Cytotoxicity Effects of Lactobacillus acidophilus on Hep G2 Cell Line and its Effects on Gene Regulation with Bax and Bcl2

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A B S T R A C T

The microorganisms most commonly used as probiotics are lactic acid bacteria, especially those of the genus Lactobacillus. In the present study, Lactobacillus acidophilus were isolated from two different pooled samples homemade curd and commercial curd, were characterized for their antiproliferative activity. The antiproliferative effects of the strains were investigated using the MTT assay with liver cancer (Hep G2) cell line. The results showed that the Lactobacillus strains had good antiproliferative effects in liver cancer cell line. Further the viability of cells was observed with the help of fluorescent microscopy by dual staining technique. Lactobacillus acidophilus can be considered as potential probiotic bacteria for humans because of their antiproliferation effect in cancer cells. In this study, effect of the samples on expression of Bax and Bcl2 gene was analysed by RT-PCR. β-Actin was chosen as an internal control to normalize the gene expression. The study indicate that the MTT assay for the strains of Lactobacillus acidophilus isolated from the sample has cytotoxicity effect. Based on the IC₅₀ value, homemade curd showed better percent of inhibition than commercial curd and Bcl2 gene showed fold up regulation when compared to Bax gene.

Keywords

Probiotic; Lactobacillus; Antiproliferation; Hep G2; Bax and Bcl2 gene

Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. The most commonly used probiotics in the food industry are Lactic acid bacteria, including the genus Lactobacillus. Probiotics have become highly recognized as supplements for human consumption because of their beneficial effects on health and well-being. For example, interference with pathogens and the prevention of infections, immune system stimulation and immunomodulation, anticarcinogenic and antioxidant activities, reduction of gastrointestinal disorders such as diarrhoea, constipation and the irritable bowel syndrome, alleviation of allergic and lactose intolerance symptoms, reduction in serum cholesterol, blood pressure and risk of gestational diabetes (Dicks LMT and Botes M, 2010).

Among these effects, the antiproliferative or
The cytotoxic effect of probiotic strains against cancer cells is a very important and relatively recent aspect. This probiotic property is important because cancer is considered as the major course of worldwide morbidity (Parisa Shokryazdan et al., 2017).

The MTT system is used to measure the activity of living cells via mitochondrial dehydrogenases. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt. This exhibits a yellow colour when prepared in media lacking phenol red. Insoluble purple formazan is formed from dissolved MTT by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells.

This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The result of the purple solution was measured spectrophotometrically. The concomitant change in the amount of formazan formed can be visualized by an increase or decrease in the cell number, indicating the degree of effects caused by the test material.

In the present study, *Lactobacillus acidophilus* were isolated from the two different pooled samples, homemade curd and commercial curd and used to investigate their antiproliferation effects against liver cancer (Hep G2) cell line and the effect of the samples on expression of Bax and Bcl 2 gene was analysed by performing RT-PCR.

The main aim and objectives of this study includes that to isolate the probiotic bacterium from homemade curd and commercial curd. Also to determine the cytotoxicity effect of the samples on Hep G2 cell line. And to analyze the gene regulation with Bax and Bcl2 gene for the samples.

### Materials and Methods

#### Collection of samples

Homemade curd and commercial curd were collected from the local outlets of Bengaluru, Karnataka, India.

#### Media preparation

MRS (De Mann, Rogosa, Sharpe) – 6.16g/L autoclaved at 121°C for 15 min. After incubation, MRS were allowed to cool and poured it into sterile petri plate.

#### Isolation of bacteria

Samples (100 µL) of homemade curd and commercial curd were serially diluted using sterile phosphate buffer. Serially diluted samples (10⁻²) and (10⁻³) were placed on MRS agar plates by spread plate technique. The plates were incubated at 37°C for 48 hrs and observed for colonies. Bacterial colonies were purified by subsequent subcultures (Sahar Karami et al., 2017).

