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Evaluation the Using of Potential Probiotic Antibacterial Against Urogental Tract Infection *In-vitro.*

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ABSTRACT

Considering the emergence of the fact that the pathogenic bacteria may be an additional negative factor influencing male fertility and worsening sperm quality. The isolated *Staph. aureus* and *E.coli* were found resistant to the antibiotics used in treatment . This study was conducted to evaluate the using probiotic of *Lactobacillus acidophilus* CL1285® alternative antibiotic against *Staphylococcus aureus* and *Escherichia coli* causing urogenital tract infections *in vitro*. The growth inhibitory effect produced by *Lb. acidophilus* CL1285 on the pathogenic *Staph. aureus* and *E.coli* was tested on solid medium using agar diffusion methods. In the latter instance, the direct effect of Lb. acidophilus and bacteriocin on pathogenic isolates led to the growth inhibition of the bacterial cells after 24 h of their incubation at 37 °C. Lb.acidophilus CL1285 bacteriocin was purified by gel filtration and it increased in specific activity by antibiogram . The purified bacteriocin recorded an apparent four polypeptides with molecular weights,63,45,38,and 30 KDa. Amino acid analysis showed that the bacteriocine consists of 14 amino acids with high content of alanine, asparagine. glycine, tryptophane and glutamic acid . The results suggest that *Lb. acidophilus* and Cell Free Supernatants (CFS) containing bacteriocin of L. acidophillus inhibited Staph. aureus and E. coli pathogenic bacteria. So *Lb. acidophillus* are meant to treat infections in male urogenital tract infections; however more studies including in vivo need to be performed.

Keywords: Antimicrobial activity; multidrug susceptibility; Antibiogram probiotic; E. coli; *Staph. aureus*; Lb. acidophillus.

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INTRODUCTION

Infections of the male genitourinary tract account for up to 15% of cases of male infertility [1]. Recent studies have shown that acute and chronic infections and consequent inflammation in the male reproductive system may compromise the sperm cell function and the whole spermatogenetic process [2]. causing qualitative and quantitative sperm alterations. Deterioration in spermatogenesis, obstruction of seminal tract and effect of spermatozoa function may be caused indirectly by activation of seminal plasma white blood cells or cellular reactions against microbial agents, as well as by direct influence of pathological bacterial strains on gametogenic cells [3]. The most frequently isolated microorganisms in male patients with genital tract infections or semen contamination is Staphylococcus aureus and Escherichia coli. The negative influence of this species on sperm quality is partially due to the following mechanisms: (a) Bacterial attachment to sperm and its effect on motility; (b) an immobilizing factor produced by some bacteria; (c) immune system recruitment, and (d) alteration of glandular function [2,4]. Lactopacillus is an important genus with Generally Regarded As Safe (GRAS) status lactic acid bacteria [5]. Some species of this genus enhance resistance against pathogens via antimicrobial mechanisms. These include competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins and other primary metabolites, such as hydrogen peroxide, carbon dioxide and diacetyl [6]. Bacteriocins are antimicrobial proteins produced by bacteria and active against gram positive and gram negative bacterial pathogens [7]. Numerous bacteriocins, such as nisin, lactobrevin, acidophilin, acidolin, lactobacillin, lactocidin and lactolin, have been reported to be produced by lactobacilli [8]. Bacteriocins are active against a wide range of food-borne pathogens, depending on their specificity [7].

The main objectives of this study was to (1) Study and evaluate the antibacterial activities of bacteriocin produced by *Lb. acidophilus* against pathogenic *Staphylococcus aureus* and *Escherichia coli* isolated from Semen of infertile patients which were identified in a previous study [9]. (2) Purify *Lb. acidophilus* CL1285 bacteriocin and (3) Characterize this bacteriocin by elucidation of its molecular mass, its amino acid composition and its quantitative effect on *Staphylococcus aureus* and *Escherichia coli.*

MATERIALS AND METHODS

Bacterial sources : The tested bacterial strain *Lb. acidophilus* CL1285 used in this study was obtained from Egypt Microbiology Culture Collection, Cairo MIRCEN, Fac. of agric., Ain Shams Univ., Cairo, Egypt . The multidrug antibiotic resistance pathogenic *Staph . aureus* and *E,coli* isolated from semen of infertile patients [9].

