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Screening of Bacteriocin Production in Lactic Acid Bacteria Isolated From Fermented Dairy Products

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Authors' contributions

This work was carried out in collaboration between both authors. Author GB designed the study, wrote the protocol and the first draft of the manuscript. Author ED managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study we aimed to screen LAB strains from different fermented dairy products by phenotypic and genotypic methods.
Methodology: 138 LAB isolates belonging to *Enterococcus* (13,76%), *Lactococcus* (18,84%) and *Lactobacillus* (67,39%) genera from fermented dairy products were used to search for their bacteriocin production and bacteriocin encoding genes were searched. LAB isolates were screened by spot-on lawn assay method against indicator microorganisms. *Geobacillus stearothermophilus* DSMZ 22, *Escherichia coli* ATCC 35218, *Bacillus cereus*, *Listeria innocua*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Micrococcus luteus*, *Lactococcus lactis* DSMZ 20729 and *Lactobacillus plantarum* DSMZ 20205. Cell-free supernatants (CFS) of LABs used for agar-well diffusion assay to confirm antibacterial activity.
Results: All the LAB strains exhibited antibacterial activity against indicator bacteria at varying degrees by spot on lawn method. None of the CFS except one belong to positive control *Lactococcus lactis* DSMZ 20729 showed inhibitory effect by agar well diffusion assay. According to PCR results which used to investigate the bacteriocin genes of LAB's , *ent*-A (5,07%), *ent*-B (2,17%), *lcn*-A (2,17%), and *pln* (1,44%) genes were detected in some of the isolates.

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Keywords: LAB; bacteriocin; spot-on lawn assay; agar well diffusion assay; PCR.

1. INTRODUCTION

Despite of the modern technology and safety systems used currently, foodborne illness and intoxications are increasing. Pathogens like *Salmonella, Campylobacter jejuni, Escherichia coli*, *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum* and *Clostridium perfringens* could contaminate the water or basic foods and can cause the various symptoms even death sometimes. Shelf-life of foods also negatively effected by microorganisms and this situation is economically harmfull. For these reasons, inhibition of microbial pathogens and spoilers in foods is important issues.

The use of chemical preservatives or additives in food or feed is not preferred way according to public tendency. Instead, consumers need high quality, safe, minimally processed food especially those one with extended shelflife by biopreservatives [1]. By the use of controlled microflora and/or antibacterial substances, ability to providing of long shelf life and safe food production is presented. Lactic acid bacteria (LAB) or substances such as bacteriocins produced by LAB gained too much attention due to be safe and natural use as the inhibitor against pathogen microorganisms in food production [2].

The bacteriocins have been grouped into three main classes as Class I bacteriocins are small modified bacteriocins (lantibiotics) with lanthionine or β-methyllanthionine residues, e.g. Nisin, Lacticin 3147, while Class II bacteriocins are non-lanthionine containing bacteriocins which include pediocin like PA-1, Enterocin AS48, Lactococcin A, etc. Bacteriolysins which classified before as Class III bacteriocins are large, heat-labile murein hydrolases with molecular weight >10 kDa and they catalyse cell-wall hydrolysis [3].

The increase in use of bacteriocins for food industry depends on identify new strains which have high bacteriocin producing capacity or broader spectrum of activity. In our study, we aimed to characterize the bacteriocin production in LAB belong to different genera (*Enterococcus*, *Lactococcus* and *Lactobacillus*) isolated from fermented dairy products. Strains were tested phenotypically against the indicator bacteria by using two methods, and genes responsible for bacteriocin production in LAB investigated by PCR.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 LAB strains

LAB strains used in the study like as follows; *Lactobacillus helveticus* (n=2), *Lb. acidophilus* (3), *Lb. delbrueckii* (28), *Lb. gasseri* (1), *Lb. uvarum* (1), *Lb. brevis* (1), *Lb. coryniformis* (1), *Lb. curvatus* (5), *Lb. kefiri* (8), *Lb. alimentarius* (2), *Lb. diolivorans* (1), *Lb. otakiensis* (1), *Lb. rhamnosus* (9), *Lb. paracasei* (5), *E. faecalis* (5), *E. durans* (3), *E. faecium* (3), *E. gallinarium* (2), *E. hirae* (1), *Lc. lactis* subsp. *lactis* (16), *S. macedonicus* (11), *S. lutitiensis* (6). They were identified according to 16S rDNA analysis [4] and propagated in Brain Heart Infusion (BHI), Man-Rogosa Sharp (MRS) or Trypticase Soy Yeast Agar (TSYA) media before activity assays.

