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PROBIOTIC EFFECTS OF HEAT KILLED AND LIVE *LACTOBACILLUS PLANTARUM* ON PATHOGENIC BACTERIA AND HEK – 293 CELL LINES

PRATHIBA SANDEEP* AND P. SARAVANA KUMARI[1]

**Research and Development centre, Bharathiar University, Coimbatore, India.*
[1]Department of microbiology, Sree Narayana guru college, Coimbatore, India.

ABSTRACT

The study was aimed to evaluate the impact of probiotic bacteria on HEK–293 kidney cell lines and action on pathogenic microbes. The heat killed and live *L.plantarum* cultures were treated on the HEK 293 cell lines and on pathogenic bacteria. The percentage of viability of the cell line was carried out by using Trypan blue dye exclusion method, by mtt assay it was proved that different concentrations of Heat killed and live strains of *L.plantarum* were treated on HEK-293 human embryonic kidney cells. The cell viability was not affected by the liver as well as heat killed strains. An antimicrobial assay was carried out to see the action on pathogens. The strain was most effective against gram positive *Staphylococcus aureus* and least effective against *Shigella flexneri.* The heat killed strain of *L.plantarum* is not harming the cell lines and works against pathogens.

KEYWORDS: HEK 293 cells, Microorganisms, Heat killed L.plantarum, Probiotics, Mtt assay.

PRATHIBA SANDEEP
Research and Development centre, Bharathiar University, Coimbatore, India

INTRODUCTION

In the past century the beneficial roles of non-pathogenic bacteria in the intestinal lumen was described. In the past decade there has been a dramatic increase in scientific work supporting the concept that there are clinical benefits by ingestion of specific nonpathogenic organisms that are called as probiotic. The use of probiotics as a food supplement has gained tremendous interest in the last few years as beneficial effects were reported in gut homeostasis and nutrient absorption but also in immunocompromised patients, supporting protection from colonization or infection with pathogenic bacteria or fungi. The scientific development aims to find the best probiotic so that the technology emerge the new kinds of probiotic that derived from live bacteria [1]. Intestinal lactic acid bacteria including various species of *Lactobacillus* interact regularly with intestine cells which include antigen presenting cells (APCs) and intestinal epithelial cells [2]. It has been reported recently that *lactobacilli* may moderate the allergic reaction by maintaining the balance between Th1 and Th2 responses. This balance is thought to be maintained by specialized subsets of T regulatory (Treg) cells that produce suppressive cytokines, such as interleukin (IL) 10 and transforming growth factor (TGF)- β, most studies have focused on the Immunomodulatory effects of viable lactic acid bacteria; however, we investigated those of heat killed lactic acid bacteria in this study. Heat killed lactic acid bacteria have the advantages of allowing a longer product shelf life, easier storage and transportation [3].

MATERIALS AND METHODS

Bacterial preparation

The Gram positive *Lactobacillus plantarum* culture was procured from ATTC cultures) ATCC- 8014) The bacteria were grown in Deman Rogosa Sharpe (MRS) broth for 24 hours 37 ℃. The whole cells were obtained by centrifugation at 6000* for 10 min which was washed twice with sterilized phosphate buffer at pH 7.2. The heat killed *Lactobacillus plantarum* was prepared by killing them by increasing heat in the water at 100 ℃ for 15 min, the mixture was mixed with 20% Heat killed Lactobacillus plantarum and 80% dextrin and used for further studies.

