IN VITRO STUDIES ON ADHESION AND THE EFFECT OF CYTOTOXICITY OF BIFIDOBACTERIUM SPP. USING CELL LINES

S.Subhashini, Assistant Prof.
J.Lavanya
Dr.S.Meignanalakshmi
Department of Biotechnology, SRM University, Kattankulathur, Tamil Nadu

Abstract

The objective of this study was to elucidate adhesion property and the effect of cytotoxicity studies of *Bifibobacterium in* vitro. *Bifidobacterium* strains were isolated from milk and milk products, around twelve strains were isolated in which four strains were identified they are- Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium bifidum and Bifidobacterium infantis. The identification was done by morphological features and biochemical tests. The isolated strains were then assayed for the adherence and antitumor activities. The Bacteria showed good adherence pattern on the HT-29 cell lines and exhibited profound inhibitory activity on cancer cell lines (Human adeno -carcinoma cell lines).

Keywords: In vitro, Cytotoxicity Of Bifidobacterium, Cell Lines

Introduction

Probiotic organisms are live microorganisms thought to be beneficial to the host organism. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Lactic acid bacteria (LAB) and **bifidobacteria** are the most common types of microbes used as probiotics; but certain yeasts and bacilli may also be used. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements.

At the start of the 20th century, probiotics were thought to beneficially affect the host by improving its intestinal microbial balance, thus inhibiting pathogens and toxin producing bacteria. Today, specific health effects are being investigated and documented including alleviation of chronic intestinal inflammatory diseases, prevention and treatment of pathogen-induced diarrhea, urogenital infections and atopic diseases

Biffidobacteria was first isolated by tissier and it was initially named as Bacillus bifidum[1].Biffidobacterium inhibits the growth of gram negative bacteria in infant. Mother's milk contains high concentration of lactose and lower quantities of phosphate. When milk ferments in the gastrointestinal track of infants the pH reduces which inhibit gram negative bacteria for growth[2-3]. Recent study has reported that the biffidobactrium strain exhibits in various types of oxic growth. Based on the growth profile under different oxygen concentration they are classified into 4 as O2- hypersensitive, O2 – sensitive, O2- tolerant and microaerophilic[4]. Biffidobacterium and lactobacillus has been isolated with beneficial effect in both human and animal health. The fructose- 6- phosphate phosphoketolase of bifid-shunt enzyme is the enzyme present in biffidobacterium which helps to identify the organism. The optimum temperature for growth is 37- 410C, growth gets inhibited below 200C and above 46^0C[5]

Most of the probiotic has the property to adhere in the surface of digestive system, large intestine colon 6. In vitro studies have been done on the ability of human probiotic to survive in low pH, bile salt and adhesion property. The adhesion of probiotic the human epithelium cells has been suggested as an important prerequisite for probiotic action. The adhesion should be maximum in the intestinal track to increase the ability for metabolism, immunomodulation, stabilize with the intestinal mucosal barrier and to provide competitive exclusion of pathogen bacteria.[6-8]. Adhesion to the intestinal cells helps to colonies with other bacteria in gastrointestinal region [9-12]. The cell culture models stimulating the human situation has been widely used for the study of specific function of the human intestinal cell [13].biffidobacteria usually colonize to human GI track at the concentration of 10^{10}CFU/g of the intestinal content[14-15].

The probiotic mediated stimulation of the immune system may be responsible for the antitumor property of biffidobacteria[16]. Epidemiological and population based studies of biffidobacterium on Brest cancer [17] and direct experimental evidence regarding anticancer efficiency of probiotic on human [18] reported the protective effect of Biffidobaterium logun. Animal studies have shown that the capacity of the microorganisms to colonize the epithelial surface depends on the capacity to bind to the epithelium and the nutritional and environmental condition. Lactobacilli adhere to squamous epithelia via acidic polysaccharides. However, it has been reported that macromolecules other than polysaccharides may be involved in the adhesion [19-20].

Materials And Methods

Microorganism was isolated from the milk and milk products and was identified as bifidobacteria. The antibiotic disc was prepared using Kanamycin (30 mcg/disc) Vancomycin- (30mcg/disc) Nitrofurantoin(

100mcg/disc) Tetracycline (30mcg/disc) Nalidixic acid (30mcg/disc) Gentamycin(10mcg/disc) Bacitricin(10mcg/disc). The cell lines are prepared in the following method:

HT29 Cell line (Human Colon Adeno Carcinoma Cell line Grade –II) Whole cell lysate

HT 29 cell lysate was prepared by homogenization in modified RIPA buffer (150 mom sodium chloride, 50 mom Trish, pH 7.4, 1 mom ethylene demine tetra acetic acid, 1 mom phenylmethylsulfonyl flouride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 5 µg/ml of aprotinin, 5 µg/ml of leupeptin). Cell debris was removed by centrifugation. Protein concentration was determined with Bio-Rad protein assay. The cell lysate was boiled for 5 min in 1 x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1%sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% b-mercaptoethanol.