#### Antiproliferation assay

#### Sample preparation

For cytotoxicity studies, organism concentration was maintained to OD 0.1 and cultured in 5 mL MRS broth for 48 hrs. Later organisms were heat killed for 10 min at 80°C. Cells were homogenized thoroughly and were centrifuged at 5000 g for 5 min. Cell free supernatants of *Lactobacillus* strain were collected and two fold dilutions of the broth was carried out using DMEM.

#### Cell culture

Hep G2 cell lines were procured from American type culture collection (ATCC), stock cells were cultured in DMEM supplemented with 10 % inactivated Fetal
Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5 % CO₂ at 37 °C until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells/well was seeded in a 96 well microtitre plate and incubated for 24 hrs at 37°C in 5 % CO₂ incubator (Maryam Poormontaseri et al., 2017).

**MTT assay**

The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0 x 10⁵ cells/mL using DMEM containing 10 % FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (50,000 cells/well) was added. After 24 hrs, the partial monolayer was formed and the supernatant was flicked off. The monolayer was washed once with medium and 100 µL of different concentrations of samples were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37 °C for 24 hrs in 5 % CO₂ atmosphere. After incubation the solutions in the wells were discarded and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 hrs at 37 °C in 5 % CO₂ atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. Doxorubicin was used as standard. The percentage growth inhibition was calculated using the following formula (Nimmy kumar et al., 2016)

\[
\% \text{ Inhibition} = \left(\frac{\text{OD of Control} - \text{OD of sample}}{\text{OD of Control}}\right) \times 100
\]

**Statistical evaluation**

**IC₅₀ Value**

The IC₅₀ of a sample was determined and the effect examined at different concentrations of samples. IC₅₀ values can be calculated by determining the concentration needed to inhibit half of the maximum biological response. IC₅₀ values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, SanDiego, CA, USA)

**Nonlinear regression**

In statistics, nonlinear regression is a form of regression analysis in which observational data are modelled by a function which is a nonlinear combination of the model parameters and depends on one or more independent variables. The data are fitted by a method of successive approximation.

**Acridine orange and ethidium bromide dual staining**

Dual acridine orange and ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope (Kuan Liu et al., 2015). 25 µL (approx. 1x10⁵ cells) of treated and untreated cells were taken separately in a micro centrifuge tubes and is stained with 5 µL of AO-EtBr (Acridine orange and Ethidium Bromide) for about 2 min followed by gentle mixing. 10 µL of cell suspension is placed onto a microscopic slide and covered with a glass coverslip and examined in a fluorescence microscope using a fluorescein filter.

**Gene regulation using Bax and Bcl2 gene**

**Extraction of RNA**

Total RNA from Hep G2 cells was extracted using TRIzol Reagent (Invitrogen,) according to the manufacturer’s instructions. The cells were washed twice with PBS and centrifuged at 2000 g for 5 min. To the cell pellet, 1 mL
of TRIzol was added in 1.5 mL eppendorf tube and vortexed. Samples were maintained at room temperature for 5 min. To the reaction mixture 0.2 mL of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 min, the resulting mixture was centrifuged at 10,000 g for 15 min at 4 °C. Upper aqueous phase is transferred to a new clean eppendorf tube and treated with 0.5 mL of isopropanol. The resultant mixture is mixed gently by inverting and incubated at room temperature for 5 min. Samples were centrifuged at 10,000 g for 10 min at 4 °C. Supernatant was discarded and the RNA pellet was treated with by adding 1 mL of 70% ethanol. The sample was mixed gently by inverting and centrifuged for 5 min at 14,000 g at 4 °C. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5 min at 55 °C. The pellet was then resuspended in 25 µL of DEPC treated water. The pellet was air dried and dissolved in DEPC treated water (D.Simms et al., 1993)

**RT PCR**

The RT step is critical for accurate quantification and the amount of cDNA produced must accurately represent RNA input amounts (S A Bustin. 2002). A reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of Bax and Bcl2 and β-Actin mRNA expressions. The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit with oligo dT primer. The reaction volume was set to 20 µL and cDNA synthesis was performed at 42 °C for 60 min, followed by RT inactivation at 85 °C for 5 min.