2.1.2 Indicator bacteria used in the study

Indicator bacteria belong to the different genera were used in spot-on lawn and agar well diffusion assays for screening bacteriocin activity (Table 1).

Table 1. Indicator bacteria used in the study

Indicator bacteria	Growth temperature	Growth media
Geobacillus stearothermophilus DSMZ 22	55℃	NA
Escherichia coli ATCC 35218	37℃	NA
Bacillus cereus	30℃	BHI Agar
Listeria monocytogenes	37℃	BHI Agar
Listeria innocua	37℃	BHI Agar
Enterococcus faecalis	37℃	BHI Agar
Micrococcus luteus	30°C	BHI Agar
Lactococcus lactis DSMZ 20729	30℃	TSYE Agar
Lactobacillus plantarum DSMZ 20205	30℃	MRS Agar

2.1.3 Primers

To investigate the known genes responsible for bacteriocin production in some LAB species primers in Table 2 were used in PCR experiments.

2.2 Methods

2.2.1 Spot-on lawn assay

To detect the antibacterial activity against indicator bacteria, spot on lawn assay and agar well diffusion assay (AWDA) were used. Spot-on lawn assay performed as mentioned in Schillinger and Lüke [9], overnight cultures of the LAB strains were spotted onto MRS agar plates and incubated 30 or 37℃ according to species to allow growth of colonies. Five mililitres of indicator bacteria was added in 5 ml soft appropriate media (BHI, MRS or TSYE with 0,5% agar), poured on LAB spotted plates and then incubated at temperature suitable for indicator. After 24h of incubation, plates were checked for inhibiton zones. Nisin producer *Lactococcus lactis* DSM20729 was used as positive control to test the bacteriocin activity.

2.2.2 Agar well – diffusion assay

LAB strains were incubated in liquid media for 24h and then centrifuged at 8 000 g for 10 min to collect the supernatant. After pH of the supernatants were adjusted to 6 by 1M NaOH or HCl, they were membrane filtered through 0.45 µm pore sized cellulose acetat filter.

Indicator bacteria suspensions were prepared at 0.5 MacFarland turbidity, streaking on BHI, MRS or Nutrient agar media by swabs and then 6 mm wells were punched by agar borer. A hundred microliters of culture free supernatnats (CFS) were added to wells, plates were incubated for 24 hours and checked for zone of inhibition after incubation. *Lactococcus lactis* DSM20729 was used as positive control to test the bacteriocin activity.

2.2.3 PCR screening of known bacteriocin genes

2.2.3.1 Genomic DNA isolation from LAB

Genomic DNAs of LAB used in the study were extracted by method described in Ronimus et al. [10] or InstaGene DNA isolation kit according to manufacturer instructions.

2.2.3.2 PCR for bacteriocin genes

Genomic DNAs isolated from LAB strains used as template to amplify the bacteriocin genes, enterocin A enterocin B for Enterococci; Lactococcin and lactisin in Lactococci; Nisin and plantaricin in lactobacilli species. Final concentrations in PCR mix as follow; PCR buffer 1X, $MgCl_2$ 2 mM, dNTP 0,2 mM, primers, 0,4 pmol each, Taq polymerase 1 Unit, and conditions are given in Table 3. Amplicons were then sequenced by Macrogen (Korea) and homologies were identified using BLAST with other known bacteriocin genes.