HepG2 Cell line : HepG2 are basically adherent, epithelial-like cells growing as monolayers and in small aggregates, have a model chromosome number of 55.Secrete plasma proteins, such as albumin, transferrin, fibrinogen, a-2-macroglobulin, plasminogen. Cells respond to stimulation with HGH. HEp G-2 cell line was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media (DMEM also works well) supplemented with 10% FBS, penicillin (100 U/ml), andstreptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.

The cells were passaged by briefly rinsing the cell monolayer with 1xPBS twice and pre-warmed (37°C) 0.05% Trypsin-EDTA solution for 5 - 7 minutes was added.Once cell layer was dispersed (5-7 min at 37°C) Trypsin was deactivated by adding an equal volume of complete growth medium with 10% FBS.

Vero Cell line (African Green Monkey Kidney Cell line): Vero cells are kept either in liquid nitrogen or at -80°C. This protocol describes how to start growing Vero cells obtained from frozen stock. After recovery from frozen stock, Vero cells usually take 2-3 passages to reach their regular growth rate, and this should be taken into account if planning to use the cells for experiments, infections, etc. It is important to note that Vero cells are anchorage-dependent cells and therefore cannot be grown in suspension

Isolation of Bifidobacteria: Several differential media were used for the isolation of bifidobaceria like Wilkins chalgen anaerobic agar, MRS agar with 0.05% of L-Cysteine HCL, Nutrient Agar with 2%glutamic acid, skimmed milk agar etc. The most ideally suited selective medium used for isolation and enumeration of Bifidobacterium was TPY (Trypticase Phytone Yeast) media because of its high specificity to strict anaerobes. It was free from antibiotics.Best results were obtained when TPY selective medium was

used. Good growth was also observed on MRS and WC Agar Bifidobacterium were isolated using serial dilution technique at 10^2 dilution. Total Number of cells found to be 2.192 X 10^2 cfu/ml

Biochemical Test: Gram Staining: When the bacteria were stained and visualised under the microscope at various resolution powers they were found to be gram positive. Gram-positive cell walls typically lack the outer membrane unlike that of Gram-negative cell walls. They are visualized as non motile, non-sporulating, pleomorphic rods.

Hydrogen Peroxide Test: The primary factor responsible for aerobic growth inhibition is proposed to be the production of H_2O_2 in the growth medium. A H_2O_2-forming NADH oxidase was purified from O_2-sensitive *Bifidobacterium* and was identified as a *b*-type dihydroorotate dehydrogenase. The kinetic parameters suggested that the enzyme could be involved in H_2O_2 production in highly aerated environments.

Motility Test: The motility test was performed using hanging drop technique using microscopic glass slide. The bacteria were found to be non-motile.

Lactose fermentation test: An isolated colony of bifidobacterium was streaked on a Petri plate containing Mckonkey agar. Appearance of pink coloured colonies indicated that lactose fermentation was positive.

Fructose-6-phosphate phosphoketolase test Methodology

Cells were grown in 10 ml of TPY broth at 37°C for 18 h and harvested by centrifugation at 5000 g for 10 minster pellet was washed twice with 5 ml of 0.5 g/l phosphate-cysteine buffer.After centrifugation, the pellet was collected in 1 ml of buffer and disrupted by ultrasonication at 0°C for obtaining crude cells extract.0.25 ml of reagents (6 mg/ml sodium flouride, 10 mg/ml sodium iodoacetate and 80 mg/ml fructose-6-phosphate) was added to the cells extract. The reaction was started by incubation 30 min at 37°C and stopped by adding 1.5 ml of hydroxylamine-HCl (13.9%).After 10 min, 1 ml of trichloroacetic acid (15%) and 1 ml of FeCl3.6H2O (5%) were added. The presence of fructose-6-phosphate phosphoketolase enzyme was revealed by the appearance of red and purple colors.

Positive results were obtained for the fructose-6-phosphate phosphoketolase test. Presence of frucose-6-phosphate enzyme was confirmed by a colour transformation, red coloration. Thus, the isolates belonging to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphatephosphoketolase enzyme in cellular extracts.