Antibacterial activity of *Lb. acidophilus* CL1285 : The inhibitory spectrum of *Lb. acidophilus* CL1285 cells and cells free supernatants (CFS) was studied against Staph . aureus and E,coli *in vitro*. The CFS was obtained after incubation for 24 hrs at 30°C in MRS broth by centrifuging cultures (6,000 rpm for 10 minutes at 4°C) . The CFS was filtered through syringe filter (0.45 µm, Millipore). The inhibitory activity of CFS was tested using agar well diffusion method.

Purification of bacteriocin : The partially purified bacteriocin obtained by ammonium sulfate was performed as described by [7,10]. One arbitrary unit (AU mL^{-1}) of crude bacteriocin preparation was defined as 5 µL of the highest dilution of PPE yielding a definite zone of inhibition of growth in the lawn of indicator organism. The highest dilution was multiplied by 200 µL (1 mL/5 µL) to obtain the arbitrary units per milliliter (AU mL-1). CFS from *Lb. acidophilus* was adjusted at pH 6.5 and was treated with solid ammonium sulfate till 50% saturation level. The mixtures were stirred for 12 h at 4°C and centrifuged at (14,000 rpm for 1 hr at 4°C). The pellets were resuspended in 1 mM potassium phosphate buffer, pH 6.5 and dialyzed against the same buffer for 24 hrs at 4°C in dialysis tubing. This partially purified bacteriocin was sterilized by filtration through syringe filter (Amicon 0.45 µm, Millipore).

Determination of the bacteriocin protein : The bacteriocin protein was performed and stored at -70°C. A modified version of the SDS-PAGE technique of Laemmli (16) was used . Samples containing an average of 330 ,ug of protein were solubilized in an equivalent volume of 0.125 M Tris HCL, pH 6.8, containing SDS (3.8%, wt/vol), 2-mercaptoethanol (18%, vol/vol), glycerol (18%, vol/vol), and bromophenol blue. Electrophoresis was performed with a 3.6% acrylamide stacking gel over a 10% acrylamide separating gel with a discontinuous buffer in a Bio-Rad Protean II system. Low-molecular-weight peptides (Pharmacia) were run as markers on

selected gels. The separated polypeptides were stained with Coomassie brilliant blue R (0.25%, wt/vol) in methanol-acetic acid distilled water (45:10:45) and destained in the same solvent mixture before being fixed in acetic acid (7.0%, vol/vol). A visual comparison of band patterns was made on transilluminated wet gels [11].

Antibiogram : The bacteriocine proteins of *Lb. acidophilus* cell cultures were separated. into *bands* using SDS-PAGE within each *lane* . In order to make the proteins accessible to bacteriocine detection . The line were separated from within the gel onto a plate made of *nutrient agar medium*. The proteins move from within the gel onto the medium . An older method of transfer involves placing a line on the plate agar medium inoculated with tested bacteria at 24 h incubation (*E.coli* and *Staph.aureus*) and then incubated at 24 h. As a result of either process, the proteins are exposed on a agar for detection in form zone inhibition.

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Antibiogram of the bacteriocin protein :. The unstained bacteriocin protein bands were extracted from SDS-PAGE gels and blotted onto MARS agar plates inoculated with *Staph .aureus* and Maconky agar plates inoculated with *E.coli* at overnight incubation period . The inoculated plates were incubated for 24 h. at 37^0C .

Amino acid composition : Amino Acids were determined using the method described by [12]. 200 µl of purified bacteriocin was hydrolyzed with 6N HCl in sealed tube, heated in oven at 100^oC for 24 hrs, to evaporate HCl. The residue was dissolved in diluting citrate buffer (pH 6.5). Chromatography was performed with an AAA 400 amino acid analyzer (Ingos Ltd., Czech Republic) equipped with an Ostion LG ANB ion exchange column. Free amino acids were separated by stepwise gradient elution using Na/K-citric buffer system (Ingos Ltd., Czech Republic). Post-column derivatization with minhydrin reagent and spectrophotometric measurement was used for determination of aminoacids and biogenic amines.