ANTIMICROBIAL ASSAY

Escreshia coli ATTC 25922,*Staphylococcus.aureus* ATTC 6538, *Salmonella enteritidis* ATTC 13076, *Shigella flexneri* ATTC12022, *Klebsiella pneumonia* ATTC 4352, *Streptococcus pnemoneae* ATTC 700669 and *Enterobacter cloacae* ATTC 13047 strains were grown on Luria broth at 37 ℃ for 12 hours. The number of viable cells was determined by an Agar plate counting method in LB agar, Assay of antimicrobial activity by well diffusion method. Cultures of *Escherichia coli* ATCC 25922, *Shigella flexneri* ATCC 12022, *Salmonella enteritidis* ATCC 13076, *Klebsiella pneumoniae* ATCC 4352, *Staphylococcus aureus* ATCC 6538, Streptococcus pneumoniae ATCC 700669 and Enterobacter cloacae ATCC 13047 were inoculated separately on the surface of Nutrient agar plates by surface spreading using a sterile cotton swab and each bacterium evenly spread over the entire surface of agar plate to obtain a uniform inoculum. The antibacterial activity of the given sample was evaluated by Agar well diffusion method whereby, wells of 6 mm diameter and 5 mm depth were made on the solid agar using a sterile glass borer. About 100 µl of the sample, of the concentration 5 mg/ml, was dispensed into respective wells. The plates were incubated for 24 hours at 37 °C. Twenty four (24) hours later, the antibacterial activity was evaluated in terms of zone of inhibition, measured and recorded in millimeters (mm)[10].

CELL CULTURE

Human Embryonic kidney cell line (ATCC - CRL-1573) was grown in DMEM-Dulbaccos modified Agar medium with L-glutamine and 4.5 g L glucose, supplemented with fetal bovine serum, 100 units of penicillin G and 0.1 mg of streptomycin sulfate in a humidified atmosphere of a 5% CO2 at a 37 ℃ [4].

MICROSCOPIC EXAMINATION OF CELLS

The cell counting chamber was prepared, mirror like polished surface of the haemocytometer and coverslips were cleaned with lens paper. Cover slip was placed over the counting surface, cell suspension was mixed and injected with a pippete 10 micro litre of the cell suspension into the wells of the coverslip. Hemocytometer was taken to a phase-contrast microscope and counted cells in the four large corner squares of the hemocytometer and divided by four, this gives an average concentration of cells per 0.1 mm^3 of suspension. The number of cells per ml were counted by multiplying the count by 10^4,to get the total cell number collected the results were multiplied by the volume in the ml that was collected[5].

CYTOTOXIC ASSAY BY MICROCULTURE TETRAZOLIUM (MTT)

The colorimetric assay is based on the capacity of mitochondria succinate dehydrogenases enzymes in living cells to reduce the yellow water soluble substrate dimethyl thiozole diphenyl tetrazolium bromide (MTT) in an insoluble coloured formazan product. This is measured spectrophotometrically. Which is measured spectrophotometrically since reduction of MTT can occur in metabolically active cells. The level of activity is the measure of viability of cells. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x105 cells/ml using medium containing 10%new born sheep serum. To each well of the96 well microliter plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microliter plates. The plates were then incubated at 37oC for 72hours in 5% CO2 incubator, microscopic examination

was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form an overall concentration 10%. The plates were incubated at 4oC for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then airdried.100 µl of 10mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5minutes. The absorbance was measured using a microplate reader at a wavelength of 540nm22. The percentage growth inhibition was calculated using following formula [6].

The percentage growth inhibition was calculated using following formula,
%cell inhibition= 100-{(At-Ab) / (Ac-Ab)} x100
Where,
At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control