Other Assays were performed including Anti-oxidant test and Anti-Biotic susceptibility

Antioxidant Assay

DPPH (biphenyl)-(2, 4, 6-trinitrophenyl iminoazanium) free radical scavenging assay

The antioxidant assay was carried out to determine the antioxidant potential of the bacterium

Incubation at dark for 30 minutes, Absorbance at 517nm (UV spectrophotometer)

DPPH (diphenyl)-(2, 4, 6-trinitrophenyl iminoazanium)

S.no	Reagent	Blank	Standard	Bifidobacterium spp.
1	Absolute Methanol	3.8ml	3.7ml	3.7ml
2	BHT	-	100µl	-
3	Sample	-	-	100µl
4	DPPH	200µl	200µl	200µl

% Anti oxidant activity (DPPH free radical scavenging) = {(absorbance at blank) – (absorbance at test) / (absorbance at blank)} X 100

Anti-Biotic susceptibility test: It is used to describe the susceptibility or sensitivity of bacteria to anti biotics. If the bacteria were sensitive to antibiotic a clear zone of inhibition or ring would be formed, various Anti-biotic including Bacitricin, Vancomycin, Tetracycline, Kanamycin etc The antibacterial sensitivity patterns of gram-positive, non-sporulating anaerobic bifidobacterium were performed Sensitivity of bifidobacterium was tested with antibacterial agents both with gram-positive and gram-negative spectrum Thus, the minimum inhibitory concentration was determined for the antibiotics at various dilutions(concentrations).

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Disc diffusion followed by serial dilutiom,10 discs of standard antibiotic concentration were taken and dissolved in 1ml of DMSO, Serial dilution was then carried out in 5 eppendorf tube is such that the concentration after each dilution reduces to half [(i.e.) 300, 150,75, 37.5]. Bifidobacterial culture inoculated on TPY broth was swapped on Muller-Hillton agar plate using sterile cotton swabs. 5 sterile discs were placed with varying concentration of the antibiotic. DMSO was taken as Control disc. The appearance of zone formation indicates the Antibiotic susceptibility thereby minimum inhibitory concentration was determined. Zone of inhibition measured in mm scale.

Adherence

Bacterial adherence is basically the establishment of the bacterial pathogen at the appropriate portal/site of entry. It corresponds to a specific interaction between a ligand being expressed on the bacterial surface (adhesins: - they are cell-surface components or appendages of bacteria that

facilitate bacterial adhesion or adherence to other cells or to inanimate surfaces. Adhesins are a type of virulence factor.) And a receptor on the epithelial cell surface. The process of bacterial adherence to the host cells is an important step in the initiation of the bacterial infection. In some bacterial species invasion may follow the adherence step. Different bacterial pathogens have evolved different strategies to gain access to the intracellular compartment. Once intracellular, the invasive bacteria can survive, multiply and thereby rapidly spread.

Invasion is aided by the production of extracellular substances called invasins that promote the immediate invasion of tissues and also possess the ability to bypass or overcome host defence mechanisms which facilitate the actual invasive process.

Preparaton Of Bacterial Sample For Adhesion

Isolated bacteria species were inoculated on MRS broth for 18hrs and the turbidity were observed in the broth due to mucous like structure .The broth containing the culture was transferred to Eppendorf tube, Cells were harvested by centrifugation at 10000g, pellet was obtained which was thrice with Phosphate cysteine buffer and re suspended with 1ml of sterile PBSF or the purpose of storage 0.25 of glycerol was added to the cell suspension Adherence was observed periodically for bacterial strains on HT-29 cells at various stages of incubation(time intervals) Initial adherence(after 4hrs of incubation in CO2 incubator at 37 C) Staining was performed using Giemsa stain and complete adhesion was observed.

Bacterial culture inoculated on the MRS broth and was incubated at 37C with 5% co2 for 18hrs followed which the cells were harvested by centrifugation at 1000g for 10mins, Supernatant was discarded and pellet was thrice with 1X sterile PBS. It was centrifuged at at 10000rpm again the supernatant was discarded and pellet was resuspended with 1ml of 1X sterile PBS. 40% of .25ml glycerol was added to store the cells at 4C, HT-29 celline were seeded on the 6 well plates (revived and subculture), Cells were discarded and washed thrice with MEM supplemented with .53 mM EDTA. Now the wash solution was discarded, 500 microlitre of prepared bacterial cell suspension was added to each of the wells. It was incubated in co2 incubator at 37C with 5% Co2 for anaerobic condition. After period of 4hrs of incubation initial bacterial bacterial adherence was observed under the microscope. Which is followed by periodic observation at 8hrs, 16hrs and 24hrs and adherence were studied. After 24hrs of observation Staining was done with Giemsa Stain for final adherence study.

Cytotoxicity Assay

In order to study the antitumor activity of a new drug, it is important to determine the cytotoxicity concentration of the drug. Cytotoxicity tests define the upper limit of the extract concentration which is non toxic to the

cell line. The concentration non toxic to the cells is chosen for antiviral assay.

After the addition of the drug, cell death and cell viability was estimated. The result was confirmed by additional metabolic intervention experiment such as MTT assay

MTT ASSAY:1 ml of medium without FCS containing each dilution of sample ranging from 1:1 to 1:64 were added to the respective wells. To the cell control well1 ml MEM without FCS was added. The plates were incubated at 37ºC for 24 hrs. After incubation the medium was removed from the wells and 200µl MTT dye was added to it and incubated for 4 hrs. After incubation 1ml of DMSO was added in each well and left for a minute. This suspension was transferred to the cuvette and O.D. values were read at 595nm by taking DMSO as a blank. Cell viability % = Mean O.D. / Control O.D. * 100

Results And Discussion

Identification of the Bifido Bacterium

Gram Staining: When the bacteria were stained and visualized under the microscope at various resolution powers they were found to be gram positive. Gram-positive cell walls typically lack the outer membrane unlike that of Gram-negative cell walls. They are visualized as non motile, non-sporulating, pleomorphic rods.

