The Evaluation of Local Strains of Lactobacilli to Produce Antimicrobial Against Pathogenic Bacteria

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SUMMARY

Three strains of lactic acid bacteria (LAB) were isolated from local dairy products and were identified using biochemical tests and confirmed by Polymerase Chain Reaction (PCR) as ***Lactobacillus delbrueckii*** **subsp.** ***bulgaricus, L. acidophilus*** **and** ***L. casci***. The LAB were tested for their antimicrobial activity as single or mixed cultures with and without different types of antibiotics against seven genera of pathogenic bacteria.

According to the results, the inhibition zones were greater when used bacterial cells than supernatant. The statistical analysis using SPSS V.11 programs was showed that the significant differences of LAB cells and supernatant when mixed with different types of antibiotics were increased at P <0.01 and 0.05 levels.

INTRODUCTION

The lactic acid bacteria (LAB) has been used for centuries in the fermentation of food not only for flavor and texture, but also due to the ability of starter – derived inhibitors to prevent the growth of spoilage and pathogenic microorganism (Abee, 1995 ; Stiles, 1996;Gurrieri *et al*., 2009). LAB has ability to produce antimicrobial substances which have inhibitory effects against closely related LAB and against spoilage and pathogenic bacteria (Dave and Shah, 1999), these inhibitors include synthesis metabolites such as acetaldehyde, diacetyl, hydrogen peroxide, organic acid and carbon dioxide (Desmazeaued, 1996). Furthermore, a great number of stains of LAB produce bacteriocins, ribosomally synthesized peptides and inhibitory enzymes that exhibit antagonistic activity against scaly related species (Gänzle *et al*., 1999; McAuliffe *et al*., 2001). Some reports shown that lactobacilli of intestinal origin exhibit antimicrobial activity that could not be attributed to either bacteriocins or organic acids (Coconnier *et al*., 1997; Silva *et al*., 1987).

Antimicrobials of LAB has been employed successfully to prevent the formation of biogenic amines (Joosten and Nunez, 1996) and have also the ability to inhibit enteropathogens in the small intestines of animals (Bernet – Camard *et al*., 1997), to pathogens causing mastitis (Ryan *et al*.,1998), growth of *Helicobacter pylori in vitro* and this inhibition was more greater when conjunction with either omeprazole or a placebo (Michetti *et al* ., 1999) and there are many strains of LAB produce antimicrobial compounds use in the food industry (Delgado *et al* .,1999), for example the antibacterial activity of

Lactobacillus plantarum LB17.2b (Boycheva,1997), which was isolated from fermented brine of table olives against Gram negative bacteria. The inhibitory effect of LAB in yoghurt starter against *Salmonella typhimurium* , *S. enteritidis* and *S. gallinarum* and soon.

Lactobacilli have antibacterial activity against some strains of *Escherichia coli, Serratia marcescens , Shigella boydii , Listeria monocytogenes , Listeria ivanovii, Listeria innocua, Staphylococcus aureus* and other genera of spoilage and pathogenic bacteria (Dembele *et al*., 1998). This study was undertaken to assess the inhibitory activity of lactobacilli against some strains of spoilage and pathogenic bacteria.

MATERIALS AND METHODS

Microorganisms

1. Lactic acid bacteria (LAB)

Three strains of LAB were obtained from Food and Dairy technology Department, college of Agriculture, University of Basrah. These strains were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* , *L. acidophilus* & *L. cascei.* Using biochemical tests and confirmed by Polymerase Chain Reaction (PCR) (by Dr. Richard K. Robinson, Food Science and Technology Dept. University of Reading, UK). LAB were propagated twice in 10% skim milk at 37 ◌°C for 16-18hrs. (Reid & Burteon, 2002). The grown bacteria were cultured in DeMan, Rogosa, Sharpe broth (MRS)(Difco) at 40-45◌°C for 18-24hrs.

2. Target bacteria

Seven genera of pathogenic bacteria (Marine bacteria Lab /Marine Environmental Chemistry, Marine Science Center, University of Basrah) were isolated from different sources of water including *Escherichia coli , Salmonella* sp., *Proteus* sp., *Klebsiella* sp., *Aeromonas* sp., *Staphylococcus* sp. and *Clostridium* sp. which were previously identified according to Holt *et al*.,(1994) have been tested for their resistance to antimicrobial activity and antibiotics. The antibiotics used in the study were : cefotaxime, Amoxicillin, clindamycin, gentamycin and ampiclox.

