Antimicrobial effect of cell-free supernatant of Lactobacillus against isolated E. coli.

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ABSTRACT

Antimicrobial activity of the cell free culture supernatants (CFCS) was tested against the EPEC and EAEC . the Zones of inhibition of probiotic (Lactobacillus) against EPEC were compared among 105,106,107 concentrations. Regarding L. acidophilus, the best zone of inhibition was obtained at 106 concentration. L. plantarum , the best zone of inhibition was obtained at 107 concentration. While the best inhibition zone of L. rhamnosus was obtained at 106 concentration and the Zones of inhibition of probiotic (Lactobacillus) against EAEC was compared among 105 , 106 and 107 concentrations. Regarding L. acidophilus, the best zone of inhibition was obtained at 107 concentration. In case of L. plantarum, the best zone of inhibition was obtained at 106 concentration. The best zone of inhibition was obtained at the concentration 106 L. rhamnosus. The Characterization of antimicrobial substances produced by Lactobacillus has been studded The result showed the bacteriocin only has inhibitory effect against .

Keywords: Pathogenic E. coli, Escherichia Coli , Diarrhea ,Probiotic, Lactobacillus spp

Introduction

Probiotic refers to harmless live normal flora microorganism that provides a health benefit on the host, when administrated in adequate amounts and it leads to have nutritional advantage [1]. Probiotic microorganisms beneficially affect human health by improving the gastrointestinal tract (GIT) micro- biota balance and the defenses against pathogens. Additional health benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis and anti-bacterial activities. There are strain most widely used as probiotics belong to genera *Lactobacillus*, *Bifidobacterium, Pediococcus, Lactococcus, Bacillus* yeast, and others [2]. Some of probiotic microorganisms are producing antimicrobial activity including organic acids e.g. lactic acid (LAs), and acetic acid (AAs), hydrogen peroxide (H_2O_2), bacteriocins, and others that inhibition of enteric pathogen [3].

A large number of studies have assessed the utility of probiotics in the prevention or treatment of certain clinical conditions; diarrheal illnesses are perhaps the best documented indication for probiotic therapy, particularly in the pediatric population. Approximately 50% to 80% of traveler's diarrhea cases are caused by bacteria, whereas theremaining cases are caused by viruses and protozoa. *E coli* are the most common cause of bacterial traveler's diarrhea. Clinical studies have shown inconsistent results in the use of probiotics for the treatment of traveler's diarrhea and the probiotics have preventive as well as curative effects on several types of diarrhea of different etiologies [4].

Materials and Methods

Lactobacillus spp was obtained from Microbiology Laboratory at College of Veterinary Medicine, Ferdowsi University by personel communication. It was used for Probiotic Bacteria.

Preparation of *Lactobacillus* cell free culture supernatants

1. Loopful bacterial culture was taken from agar plate, inoculated in MRSbroth and cells were grown to mid exponential phase for 24 h at 37°C under anaerobic condition.
2. The optical density of the standard cell suspension was adjusted with turbidity equals to McFarland standard no. 0.5.
3. To prepare the supernatant, 0.1 ml from this standard cell suspension was transferred to tube containing MRS broth then incubated for 24 hr., at37°C., following of incubation.
4. The bacterial culture was subjected to centrifugation (10,000xg for 15 min, 4°C), filtered through sterilized 0.22 mm pore size membrane (Millipore)then plating on MRS agar showed no lactobacilli growth.
5. This freshly prepared cell free supernatant (stock solution) was used to check the inhibitory activity [6].

Antimicrobial activity of *Lactobacillus* isolates

Antimicrobial activity of *Lactobacillus* isolates were carried out according to the agar well diffusion assay as described previously [3]. Diarrheagenic *E. coli* were cultured in nutrient broth for 24 hours, and then cultured on nutrient agar by streaking technique.

1. Different concentrations of cell-free culture supernatant (CFCS) (100 µL) were placed into the wells of the nutrient agar and incubated at 37°C for 18 hours and the sterile MRS broth was used as negative control.
2. The diameter of the clear zones around each well was measured wider than 6 mm was considered as positive.

Characterization of antimicrobial substances producedby *Lactobacillus*.

The supernatant was aliquoted into five tubes:

A-First tube was treated with 1 mg /mL trypsin to determine the bacteriocin production.

B- Second tube was adjusted to pH 6.5 ± 0.1 with NaOH.

C-Third tube was treated with 0.5 mg /mL catalase for 30 min at 25 °C to determine hydrogen peroxide production.

D- Fourth tube was adjusted to pH 6.5 ± 0.1, treated with catalase andtrypsin.

