonu amaçlanmıştır. Gıdalardan izole edilen toplam 12700 LAB'nin antimikrobiyel etkisi test edilmiştir. Bu izolatlardan sadece 601 adedi çalışmada kullanılan indikatör suşlara karşı antimikrobiyel etki göstermiştir. LAB'nin ürettiği laktik asitin ve hidrojen peroksitin güçlü antimikrobiyel etkisi göz önüne alındığında bu etkinin ortadan kaldırılması için katalaz ilavesiyle birlikte ortam nötrlendiğinde antimikrobiyel etkisini sürdüren sadece 35 izolat olduğu belirlenmiştir. İzolatların etkisi proteolitik enzimlerin varlığında kaybolmuştur. Üretilen antimikrobiyel etkili metabolitlerin gıda üretim ve muhafaza sıcaklıklarında ve geniş pH (pH 2-12) aralığında aktivitelerini sürdürdükleri gözlenmiştir. Kaşar peynirinden izole edilen *Lactococcus lactis ssp. lactis* suşunun ürettiği bakteriyosin kısmi olarak saflaştırılarak tanımlanmaya çalışılmıştır. İzole edilen bakteriyosinojenik suşların Gram-negatif ve Gram-pozitif indikator mikroorganizmalara karşı oldukça güçlü antimikrobiyel etkiye sahip olduğu belirlenmiştir. Bakteriyosinojenik LAB ve/veya onların bakteriyosinleri gıda kaynaklı patojenlerden özellikle *Listeria monocytogenes*'in inhibisyonu için doğal bir katkı maddesi olarak kullanılabilir.

Anahtar sözcükler: *Hayvansal gıdalar, Laktik asit bakterileri, Bakteriyosin, Antimikrobiyel aktivite*

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INTRODUCTION

Lactic acid bacteria (LAB) can produce antimicrobial substances with the capacity to inhibit the growth of pathogenic and spoilage microorganisms. Organic acids, hydrogen peroxide, diacetyl and bacteriocins are included among these compounds [1]. Antimicrobial proteinaceous compounds produced by bacteria that are active against other bacteria, despite varying greatly in chemical nature, mode of action and hot specificity, have traditionally been defined as bacteriocins [2]. Bacteriocins and lantibiotics produced by LAB are extremely important in preventing the growth of spoilage and pathogenic bacteria, and have been subject of extensive studies in recent years because of their potential use as novel, natural food preservatives [3]. They have an inhibitory effect only on closely related species and on other Gram-positive organisms. Those produced by LAB are of particular interest due to their potential application in the food industry as natural preservatives [3,4].

This paper reports the identification of the bacteriocin producing strain isolated from several foods, and physical and chemical properties, and action mode of the bacteriocin.

MATERIAL and METHODS

Indicators strains

The bacteria chosen as indicators were *Lactobacillus casei, Lb. cellebiosis, Leuconostoc paramesenteroides, Streptococcus lactis,* (isolated from kefir samples in our laboratory), *Micrococcus luteus, L.monocytogenes, Staphylococcus aureus, Bacillus subtilis, Salmonella enteritidis, Yersinia enterocolitica* O: 3 (Refik Saydam Hygiene Center, Turkey).

Samples

450 samples of a variety of milk and meat products obtained from different manufacturers in Turkey were analyzed. They included raw milk, yoghurt, butter, cream, tulum cheese, kashar cheese, white cheese and fermented sausage. Samples of kefir were made using with kefir grains in our laboratory [5].

Isolation of bacteriocin-producing lactic acid bacteria from samples of food

For isolation of LAB the serial dilutions of the samples were inoculated (pour plate) onto Chalmers agar [6], M17 agar and MRS agar. Followed by aerobic incubation at 30ºC for 48–72 h to allow the colonies to develop. From MRS agar, M17 agar and modified Chalmers agar, five colonies were randomly taken from each sample. Tests were carried out on each isolate. All isolates were investigated to determine their colony morphology, cell morphology, and Gram staining, catalase, and gas production from glucose. These tests were conducted as a preliminary step in characterization of the selected isolate [7,8].

