Antibacterial activity of probiotics against pathogenic bacteria contaminate some personal care products

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Abstract:

Probiotics have attained popularity among consumers worldwide as a natural approach to maintain or restore human health. Many products are already available in the market place with even more potential candidates in development. Knowledge gained from the Human Microbiome Project supports the utility of probiotics to achieve a balanced microbial community and potentially reduce or alleviate certain disorders. Most of the research studies and commercial probiotic products have focused on personal care products, which consist of over- the-counter drugs, cosmetics, and other consumer products, are used by almost everyone on a daily basis. Although the understanding and development of probiotics in personal care products is lagging behind the rapid progress that has been made in food products. This paper reviews recent findings related to microbial communities in the skin, where a large number of personal care products are applied and discusses the opportunities and challenges of probiotics in these products. The Probiotic bacteria (LAB) were obtained from Egypt Microbiology Culture Collection (MIRCEN), Fac. of agric., Ain Shams Univ., Cairo, Egypt and screened for the production of bacteriocin. The strain isolated gave an inhibitory activity against several Lactic acid bacteria (LAB). The strain was identified as *Lactobacillus acidophilus CL1285 and Befedo-bacterium sp.* The study showed that the inhibitory activity was not caused by hydrogen peroxide, organic acids or bacteriophage. The bacteriocin production reached the maximum level after 10 hours of incubation with an activity of 200 AU/ml. The bacteriocin was sensitive towards trypsin, α-chymotrypsin, β-chymotrypsin, α-amylase and lysozyme.

Keywords: probiotics, antibacterial activity, pathogenic bacteria, care products

Introduction

The concept of probiotics was proposed in the early 20th century by Elie Metchnikoff who postulated that ingested microorganisms could confer health benefits for humans (Huang and Tang, 2015). The incorporation of probiotics into cosmetics and skin care products provides a distinct perpetual marketing appeal enhancing brand differentiation. The major brands like: Garnier, Dr. Ohhira, Align, Culturelle, L'Occitaine, Murad, Sonya Dakar, Lierac, Bliss, Mama Mio, Beauty Cycle and Éminence have launched their products with probiotic claims. Probiotic skin care is the first professional line of anti-aging products formulated to harness the power of natural probiotics and botanicals to give a firmer, smoother and younger-looking skin. This innovative allows formulators to develop products for the next generation of anti-aging cosmetics *(http://beautystat.com/site/skincare/what-are-probiotics-and-the-best-probiotic-skincare-and-cosmetic-beauty-products/Theresa, 2012).*

Lactic acid bacterial (LAB) strains are potentially promising as they generate bactericidal bioactive peptides (bacteriocins) and enzymes able to control biofilm formation and growth of pathogens. Nisin is the best defined bacteriocin (Huttunen *et al*., 1995) produced by *Lactococcus* species that has been approved for using in food products (Hansen, 1994; Millette *et al*., 2007) and in *Lactobacillus* species. The *Lb. acidophilus* produces lactacin B or F, whereas *Lb. casei* B80 produces casein 80 (Rammelsberg and Radler 1990; Klaenhammer, 1993). Certain LAB strains have been reported to be highly antagonistic to biofilm-forming *S. aureus* (Ammor, *et al*, 2006). *Lactobacillus* has a long history of safe use in the dairy industry. Over the past few decades there has been increased impetus to introduce new *Lactobacillus*.

Four types of LAB strains have been studied as competitive inhibitors of pathogenic organisms (Massi, *et al*., 2004). Beneficial effects conferred by Lactobacilli, including inhibition of gram negative and positive bacteria were described by Maragkoudakis *et al*., (2006); Charlier, *et al*., (2008) who found *Lactococcus* lactis had a specific antagonistic effect against *S. aureus*. Antimicrobial activity produced by LAB strains appears to be unrelated to the acidification of the medium. LAB strains were reported to exert a strong

inhibitory effect on S. aureus growth in milk. Several suggestions have been proposed for inhibition of *S. aureus* by LAB, includeing production of bacteriocins, hydrogen peroxide and organic acids (Lin *et al*., 2006; Hernandez *et al*., 2005).