Preparation of inoculum

From LAB and target bacteria which grown on MRS agar and nutrient agar respectively at 37◌°C for 24 hrs, ten colonies were transferred to test tubes containing 5ml of nutrient broth and incubated at 37◌°C for 4-6 hrs. Initial bacterial concentrations($1x10^7$ cfu/ml) were estimated by the McFarland (bioMerieux S.A.France) turbidity using a spectrophotometer(A_{625}) as described by Senol *et al*.,(2007) and the viable cells were counted by MRS agar.

Determination of antimicrobial activity

1. Bacterial disk diffusion methods

A- Bacterial Biomass:

The antimicrobial activity of LAB were tested singly or mixed with two or more species or mixed (v/v) with different types of antibiotics. By spreading 0.1ml of target bacterial broth on nutrient agar , and left for 15 min to dry at room temperature, 6 to 8 wells were done with cork porer (7 mm in diameter). Using microsyring 50 µl of LAB transferred to the wells and incubated at 37°C for 18- 24 hrs. The diameter of inhibition zones were measured according to Baron and Finegold (1990).The antimicrobial activity of LAB were tested as following:

1- The antimicrobial effect of each LAB strain alone against the target bacteria.
2- The antimicrobial effect of each two mixed LAB strains against the Target bacteria.
3- The antimicrobial effect of the mixture of the three LAB strains against the target bacteria.
4- The antimicrobial effect of each antibiotic alone or mixed with one LAB strains or with each two LAB strain or with each two LAB strains or mixed with the three LAB strains.

B – Bacterial Supernatant:

The lactobacilli broth was centrifuged at 2000 rpm for 10 min followed by filtering the supernatant through 0.45µm filter paper. The supernatant were tested for their antimicrobial activity (Baron and Finegold,1990).

2. Determination of Minimum Inhibitory Concentration (MIC)

This method was used according to Baron and Finegold (1990) to determine the minimum inhibitory concentration of LAB.MICs were determined by broth dilution method in MRS broth supplemented with different concentrations of antibiotics and inoculated with the bacterial culture, after 24h incubation at 37°C,the Minimum Inhibitory Concentration (MIC, in µg/ml) of the target bacteria was determined by turbidometry.It corresponded to the lowest antibiotic concentration that inhibited bacterial growth.

Statistical analysis:

The results were analyzed using SPSS V.11 (2001) program at 0.1 and 0.05.

RESULTS AND DISCUSSION

All LAB (single and multiple) were found to produce inhibition zones toward target bacteria, this is in agreement with Babic` *et al*., (2011)who stated that one of the most important characteristics of functional starter cultures is inhibition of pathogenic microorganisms. The bacterial cells showed antimicrobial activity more than supernatant as in table (1) especially for *Clostridium* and this agree with Chae *et al*.,(2009) who attributed this to the structural disruption in the cell envelope of lactobacilli supernatant leading to lack many antibacterial compounds which found in the whole lactobacilli cells.

Table (2) appeared that the mixed cefotaxime and LAB cells were decreased in MIC values for *Proteus* and the MIC values were decreased for *E.coli* excepted in three cases (cefotaxime and *L.bulgaricus* ; *L. cascei* and mixed culture of *L.acidophilus* and *L.bulgaricus*). *Clostridium* sp. was not affected only if cefotaxime mixed with *L.bulgaricus* and *L. cascei*. For *Staphylococcus* sp. there were an increasing in MIC value from 2µg/ml to 4µg/ml especially with cefotaxime and *L. cascei* while there was an increasing from 16 µg/ml to32µg/ml for *Salmonella* sp., this agree with Charlier *et al*.(2009)

Aeromonas sp. were showed no change in MIC values in all cases except with cefotaxime and mixed cultures of *L.bulgaricus* and *L. cascei* while there was no inhibition zone against *Klebseilla* sp., this agree with Chae *et al*.(2009)

The cefotaxime was mixed with *L. acidophilus.* and *L. bulgaricus* the MIC value was increase from 8µg /ml to16 µg /ml in one case and showed decreasing in another and this is agreed with results of Boycheva (1997). The antibiotics amoxycillin and clindamycin has not affected on target bacteria (Table 3&4) alone or with LAB, only in some cases and that is in agreement with EL-Sawah (1999).