Hydrogen Peroxide Test: The primary factor responsible for aerobic growth inhibition is proposed to be the production of H2O2 in the growth medium. A H2O2-forming NADH oxidase was purified from O2-sensitive Bifidobacterium and was identified as a b-type dihydroorotate dehydrogenase. The kinetic parameters suggested that the enzyme could be involved in H2O2 production in highly aerated environments.

Fructose-6-phosphate phosphoketolase test: Positive results were obtained for the fructose-6-phosphate phosphoketolase test. Presence of frucose-6-phosphate enzyme was confirmed by a colour transformation, red coloration. Thus, the isolates belonging to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphatephosphoketolase enzyme in cellular extracts.

Lactose Fermentation Test: An isolated colony of bifidobacterium was streaked on a Petri plate containing Mckonkey agar. Appearance of pink coloured colonies indicated that lactose fermentation was positive.

Antibiotic Susceptibility Test

Different type of antibiotics were used against the isolated Bifidobacterium species which includes Bacitricin, Vancomycin, Tetracycline, Kanamycin, Nitrofurantoin, Nalidixic acid and Gentamycin

Antibiotic susceptibility test for the determination of minimum inhibitory concentration based on the results obtained using Muller Hilton Agar

Anti Microbial agent	symbol	Disc concentration (mcg/disc)	Diffused Concentration (10disc/ml DMSO) (300)	Serial dilution 1 (150)	Serial dilution 2 (75)	Series dilution 3 (37.5)
Tetracycline	T	30	21	19	16	15
Kanamycin	K	30	18	16	15	13
Nitrofurantoin	NF	100	19	16	14	13
Bacitricin	B	10	6	4	3	2
Nalidixic Acid	NA	30	12	10	9	7

The antibacterial sensitivity patterns of gram-positive, on-sporulating anaerobic bifidobacterium were performed. Sensitivity of bifidobacterium was tested with antibacterial agents both with gram-positive and gram-negative spectrum. Thus, the minimum inhibitory concentration was determined for the antibiotics at various dilutions(concentrations) .The obtained results have been compared and analyzed with the previous results obtained for Bifidobacterium antibiotic susceptibility for the determination of minimum inhibitory concentration. Resistance was shown more resistance towards Baciticin and Gentamycin. More sensitive to Tetracycline and Nalidixic acid Nitrofurntonin shown the intermediate sensitivity.

Antioxidant assay

Time Interval(mins)	OD of blank at 517nm	OD of Sample at 517nm	Percentage of DPPH free radicals	mean
0-5	0.192	0.114	40.62	33.07
5-10	0.192	0.119	38.02	33.07
10-15	0.192	0.127	33.85	33.07
15-20	0.192	0.132	31.25	33.07
20-25	0.192	0.138	28.13	33.07
25-30	0.192	0.141	26.57	33.07

Formula used

% Anti oxidant activity (DPPH free radical scavenging) = {(absorbance at blank) – (absorbance at test) / (Absorbance at blank)} X 100

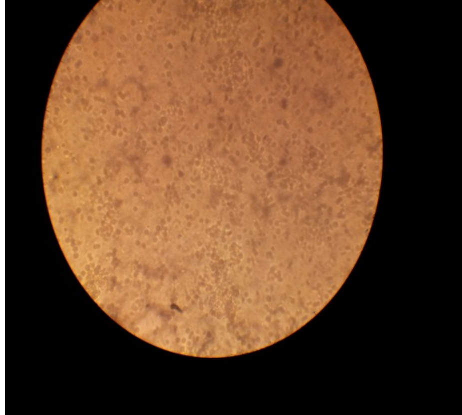
% Antioxidant activity through DPPH free radical scavenging:-

Bifidobacterium spp : - 33.07%

Thus the anti oxidant property found to be a moderately good for the isolated Bifidobacterium spp.