Bacteriocins have large diversity, and the genes encode ribosomal synthesized antimicrobial peptides or proteins, which kill other related (narrow spectrum) or non-related (broad spectrum) microbiotas is one of the inherent defense system weapon of bacteria (Cotter *et al*., 2005). More than 99% of bacteria can produce at least one bacteriocin, most of bacteriocins are not identified (Riley and Wertz, 2002). The killing ability of bacteriocins is considered a successful strategy for maintaining population and reducing the numbers of competitors to obtain more nutrients and living space in environments. Unlike most antibiotics, bacteriocins are ribosomal synthesized and sensitive to proteases, generally harmless to the human body and environment (Yang *et al*., 2014).

Recently, there has been much interest in bacteriocin synthesized by LAB. The bbacteriocins are defined as bioactive peptides or protein with an antimicrobial activity towards gram positive bacteria including closely related species and/or food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocyto*genes (Nettles, and Barefoot 1993). The use of bacteriocin or bacteriocin producing culture as potential 'bio-preservatives', and possibly for replacing chemical preservatives has received much attention. The purpose of this study is to screen lactic acid bacteria isolated from Malaysian traditional fermented food such as "tempeh", "tapai" and "tempoyak" for their ability to produce bacteriocin which exhibit antagonistic activity against indicator strains.

Material and methods

Bacterial sources: The probiotic bacteria tested l strain *(Lactobacillus acidophilus* CL1285 and *Befedo-bacterium* sp.) which obtained from the Egyptian Microbiology Culture Collection (MIRCEN), Fac. of agric., Ain Shams Univ., Cairo, Egypt. The multidrug antibiotic resistance pathogenic *E,coli ; Ps. aeruginosa and S. aureus* were isolated from traditional cosmetic products. The inhibitory spectrum of *Lb. acidophilus* CL1285 and *Befedobacterium* sp. cells were studied against the isolated pathogenic

bacteria in vitro. The inhibitory activity was tested using agar well diffusion method according to NARMS (2002)**.**

Purification of bacteriocin: The bacteriocin, cells free supernatant (CFS), was obtained after incubation of MRS broth inoculated with *Lb. acidophilus* for 24 hrs at 30 ^{o}C in centrifuging cultures (6,000 rpm for 20 minutes at 4^{o}C). The CFS was filtered through syringe filter (0.45 µm, Millipore). The partially purified bacteriocin obtained by ammonium sulfate was performed as described by (Enan *et al.,* 2014). One arbitrary unit (AU ml^{-1}) of crude bacteriocin 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 was adjusted at pH 6.5 and treated with solid ammonium sulfate till 50% saturation level**.** The mixtures were stirred for 12 h at 4^{o}C and centrifuged at (14,000 rpm for 1 hr. at 4^{o}C). The pellets were re-suspended in 1 mM potassium phosphate buffer, pH 6.5 and dialyzed against the same buffer for 24 hrs at 4^{O}C in dialysis tubing. This partially purified bacteriocin was sterilized by filtration through syringe filter (Amicon 0.45 µm, Millipore).

Determination of bacteriocin protein: The bacteriocin protein was performed by the method of Lowry *et al*. (1951), and a modified version of the SDS-PAGE technique of Laemmli (1970) was used. Samples containing ~ 330 µg 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 trans- illuminated wet gels.

Amino acid composition: Amino Acids were determined using the method described by El- Hawary *et al* (2014). A 200µl of purified bacteriocin was hydrolyzed with 6N HCl in

sealed tube, heated in oven at 100^{o}C 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 ninhydrin reagent and spectrophotometric measurement was used for determination of amino acids and biogenic amines.

Antibiogram of bacteriocin protein: The bacteriocin proteins of *Lb. acidophilus* cell cultures were separated into bands using SDS-PAGE within each *lane* to make the proteins accessible to bacteriocin detection. The line was 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 (NCCLS, 1999).

