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Detection of genes encode for producing Bacteriocins in Lactobacillus genus isolated from local Iraqi dairy products

Rahem E. Alziadi [*1]

Abstract

This study was aimed to isolate and identify *lactobacillus* bacteria from some dairy products, antibiotic susceptibility and supernatants antimicrobial activity was tested, then detection for genes encoded to produce bacteriocins. morphological, microscopically and biochemical tests results showed that eight isolates were *lactobacillus,* antibiotic susceptibility test showed that most *Lactobacillus* isolates appeared multi resistant to more than one of antibiotics and sensitive to chloramphenicol while several of them are sensitive to others antibiotics , also results showed varying inhibitory effect of all *Lactobacillus* isolates supernatants in growth of pathogenic bacteria tastes, the highest inhibition zone that observed was against *Escherichia coli O157:H7, Salmonella typhimiurum, Enterococcus faecalis, Clostridium spp*, and *Staphylococcus aureus*, while weaker inhibition zone of *Lactobacillus* Isolates that observed was against *Klebsiella sp* and *Pseudomonas aeruginosa,*. DNA was extracted from isolates and PCR reaction was performed using primer specific for gene producing bacteriocins, this gene was produced PCR products with molecular weight ≈ 4000pb. The results showed that bacteriocins gene appeared in all *lactobacillus* isolates, therefore, such these *Lactobacillus* can be used as probiotic and as bio-preservative in food.

Keywords: Robiotics; *Lactobacillus*; Bacteriocins; Dairy products

* Correspondence author: rahem_enad@mu.edu.iq
[1]Al Muthanna University/College of pure science
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Introduction

Lactobacilli genus belong to group of bacteria that has Unique abilities like production of lactic acid, enzymes such as β-Galactosidase and bacteriocins as a natural metabolic substance [1]. this genus present widespread in dairy, fish, vegetable and grains products, also found in normal vaginal flora and protect the vagina from urinary tract infection, in fact, many species are capable of colonizing certain parts of the body, like oral cavity, gastrointestinal and urinary-genital tract, where they play an important role in health and competitive exclusion of pathogenic bacteria [2]. bacteriocin is plentiful and varied group of ribosomal synthesized antimicrobial peptides produced by bacteria and antimicrobial agent potential of inhibiting some pathogenic bacteria growth [3]. Traditionally, bacteriocin production has been considered an important feature in the selection of probiotic bacteria strains, but until recently, few studies have definitively demonstrated the impact of bacteriocin production on the ability of a strain to compete within complex microbial flora and influence the host health, so *lactobacillus* has gained special attention nowadays, due to the production of bacteriocins or peptide like bacteriocins [4]. there is more evidence suggests that bacteriocins may be have several mechanisms within the gastrointestinal tract, bacteriocins may directly inhibit or competing with pathogens, or modify the composition of bacteria and affect the host immune system [5]. bacteriocins are a kind of ribosomal synthesized antimicrobial peptides and biologically active proteins produced by bacteria, which can kill or inhibit bacterial strains closely-related or non-related to produced bacteria but will not harm the bacteria themselves by specific immunity proteins, while antibiotics are chemicals that inhibit the growth of bacteria, produced by different organisms (organisms and microorganisms, plants and fungi) [6]. many bacteriocins are ribosomal synthesized, there are many others that are non-ribosomal synthesized, commonly divided into three groups: class I – the antibiotics; class II – the heat stable unmodified bacteriocins; class III the larger heat stable bacteriocins. These compounds are variety in sizes, structures, physicochemical properties, and inhibitory spectrum, with large diversity [7].

Materials and methods

Collection of dairy products

Local dairy products samples were collected (Fresh laban Kanoon, sheep milk soft cheese, cow milk soft cheese, Fresh cow milk and Fresh sheep milk) from commercially markets in sterile containers and were transported to the lab.

Isolation and Identification of *Lactobacillus* genus

One ml of collected dairy products were cultured in 9 ml MRS- L. Cysteine-HCL broth (Oxoid, England), then, incubated at 37^{o}C for 48 – 72 hrs. serial dilution that was made for these samples in MRS broth. 0.1 ml of last dilution was spreader on MRS – $CaCO_3$ (Oxoid, England), and incubated an aerobically at 37^{o}C for 24 hrs. the raised colony, which surrounded by clear zone was transferred on MRS agar (pH 5.5) for purification. then, several steps were applied for purity, bacterial isolates were identified based on the colony characteristics, gram staining, biochemical characterization and production of catalase, acid and curd in litmus milk, ammonia from arganine, and growth at 15°C and 45°C in MRS broth. the identification of the genus *Lactobacillus* was confirmed according to [8] and as described by [9].