### Table.A Primer: details

| Gene       | Primer pair | Sequence               | Tm   | Amplicon size (bp) |
|------------|-------------|------------------------|------|--------------------|
| β – Actin  | FP          | TCCTCCTGAGCGCAAGTACTCT | 62.1 | 153                |
|            | RP          | GCTCAGTAACAGTCGCCCTAGAA| 62.4 |                    |
| Bax And Bcl2 | FP          | GGGAGGTCAGGTGTCCTATTG  | 55.88| 152                |
|            | RP          | TGCTCTCGGGATAGTCACCA   | 53.83|                    |
|            | FP          | GGTGTCCATTAGTGTCACCA   | 54   |                    |
|            | RP          | GCAAGTACTCTCAGTAA      | 53   |                    |

FP : Forward primer ; RP : Reverse primer

**PCR**

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1 µM of each complementary primer specific for Bax and Bcl2 and β-Actin (internal control) sequence. The samples were denatured at 94 °C for 5 min, and amplified using 35 cycles of 94 °C for 30 seconds each, and for Bax and Bcl2 annealing temperature was set to 49 °C and for β-Actin the annealing temperature was set to 55 °C for 30 seconds and elongation at 72 °C for 1 min followed by a final elongation at 72 °C for 10 min. The optimal numbers of cycles have been selected for amplification of these genes experimentally so that amplifications were in the exponential range and have not reached a plateau. Ten microliters of the final amplification product
were run on a 2 % ethidium bromide-stained agarose gel and photographed. Quantification of the results were accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-Actin intensity levels.

**Results and Discussion**

**Isolation of bacteria**

The bacteria were isolated from homemade and commercial curd and their colonies were counted using colony counter. The results of the bacterial colonies are shown in Table-1.

The Figure 1 and Figure 2 shows the growth of bacteria from homemade and commercial curd respectively.

**Antiproliferative effect**

**Percent of inhibition**

The percent of inhibition of the samples homemade and commercial curd on the Hep G2 cell line is tabulated in Table-2.

The results of the MTT assay, in accordance with the percent of inhibition and concentration of the samples denotes that, the percent of inhibition is directly proportional to the concentration of the samples and hence homemade curd shows greater percent of inhibition than that of commercial curd.

Doxorubicin was chosen as a positive control. The proliferation of the cancer cells shall be inhibited by the samples showing lower value for the IC50. Standard Doxorubicin showed an IC50 value of 18.69 µM inhibition in Hep G2 cells. Commercial curd and homemade curd showed 54.20 % and 62.44 % inhibition at higher concentration of cell free supernatant.

**Acridine orange and ethidium bromide dual staining using HepG2 cell line**

Acridine orange stains both live and dead cells whereas Ethidium bromide stains only cells that have lost membrane integrity. Live cells were visualized green. Early apoptotic cells stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells also incorporate Ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin.

**Standard doxorubicin 25µM**

Dual staining was examined under a fluorescent microscope. Normal cells are seen with circular nucleus uniformly distributed in the center of the cell which is seen in the control Fig 5 and 6. Fig 7, 8, 11 and 12 are showing Early stage apoptotic cells, where cell’s nucleus is showing yellow-green fluorescence by Acridine orange (AO) staining and concentrated into a crescent or granular that located in one side of cells.

Staining was localized asymmetrically within the cells. In Fig 9, 10, 13 and 14 it is seen that the nucleus of cells showed orange fluorescence by EtBr staining and gathered in concentration and located in bias. This is late apoptotic phase. Cells that have taken up complete EtBr are the dead cells as in Fig 15 and 16.

The bacteria were isolated from homemade and commercial curd and their colonies were counted using colony counter. The percent of inhibition were calculated on the samples homemade and commercial curd with Hep G2 cell line. Dual staining technique (AO/EtBr)
was done under the fluorescent microscope to visualize the viable cells. Staining was localized asymmetrically within the cells, cells that have taken up complete EtBr are recognized as dead cells. Further the study was followed for gene regulation by Bax and Bcl2 gene with β-Actin as housekeeping gene. The study concludes that Bcl2 gene showed more fold up regulation when compared with Bax gene on the pooled homemade curd sample.