Table 2. Primers for some known bacteriocin genes

Genes	Primers	Fragman size (bp)	Reference
Enterocin A	F: GGT ACC ACT CAT AGT GGA AA R: CCC TGG AAT TGC TCC ACC TAA	138	[5]
Enterocin B	F: CAA AAT GTA AAA GAA TTA AGT ACG R: AGA GTA TAC ATT TGC TAA CCC	201	[5]
Lactococcin A	F: CAA TCA GTA GAG TTA TTA ACA TTT G R: GAT TTA AAA AGA CAT TCG ATA ATT AT	771	[6]
Lacticin 481	F: TCT GCA CTC ACT TCA TTA GTT A R: AAG GTA ATT ACA CCT CTT TTA T	366	[6]
Nisin	F: GGA TTT GGT ATC TGT TTC GAA G R: TCT TTC CCA TTA ACT TGT ACT GTG	598	[7]
Plantaricin	F: GGC ATA GTT AAA ATT CCC CCC R: CAG GTT GCC GCA AAA AAA G	428	[8]

Table 3. PCR conditions for screening bacteriocin genes

Genes	PCR conditions			
ent-A	Initial denaturation	95℃	5 min	
	Denaturation	95℃	30 sec	
	Annealing	58℃	30 sec	35 cycles
	Extension	72℃	30 sec	
	Final extension	72℃	5 min	
	[11]			
ent-B	Initial denaturation	95℃	5 min	
	Denaturation	95℃	30 sec	
	Annealing	56℃	30 sec	35 cycles
	Extension	72℃	30 sec	
	Final extension	72℃	5 min	
	[11]			
lcn-A	Initial denaturation	95℃	5 min	
	Denaturation	92℃	2 min	
	Annealing	38℃	2 min	30 cycles
	Extension	72℃	2 min	
	Final extension	72℃	5 min	
	[6]			
lac-481	Initial denaturation	95℃	5 min	
	Denaturation	92℃	30 sec	
	Annealing	51℃	30 sec	26 cycles
	Extension	72℃	1 min	
	Final extension	72℃	5 min	
	[6]			
nisin	Initial denaturation	95℃	5 min	
	Denaturation	92℃	2 min	
	Annealing	41℃	2 min	26 cycles
	Extension	72℃	2 min	
	Final extension	72℃	5 min	
	[7]			
pln	Initial denaturation	95℃	5 min	
	Denaturation	94℃	3 min	
	Annealing	53.2℃	1 min	30 cycles
	Extension	72℃	1 min	
	Final extension	72℃	5 min	
	[8]			

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Screening of LAB for production of antibacterial compounds

As a result of agar spot test, all LAB strains showed inhibitor effect against some indicator bacteria at varying degrees (Fig. 1).

When strains tested for antibacterial activity by agar well diffusion method none of them showed inhibition against indicator bacteria except for *L. lactis* DSM 20729 which used as positive control (Fig. 2.)

3.1.2 PCR detection of bacteriocin genes and sequencing

All LAB strains tested for bacteriocin activity in this study were also searched for some known genes responsible for bacteriocin production. *ent*A and *ent*B genes were found in seven and three *Enteroccus faecium* strains respectively. *lcn*-A gene was detected in three *Lactococcus lactis* subsp. *lactis* strains while *pln* gene was found in two *Lactobacillus plantarum* (Table 3). Electrophoresis images of amplicons were given in Figs. 3, 4, 5 and 6. DNA sequences of amplicons obtained by PCR were analyzed with BLAST and homologies with the other bacteriocin genes were given in Table 4.