Detection of antagonistic activity

Three methods were used to determine antimicrobial activity. Antimicrobial activity was determined in both colonies of LAB and culture supernatant.

Method 1: One of the methods, briefly, Mueller Hinton (Oxoid) agar plates was seeded with a bacterial lawn of indicator strains at $1x10^5$ CFU/ml concentration. Then, colonies of LAB isolates spotted on the agar plates. The plates were incubated 24h at 30ºC, antimicrobial activity being detected as translucent halos in the bacterial lawn surrounding the colonies [9].

Method 2: Culture supernatant (CS) was obtained by centrifuging (Hettich, Universal 32 R) the culture at 10.000 x g for 10 min at 4ºC. Mueller Hinton (Oxoid) agar plates were seeded with a bacterial lawn of indicator strains at a $1x10^5$ CFU/ml concentration. After this step, two different processes were applied; Agar disk diffusion methods [10] and Well-infusion methods [11].

Method 3: Microtiter plate method was used to determine antimicrobial activity.

Bacteriocin assays

LAB strains were grown in MRS broth at 30ºC for 18 h. A cell-free solution was obtained by centrifuging the culture at 10.000 x g for 10 min at 4ºC. The supernatant fluid was then adjusted to pH 6.5-7 with 5 N NaOH or 5 N HCl, so as to rule out inhibition through the production of organic acids. The supernatant fluid was filtered through a syringe

filter with a pore size of 0.22 µm (Millipore). If antagonism was present, the possible inhibitory action of hydrogen peroxide was eliminated by the addition of a sterile solution of catalase (1 mg/ml-Sigma) at 25ºC for 2 h. The samples were incubated at in water bath at 60ºC for 10 min. Any residual antimicrobial activity to treated extract was determined by the agar disk method and well-diffusion method [8,11,12].

If inhibition continued to be present when the extract added the wells had been neutralized and treated with catalase, this was taken to be due to the production of bacteriocin or bacteriocin-like compounds. The proteinaceous nature of the active extract was evaluated by treating this extract with a 1 mg/ml final concentration of proteolytic enzymes [α-chymotrypsin (Sigma SIC 4129), Protease (Sigma P4860), Trypsine (Sigma T1426)]. All these enzymes solution were filter-sterilized through a syringe filter with a pore size of 0.22 µm (Millipore). An untreated culture extract and enzyme solution (in buffer only) served as controls. The samples and controls were incubated at 37ºC for 2 h and water bath at 60ºC for 10 min to inactive the enzymes. Any residual antimicrobial activity to treated extract was determined by the agar disk method and well-diffusion method [12,13].

Sensitivity of bacteriocin-like substance to pH and heat

The bacteriocin or bacteriocin-like activity of the cell free culture supernatant was analyzed after heat-treatment and at different pH values. The residual antimicrobial activity was determined by the agar disk method and well-diffusion method. The sensitivity of the active substance to different pH was estimated by adjusting the pH of supernatant samples to pH 2–12 with NaOH or HCI and testing against the indicator strains after 37ºC 2 min incubated. The pH-treated samples were neutralized to pH 6.5–7 before measuring the residual activity. The sensitivity of to heat was tested by heating cell free supernatant samples to at 4ºC for 7 days, at 65ºC for 30 min, at 121ºC for 15 min and testing the residual activity by the agar disk method and well-diffusion method [13,14].

Identification of LAB

Isolates were identified as follows: hydrolysis of arginine, growth in 6 and 10% NaCI broth, dextran production, growth at pH 4–9 in 0.1% methylene blue and growth at 0, 10, 15 and 45ºC, growth in Acetate agar, Voges prouskauer test and the fermentation of carbohydrates test. The phenotypic identification of LAB strains was carried out by means of miniaturized API 50 CH biochemical test (Biomerieux). The result of the identification tests were interpreted using the APILAB PLUS software (Biomerieux) [7,15-17].