Preparation of culture supernatants:

Isolates were grown in MRS broth (pH 5.5) at 37 ºC for 18-20 hrs. the *Lactobacilli* culture was centrifuged at 10,000 rpm for 5 min, and then the supernatant was adjusted to pH 5.5±1 with 1N NaOH to eliminate the putative effect of produced organic acids then filtration with Millipore (0.22 µm pore size) [10].

Antibiotic susceptibility of *Lactobacillus* isolates

Conducted the sensitivity of *lactobacillus* isolates to antibiotics by following the method of [11] with some modified by using antibiotics tablets which included (ampicillin 10µg, cephalexin 30µg, chloramphenicol 30µg, gentamicin 10µg, tetracycline 30µg, erythromycin 15µg, amoxicillin 25µg and cefotaxime 15µg) (Oxoid, England), *Lactobacillus* isolates were spreader on MRS agar (Oxoid, England) by streaking, antibiotic discs were placed on the agar surface and incubated for 24 hrs. at 37C° under microaerophilic condition.

Antimicrobial activity of *Lactobacillus* isolates

Screening for antimicrobial activity in the tested supernatants against several indicator pathogenic bacteria was performed. indicator pathogenic bacteria was used in this study

provided from the laboratories of bio – technological and food science department/college of Agriculture / Baghdad University, gram negative bacteria were: *Escherichia coli O157:H7*, *Pseudomonas aeruginosa*, *Salmonella typhimiurum*, *Klebsiella spp*, *Enterococcus faecalis*, while gram positive bacteria were: *Clostridium spp*, *Staphylococcus aureus*. All these indicator bacteria are pathogenic for human and animals, also considered as important food pathogens that cause damage for some food [12]. Well diffusion assay was used to study antimicrobial activity according to method described by [13]. aliquots of 50 µl of the sterile supernatant were placed in 6 mm diameter wells on Muller-Hinton-agar (Oxoid, England) plates previously seeded with the respective indicator bacteria. after 16-18h of incubation at 37°C, the diameters of growth inhibition zones were measured, two plates were done for each isolate.

Detection of genes encode for produce bacteriocins

DNA extraction

DNA was extracted from the elected isolated cells according to the protocol of i-genomics CTB DNA Extraction Mini kit (cat no.17341) which supplied by intron Korea, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

Primer and PCR Technique

PCR reaction was performed using primer specific for gene producing bacteriocins (Bioneer /Korea). the primer set bacteriocin forward sequence was 5 - AAG AGT TTG ATC CTG GCT CAG - 3 and the reverse was 5 - CTA CGG CTA CCT TGT TAC GA - 3 [14] , the primers were used in PCR to amplify a 4000 bp DNA fragment of the lactocin bacteriocins gene from chromosomal DNA of the isolates, The PCR reaction was performed in a thermal cycler (Techne Thermocycler, Applied, USA), optimization of polymerase chain reaction was accomplished after several trial. the PCR mixtures were prepared according to [14] , each PCR mixture (25 µl) contained a reaction mix of 12.5 µl Master Mix 1 µl of each primer,2 µl of DNA sample and 8.5 µl of D.W . each PCR cycling profile consisted of an initial denaturation time of 3 min at 95°C was followed by an amplification for 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 61°C) and extension steps (2 min at 72°C) and the holding time was 3 min at 15°C. PCR products were analyzed on agarose gel (1%) using horizontal electrophoresis unit(Bioneer, Korea) at 5v/cm for two hours after ethidium

bromide staining, then DNA bands were visualized by using U.V transilluminator (Vilber-Lourmat, France) at 365 nm [15].