Table 1 Bacterial colony counts of samples

| Sample             | Dilutions | Colony counts (CFU/g) |
|--------------------|-----------|-----------------------|
| Homemade Curd      | $10^{-2}$ | 267                   |
|                    | $10^{-3}$ | 158                   |
| Commercial Curd    | $10^{-2}$ | 537                   |
|                    | $10^{-3}$ | 341                   |

Table 2 Percent of inhibition of curd samples

| Hep G2 cell line | Samples        | Concentration in % | OD at 590nm | % Inhibition |
|------------------|----------------|--------------------|-------------|-------------|
| Control          | 0.00           | 0.583              | 0.00        |
| Commercial curd  | 1.56           | 0.510              | 12.52       |
|                  | 3.13           | 0.458              | 21.44       |
|                  | 6.25           | 0.376              | 35.49       |
|                  | 12.50          | 0.341              | 41.51       |
|                  | 25.00          | 0.304              | 47.86       |
|                  | 50.00          | 0.267              | 54.20       |
| Homemade curd    | 1.56           | 0.502              | 13.83       |
|                  | 3.13           | 0.432              | 25.90       |
|                  | 6.25           | 0.380              | 34.82       |
|                  | 12.50          | 0.297              | 49.06       |
|                  | 25.00          | 0.253              | 56.60       |
|                  | 50.00          | 0.219              | 62.44       |
**Table 3** IC₅₀ of the *Lactobacillus* strain supernatants on Hep G2 cell line

| Hep G2 cell line Samples | Conc. µM | OD at 590nm | % Inhibition | IC₅₀ µM |
|--------------------------|----------|-------------|--------------|---------|
| Control                  | 0        | 0.583       | 0.00         | 18.69   |
| Doxorubicin              | 3.13     | 0.403       | 30.82        |         |
|                          | 6.25     | 0.366       | 37.16        |         |
|                          | 12.5     | 0.294       | 49.57        |         |
|                          | 25       | 0.223       | 61.67        |         |
|                          | 50       | 0.176       | 69.73        |         |
|                          | 100      | 0.111       | 80.89        |         |

**Table 4** Relative expression of Bax and Bcl2 gene in homemade curd

| Gene  | Samples (µL) | Band Intensity Of PCR Amplicon Of Genes | Normalised | Relative Gene Expression |
|-------|--------------|----------------------------------------|------------|--------------------------|
|       |              | β-Actin                               | Bax and Bcl2 |                           |
|       |              |                                        |            |                          |
| Bax   | 25           | 21786.57                               | 4915.2     | 0.226                    | 0.210                     |
|       | 12.5         | 20556.18                               | 16778.76   | 0.816                    | 0.759                     |
|       | Control      | 16661.52                               | 17919.18   | 1.075                    | 1                         |
| Bcl2  | Control      | 21380.69                               | 16078.93   | 0.752                    | 1                         |
|       | 12           | 17729.28                               | 11083.71   | 0.625                    | 0.831                     |
|       | 25           | 16323.66                               | 5469.08    | 0.335                    | 0.446                     |

**Table 5** Relative expression of Bax and Bcl2 gene in commercial curd

| Gene  | Samples (µL) | Band Intensity Of PCR Amplicon Of Genes | Normalised | Relative Gene Expression |
|-------|--------------|----------------------------------------|------------|--------------------------|
|       |              | β-Actin                               | Bax and Bcl2 |                           |
|       |              |                                        |            |                          |
| Bax   | 50           | 14189.40                               | 6194.32    | 0.44                     | 0.409                     |
|       | 25           | 15784.27                               | 8547.76    | 0.54                     | 0.507                     |
|       | Control      | 16867.00                               | 18023.49   | 1.07                     | 1                         |
| Bcl2  | Control      | 14266.15                               | 15215.47   | 1.07                     | 1                         |
|       | 25           | 15280.23                               | 11787.57   | 0.77                     | 0.723                     |
|       | 50           | 14229.38                               | 8705.61    | 0.61                     | 0.574                     |
Fig. 1 Homemade Curd plating of $10^{-2}$ and $10^{-3}$