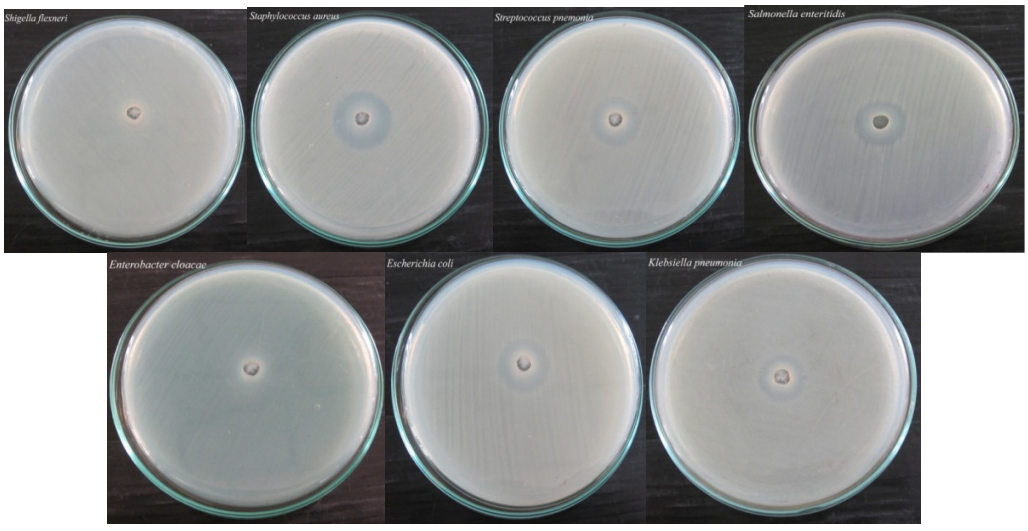
RESULTS

Assay of antibacterial activity by well diffusion method
The cells were most effective against gram positive *Staphylococcus aureus* and least effective against *Shigella flexneri.*

Table1
Antimicrobial activity of heat killed probiotics

Name of the Sample	Organisms Used	Zone of Inhibition(mm)
Lactobacillus plantarum	*Escreshia coli*	17
	Shigella flexneri	11
	Salmonella enteritidis	17
	Klebsiella pneumonia	16
	Staphylococcus.aureus	22
	Streptococcus pnemoneae	18
	Enterobacter cloacae	12

Antimicrobial assay results



HEK -293 CELL CULTURE
Human embryonic kidney cell line (HEK 293T) (ATCC (www.atcc.org – catalog # CRL-1573) were grown in DMEM (Dulbecco s modifications of egal s medium with L-glutamine & 4.5G/L glucose) without contamination.

MICROSCOPIC EXAMINATION OF CELL LINES

Cell viability was confirmed under phase contract microscope, Hemocytometric count of cell lines were shown in live large cells than dark dead Repeated counting shown that above 95 % of cells were live so the cell line were selected for further studies.