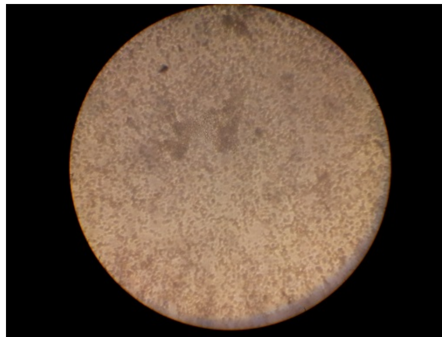
ADHESION ASSAY OF Bifidobacterium longum

In vitro Adherence of Bifidobacterium on HT29 cell lines after 4hrs



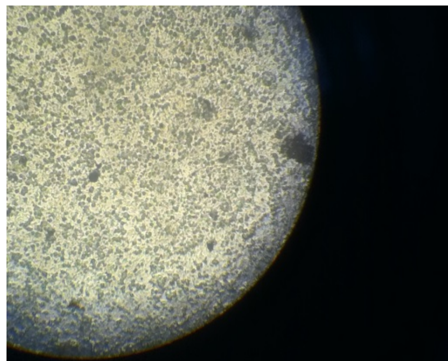
After 4hrs of incubation period they have just began to adhere to the HT-29 Cells

After 8hrs



Slight change in the morphology of the HT29 cells were observed due to clumps of bifido bacteria formed on the epithelial surface

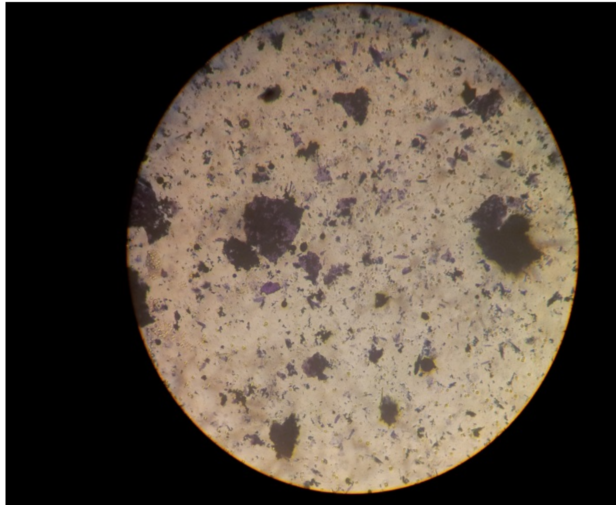
After 16hours



At the 16hrs colonies of Bifidobacterium were observed around the HT29 cell lines

After 24hours

Bacterial cells

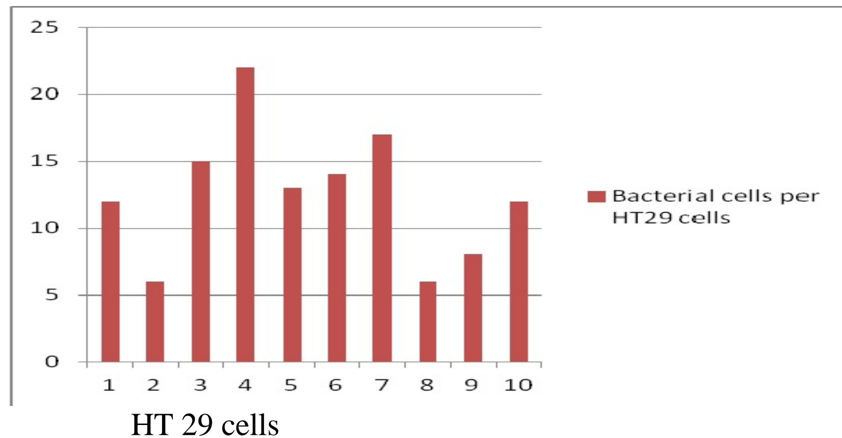


After 24 hours of incubation, the cells were discarded and washed thrice with 1X sterile PBS again it was discarded dried and viewed under the microscope

NUMBER OF BACTERIAL CELLS PER HT29 Cell line

S.no	HT-29 cells	Number of cells
1	1	12
2	2	6
3	3	15
4	4	22
5	5	13
6	6	14
7	7	17
8	8	6
9	9	8
10	10	12

ADHERENCE PATTERN SHOWN BY Bifidobactrium STRAINS ON HT-29 CELLS:



HT 29 cells

Above FIG shows the adherence pattern of Bifidobacterium strains towards the HT-29 Cells. The 4th cell shows the maximum adherence whereas the 2nd and 8th cells shows minimum adherences

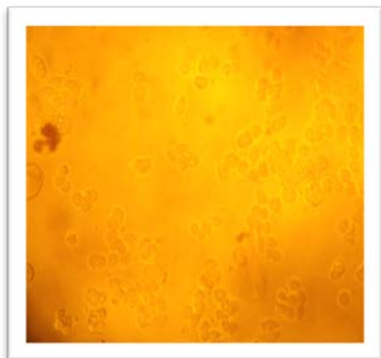
CytoToxicity studies on Various Cell lines

Inhibitory Effects of Bifidobacterium on HT-29 Cell Viability (MTT Assay)