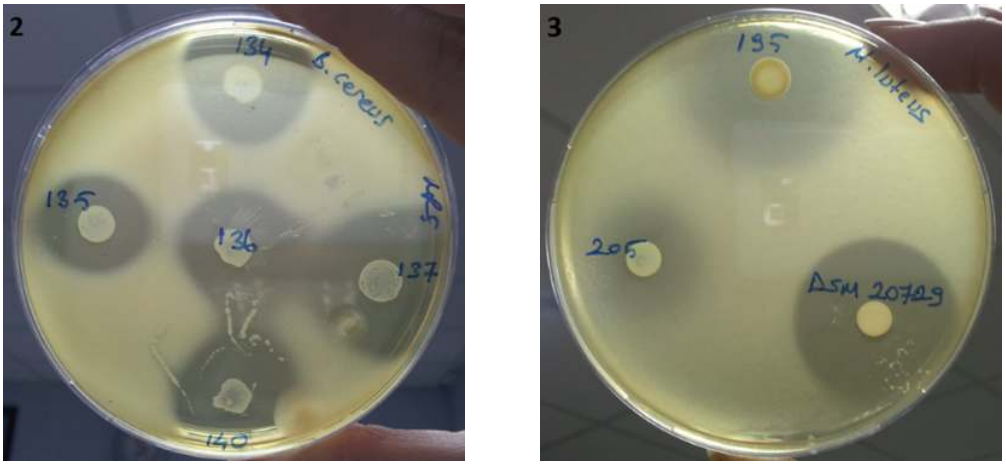

Fig. 1. Inhibition of *B. cereus* and *M. luteus* by some LAB strains in the spot-on lawn assays. Nisin producer *L. lactis* DSM20729 was used as positive control

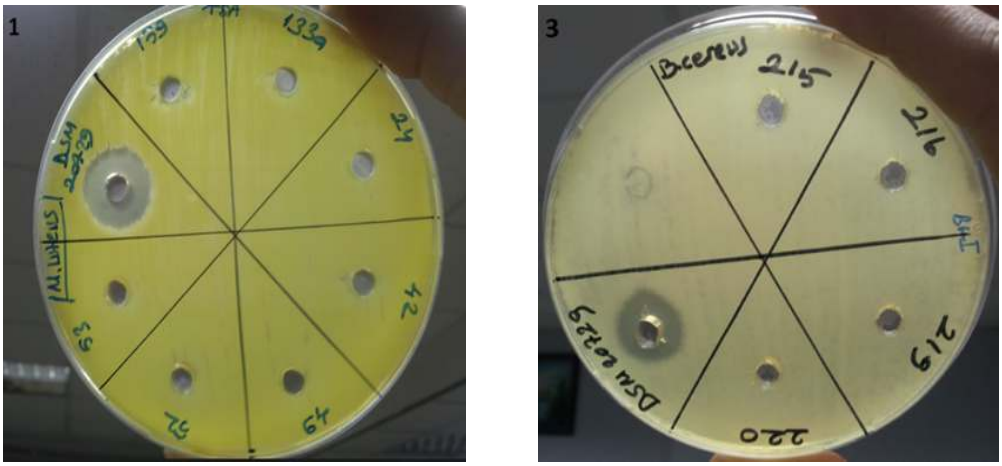

Fig. 2. Agar well diffusion assay results. Among the tested LAB strains, only positive control strain, *L. lactis* DSM 20729 showed antibacterial acitivity

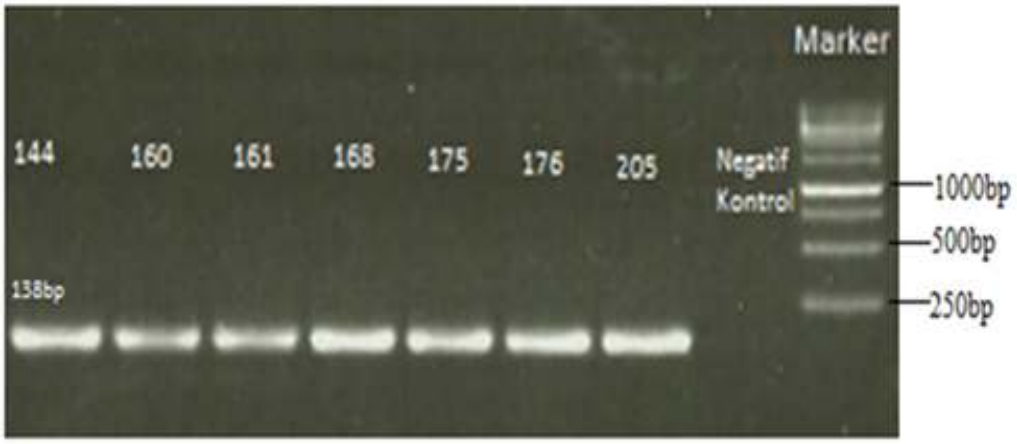

Fig. 3. *ent*A positive isolates *(E. faeci.*, n=7)