E- Fifth tube was used as positive control (non-treated). Antimicrobial activity was carried out according to the agar well diffusion assay[7].

Statistical Analysis

The data of the present study was analyzed statistically by statistic package for social science (SPSS) version 27 program using chi-square test (X^2) and two-way ANOVA & Least significant differences (LSD). The level of significance was set to 5%. $P<0.05$ was considered significant while $P>0.05$ was considered as non-significant [7].

Results and Discussion

Antimicrobial activity of the cell free culture supernatants (CFCS) was tested against the EPEC and EAEC .The cell- free neutralized supernatant (CFS) of Lactobacilli (10^5,10^6,10^7) were inhibited the growth all pathogenic *E.coli* isolates by well diffusion method. It was also noticed that, L. acidophilus showed the strongest Antimicrobial activities against pathogenic EPEC with different degrees of inhibition zones in comparsion with each of *L. rhamnosus* and *L. Plantarum* , while*, L. rhamnosus* revealed strongest Antimicrobial activity against pathogenic EAEC table (1 and 2).

Table (1): Number of inhibited isolates of EPEC (N=15) with cell free supernatant (CFS) of probiotic *Lactobacillus spp*

Lactobacillus spp	Number isolates of inhibition and %			X^2	P value
	10^5	10^6	10^7		
L. acidophilus	7(46.66)	10(66.66)	4(26.66)	4.82	0.09
L. plantarum	0(0)	8(53.33)	2(13.33)	13.37	0.001*
L. rhamnosus	0(0)	7(46.66)	3(20)	9.51	0.009*

X^2	16.57	1.26	0.833		
P value	0*	0.53	0.659		

10^5,10^6,10^7= cell free supernatant concentration

*** Significantly difference at P<0.05**

Table (2): Number of inhibited isolates of EAEC (N=14) with cell free supernatant (CFS) of probiotic Lactobacillus spp

Lactobacillus spp	Number isolates of inhibition and%			X^2	P value
	10^5	10^6	10^7		
L. acidophilus	0(0)	13(92.85)	3(21.42)	28.06	0*
L. plantarum	1(7.14)	6(42.85)	0(0)	10.62	0.005*
L. rhamnousus	2(14.28)	11(78.57)	5(35.71)	12.25	0.002*
X^2	2.15	9.1	5.86		
P value	0.341	0.011*	0.053		

10^5,10^6,10^7= cell free supernatant concentration

*** Significantly difference at P<0.05**

Zones of inhibition of probiotic (Lactobacillus) against EPEC were compared among 10^5,10^6,10^7 concentrations. Regarding *L. acidophilus*, the best zone of inhibition was obtained at 10^6 concentration. *L. plantarum* , the best zone of inhibition was obtained at 10^7 concentration. While the best inhibition zone of *L. rhamnosus* was obtained at 10^6 concentration, as shown in table (3)

Table (3): Zones of inhibition for EPEC (N= 15) with a cell free supernatant of probiotic *Lactobacillus spp*

Lactobacillus spp	**Zones of inhibition for EPEC**		
	10^5	**10^6**	**10^7**
L. acidophilus	19.92±0.72Aa	30.55.±2.7Ab	25.92±1.97Ac
L. plantarum	0±0Ba	16.9±0.90Bb	34.3±1.88Bc
L. rhamnosus	0±0Ba	36±1.56Cb	24.15±1.51Ac
LSD(P<0.05)			2.58

10^5,10^6,10^7= Diameter of inhibition zone (mm) i

Means with different capital letters n the same column and small letters in The same row are significantly different.

Zones of inhibition of probiotic (*Lactobacillus*) against EAEC was compared among 10^5 , 10^6 and 10^7 concentrations. Regarding *L. acidophilus*, the best zone of inhibition was obtained at 10^7 concentration. In case of *L. plantarum*, the best zone of inhibition was obtained at 10^6 concentration. The best zone of inhibition was obtained at the concentration 10^6 *L. rhamnosus*, as shown

Table (4): Zones of inhibition for EAEC (N= 14) with a cell free supernatant of probiotic Lactobacillus spp

Lactobacillus spp	**Zones of inhibition for EAEC**		
	10^5	**10^6**	**10^7**
L. acidophilus	0±0Aa	28.28±0.75Ab	42±1Ac
L. plantarum	19±1Ba	38.57±0.92Bb	0±0Bc
L. rhamnousus	15±0.57Ca	23.5±1.3Cb	20±0.31Cc
LSD(P<0.05)			1.38

10^5,10^6,10^7= Diameter of inhibition zone (mm)

Means with different capital letters in the same column and small letters in the same row are significantly different

