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Original Article

Analysis of Antibiotic Resistance and Antimicrobial Effects of *Enterococcus faecium* and *Lactococcus lactis* Isolated from Khorramabad Traditional Cheeses

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Abstract

Enterococcus and *lactococcus* are Gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to differentiate from *streptococci* on physical characteristics alone. *Enterococcus faecium* because have concern antibiotic resistant, consider difficult as probiotic. The results of an assay show that a probiotic *E. faecium* strain might be a potential recipient of Vancomycin resistance genes. For analysis of this concept, 13 samples of traditional cheeses were collected from different areas in Khorramabad city and identified with using phenotypic methods, and then the bacteriocin was extract from indentified bacteria. Agar diffusion method was used to assay the antimicrobial activities of bacteriocin produced by isolated bacteria against *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *E. coli*, *Bacillus cereus* and *Bacillus subtilis*. On the other hand, antibiotic resistance of these bacteria was test using antibiogram method. The results showed that some of bacteria such as *P. aeruginosa*, *S. aureus*, and *E. coli* are intermediately resistance while *P. vulgaris* was completely sensitive and *B. cereus* and *B. subtilis* were resistant. Also, the *Enterococcus faecium* was resistant to kanamycin and trimethoprim antibiotics and intermediatly to clindamycin and tetracycline, and sensitive to amoxicillin and erythromycin. *Lactococcus lactis* was sensitive to trimethoprin, amoxicillin, tetracycline, and erythromycin while was resistant to kanamycin and clindamycin. In this study both bacteria *enterococcus faecium* and *lactococcus lactis* had an inhibitory effect on pathogenic bacteria and, these bacteria also have an appropriate antibiotic resistance to most antibiotics.

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Introduction

Enterococcus faecium and *Lactococcus lactis* are Gram-positive cocci bacteria in dairy products that are homofermentative done. These bacteria can produce bacteriocin against pathogenic bacteria that cause the loss of pathogenic bacteria in the body, so can be used as probiotic bacteria. Generally, The World Health Organization's 2001 definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host [1-3].

Enterococci are involved in the deveopment of the typical organoleptic characteristics of a variety of fermented foods such as cheeses, fermented sausages and vegetables, in some cases, they are the predominant lactic acid bacteria [4].

In the following decades, intestinal lactic acid bacterial species with alleged health beneficial properties have been introduced as probiotics including *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus johnsonii*. these bacteria used in food for controlling spoilage microorganism or against pathogens in the body. In recent years for many probiotics including *Enterococcus* and *Lactococcus* is reported that have compounds as bacteriocin that act against pathogenic bacteria [5, 6].

For example in a study that was conducted by savadogo *et al*. lactic acid antibacterial activity against pathogenic bacteria including *Bacillus cereus*, *E. coli*, *Entrococcus feacalis*, *Staphylococcus aureus*, was positive with inhibition zone between 8 to 12 mm [7]. Bacterial bacteriocins are classified into three main groups: i) lantibiotics: small peptides <5 kDa, ii) nonlantibiotic: low molecular weight (<10 kDa), and iii) nonlantibiotic: large molecular weight (>30 kDa). Nisin is as a typical example that produce by *Lactococcus lactis*. Generally, nisin is an antimicrobial peptide produced by certain *Lactococcus species* which has been accepted as a safe and natural preservative in more than 50 countries [8, 9].

The use of probiotics or their antimicrobial compounds in foods are new approach for controlling of food borne pathogens such as *Listeria monocytogenes* [10].

The purpose of this study was analysed the traditional cheese dairy producted in Khorramabad to existence *Lactococcus lactis* and *Enterococcus faecium* and antibacterial effects of cell-free supernatants (CFS) and using them against *Pseudomonas aeruginosa*, *Listeria*

monocytogenes, *Proteus vulgaris*, *Staphylococcus aureus*, *E. coli*, *Bacillus cereus*, *Bacillus subtilis* and *Streptococcus feacalis* as pathogenic bacteria and also antibiotic resistance to common antibiotics was checked.

Materials and Methods

Isolation and identification

A total of 13 samples were collected from traditional cheeses of Khorramabad city. This study was performed in three stages including separation, identification, antibacterial assay and antibiotic sensitivity testing for *Enterococcus faecium* and *Lactococcus lactis*. In the first stage for isolation, samples was collected from village that product traditional cheese. After transfer them to laboratory using the transporter medium, samples were diluted with sodium citrate, and for isolation of Enterococcus and *Lactococcus*, samples plated in MRS agar medium (pH 6.2-6.8) and M17 agar (pH 7.15) containing cyclohexamide. MRS plates were incubated under microaerophilic conditions and m17 incubated in aerobic condition. All plates were incubated for 3 days at 37ºC.