RESULTS

Bacterial strains and culture media: The pathogenic bacteria (Staph . aureus and E,coli) isolated from semen of infertile patients were characterized and identified previously [9]. *Staph.aureus* and E.coli isolates maintenance on nutrient plate agar medium, one colony from each of isolates was cultured on parker agar and Ma Conky agar media . *Lb. acidophilus* strain obtained from Cairo MIRCEN, Cairo, Egypt was sub cultured on MRS broth it inhibited the pathogenic bacteria (Staph . aureus and E,coli) isolated from semen of infertile patients . The inhibition of *Staph. aureus* & *E. coli* isolates by *Lb. acidophilus* are seen in fig. (1) and noted in table (1) . It was noticed that *Staph. aureus* was more sensitive organism than *E.coli* . They showed inhibition zones of about 13-20 mm in diameter, while *E. Coli* showed inhibition zones between 5-15 mm in diameter.

Table (1): Antibacterial activity of *Lb. acidophilus* against *Staph. aureus* and *E. Coli* by agar well diffusion method .

Diluted *Lb. acidophilus* (CFS)	*S. Aureus*	*E. Coli*
	Diameter of inhibition zone (mm)	Diameter of inhibition zone (mm)
Control (No probiotic)	Normal growth	Normal growth
1 (4th CFS concentration)	13	5
2 (3rd CFS concentration)	16	10
2 (3rd CFS concentration)	18	12
4 (1st CFS concentration)	20	15

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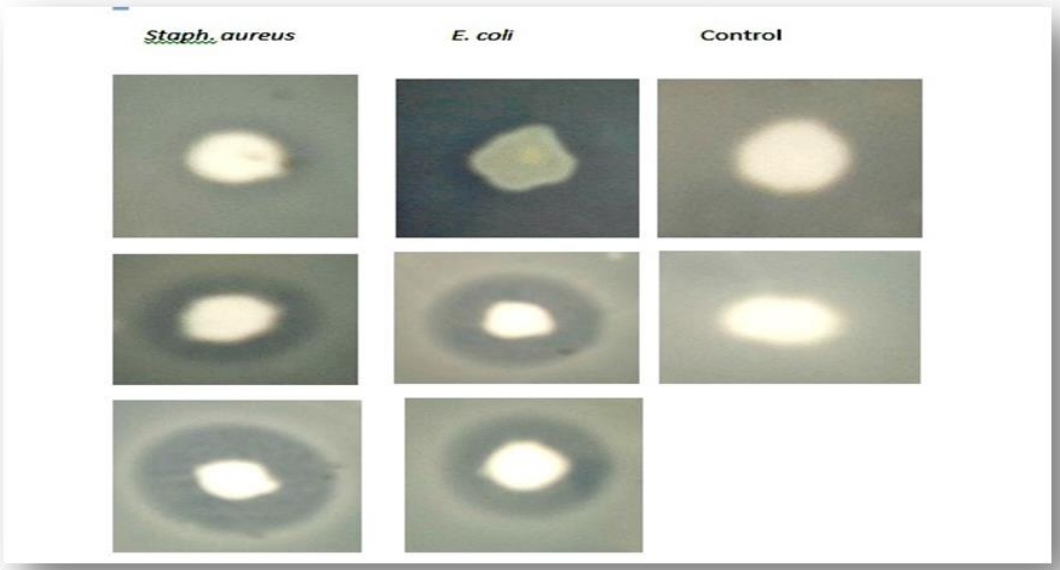

Fig. 1. Antibacterial activity of *Lb. acidophilus* cells at different concentrations (1,2,3,) against (A) *Staph. aureus* and (B) *E. Coli* isolates.

Antimicrobial activity of bacteriocin produced by *Lb. acidophilus* in PPE was studied against sensitive bacterial by critical dilution assays. Results are given in Table 2; by titration of PPE, about 4.00-7.00 and 3.47-6.00 CFU mL-1 were obtained with *Staph. aureus* and *E. coli*, respectively.