The gentamycin had limited effect on pathogenic bacteria especially *Staphylococcus* sp and *Proteus* sp.(Table 5) while Uraz and Simsek(1999) found that gentamycin with single and mixed cultures of LAB had effected on *C.perfringens*. in table (6) the antibiotic ampiclox with LAB had variable effecting on target bacteria ,this agree with Kushiro *et al*.(2009)

In general the effecting of LAB single or mixed culture with or without antibiotic may be due to the whole components of the cells which contain bacteriocin (Nes *et al*., 1996), hydrogen peroxide as toxic materials (Desmazeamed, 1996), the most frequently found pathogens, e.g.*S.aureus*,

E.coli and *Salmonella* spp.can be suppressed by a combination of low pH, competition with lactobacilli for substrate (Babic` *et al*., 2011).

Results of this study is in agreement with Timmerman(2006) who reported that the combination of some LAB strains with certain antibiotics resulted in a wider antimicrobial pectrum as compared with antibiotics alone. So the statistical analysis of results was showed significant differences ($P < 0.01$) for LAB cells and supernatant the significant differences were increased ($P < 0.01$ and 0.05) when LAB mixed with antibiotics but also in some cases and against some pathogenic bacteria their were no significant differences.

Table (1): Antimicrobial activity of Lactobacilli and Lactobacilli supernatant against target bacteria.

Lactobacilli type	Diameter of inhibition zone (mm)													
	***Proteus* sp.**		***E. coli***		***Clostridium sp.***		***Staphylococcus sp.***		***Salmonella sp.***		***Aeromonas sp.***		***Klebsiella sp.***	
	Bac.*	**Sup.****	**Bac.**	**Sup.**	**Bac.**	**Sup.**	**Bac.**	**Sup.**	**Bac.**	**Sup.**	**Bac.**	**Sup.**	**Bac.**	**Sup.**
Lb.a	9	7	7	5	-	-	8	6	6	3	6	2	6	3
Lb.b	13	7	11	5	4	-	14	7	6	4	8	5	7	2
Lb.c	11	7	10	4	5	-	12	6	7	3	9	4	8	3
Lb.a + Lb.b	16	8	14	6	5	-	15	7	8	2	8	2	6	5
Lb.a + Lb.c	19	7	14	5	7	-	18	6	10	6	11	6	9	7
Lb.b + Lb.c	21	11	19	10	10	-	21	7	13	7	17	7	15	5
Lb.a + Lb.b+ Lb.c	17	9	20	9	8	-	20	7	15	6	18	8	15	7

*Bac.: Bacteria. ; **Sup.: Supernatant. ; Lb.a: *Lactobacillus acidophilus.* ; Lb.b: *Lactobacillus delbrueckii* subsp. *bulgaricus* ; Lb.c: *Lactobacillus cascei*.;

Table (2): The minimum inhibitory concentration (MIC) of Cefotaxime and Lactobacilli against target bacteria.

Target Bacteria	Cefotaxime (MIC mg/ml)							
	Cef	Cef Lb.a	Cef Lb.b	Cef Lb.c	Cef Lb.a+Lb.b	Cef Lb.a+ Lb.c	Cef Lb.b+Lb.c	Cef Lb.a+Lb.b+Lb.c
***Proteus* sp.**	4	2	2	4	2	2	2	2
E. +coli	8	4	8	8	8	2	2	4
***Clostridium* sp.**	-	-	-	-	32	-	8	32
***Staphylococcus* sp.**	2	2	2	4	2	2	2	2
***Salmonella* sp.**	16	16	8	32	8	16	8	8
***Aeromonas* sp.**	8	8	8	8	8	8	4	2
***Klebsiella* sp.**	8	-	-	16	8	8	4	8

Cef :Cefotaxime.

Table (3): The MIC of Amoxicillin and Lactobacilli against target bacteria.

Target Bacteria	**Amoxicillin** (MIC mg/ml)							
	Amo.	Amo. Lb.a	Amo. Lb.b	Amo. Lb.c	Amo. Lb.a+Lb.b	Amo. Lb.a+ Lb.c	Amo. Lb.b+Lb.c	Amo. Lb.a+Lb.b+Lb.c
***Proteus* sp.**	-	32	32	8	8	2	4	2
E.coli	-	-	-	16	16	16	16	32
***Clostridium* sp.**	-	-	-	-	-	-	-	-
***Staphylococcus* sp.**	-	32	32	8	8	4	8	4
***Salmonella* sp.**	-	-	-	-	16	16	-	16
***Aeromonas* sp.**	-	-	-	-	16	8	16	4
***Klebsiella* sp.**	-	-	-	-	-	-	-	-

Amo.: Amoxicillin.