Results and discussion

Isolation and Identification of *Lactobacillus spp.*

From the total sixty that collected, eight Local dairy products samples gave colonies appeared pale, round shape, soft, mucoid, convex and surrounded by inhibition zone after cultured on MRS contained 1% $CaCO_3$. isolates were gram-positive bacilli when examined microscopically and negative to catalase test except one isolate gave few bubbles when hydrogen peroxide was added, also gave negative results for gelatin test, they were able to produce clot on litmus milk medium, unable to produce ammonia from arginine, unable to grow in 15^0C while, all grown in 45^0C except one isolate, which grew slowly at such temperature, all isolates were able to ferment glucose and lactose. But unable to ferment xylose and mannitol [8].

Antibiotic susceptibility of *Lactobacillus* isolates

Results in Table (1) showed that most *Lactobacillus* isolates showed multi resistant to more than one of antibiotics, *Lb.1, Lb.4, Lb.7,* and *Lb.8* can be resistant to three antibiotics, *Lb.2, Lb.3,* and *Lb.5*can be resistant to two antibiotics and *Lb.6* for one antibiotics. Also, most *Lactobacillus* isolates were sensitive to chloramphenicol while several of them were sensitive to other antibiotics. Several genes among *Lactobacilli* have been reported responsible for antibiotic resistance properties like chloramphenicol resistance genes (chloramphenicol acetyltransferases CAT) [16] In addition, erythromycin resistance genes, [17] tetracycline resistance genes [18] Aminoglycoside resistance genes [19], and β-lactam resistance genes (blaZ) [20].

Table 1.

Antibiotic susceptibility test of *Lactobacillus* isolates

***Lactobacillus* isolates** / **Antibiotics**	mpicillin	ephalexin	hloramphenicol	entamicin	etracycline	rythromycin	moxicillin	Cefotaxime
Lb.1	R	S	S	R	S	S	S	R
Lb.2	S	R	S	S	S	R	S	S
Lb.3	S	R	S	R	S	S	S	S
Lb.4	R	S	S	S	R	S	S	R
Lb.5	S	S	S	S	S	S	R	R
Lb.6	S	R	S	S	S	S	S	S
Lb.7	S	S	S	R	S	S	R	R
Lb.8	S	R	S	R	S	R	S	S

R: resistant, S: sensitive

Antimicrobial activity of *Lactobacillus.* Isolates

Results showed varying inhibitory effect for all isolates supernatants on pathogenic bacteria which tested, **(**Table 2) the highest inhibition zone observed was against *Escherichia coli O157:H7*, *Staphylococcus aureus, Enterococcus faecalis*, *Salmonella typhimiurum*, and *Clostridium spp*, while weaker inhibition zone of *Lactobacillus* Isolates supernatants was observed against *Klebsiella sp* and *Pseudomonas aeruginosa*. *Lactobacillus* produced bacteriocins that had broad spectrum of inhibition against pathogenic bacteria, like *Escherichia coli* and *Enterococcus faecalis*, [21, 22]. *Lactobacillus* is containing different mechanisms underlying antibacterial activity includes lowering of pH and production of lactic acid and antibacterial compounds, including bacteriocins and non-bacteriocin, and non-lactic acid molecules Some investigations had been declared the ability of bacteriocins to inhibit pathogenic bacteria like *E. coli*, *Pseudomonas* and *Klebsiella* [23].

Table 2.

Antimicrobial activity of *Lactobacillus* isolates against some pathogenic bacteria

Pathogenic isolates bacteria / Diameter of inhibition zone (mm)	*Lactobacillus isolates*							
	Lb.1	*Lb.2*	*Lb.3*	*Lb.4*	*Lb.5*	*Lb.6*	*Lb.7*	*Lb.8*
Escherichia coli O157:H7	16	14	17	16	16	13	18	14
Pseudomonas aeruginosa	8	-	9	7	-	7	10	9
Staphylococcus aureus	15	14	13	10	14	12	14	13
Enterococcus faecalis	16	17	20	18	14	15	19	17
Klebsiella sp.	9	7	-	9	-	7	9	11
Salmonella typhimiurum	20	19	16	19	17	14	19	17
Clostridium sp.	16	14	19	15	16	12	16	14

*each number represent rate of two repeated

*- no inhibition

Lactobacillus genus is wide bacteria that widespread in dairy, fish, vegetable and grains. also found in normal vaginal flora and protect the vagina from urinary tract infection. In fact, many strains of this genus are capable of colonizing specific parts of the body, like oral cavity, gastrointestinal and uro-genital tract, where they play an important role in enhance health and competitive exclusion of pathogen [2]. bacteriocins may act as antimicrobial or killing peptides, directly inhibiting competing strains or pathogens [24]. bacteriocins may work as peptides indicates, either indicate other bacteria through quorum sensing and across bacteria communities or the host immune system cells signals [25].