Table (2). Growth of isolated *Staph .aureus* and *E. coli* in nutrient broth medium with and without partially purified bacterocin.

Time (min)	Log (CFU ml^{-1})			
	Staph.aureus		*E.coli*	
	Control	Treatment	Control	Treatment
0	7.00	7.00	6.00	6.00
5	7.10	6.90	6.15	5.85
10	7.36	6.73	6.45	5.62
15	7.58	6.58	6.67	5.51
20	7.63	6.19	6.82	5.30
25	7.79	5.85	7.05	5.12
30	7.92	5.26	7.18	4.95
35	8.14	5.00	7.32	4.72
40	835	4.85	7.59	4.49
45	8.58	4.65	7.75	4.25
50	8.62	4.37	8.05	4.00
55	8.75	4.23	8.25	3.75
60	9.00	4.00	8.58	3.47

SDS-PAGE profiles of total bacteriocin proteins produced from Lactobacillus are presented in (Fig. 3). SDS-PAGE analysis revealed 4 protein bands with different molecular weights ranged from 75 to 20 kDa as shown in (Table 3). These bands were varied in molecular weight 60, 40,38 and 20 kDa).

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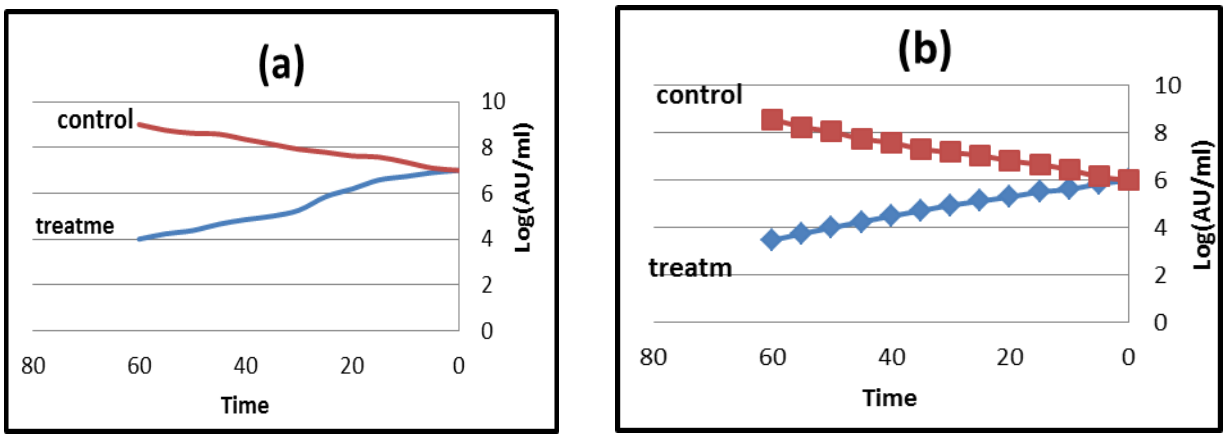

Fig. (2). Curve gram showing Growth of *Staph.aureus* (a) and *E.coli* (b) in broth media with bacteriocin (treated) or without partially purified bacteriocin (control) .

Bacteriocin purified : Cell free suspension (CFS) from *Lb.. acidophilus* treated with 40% saturation ammonium sulphate suspension and stirred for 12 h at 4°C then centrifuged at 12000 rpm for 1 h at 4°C .The precipitate was resuspended in 10 mM phosphate buffer, pH 6.5 and dialysed against the same buffer for 24 h. This partially purified bacteriocin was sterilized by filtration through filters (0.45 µm, Millipore, Amicon) . It was applied to a 200 mL column (4 cm interior diameter) of Sephadex G200-50 (Sigma) equilibrated with 1 M potassium phosphate buffer, pH 6.5, at room temperature. Elution was started with the same buffer and 5 mL fractions were collected and were monitored for A 280 nm . The bacteriocin activity (AU mL^{-1}) using *Staph.aureus* and *E.coli* .