Partial purification of bacteriocin

For purification, the sterilized neutralize supernatant was saturated with 80% (w/v) ammonium sulfate (Merck), and held for 18h with stirring at 4ºC. The samples were centrifuged at 15.000 x g for 30 min at 4ºC and pellet was resuspended in 10 ml 10mM sodium phosphate buffer (Merck), pH 6.5. Further purification included dialysis using a Spectra/Por dialysis membrane with a 1 kDa cut-off (Spectra/Por 7 – 132104a) against the same buffer, overnight at 4ºC [18,19].

For purification of dialyzed was used ion-exchange column. The sample was applied to a column (2.5 by 50 cm) of carboxymethyl-cellulose (CM52 Whatman 4037050) prequilibrated with 50mM sodium phosphate buffer (Merck), pH 6.5. After being washed with buffer and 0.3 M NaCI. Antimicrobial activity of filtrate (anionic protein) and precipitate (cationic protein) were tested by the agar disk method and well-diffusion method [20].

Protein concentration of the bacteriocin fractions were determined by using the protein assay kit (Sigma BCA1 and B 9643) with bovine serum albumin as the Standard.

Determination of molecular weight

The apparent molecular masses of bacteriocin were estimated by the Tricine-SDS-PAGE method [21]. Electrophoresis was performed at 30–90 V for 2.5 h using 10–16% gradient Tris-Tricine SDS PAGE gels and polypeptide SDS Standard (BioRad 161-0326) as molecular weight standard. Gel was stained with both Coommasie blue stain and silver stain [22]

RESULTS

A total of approximately 13500 colonies isolated in each culture medium were examined for their strongest antibacterial activity against one of the

twelve indicator strains. A total of 601 colonies, showing antibacterial activity against at least one of the indicator organisms, were isolated. Results showed that 35/601 LAB cultures retained activity in the cell-free supernatants after neutralization (pH 6.5–7). All of them showed powerful anti-listerial activity, especially against *L. monocytogenes, S. aureus, B. subtilis* and *M. luteus* but not against strains of *E. coli, Y. enterocolitica* and other Gram negative indicator strains. Only three strains showed antimicrobial activity against both Gram negative strains and other strains.

Effects of enzymes, pH and temperature

Complete inactivation of antimicrobial activity was observed after treatment of bacteriocin-containing cell-free supernatants with α-chymo-trypsin, protease and trypsine, confirming its proteinaceous nature. Treatment of the bacteriocin with catalase did not result in any changes of antimicrobial activity, indicating that the inhibition recorded was not hydrogen peroxide. While some the bacteriocin-containing cell-free supernatants were stable in wide pH range from 2 to 12 or heat treatment, other was instable (Data not shown).

According to result of test, when LAB to classify, 15 of this 35 isolates were *Lb. plantarum,* 12 of isolates *Lc. lactis ssp. lactis*, four of isolates *Lb. brevis*, three of isolates *Lb. paracasei ssp. paracasei*, one of isolates *Leuc. mesenteroides ssp. mesenteroides* were as identified. Thus far a great number of LAB isolates were worked however bacteriocins were identified very hard. Because of difficultly of partial purification of bacteriocin only one bacteriocin which has a strong antilisterial activity was partially purified from a total of 35 isolates.

Partial purification of bacteriocin

The bacteriocin produced *Lc. lactis ssp. lactis* was partially purified from the supernatant fraction of cultures by 80% ammonium sulfate pre-cipitation. For partial purification, ion-exchange column was used. Bacteriocin was determined cationic. The apparent molecular masses of bacteriocin were estimated by the Tricine-SDS-PAGE method. SDS-PAGE method was un-successful in samples containing small sized protein. Resolution of the small peptides was difficult because they appeared as faint, diffuse bands, Tricine-SDS-PAGE offer high resolution, especially for the small protein and peptide range. Samples of bacteriocin on Tricine-SDS-PAGE gel could not be stained with Coomassie blue dye but could be stained with silver (Data not shown). Silver-stained Tricine-SDS-PAGE gel showed several moderately sized protein bands as well as a prominent band of 2.5 kDa. While coomassie blue-stained used methanol and acetic acid for fixation, silver-stained used formaldehyde as strong agent for fixation.