Normal

High Toxicity

Medium Toxicity



Low Toxicity

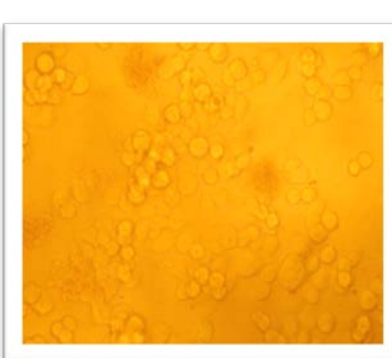
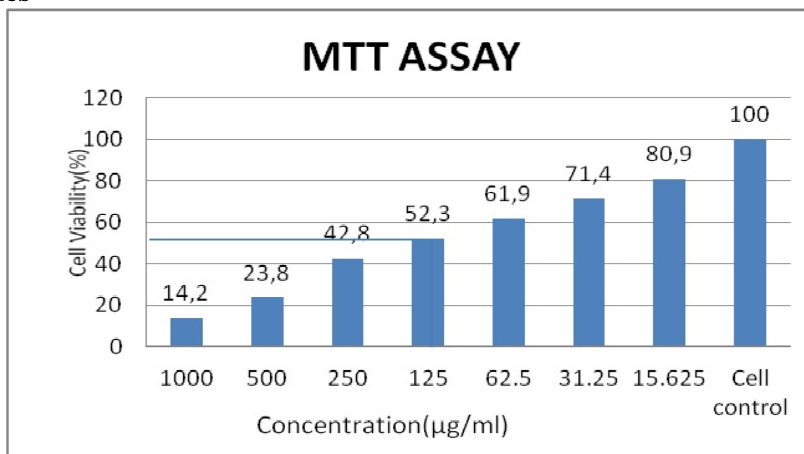


Table 1 In vitro Cytotoxicity effect of lactobacillus HT29 cell lines

S.no	Concentration (µg/ml)	Dilutions	Absorbance (OD)	Cell viability (%)
1	1000	Neat	0.03	7.14
2	500	1:1	0.12	28.5
3	250	1:2	0.18	42.8
4	125	1:4	0.21	50.0
5	62.5	1:8	0.25	59.5
6	31.25	1:16	0.27	64.2
7	15.62	1:32	0.30	71.4
8	Cell Control	-	0.42	100

Graphical representation of Cytotoxicity effect of lactobacillus on HT29 cell lines

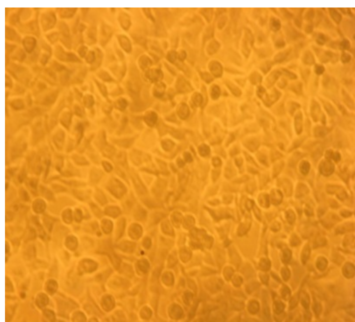


Inhibitory Effects of Bifidobacterium on HT29 Cell Viability (MTT Assay)

In this study HT29 cell lines were used as the cell model of human colon adeno carcinoma cell Grade II to evaluate the inhibitory effects of Bifidobacterium in vitro. Bifidobacterium strains reduced the cancer cell viabilities to a range of 7.14% -71.4 %.At 1:4 dilutions Bifidobacterium could able to reduce the cell viability of 50%.At no dilution Bifidobacterium reduced maximum cancer cell viability. With 1:32 & 1:64 dilutions strongest inhibitory effects were seen i.e. 64.2 % & 71.4% cell viability, showing maximum cell viability and minimum anticancer activity of Bifidobacterium stains. The disintegration of monolayers was seen under the inverted microscope after the incubation of Bifidobacterium stains .The above graphical figure demonstrate the effect of Bifidobacterium exposure on the viability of the human colon carcinoma cell lines measured by the MTT assay

Inhibitory Effects of Bifidobacterium on HEpG2 Cell Viability (MTT Assay)

Normal HEpG2 cell line High Toxicity



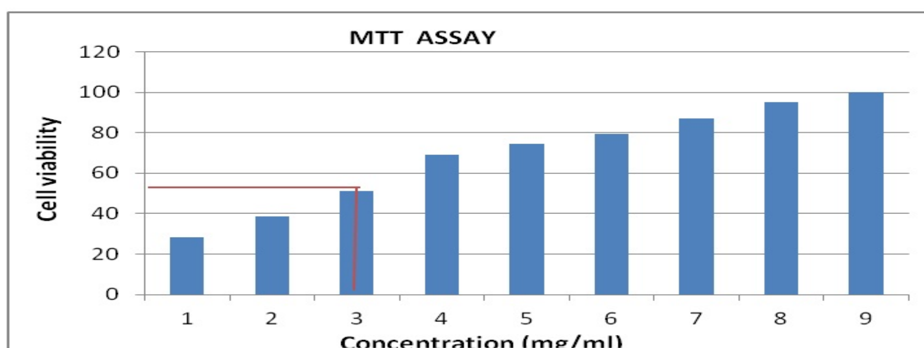
Medium Toxicity

Low Toxicity

Table 2 In vitro Cytotoxicity effects of lactobacillus HEpG2 cell lines

S.No	Concentration (mg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	10	Neat	0.11	28.20
2	5	1:1	0.15	38.46
3	2.5	1:2	0.20	51.28
4	1.25	1:4	0.27	69.23
5	0.625	1:8	0.29	74.53
6	0.312	1:16	0.31	79.48
7	0.156	1:32	0.34	87.17
8	0.078	1:64	0.37	94.87
9	Cell control	-	0.39	100