Table (4): The MIC of Clindamycin and Lactobacilli against target bacteria.

Target Bacteria	**Clindamycin** (MIC mg/ml)							
	Cli.	**Cli. Lb.a**	**Cli. Lb.b**	**Cli. Lb.c**	**Cli. Lb.a+Lb.b**	**Cli. Lb.a+ Lb.c**	**Cli. Lb.b+Lb.c**	**Cli. Lb.a+Lb.b+Lb.c**
***Proteus* sp.**	4	2	2	2	2	2	2	2
E. coli	-	-	32	-	8	8	16	8
***Clostridium* sp**	-	-	-	-	-	-	-	32
***Staphylococcus* sp**	8	8	2	8	4	4	2	2
***Salmonella* sp.**	-	-	-	-	8	16	16	8
***Aeromonas* sp.**	-	-	32	32	4	4	8	2
***Klebsiella* sp**	-	-	-	-	32	-	-	8

Cli..: Clindamycin.

Table (5): The MIC of Gentamycin and Lactobacilli against target bacteria.

Target Bacteria	**Gentamycin** (MIC mg/ml)							
	Gen.	Gen. Lb.a	Gen. Lb.b	Gen. Lb.c	Gen. Lb.a+Lb.b	Gen. Lb.a+ Lb.c	Gen. Lb.b+Lb.c	Gen. Lb.a+Lb.b+Lb.c
***Proteus* sp.**	2	2	2	4	2	2	2	2
E.coli	6	8	8	8	8	8	2	4
***Clostridium* sp.**	-	-	-	-	-	32	32	16
***Staphylococcus* sp.**	2	2	2	2	2	2	2	2
***Salmonella* sp.**	16	8	16	-	16	8	8	4
***Aeromonas* sp.**	8	2	4	10	2	2	4	2
***Klebsiella* sp.**	-	-	-	-	-	16	4	8

Gen.: Gentamycin.

Table (6): The MIC of Ampicloxn & Lactobacilli against target bacteria.

Target Bacteria	**Ampicloxn** (MIC mg/ml)							
	Amp.	Amp. Lb.a	Amp. Lb.b	Amp. Lb.c	Amp. Lb.a+Lb.b	Amp. Lb.a+ Lb.c	Amp. Lb.b+Lb.c	Amp. Lb.a+Lb.b+Lb.c
***Proteus* sp.**	2	2	2	4	2	2	2	2
E. coli	8	8	8	32	2	2	2	2
***Clostridium* sp.**	-	-	-	-	16	32	16	8
***Staphylococcus* sp.**	2	2	2	4	2	2	2	2
***Salmonella* sp.**	4	8	8	16	4	2	8	4
***Aeromonas* sp.**	2	2	2	4	2	2	2	2
***Klebsiella* sp.**	16	4	-	-	8	8	8	4

Amp.: Ampiclox

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تقييم الفعالية الحيوية لعتر محلية من بكتريا Lactobacilli ضد البكتريا المرضية

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الخلاصة

عزلت ثلاث عتر لبكتريا حامض اللاكتيك من منتجات الألبان المحلية ، وشخصت باستخدام الاختبارات الكيموحيوية وجهاز Polymerase Chain Reaction (PCR) على إنها *L. acidophilus , L. casci, Lactobacillus delbrueckii* subsp. *bulgaricus,*. فحصت الفعالية التثبيطية للبكتريا بصورة منفردة أو كمزارع خليطه بوجود أو عدم وجود أنواع مختلفة من المضادات الحياتية ضد سبعة أجناس من البكتريا المرضية.

طبقاً للنتائج المستحصلة تبين إن منطقة التثبيط تكون اكبر عند استخدام الخلايا منها عند استخدام العالق البكتيري. استخدم نظام SPSS V.11 لتحليل النتائج إحصائيا عند المستوى (0.01 , 0.05) ووجد ان فعالية التثبيط لبكتريا حامض اللاكتيك تزداد معنوياً سواء للخلايا البكترية أو عندما تمزج مع المضادات الحياتية.