This result is agree with Davoodabadi *et al*. (2020) [9]. who demonstrated that the Lactobacillus characterized by inhibitory activity against the diarrheagenic *E. coli.* These strains could be used as probiotic to help in preventing the intestinal infections caused by diarrheagenic *E. coli*. A similar results from study by [10]. included isolates of *L. casei,L. delbrueckii, L. fermentum, L. plantarum, and L. pentosus,* that used the antibacterial activities of cell-free supernatants (CFSs) test for all the pathogenic isolates. The test was performed through standard agar-well diffusion assay, against human. Other study suggested that none of lactobacilli cell-free supernatant (CFS) has an inhibitory activity against four pathogens, these pathogens are *Staphylococcus aureus*, *Listeria monocytogenes*, *E.coli*, and *Klebsiella pneumoniae* [11].

Study in Babylon University, Iraq, compatible with study when isolated six isolates of *L. acidophilus* and study antimicrobial effect of *L. acidophilus* against some pathogenic bacteria, such as *E.coli,P. aerogenosa, A. hydrophila, P.vulgaris, S. aureas, S. epidermidis, S. pyogenes,* and *B. subtilis*. The results clearly suggest that the cell-free- supernatants have an inhibitory influence on the indicator pathogenic strains [12].. The result disagree with Gaspar *et al*., (2018) [13]. that demonstrated *L. acidophilus* inhibited *Streptococcus agalactiae P. aeruginosa* and no antimicrobial effect on and against the strains of *E. coli*, *S. aureus* and *Candida albicans*.

Antimicrobial activity of *Lactobacillus* isolates have the ability to produced antimicrobial substances, such as organic acids, hydrogen peroxide and bacteriocins, the most effective form bacteriocins is one produced from LAB. The bacteriocin possible mechanism of resistance in Gram-negative and some Gram-positive bacteria could be related with the barrier properties of the outer membrane and the cell wall [14]. Other strategies used by Lactobacilli are the iron inhibitory effect for *E.coli* and other pathogens by the regulation of intracellular iron concentration [15]. This effect take place due to the iron metabolic need in Lactobacilli, the iron plays an important role in the pyrimidine and purine metabolism, therefore *lactobacillus* utilize it during the growth in medium containing concentration of iron.

Characterization of antimicrobial substances produced by *Lactobacillus*

Lactobacillus isolates has antimicrobial activity due to the ability of producing antimicrobial substances, such as organic acids, hydrogen peroxide, bacteriocins and other inhibitory substance produced by *Lactobacillus* with similar broad spectrum activity [16].

The result showed the bacteriocin only has inhibitory effect against pathogenic *E. coli,* because when addition the trypsin to crude supernatant, the antimicrobial activity was inactivated (no inhibition zones)(Figure 3-11,12), but without trypsin, the result is reversed while organic acid and hydrogen peroxide have no role in inhibition because the antimicrobial activity (inhibition zones) was continues until when adjusted pH to 6.5 ± 0.1 with NaOH and treated with 0.5 mg /mL catalase.

Similar result by Al-Mndalawi (2019) [17]. showed that bacteriocin production by *L. acidophilus* has strong inhibitory effect on the growth ofthe indicator pathogens and the inhibition was measured against *P. aerogenosa, E. coli* and *S. aureus.* In another study, bacteriocin was isolated from *L. acidophilus* and its antimicrobial activity against antibiotic resistant bacteria isolated from throat of patients having upper respiratory tract infections was studied, and the results found that the bacteriocin was effective against all test antibiotic resistant isolates [18].

The majority of the previous result demonstrated the bacteriocin was responsible for inhibitory effect against pathogenic *E.coli*. In another study the antibacterial properties of

bacteriocin were found to be stronger against Gram-positive bacteria (*S. aureus* and *Listeria monocytes*) than Gram-negative bacteria (*Salmonella typhi* and *E. coli)*. The possible reason for that is due to environmental factors leading less optimal growth and therefore, decreases the bacteriocin activity which in the end means less inhibition to indicate bacterial growth [19]. While a more recent study in 2018 suggested that the bacteriocin has inhibitory activity against gram-positive as well as gram-negative indicator strains (Sharma *et al*., 2018).