Graphical representation of Cytotoxicity effect of Bifidobacterium on HEpG2 Celline



Inhibitory Effects of Bifidobacterium on HEpG2 Cell Viability (MTT Assay)

In this study HEpG2 cell lines were used as the cell model of human liver hepato carcinoma cell to evaluate the inhibitory effects of Bifidobacterium in vitro. Bifidobacterium strains reduced the cancer cell viabilities to a range of 28.2% -98.47 %.At 1:4 dilutions Bifidobacterium

could able to reduce the cell viability of 50%.At no dilution Bifidobacterium reduced maximum cancer cell viability. With 1:32 & 1:64 dilutions strongest inhibitory effects were seen i.e. 87.17% & 94.87% cell viability, showing maximum cell viability and minimum anticancer activity of Bifidobacterium stains. The disintegration of monolayers was seen under the inverted microscope after the incubation of Bifidobacterium stains .The above graphical figure demonstrate the effect of Bifidobacterium exposure on the viability of the human colon carcinoma cell lines measured by the MTT assay

Inhibitory Effects of Bifidobacterium on Vero Cell Viability (MTT Assay)

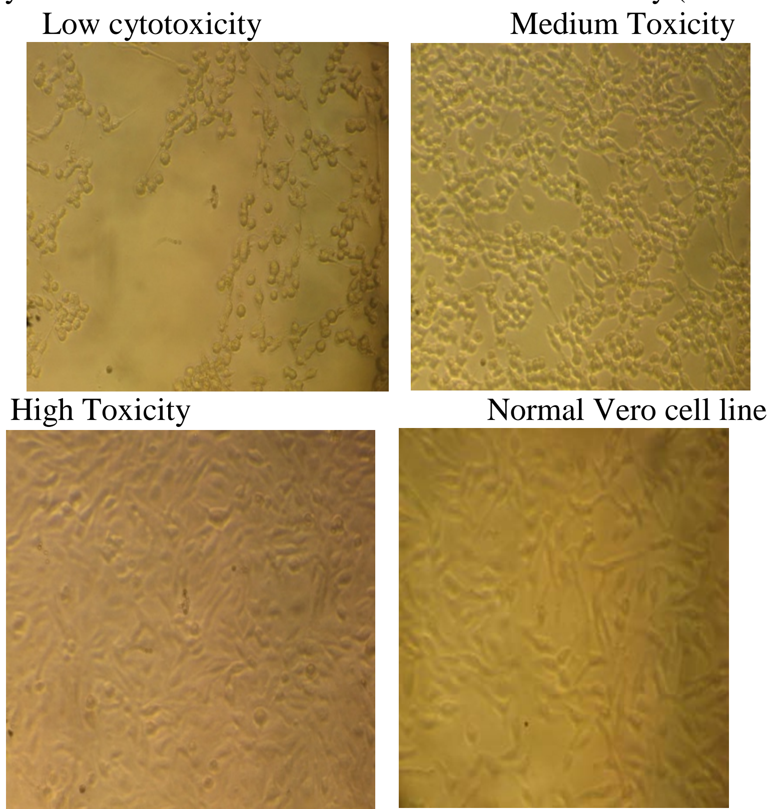
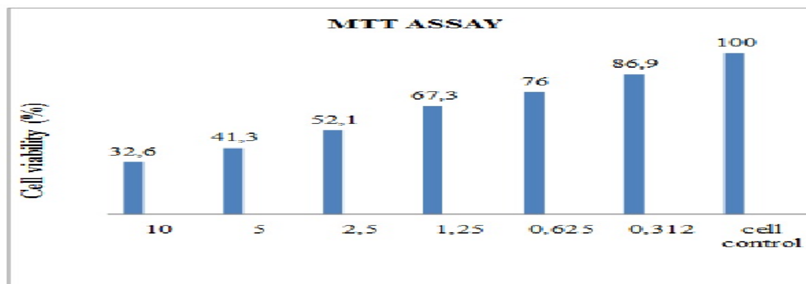


Table 3 In vitro Cytotoxicity effects of lactobacillus Vero cell lines

S.No	Concentration (mg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	10	Neat	0.15	32.6
2	5	1:1	0.19	41.3
3	2.5	1:2	0.24	52.1
4	1.25	1:4	0.31	67.3
5	0.625	1:8	0.35	76.0
6	0.312	1:16	0.40	86.9
7	Cell control		0.46	100

Graphical representation of Cytotoxicity effect of Bifidobacterium on Vero Cell line



Effects of *Bifidobacterium* on Vero Cell Viability (MTT Assay)