DISCUSSION

In this study, strains producing bacteriocins were isolated from local foods and antimicrobial compounds produced by those strains were partially purified.

There are many methods for determine antimicrobial activity of LAB. In this study, using broth culture of LAB to determine antibacterial activity gave better results than colonies of them on solid medium. In this study, results showed that incubation period must not 12-24 h. Antimicrobial activity of isolates was not determined after 12-24 h. Antimicrobial activity may be lost at the end of six hours period possibly due to purity of supernatant.

Although 31500 colonies were isolated from foods, bacteriocin productions among the LAB isolated were detected in thirty-five strains. Various factors may have influence, such as culture medium, condition of incubation, target microorganisms, variability and sensitivity of methods used to for determine antimicrobial activity.

Bacteriosinogenic strains showed strong anti-microbial activity against Gram positive indicator microorganism but only three of 35 showed activity against Gram-negative bacteria. Nisin exhibits antimicrobial activity towards a wide range of Gram-positive bacteria, and is particularly effective against spores. It shows little or no activity against Gram-negative bacteria, yeast and moulds [23]. However, Vignolo et al.[11] reported bacteriocins of LAB against Gram-negative bacteria, contrary to other researchers [24,25].

Total 12 *Lc. lactis ssp. lactis* strain produced substrate show that one from the other different data against tolerant test, activity test. The production

of more than one bacteriocin by a single LAB strain has been reported previously. Several bacteriocins were produced by *Lc. lactis ssp. lactis*. For example lactococcin, lacticin, nisin [26-28].

Lc. lactis ssp. lactis isolated from kefir showed strong antimicrobial activity against *E. coli*. In addition most of those isolates continued to demonstrate antimicrobial activity indicator microorganisms at wide pH range from 2 to 12 and heat treatment (121ºC for 15 min). Despite the fact that LAB-bacteriocins shows little or no activity against Gram negative bacteria, this isolate is important which showed strong antimicrobial activity against *E. coli*. Antimicrobial substance of *Lb. plantarum* species in identified this research showed variable sensitivity at heat and pH treatment. *Lb. plantarum* was identified in almost all analyzed sample foods. *Leuc. mesenteroides ssp. mesenteroides* isolated from butter and *Lb. brevis* isolated from butter, cream and kefir showed strong antimicrobial activity against *L. monocytogenes* and *S. aureus* but not against strains of *E. coli, Y. enterocolitica* and other Gram negative indicator strains. The sensitivity to temperature, pH and enzymes and inhibitory effects against indicator microorganisms of antimicrobial compounds produced by species is varied in strains.

Our study result compared to the results of other researches differed. This distinction being greatly influenced by several factors such as origin of isolates, different antimicrobial substance could be produced by bacteria, variable methods for determination of antimicrobial affect and sensitivity of methods, bacteriocin dose and degree of purification, physiological state of the indicator cells (e.g. growth phase) and experimental conditions (e.g. temperature, pH, presence of agents disrupting cell wall integrity and other antimicrobial compounds, etc.), variable methods for purification of bacteriocin and sensitivity of methods.

Many studies on the activity of bacteriocins against target strains were done in laboratory media and not in foods. There are intrinsic factors in foods that could cause reduced activity of a bacteriocin. While in-vitro tests can give a good deal of information on antimicrobial performance, they cannot necessarily duplicate all the variability, which might exist in a food. Therefore, once it has been determined that the antimicrobial performs well in an in-vitro situation, it should be applied to a food system. Several studies show that different results were obtained while bacteriosinogenic-LAB used as food preservative in food system or in vitro. The production of a certain bacteriocin in laboratory media does not imply its effectiveness in a food system. This subject was needed overmuch research. The study of bacteriocins as preservatives in foods can be misleading and confusing unless they have been fully characterized.

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