Fig. 2 Commercial curd plating of $10^{-2}$ and $10^{-3}$

Figure 3 Bar graph for percent of inhibition
MTT assay using Hep G2 cell

\[ IC_{50} = 18.69 \]

Figure 4 Percentage inhibition of doxorubicin

Control HepG2 cells

Figure 5 Normal cells - treated cells

Figure 6 Normal cells - untreated cells
Conc. 12.5% of homemade curd

Figure 7 Early stage apoptotic phase - treated cells

Conc. 25% of homemade curd

Figure 8 Early stage apoptotic phase - untreated cells

Figure 9 Late stage apoptotic phase - treated cells
Figure 10 Late apoptotic phase - untreated cells

Conc. 25% of commercial curd

Figure 11 Early apoptotic cells - treated cells

Figure 12 Early stage apoptotic cell - untreated cells
Conc. 50% of commercial curd

Figure 13 Late stage apoptotic phase - treated cells

Figure 14 Late stage apoptotic phase - untreated cells

Standard Doxorubicin 25µM

Figure 15 Dead cells - treated cells
Figure 16 Dead cells - untreated cells

Comparison of amplicons - Amplicons of homemade curd

![Image of DNA ladder and amplicons]

Figure 17 Amplification of β-Actin gene in Hep G2 cell line
Lane 1 - DNA Ladder; Lane 2 - 500 μL; Lane 3 - 250 μL; Lane 4 - Control; Lane 5 - Control, Lane 6-250 μL, Lane 7 - 500 μL, Lane 2,3,6,7 - Samples with β-Actin gene

![Image of DNA ladder and amplicons]

Figure 18 Amplification of Bax and Bcl2 gene in Hep G2 cell line
Lane 1 - DNA Ladder; Lane 2-500 μL; Lane 3-250 μL; Lane 4- Control; Lane 5- Control, Lane 6-250 μL, Lane 7- 500 μL, Lane 2,3 - Samples with Bax gene; Lane 6,7 - Samples with Bcl2 gene
Amplicons of commercial curd

**Figure.19** Amplification of β-Actin gene in Hep G2 Cell line
Lane 1 - DNA Ladder; Lane 2– 500 μL; Lane 3-250 μL; Lane 4- Control; Lane 5- Control, Lane 6-250 μL, Lane 7- 500 μL, Lane 2,3,6,7 - Samples with β-Actin gene

**Figure.20** Amplification of Bax and Bcl2 gene in Hep G2 Cell line
Lane 1 - DNA Ladder; Lane 2– 500 μL; Lane 3-250 μL; Lane 4- Control; Lane 5- Control, Lane 6-250 μL, Lane 7- 500 μL, Lane 2,3 - Samples with Bax gene; Lane 6,7 - Samples with Bcl2 gene

**Figure.21** Fold regulation of Bax and Bcl2 gene in homemade curd
In conclusion, our study disclosed that the probiotic bacteria was isolated from two different sources such as homemade curd and commercial curd and was analyzed for their antiproliferative activity by MTT assay on Hep G2 cell line. Based on the IC\textsubscript{50} value, homemade curd shows more percent of inhibition than commercial curd. In this study, effect of Homemade curd and commercial curd for expression of Bax and Bcl2 gene was studied in Hep G2 cell by RT-PCR. The internal control β-Actin was used to normalize the gene expression. Bcl2 showed fold up regulation when compared to Bax. The results of this study revealed that the MTT assay for the probiotic bacterium isolated from the sample has better cytotoxicity effect.

**Acknowledgement**

We are thankful to Skanda Life Science Pvt.Ltd. Bengaluru, for providing us the laboratory facilities to carry out this project.

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How to cite this article:
Ramesh Babu. N. G., K. A. Simrah Fathima, V. Nandhini and Nandhini. V. 2020. Cytotoxicity Effects of Lactobacillus Acidophilus on Hep G2 Cell Line and its Effects on Gene Regulation with Bax & Bcl2. Int.J.Curr.Microbiol.App.Sci. 9(05): 891-906. doi: https://doi.org/10.20546/ijcmas.2020.905.098