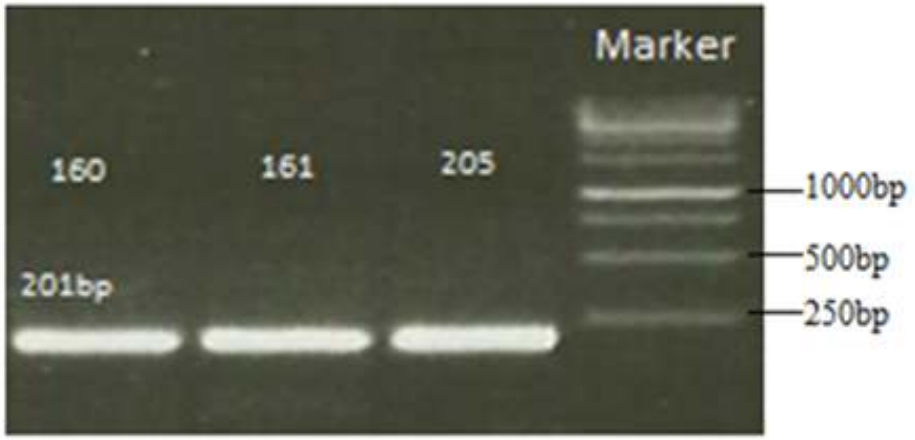

Fig. 4. *ent*B positive isolates (*E.faeci.*, n=3)

Fig. 5. *lcn*-A positive isolates (*L. lactis* subsp. *lactis*, n=3)

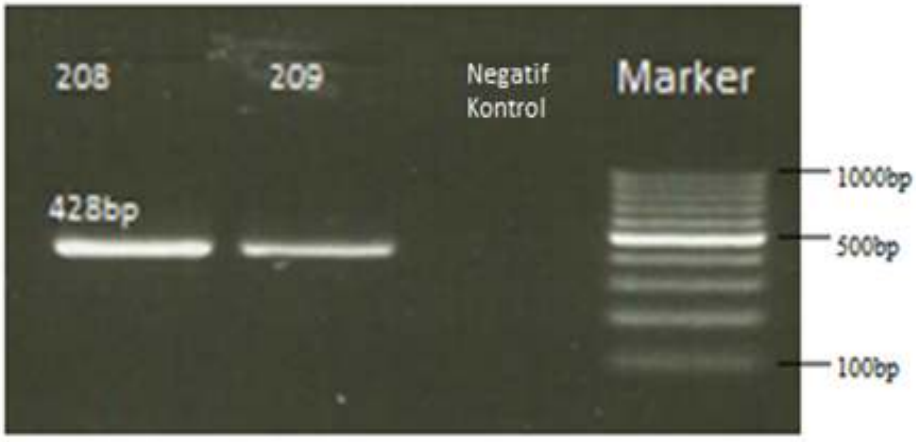

Fig. 6. *pln* positive isolates (*L. plantarum*, n=2)

Table 4. Detected bacteriocin genes in LAB strains as a result of PCR and BLAST results

Isolate number	Species	Detected gene	Homology (%)
GLM 144	*Enterococcus faecium*	*ent*A	98
GLM 160	*Enterococcus faecium*	*ent*A, *ent*B	100, 98
GLM 161	*Enterococcus faecium*	*ent*A, *ent*B	99, 98
GLM 168	*Enterococcus faecium*	*ent*A	98
GLM 175	*Enterococcus faecium*	*ent*A	99
GLM 176	*Enterococcus faecium*	*ent*A	97
GLM 205	*Enterococcus faecium*	*ent*A, *ent*B	97,94
GLM 134	*Lactococcus lactis subsp. lactis*	*lcn*A	94
GLM 135	*Lactococcus lactis subsp. lactis*	*lcn*A	96
GLM 136	*Lactococcus lactis subsp. lactis*	*lcn*A	95
GLM 208	*Lactobacillus plantarum*	*Pln*	99
GLM 209	*Lactobacillus plantarum*	*Pln*	98