Protein profile of bacteriocin : SDS-PAGE profiles of total bacteriocin proteins produced from *Lb . acidophilus* are presented in (Fig. 2). SDS-PAGE analysis revealed 4 protein bands with different molecular weights ranged from 75 to 20 kDa. These bands were varied in molecular weight 60, 40,38 and 20 kDa).

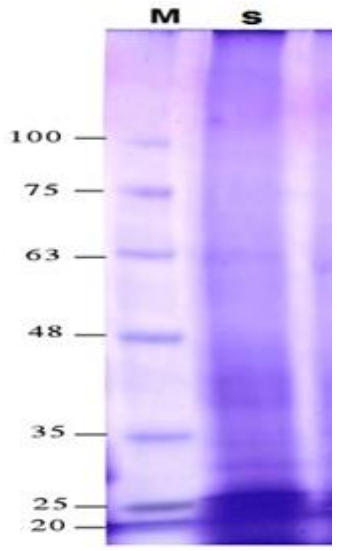

Fig. (3) . Electogram showing Acrylamide gel (12%) of Protein pattern of bacteriocin produced from Lactobacillus by SDS-PAGE and determined molecular weight with protein marker (M).

Antibiogram of bacteriocin: The 10 mL of fraction No. 5 containing the highest bacteriocin activity were pooled from the column and were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In another round of the experiment, the unstained SDS-PAGE was overlaid with parker agar and Ma Conky agar media containing *Staph.aureus* and *E.coli and* incubated for 24-48 h at 30°C. The inhibition obtained in lawn *Staph.aureus* and *E.coli* were evaluated .

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Fig. (4). Antibiogram supressed growth of each *E.coli* and *Staph. aureus* on SDS-PAGE containing *Ib. acidophillus* bacteriocin.

The amino acid composition of the purified bacteriocine of *Lb. acidophilus* which was pooled from ion exchange chromatography are recorded in Table(3). fourteen amino acids were detected with different percentage and amount in the purified bacteriocine. The higher amino acids amount obtained can be recorded high content of alanine, asparagine. glycine, tryptophane and glutamic acid comparable of thoreonine, serine, proline, methionine, isoleucine, tyrosine, phenyl alanine and histidine. Therefore, the antimicrobial compound produced by *Lb. acidophilus* was proved to consists of antimicrobial protein bacteriocine.

Table (3): Amino acid composition of Bacteriocine produced by Lb. acidophilus.

Amino acids	Percentage (%)	Amount ($mgml^{-1}$)	Amino acids	Percentage (%)	Amount ($mgml^{-1}$)
Alanine	4.75	12.3	Lycine	2.06	4.0
Argnine	0.70	1.65	Methionine	1.02	2.75
Asparagine	2.56	5.85	Proline	0.49	1.2
Glutamic acid	4.18	9.55	Phenylalanine	0.79	1.58
Glycine	5.96	18.21	Serine	1.25	2.85
Histidine	0.64	1.15	Threonine	0.49	0.75
Leucine	2.25	4.75	Tyrosine	0.59	1.25

DISCUSSION

This study shows that the *Lb. acidophilus* CL1285® produce antimicrobial components that can inhibit the growth and eliminate pathogenic bacteria which were isolated from semen of infertile patients. To our knowledge, these results provide direct evidence that staph. Aureus and E. coli strains collected from a variety of infertile male patients were vulnerable to the antimicrobial action expressed by *Lb. acidophilus* when tested *in vitro*.

The interaction between Lactic acid bacteria and staph. Aureus and E. coli, in mixed liquid culture, can be bactericidal for those pathogenic microorganisms.The objective of our research was to increase our knowledge on bactericidal effect produced by mixed *Lb. acidophilus* against pathogenic infections that may cause male infertility. In addition, for the agar diffusion spot test, we standardized and optimized bacterial cultures and the inoculation method. This method included a preparation of conditions and cell concentrations for Lb. acidophilus, which can be used for elimination of the pathogenic cells from mixed co-culture [13]. The dimensions of the inhibitory zones are related to the concentration of *Lb. acidophilus* cells and the variance in sensitivity of each tested pathogenic microorganism separately. But with both of these tested bacteria, *Lb. acidophilus* was effective in the production of antibacterial agent activity [14].