Detection of genes encode for produce bacteriocins

lactobacillus isolates from Dairy products samples were detected for gene encoding for bacteriocins by amplified extraction DNA with bacteriocin gene primers. Results in Figure (1) showed that bacteriocins gene bands appeared in all *lactobacillus spp* isolates with molecular weight 4000pb. So, all *lactobacillus* isolates in this study can produce antimicrobial compounds which can be similar to bacteriocins and the isolates possessed

genes encoding bacteriocins, it is conceivable that the ingredient in the tested supernatants could include bacteriocin because they have bacteriocins genes. [26] tested 9 isolates of *lactobacillus* for bacteriocin gene and demonstrated that 8 out of the 9 tested isolates produced the 3500- 4000 bp in the PCR products. *Lactobacillus* isolates possess the ability to produce inhibitor substances, such as, bacteriocin and bacteriocin like substances, so isolates possess antimicrobial activity against gram positive and gram-negative bacteria according to studies and previous research [27].

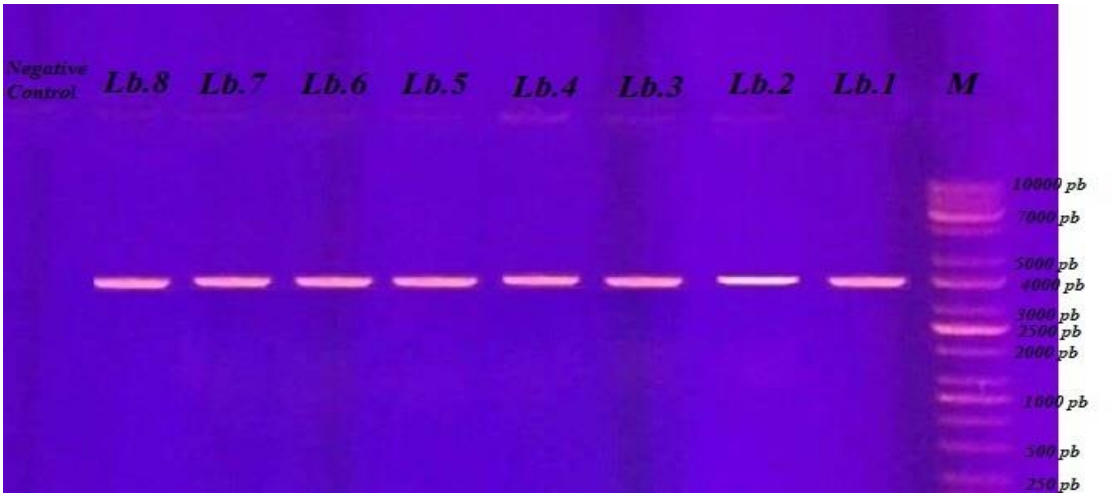

Figure 1.

Gel electrophoresis for amplification genes encoded to produce bactriocins in *Lactobacillus* isolates. Electrophoresis was performed on 1% agarose gel and run with a 5v/cm current for 2 hrs. With (10000 bp) ladder, Line *Lb.1 – Lb.10 Lactobacillus* isolates and Negative control.

the antimicrobial activity against pathogenic bacteria is mostly due to the bactericidal effect or\and protease sensitive [28]. other studies have indicated that antagonistic effects towards pathogens could be related to bacteriocins and the production of organic acids and hydrogen peroxide [29]. However, a bacteriocins of *Lactobacillus* activety against *E. coli* and *Salmonella typhimurium* have been reported [30] also bacteriocins inhibit the growth of a broad spectrum pathogenic bacteria and spoilage bacteria as well as yeast therefore, such these *Lactobacillus* can be used as probiotic [4]. More that, these probiotic bacteria have ability to produce bacteriocins which can be used widely in food, animal husbandry, and

medicine. [31] due to the bacteriocin producing bacteria presence in fermented meat, they were considered as a natural food additive [32].

Conclusion

Lactobacillus bacteria that isolated from dairy products can produce bacteriocins or protein like bacteriocin as therapeutics and bio-preservative agents inhibit the growth of pathogenic and spoilage bacteria.

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