Measurement of potential cytotoxicity of bacteriocin: Potential cytotoxicity of bacteriocin was tested using the method described by Skehan *et al.,* (1990). PHK-A cells (104 cells/well) were plated in 96- multi well plate for 24 hrs, before treatment to allow attachment of cell to the wall of the plate. Different concentrations of the bacteriocin (0, 10, 50, 75 and 100 µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Treated cell monolayers with bacteriocin were incubated for 48 hrs at 37^{o}C and in atmosphere of 5% CO_2. After 48 hrs, the cells were fixed, washed and stained with sulfo-rhodamine- B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader at 480 µm. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified bacteriocin.

Results

Bacterial strains: The pathogenic bacteria (S. aureus *Ps. aeruginosa* and *E coli*) isolated from Traditional cosmetic products were characterized and identified previously. *St.*

aureus; *Ps. aeruginosa* and *E. coli* isolates maintenance on nutrient plate agar medium. One colony from each of isolate was cultured on Parker agar nutrient, and MacConky agar media.

Antibacterial activity

Bacterial cells: The inhibition of *E. coli*; *Ps. aeurgenosa and S. aureus* isolates by *Lb. acidophilus* are seen in figure (1) and table (1). It was noticed that *S. 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 probiotic bacteria against *E. Coli; Ps. aeruginosa and S. aureus* by agar well diffusion method.

Diluted (CFS)	Inhibition zone (mm)					
	E. Coli		*Ps. aeruginosa*		*S. Aureus*	
	Lb.(CFS)	*B.*(CFS)	*Lb.*(CFS)	*B.*(CFS)	*Lb.*(CFS)	*B.* (CFS)
Control (No probiotic)	N. G.	N. G.	N. G.	N. G.	N. G.	N. G.
1 (4^{th})	5	3	5	4	9	5
2 (3^{rd})	6	9	8	6	6	8
2 (2^{nd})	6	9	7	8	7	8
4 (1^{st} concentration)	8	9	6	9	8	9

Lb-= *Lb acidophilus*; B= *Bifidobacterium* sp and N.G.= normal growth

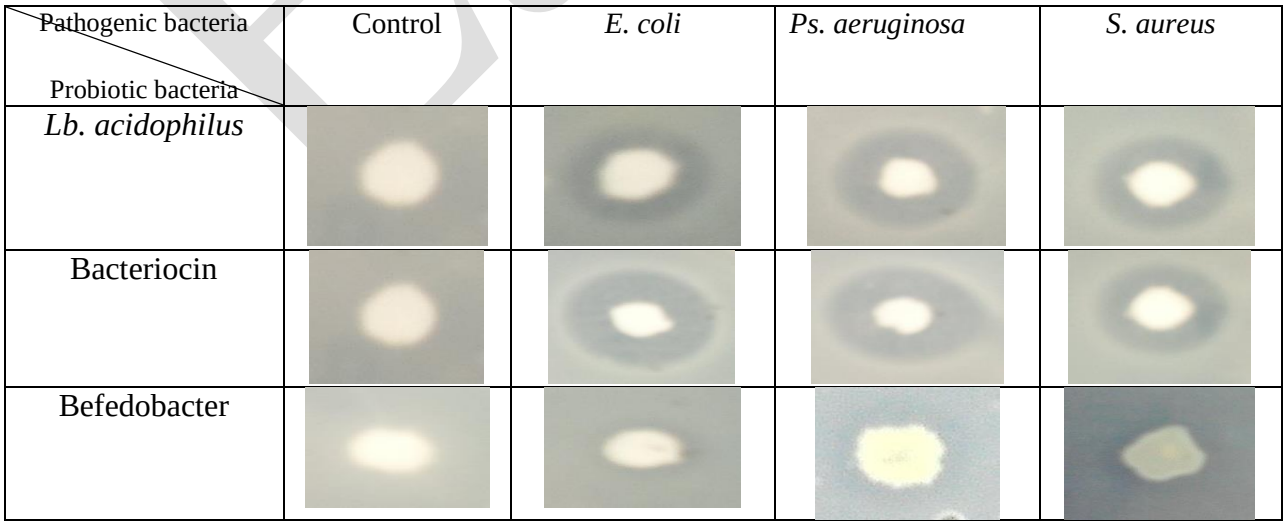

Fig. 1. Antibacterial activity of probiotic bacteria cells and bacteriocin at MIC (100 µg/ml) on pathogenic bacteria contaminated cosmetic products.