In this study Vero cell lines were used as the cell model of Green African monkey kidney cell line to evaluate the inhibitory effects of Bifidobacterium in vitro. Bifidobacterium strains reduced the cancer cell viabilities to a range of 32.6% -86.9 %.At 1:3 dilutions Bifidobacterium could able to reduce the cell viability of 50%.At no dilution Bifidobacterium reduced maximum cancer cell viability. With 1:8 & 1:16 dilutions strongest inhibitory effects were seen i.e. 76.0% & 86.9% cell viability, showing maximum cell viability and minimum anticancer activity of Bifidobacterium stains. The disintegration of monolayers was seen under the inverted microscope after the incubation of Bifidobacterium stains .The above graphical figure demonstrate the effect of Bifidobacterium exposure on the viability of the human colon carcinoma cell lines measured by the MTT assay

References:

1. Tisser H 1900.Recherches sur la flore intestinale normale et pathologique de nourrisson pp1-25. Thèse de paris
2. Schelle, Mark A.; Karmirantzou, Maria; Snel, Berend; Vilanova, David; Berger, Bernard; Pessi, Gabriella; Zwahlen, Marie-Camille; Desiere, Frank et al. (2002). "The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract". *Proceedings of the National Academy of Sciences* 99 (22): 14422–7. Doi: 10.1073/pnas.212527599. PMC 137899. PMID 12381787. //www.ncbi.nlm.nih.gov/pmc/articles/PMC137899/.
3. Mayo, Baltasar; van Sinderen, Douwe, eds. (2010). *Bifidobacteria: Genomics and Molecular Aspects*. Caister Academic Press. ISBN 978-1-904455-68-4.
4. Sonomoto, Kenji; Yokota, Atsushi, eds. (2011). *Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research*. Caister Academic Press. ISBN 978-1-904455-82-0.
5. Gavini 1996 Characterization of the lactose transport system in the strain Bifidobacterium bifidum DSM 20082.

6. Krzewinski F, Brassart C, Gavini F, Bouquelet S. Curr Microbiol. 1996 Jun;32(6):301-7. Salminen S., Wright A., and Ouwehand A. "*Lactic Acid Bacteria*, Marcel Dekker," New York, 2004.
7. Ouwehand A., Kirjavainen V., Shortt C., and Salminen S. "Probiotics: Mechanisms and established effects," *International Dairy Journal*, vol. 9, pp. 43-52., 1999
8. Saarela M., Mogensen G., Fonden R., Matto J., and Mattila-Sandholm T."Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology," vol. 84, pp.197-215., 2000.
9. Larsen N., Nissen P., and Willats W." The effect of calcium ions on Adhesion and competitive exclusion of Lactobacillus ssp. and E. coli O138," International Journal of Food Microbiology, vol. 114, pp. 113–119., 2007.
10. Tannock G.W. "Microecology of the gastrointestinal tract in relation to Lactic acid bacteria," *International Dairy Journal,* 5: 1059– 1070. 1995
11. Salminen S., Wright A., and Ouwehand A. "*Lactic Acid Bacteria*, Marcel Dekker," New York, 2004.
12. Saarela M., Mogensen G., Fonden R., Matto J., and Mattila-Sandholm T."Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology," vol. 84, pp.197-215., 2000.
13. Larsen N., Nissen P., and Willats W." The effect of calcium ions on adhesion and competitive exclusion of Lactobacillus ssp. and E. coli O138," International Journal of Food Microbiology, vol. 114, pp. 113– 119. 2009
14. Servin A., and Coconnier M. "Adhesion of probiotic strains to the Intestinal mucosa and interaction with pathogens," *Best Practice and Research Clinical Gastroenterology*, vol.5, pp. 741–754., 2003
15. Salminen S., Wright A., and Ouwehand A. "*Lactic Acid Bacteria*, Marcel Dekker," New York, 2004.
16. Jiang T., Mustapha A., and Savaiano D.A." Improvement of lactose Digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*," *Journal of Dairy Science*, vol. 79, pp. 750–757. 1996.
17. Rafter, J. (2004). The effects of probiotics on colon cancer development. *Nutrition Research Reviews*; 17: 277-284.
18. YAN YIN,[1] LEI KOU,[1] JIAN-JUN WANG,[1] and GEN-XING XU[2,3] Therapeutic efficacy of *Bifidobacterium longum*-mediated human interleukin-2 with endostatin or TRAIL in transplanted tumors in mice Exp Ther Med. 2012 March; 3(3): 481–486. Published online 2011 December 19. Doi: 10.3892/etm.2011.421
19. G. W. Tannock (Ed.): A fresh look at the intestinal microflora. In: *Probiotics: A Critical Review*, Horiozon Scientific Press, Wymondham (1999) pp. 5–14.
20. D. C. Savage, *Annu. Rev. Microbiol. 31* (1977) 107–133.Savage 1984.