3.2 Discussion

In this study we aimed the screening and genotypic characterization of bacteriocins from LAB isolated from different fermented dairy products, e.g. yoghurt, cheese, kefir samples sold in bazaars or markets in Aydin. All of LAB isolates displayed inhibitory effect against tested food borne or illness related indicator bacteria at various degree by spot-on lawn assay. However, when culture supernatants of LAB strains tested by AWDA, none of them were found with antibacterial activity. This situation may explain with many reasons according to bacteriocin experiments done before by different researchers. Rubio et al. [12] isolated totally 109 lactic acid bacteria from infant faeces and tested their bacteriogenic activity by agar spot test and agar well diffusion methods. By agar spot test inhibitory zones observed against variety of pathogens and reserachers reported that inhibition of pathogens was not achieved with neutralized and pasteurized supernatants indicates that the inhibitory activity was related to acid production.

In another study probiotic traits included antimicrobial activities of Lactobacilli strains isolated from raw cow milk were searched. Inhibitory effect was observed in all strains when they tested by agar spot test against pathogens (*L. innocua* ATCC 33090, *S. aureus* ATCC 25923, *S. aureus* ATCC 25922, *S. aureus, S. mutans* DSM 20523, *E. faecalis* ATCC10541, *E. coli* ATCC 13706, *E. coli*) [13]. On the other hand as a result of agar well diffusion method only one strain among fifteen Lactobacilli had displayed antimicrobial activity.

Algeria et al. [14], tested bacteriocin production of *L. lactis* strains isolated from traditional Spanish cheeses made from raw milk. It was found that, variable number of the sixty strains inhibited the different indicator microorganisms by agar spot test, while only 17 isolates showed clear zones in agar well diffusion experiments. According to researchers results were not surprised because many of antimicrobial compounds related to colonies on agar media like H_20_2 and fatty acids are responsible for the inhibitory effect.

Researchers found similar results with variety of LAB isolates which they were found as negative in the well diffusion assays [9,10]. They indicated that diffusion of bacteriocins, aggregation, protease activity and concentration are important factors for bacteriocin activity in the agar well diffusion tests.

Many studies reported the presence of bacteriocin genes of food, human, environment or animal origin [14-19]. In our experiments, *ent*A (5,07%), *ent*B (2,17%), *lcn*A (2,17%) and *pln* (%1,44) genes detected in some of *Enterococcus, Lactococcus* and *Lactobacillus* strains which they did not exhibit inhibitory effect in agar well diffusion assay.

One of the reason of antibacterial activity could not be detected in strains which carry structural bacteriocin genes might be due to inhibitory effect of LAB depends on indicator strains and methods used in experiments [20]. Because of some bacteriocins have very narrow targets, it is a key point to choose sensitive indicator strains It is also possible that encoded bacteriocin in chromosome or plasmid may not be expressed. The detection of bacteriocin genes does not necessarily mean that the related bacterium produces antimicrobial peptid and in a similar manner a lack of detectable antimicrobial activity

does not necessarily mean that bacteriocin production genes are defective. Also, bacteriocin production often setted by a system, for example by a two-component regulatory mechanism beside of the other regulation mechanisms (Nes, 1999). Bacterial genomes sometimes only encode a part of bacteriocin production system or mutations could inactivate the function of bacteriocin genes [21].

In the event as reported by Odeyemi et al. [22] bacteriocin screening tests can be grouped as preliminary and confirmation assays. While preliminary tests e.g. agar spot or spot on lawn, have been usefull to detect the potential of producer strains to inhibit the indicator strains, confirmation assays are important for control of former test results.

4. CONCLUSION

Our study can contribute the next researches for biopreservation of foods by using bacteriocins produced by LAB from different origins.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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