Biochemical and physiological tests

Orla-Jensen achieved basis of classification of LAB [11]. Their work has had a large impact on the systematic of LAB. Based on the first stage doing physiology and biochemical tests for cocci forms. Each isolate was activated in 5 ml MRS broth for 24 h at 37 ºC before use. Therefore, overnight cultures were used during all the identification procedures.

For all tests a negative control (non-inoculated media) was also used and respective reference strains were also included in the experiments as positive controls. Cocci-shaped bacteria, gram-positive, catalase-negative were used for the identification.

Gas production from glucose

For this purpose, citrate lacking MRS broth and inverted Durham tubes were used. 50 µl of overnight cultures were transferred into the 8 ml test media. After incubation for 3 days at 37 ºC, gas production from glucose was analysed.

Growth at different temperatures

50 µl of overnight cultures were transferred into the 5 ml temperature test media. After inoculation, they were incubated for 3 days at 10ºC, 40ºC or 45ºC. Cells growth at any of these temperatures was dedectedby the change in the color of the cultures, from purple to yellow.

Growth at different NaCl concentrations

50 µl of overnight cultures were transferred into the tubes which contain 5 ml NaCl test media. Isolates were tested for growth at 2%, 4% or 6.5% NaCl concentrations. They were incubated for 3 days at 37ºC. The change of the color from purple to yellow taken as the evidence for cell growth.

Arginine hydrolysis and gas production from citrate

In order to perform this test, 5 ml of Reddy broth and inverted Durham tubes were used. 50 µl of overnight cultures were inoculated into the Reddy broth and were then incubated for 5 days at 37ºC.

Arginine Hydrolysis

The cultures which utilize arginine, change the color of the broth first to yellow due to the lactic acid production and then to violet because of the ammonia production. On the other hand, the cultures which do not utilize arginine assume a deep-yellow color by producing lactic acid only.

Fermentation of carbohydrates

Isolates were also characterized on the basis of their sugar fermentation profiles. All the reactions were performed by using 96-well microtitre plates. Fifteen different sugars were used. For each test, strains were inoculated in 5 ml MRS broth (50 ml/L), and were then incubated for 24 h at 37ºC in order to obtain overnight cultures. After this, cultures were centrifuged for 10 min at 10000 rpm. Pelleted cells were washed and resuspended in MRS (without glucose) containing bromocresol purple as the pH indicator.

Forty µl of filter sterilized (0, 22µm, Millipore) 10% sugar solutions were pipetted into each well. On to the sugar solutions, 160 µl of suspended cells were added. Thus, 2% final sugar concentration was obtained.

Dublicate reactions were prepared for each of the sugar fermentations experiment. After 24 h incubation at 37 ºC, when the sugar fermentation was taken place, the color changed from purple to yellow and turbidity was increased. Glucose fermentation included to positive control, and samples without sugar were used as negative control.

Antibacterial assay

Agar well diffusion method was used to detect antimicrobial activities of CFSs produced from isolated bacteria. After the preparation of pure culture in MRS agar, 5 ml of the culture medium was prepared in MRS broth and bacterial cells were removed by centrifuging the culture at 10000 rpm for 5 min and the supernatant was filtered using 0.22 µm membrane filter. Paper discs were treated in a CFS solution.

On the other hand indicator strains such as *Pseudomonas aeruginosa* (PTCC 1430), *Proteus vulgaris* (ATCC 1312), *Staphylococcus aureus* (ATCC 64542), *E. coli* (ATCC 2143), *Bacillus cereus* (ATCC 1015), *Bacillus subtilis* (ATCC 1156) and *Sterptococcus feacalis* (PTCC 1237) were inoculated in the TSB (0.5 on the McFarland scale). Then indicator strains were swapped with sterile culture in Muller Hinton Agar (MHA).

Then paper disk were dipped into the medium and were placed in the plates with gaps between them and the walls. And then inhibition zone was measured after incubation incubated for 24 h at 37°C [12].

Disc diffusion method (Becton & Dickinson) was used according to Clinical and Laboratory Standards Institute [CLSI 2010, formerly National Committee for Clinical Laboratory Standards (NCCLS)] guidelines [13 -15].