In this study, further study was done on *Lb. acidophilus* bacteriocin. PPE of *Lb. acidophilus* inhibited both pathogenic gram positive (Staph. Aureus) and gram negative (E. Coli) bacterial models. This supported previous results on bacteriocin activity against sensitive bacterial species within the same genus [15]. Different spectra of inhibitory action may be obtained depending on the bacteriocin producing strain, the indicator strain and also the method used for bacteriocin detection [16]. The accepted mode of bacteriocin action on both gram-positive and gram-negative bacteria is the adsorption of bacteriocin on cell surface, inducing pore formation. This is resulted in leakage of cell electrolytes which is ended by cell death [16].

The results (Table 1) compared the diameters of pathogenic strains-inhibited zones on Petri dish assays, it is evident that they are slightly different with both tested pathogenic microorganisms, which leads to the conclusion that the sensitivity of these strains to *Lb. acidophilus* inhibitory activity is also similar.

The agar diffusion method was primarily used to study the effects on the production of antimicrobial compounds by *Lb. acidophilus* cultures, which were mixed with Staph. Aureus and *E. Coli* isolates.Table 1 clearly demonstrate that the *Lb.acidophilus* inhipited Staph. Aureus with inhipition zone diameter range (13-20 mm) rather than *E. Coli which showed* inhipition zone diameter range (9-17 mm). But with both of them it was clearly observed that *Lb. acidophilus* expressed excellent antibacterial effects.

During the next step of our study, we compare the broth media of pathogenic tested microorganisms without and with lactic acid bacteria (control and treated groups respectively) to test antimicrobial activity against Staph. Aureus and *E. Coli* as pathogenic clinical isolates, using well-defined cell concentrations and standardized culture conditions, was investigated. In mixed or pure cultures of LAB and MRSA, the concentration of bacterial cells was determined by standard of CFU counting [17] *(Table 2).* The data show that *Lb. acidophilus* produced the antibacterial compounds reducing the number of pathogenic cells more than 5 log10 CFU of Staph. Aureus population and more than 4 log10 CFU of E. Coli within 24 h at 37 °C (Table 2). As described, these experiments prove the bactericidal activity of *Lb.acidophilus* mixture against pathogenic strains that may cause male infertility. This phenomenon can have a practical application if it can be performed in vivo.

The global activity of mixed microbial populations is determined by the presence and function of each species, which are strongly influenced by interactions among the different partners.However, current knowledge of microbial physiology is generally based on pure culture studies and conditions, that are different from those encountered in a complex ecosystem. Consequently, performing mixed culture studies is essential to get closer to the reality of complex populations that exist in hospitalized humans.

The results presented in this paper are in contrast with those published in previous studies that reported multiple difficulties related to their experimental procedures, which attempted to demonstrate probiotic activity of *Lb. acidophilus* strains [18,19]. Contrary to those studies, we were able to develop better methods and thus, did not observe the variability in LAB antibacterial activity which was more stable and lower than that reported by the above cited authors. In addition, the data reported in this study using the agar diffusion test was confirmed by testing cell viability in liquid medium (Table 1,2). The LAB anti-pathogenic strains that were investigated in this study have been used earlier against pathogenic organisms [20,21]. The results obtained by [20]. showed that the whey isolated from fermentation of milk transformed by strains CL1285®*Lb. acidophilus* and *Lb. casei*(presently designated as LBC80R) was able to inhibit the growth of pathogenic bacteria such as *S. aureus* (MSSA) by 85%, *Listeria monocytogenes* by 78% and E coli 0157:H by 77%. The results obtained by [21], showed that daily intake of commercial Bio-K+ International Inc. product containing *Lb. acidophilus* CL1285® and *Lb. casei* LBC80R was a safe and effective means of preventing antibiotic-associated diarrhea (AAD) caused by *Clostridium difficile* in hospitalized patients.

Finally, even though there is a growing demand for products marketed as “probiotics”, as [22] list would suggest, relevant scientific data does not always follow. Consequently, well-documented scientific research on these products is still necessary.

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