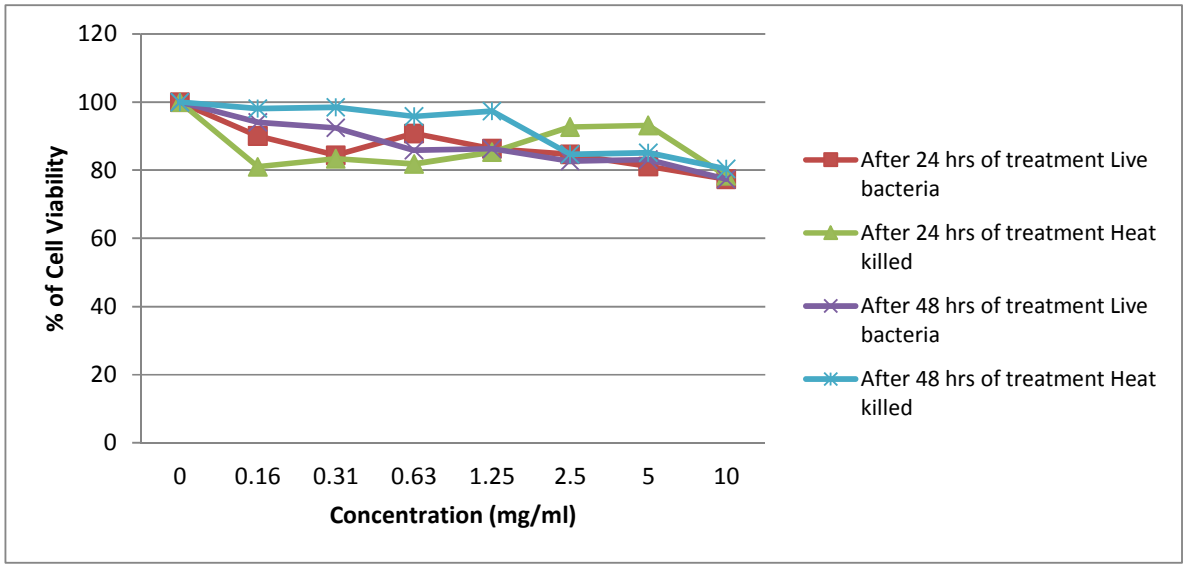
CALCULATION OF CELL VIABILITY

No. of Viable Cells Counted x 100 = % viable cells
Total Cells Counted - (viable + dead)

Cytotoxic assay by MTT:				
Concentration(mg/ml)	After 24 hrs of treatment		After 48 hrs of treatment	
	Live bacteria	Heat killed	Live bacteria	Heat killed
0	100	100	100	100
0.16	90.06	81.056	94.056	98.056
0.31	84.42	83.418	92.418	98.418
0.63	90.81	81.809	85.809	95.809
1.25	86.32	85.324	86.324	97.324
2.5	84.65	92.653	82.653	84.653
5	81.13	93.126	83.126	85.126
10	77.35	78.345	77.345	80.345

The profile of cell growth after treatment with the Live *Lactobacillus plantarum* and heat killed *lactobacillus plantarum* are presented in graph. From the graphs (1,2,3,4)it was found that application of heat killed and live culture of L.plantarum has no significant reduction in the number of viable cells when concentrations in both cases around 20-25 % of reduction in cell growth were recorded. Cell lines growth were not much varied between the treatment of Heat killed strains and live strains an average variation of 5% was recorded in given results.

Graph 1



DISCUSSION

The cells were most effective against gram positive *Staphylococcus aureus* and least effective against *Shigella flexneri.*586 Lactobacillus was ineffective against all the multi-drug tested strains demonstrating the current problem in the treatment of multiple drug resistant nosocomial infections. Some of the metabolites like organic acid produced from bacteria have been prevented the growth of other pathogenic bacteria[7].Human Embryonic kidney cell line (ATCC - CRL-1573) was grown in DMEM-Dulbaccos modified Agar medium with L-glutamine and 4.5 g L glucose, supplemented with fetal bovine serum, 100 units of penicillin G and 0.1 mg of streptomycin sulfate in a humidified atmosphere of a 5% CO2 at a 37 ˚c. Since probiotic strains have been reported to possess anti-inflammatory and immunostimulatory effects, an effort was made in this study to isolate human- derived Lactobacillus strains with these potential probiotic properties. Cell viability was confirmed under phase contract microscope. Repeated count shown that above 99% of the cells were live. So the cell lines were selected for further studies. The proliferation of cell growth after treatment with live *L.plantarum* and heat killed *L.plantarum* are presented in graph (Graph 1,2,3,4) from

the graph it was found that heat killed and live cultures of L.plantarum has no significant reduction in number of viable cells. When concentration increased in both cases around 20-25 % of reduction in cell growth were recorded. Cell line growth was not much varied between heat killed and live ones. An average of 5 % was recorded from the given results. Previous study on587 *L. acidophilus* 606 inhibit cancer cell proliferation but are much less cytotoxic to normal cells [8] .Considering that vaginal lactobacilli colonize the cervix of healthy adults, we compared the effects of these lactobacilli between normal and tumor cervical cell lines. Interestingly, live lactobacilli co-cultured with a normal cervical cell line (HNCF) did not show any cytotoxic effect after 24 h, but were potent growth inhibitors of cervical tumor cells (HeLa). On comparing the effects of LS on these cell lines, it was concluded that although both supernatants had cytotoxic effects, they were much less effective against normal cells[9].

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