The potential cytotoxicity of bacteriocin: The bacteriocin had no potential cytotoxicity on cell monolayer's of PHK-A cells plate at different concentration (0, 10, 50, 75 and 100 µg/ml). It was found no change in colour intensity by ELISA reader at 480 µm. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified bacteriocin table (2) and Fig.(2)

Table 2. Potential cytotoxicity of bacteriocin on BHK-A at different concentration in vitro

Concentration of bacteriocin	PHK-A cells
0.000	1,ooo
10,000	0.903
50,000	0.889
75,000	0.924
100,000	0.938

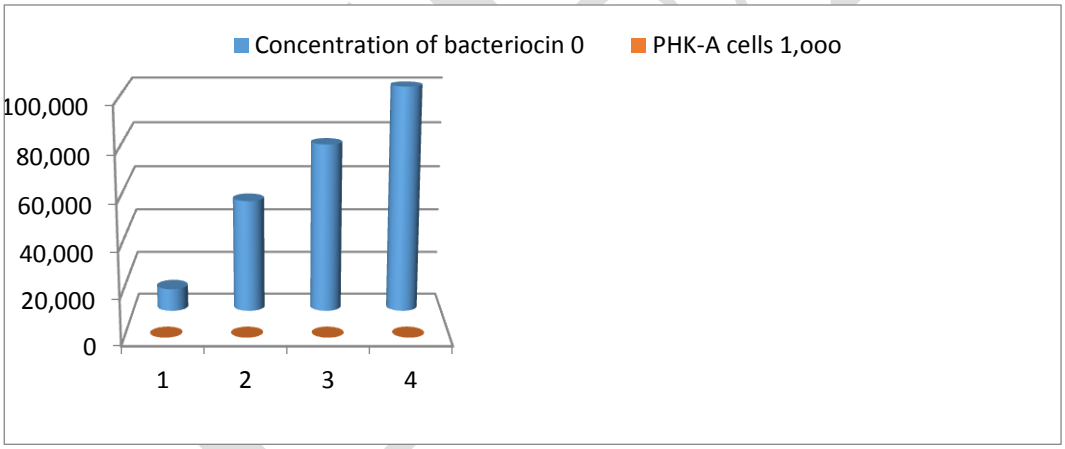

Fig 2. Histogram showing the bacteriocin not has potential cytotoxicity on cell monolayer's of PHK-A cells plate at different concentration (0, 10, 50, 75 and 100 µg/ml)

Bacteriocin: 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), about 4.23-7.00, 4.00- 7.00 and 3.47-6.00 CFU ml^{-1} were obtained with *E. coli* ; *Ps. aeruginosa* and *Staph. Aureus;* respectively.

Table (3). Growth of isolated Staph aureus and E. coli in nutrient broth medium with and without partially purified bacteriocin