Zhao *et al*. 2020 researched the bacteriocin **(**Lactobacillin) produced by *L. acidophilus,L.rhamnosus* antibacterial mechanism against *E. coli,* the suggested mechanism is via cell membrane damage and intracellular material leakage. In conclusion, "pores formation" theory might be applied also to Lactobacillin with regard to Gram negative bacteria. The bacteriocin bactericidal activity against the *E. coli* was observed in this study. This activity could be result of the direct contact with the cell membrane, and the contact disrupt the membrane potential toward destabilize the cytoplasmic membrane, moreover, instability of themembrane lead to pore/ hole formation in membrane that lead to inhibit cell growth and activates cell death process [20]

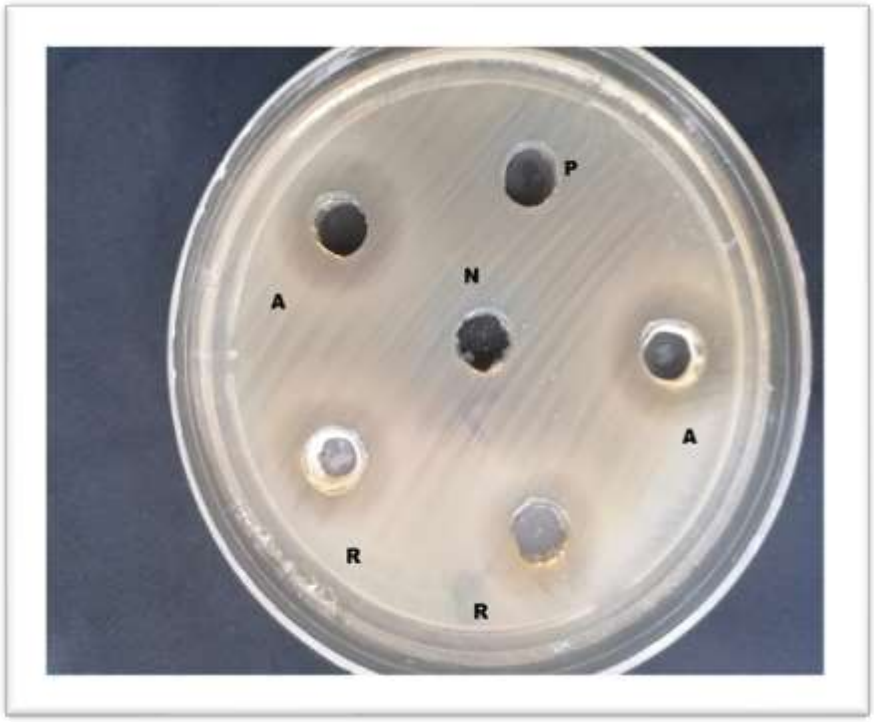
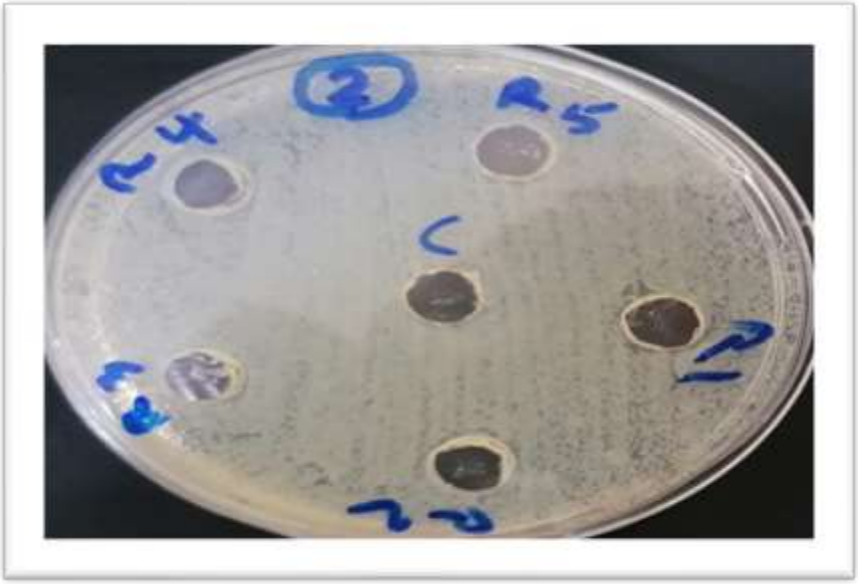


Figure (1): Antimicrobial Activity of Cell Free Culture Supernatants of the Lactobacillus(10^6) concentrations isolates Against *Diarrheagenic Escherichia coli* with no treatment
Abbreviation: p(*L. plantarum*); A(*L.acidophilis*) ;R(*L.rhamnosus*);N(Negative control sterile MRS broth).



Figure(2):CFCS treatment with Trypsin(no inhibition zone)

Conclusions

Phenotypically, cell free culture supernatant of *Lactobacillus spp* inhibited the growth of pathogenic *E. coli*

Conflict of Interest: *None*

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