Antibiotic discs including trimethoprim (15µg), amoxicillin (10µg), tetracycline (30µg) erythromycin (15µg), kanamycin (30µg) and clindamycin (2µg), rifampin (5µg) and vancomycin (30µg) was used. Inhibition zone was measured accurately, then the results were analyzed using spss software.

Results

In this study, the antimicrobial effects of *Enterococcus faecium* and *Lactococcus lactis* bacteria isolated from 13

samples were evaluated against some pathogenic bacteria. From 13 cheese samples, the follow results were obtained:

Morphological examination

Isolates were analyzed under the light microscope. At this step, cell shape (like cocci, ovoid, rod) and their arrangements (like diplo form, chains form, tetrad form) were examined after simple staining.

Physiological and biochemical tests

In order to identify the shape isolated were used from bergey Table. The isolates that were able to metabolize citrate, were listed. Sugar fermentation profiles provided identification at species level for our typical *Enterecoccal* isolates. Results showed that the maximum diameter of inhibition zone for *E. faecium* isolates was against *S. aureus* (12 mm) and lowest inhibition zone was against *Listeria* monocytogene (7 mm) while *B. cereus* and *E. coli* were resistant to them. Also the maximum diameter of inhibition zone for *Lactococcus lactis* isolates was and against *S. aureus* (12mm) and lowest inhibition zone was against *B. subtilis* (Table 1).

On the other hand *Enterococcus faecium* was resistance to rifampin ,trimethoprim and kanamycin antibiotics, intermediate resistance to clindamycin, and tetracycline and sensitive to amoxycillin, vancomycin and erythromycin.

Table 1. Activity spectrum of cell-free supernatants of *Enterococcus faecium* and *lactococcus lactis* against several pathogenic bacteria.

Isolates / Indicator strain	***Enterococcus faecium***	***Lactococcus lactis***
Pseudomonas aeruginosa	8.0 ± 0.5	8.0 ± 0.3
Staphylococcus aureus	12.0 ± 0.7	12.0 ± 0.5
E. coli	-	-
Bacillus cereus	-	-
Bacillus subtilis	8.0 ± 0.37	7.0 ± 0.5
Proteus vulgaris	10.0 ± 0.4	10.0 ± 0.0
Streptococcus faecalis	10.0 ± 0.5	10.0 ± 0.5
Listeria monocytogenes	7.0 + 0.0	8.0 + 0.2

The values are means ± standard deviations for duplicate.
- : inactive.

Lactococcus lactis was sensitive to trimethoprim, amoxicillin, tetracycline, rifampin and erythromycin while for kanamycinwas intermediate and resistance to vancomycin and clindamycin (Table 2).

Discussion

Lactic acid bacteria isolated from dairy products, which used abundance in the microbial balance and enhance beneficial microbes in their foods.

Table 2. Antibiotic resistance pathern of *Enterococcus faecium* (a) and *lactococcus lactis* (b) isolates

a

	Antibiotics							
	VAN	KAN	AMO	TTC	RIF	ERY	CC	SXT
Resistant		R			R			R
Intermediate				I			I	
Sensitive	S		S			S		

b

	Antibiotics							
	VAN	KAN	AMO	TTC	RIF	ERY	CC	SXT
Resistant	R						R	
Intermediate		I						
Sensitive			S	S	S	S		S

VAN : Vancomycin ; AMO: Amoxicillin; KAN: Kanamycin; RIF: Rifampicin
CC: Chloramphenicol; ERY: Erythromycin; SXT: Sulfamethoxazole-Trimethoprim;
TTC: Tetracycline

These bacteria are able to withst and harsh conditions such as salt, oxygen lack, pH. They also produce antimicrobial substances have the ability to deal with sensitive pathogens. Bacteriocin produced by these bacteria can good replace for chemical preservatives in foods.

This study showed that *E. faecium* and *L. lactis* had an inhibitory effect on pathogenic bacteria and improve infection or prevent infection in the body, these bacteria also had the appropriate antibiotic resistance against most antibiotics that due to having this benefit effects could serve as an adjuvant, added to food specially dairy products consumed and increases safety against pathogenic bacteria.

Nisin was the first bacteriocin that used commercially. It with Nisaplin brand in more than 50 countries, have licensed as a food additive.

Similar to this property, in this study it was found that extracellular metabolites of lactic acid bacteria that bacteriocin is one of them, were able to prevent the growth of pathogenic bacteria.

Conclusion

According to this study *E. faecium* and *L. lactis* as a good candidate can be used as probiotic bacteria for food safty and a biocontrol tool aginst pathogenic bacteria.

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