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

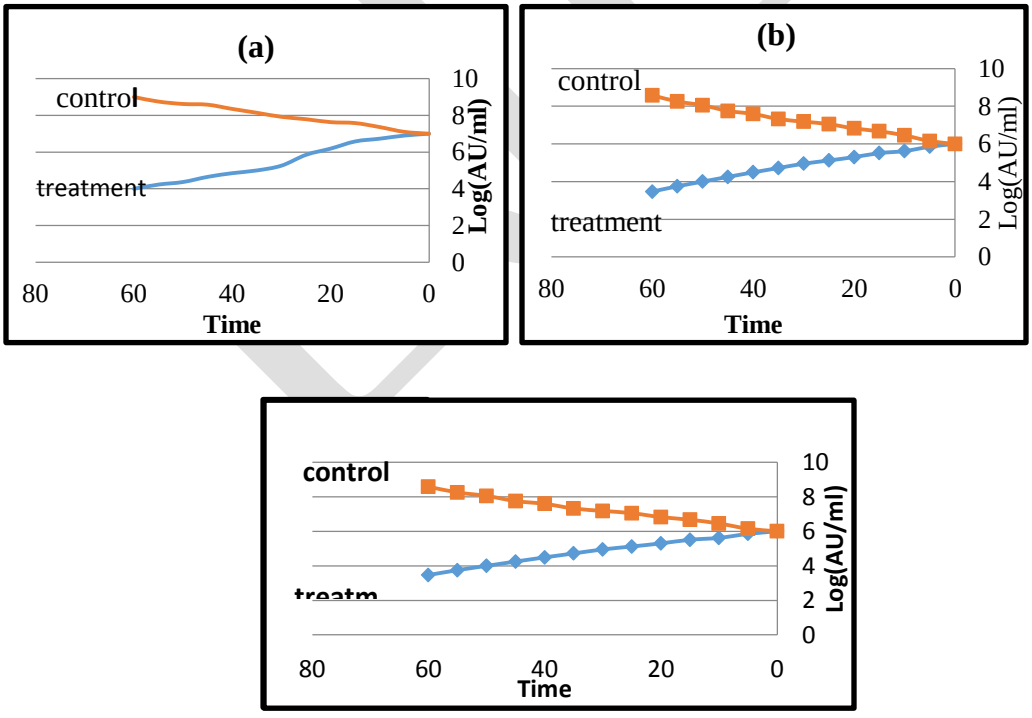

Fig. 3. Curve gram showing Growth of *Staph. aureus* (a) and *E. coli* (b) in broth media with bacteriocin (treated) or without partially purified bacteriocin (control).

Bacteriocin purified: Cell free suspension (CFS) from *Lb. acidophilus* treated with 40% saturation ammonium sulphate suspension and stirred for 12 h at 4^{O}C then centrifuged at 12000 rpm for 1 h at 4^{O}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.

Protein profile of bacteriocin: SDS-PAGE profiles of total bacteriocin proteins (CfS) produced from *Lb. acidophilus* are presented in (Fig. 4). SDS-PAGE analysis revealed 2 protein bands with different molecular weights ranged from 55 to 45 kDa compared with *Lb. acidophilus (BC).*

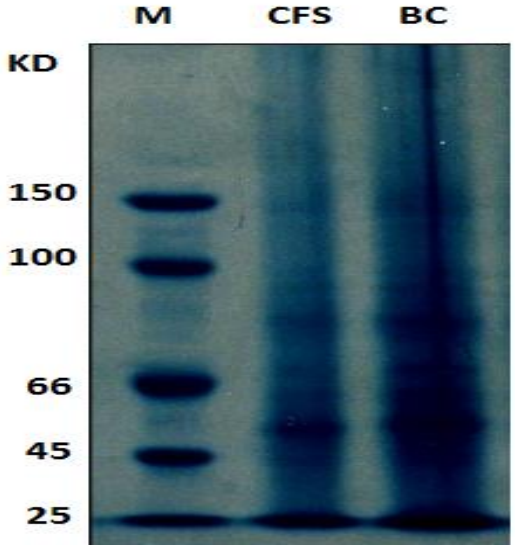

Fig. 4. Electrogram of acrylamide gel (12%) showing Protein pattern of Bacteriocin (CFS) produced from *Lb. acidophilus* (BC) by SDS-PAGE and determined molecular weight with protein marker (M) and BC

Antibiogram of bacteriocin: The 10 ml of bacteriocin were pooled from the column and were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The unstained SDS-PAGE was overlaid with nutrient agar plate inoculating with *E.coli; Ps. aeruginosa and Staph. aureus* and incubated for 24-48 h at 30^{O}C. The inhibition zone was formed around bacteriocin band in lawn *E.coli, Ps. aeruginosa and Staph. aureus* (fig. 5).

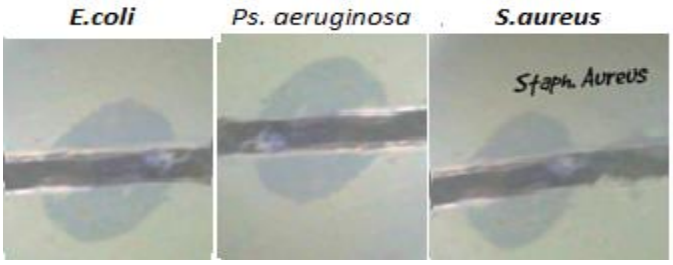

Fig. 5. Antibiogram suppressed growth of each *E. coli, Ps. aeruginosa and Staph. aureus* on SDS-PAGE containing *lb. acidophilus* bacteriocin.

The amino acid composition of the purified bacteriocin was pooled from ion exchange chromatography are recorded in Table (4). Seventeen amino acids were detected with different percentage and amount. The higher amino acids amount obtained can be recorded high content (0.16-0.33 g ml^{-1}) of alanine, proline. glycine, and glutamic comparable (0.005-0.07 g ml^{-1}) of threonine, serine, methionine, isoleucine, tyrosine, phenyl alanine Leucine, Lysin , Cystine, Valine, Arginine, Aspartic and histidine. Therefore, the antimicrobial compound produced by *Lb. acidophilus* was proved to consist of antimicrobial protein bacteriocin.

Table 4: Amino acid composition of Bacteriocin produced by Lb. acidophilus

Amino acids	Amount (gml^{-1})	Amino acids	Amount/(gml^{-1})
Cystine(CYS)	0.005		
Valine(VAL)	0.07	Isoleucine(ILE)	0.02
Alanine (ALA)	0.16	Lysine(LYS)	0.06
Arginine (ARG)	0.05	Methionine(MET)	0.01
Aspartic(ASP)	0.01	Proline(PRO)	0.19
Glutamic(GLU)	0.19	Phenylalanine(PHE)	0.04
Glycine (GLY)	0.33	Serine(SER)	0.02
Histidine(HIS)	0.01	Threonine(THR)	0.01
Leucine (LEU)	0.05	Tyrosine (TYR)	0.03

Discussion

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. This study shows that the *Lb. acidophilus* CL1285 produce antimicrobial components that can inhibit the growth and eliminate pathogenic bacteria which were isolated from commercial

cosmetics. To our knowledge, these results provide direct evidence that *S. aureus; Ps. aeruginosa* and *E. coli* strains were variable to the antimicrobial action expressed by *Lb. acidophilus* when tested *in vitro*. The interaction between lactic acid bacteria and *S. aureus, Ps. aeruginosa* and *E. coli* in mixed liquid culture, can be bactericidal for those pathogenic microorganisms. 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 (Martins and Cunha, 2007). 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 (Matto *et al*., 2006).

In this study PPE of *Lb. acidophilus* inhibited both pathogenic gram positive (S. aureus) and gram negative (*Ps. aeruginosa and E. coli*) bacterial models. This supported previous results on bacteriocin activity against sensitive bacterial species within the same genus (Kang and Lee, 2005). 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 (Drider *et al*., 2006). 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 (Alvarez-Cisneros *et al*., 2011).

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 demonstrates that the *Lb. Acidophilus* inhibit *Staph. aureus* with inhibition zone diameter range (13-20 mm) rather than *E. coli* which shows inhibition 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 *S. aureus* and *E. coli* as pathogenic clinical isolates, using well-defined cell concentrations and standardized culture conditions were investigated. In mixed or pure cultures of LAB and MRSA, the concentration of bacterial cells was determined by standard of CFU counting (Karska-Wysocki *et al*., 2010). The data show that *Lb. acidophilus* produced the antibacterial compounds reducing the number of pathogenic cells more than 5 log10 CFU of *S. aureus* population and more than 4 log10 CFU of *E. coli* within 24h at 37 ^{o}C. 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 results 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 (Ammor *et al*., 2006; Normanno *et al*., 2007). 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 (Tables 1& 2).

The LAB anti-pathogenic strains that were investigated in this study have been used earlier by Millette *et al.* (2006) and Beausoleil *et al.* (2007) against pathogenic organisms. The results obtained by Millette *et al*. (2006) showed that the whey isolated from fermentation of milk transformed by strains CL1285®*Lb. acidophilus* and *Lb. casei* was able to inhibit growth of pathogenic bacteria such as *S. aureus* (MSSA) by 85%, *Listeria monocytogenes* by 78% and *E coli* 0157:H by 77%. Beausoleil *et al.* (2007) 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 Rodgers' (2008) 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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