International Journal for Pharmaceutical Research Scholars (IJPRS)

V-4, I-1, 2015 ISSN No: 2277 - 7873

REVIEW ARTICLE

Comparison of Immunomodulatory Activity of Probiotic Bacteria and their DNA: A Study Conducted with *L. acidophilus* NCDC343 & *L. casei* Isolated from Yakult

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Manuscript No: IJPRS/V4/I1/00022, Received On: 04/02/2015, Accepted On: 13/02/2015

ABSTRACT

The increasing knowledge about the immune response mechanisms in modulating many diseases has raised interest in application of probiotics as immunomodulators. The beneficial health effects of probiotics are well known and many probiotic products are commercially available now as nutraceuticals. Though, probiotics have GRAS (Generally Regarded as Safe) status, yet their use may be limited and concentration dependent in aged and immunocompromised persons. Recent advances in immunology and molecular biology lead towards finding that the DNA of the bacteria is capable of immune activation. The preliminary experiments revealed that bacterial DNA is a better immune enhancer than its whole cell and that the bacterial genomic DNA is a good adjuvant. Hence, the present study was conducted to compare the immunomodulatory potential of *Lactobacillus acidophilus* NCDC343 (LA 343) [a strain selected after screening four strains of *L. acidophilus* (LA) for their immune activity] with that of *Lactobacillus casei* strain Shirota (LcS) [isolated from Yakult (commercial probiotic drink)] and their DNA. The results indicated that DNA has more profound effect as immunopotentiator than the whole cells. Both the probiotic strains i.e. LA 343 and LcS showed comparatively similar bioactivity; however, the isolated DNA of LcS showed maximum immunomodulatory activity. It is concluded that probiotic DNA is potent and safe immunopotentiator and may replace the whole bacterial cells or may act in synergism with standard drug available in the market after conducting experimental studies in animals.

KEYWORDS

Probiotics, CpG-DNA, Immunomodulatory Agent, Yakult, NBT, iNOS, Bactericidal Activity

INTRODUCTION

The changing life style, the food habits and the industrialization, the use of chemicals and chemical based drugs has resulted in the modulation of immune response and in turn rise in infectious diseases as well as non infectious immune related disorders like Diabetes, Hypercholesterolemia, Rheumatoid Arthritis, etc.

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This demands the need for the development of efficient immunomodulators which can be employed to prevent or cure the diseases. The chemical based drugs employed to cure these diseases may have the side effects and are costly. Moreover, consumer awareness about the harmful effects of chemical drugs raised a need to search natural / alternative therapies for the treatment of diseases. Immunotherapy is one of the alternative ways of modification of diseases.

Probiotics, the beneficial bacteria have been proved to have immunostimulating efficacy[1]. These bacteria have GRAS status, which made

them most consumer acceptable alternative therapeutic agent. Some bacterial cell components such as peptidoglycans, lipoteichoic acid, etc, secreted soluble substances play role in immunomodulation responses[2]. The live probiotic bacteria[3] as well as heat killed probiotic bacteria[4] possess immunomodulatory functions. It was further proved that bacterial DNA acts as a strong vaccine adjuvant, for inducing humoral immunity[5,6].

The vertebrate immune system recognizes 'CpG motifs' of probiotic DNA as foreign and trigger protective immune responses which are strongly Th1-based. Structural difference between bacterial and eukaryotic DNA apparently account for the ability of bacterial DNA to serve as an immune activating agent. Specifically, bacterial DNA is thought to activate inflammatory cells because of its high content of short sequences with unmethylated CpG dinucleotides[7]. Probiotic DNA could act as immunomodulatory agent[8,1].

Hence, the present study was conducted to screen out the probiotic strain having maximum immune activity and to compare the immunomodulatory potential of probiotic whole cells, isolated LcS bacteria (of commercial probiotic drink Yakult) with their isolated DNA.

MATERIAL AND METHODS

Probiotic Test Strains

Four probiotic strains of *Lactobacillus acidophilus* i.e. *L. acidophilus* NCDC291 (LA 291), *L. acidophilus* NCDC343 (LA 343), *L. acidophilus* NCDC600 (LA 600), *L. acidophilus* NCDC702 (LA 702) were procured from NDRI, Karnal.

The cultures so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37°C. Bacterial cultures were grown and maintained for further use. The procured probiotic strains were studied for their growth curve characteristics. *Lactobacillus casei* strain Shirota (LcS) was isolated from Yakult (a commercial probiotic drink).

Screening and Selection of Probiotic Strain with Maximum Immunomodulatory Potential

Test Strains

The four probiotic strains i.e. LA 291, LA 343, LA 600 and LA 702 were tested for *in vitro* immunomodulation. The strains after appropriate growth in the MRS broth, were collected and centrifuged at 4000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was washed twice with PBS (pH 7.4). Finally, the cells were suspended in 2 ml PBS, counted and standardized as 1 x 10^9cells ml^{-1} for each strain.

Total Splenocyte Isolation from Spleen

Splenocytes were isolated by teasing the tissue. The cells were centrifuged (400 × g for 10 minutes at 4°C) and lysed by using the ACK lyses solution (0.5M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.4). The splenocytes which were obtained were washed thrice in PBS, counted and adjusted for cell number (2 x 10^6cells/ml) in MEM followed by incubation with the test strains for 1 hour and thereafter assayed for their immune activity *in vitro* by employing the following tests: the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test, bactericidal activities.

The Nitroblue Tetrazolium Reduction Assay[9]

The splenocyte suspension was incubated with yellow colored NBT dye, leading to the ingestion of blue colored formazon by splenocytes. This complex was extracted using dioxan and the intensity of blue color was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as the blank. The results were expressed in percentage dye reduction.

Inducible Nitric Oxide Synthase Activity[10]

The inducible nitric oxide synthase activity in the splenocyte suspension was evaluated using arginine as a substrate. The colour which was developed (indicating the presence of citrulline, due to the conversion of arginine into citrulline by the enzyme iNOS) was measured spectrophotometrically at 540nm against RPMI and the Griess reagent as the blanks and the

results were expressed as the percentage iNOS activity.

Bactericidal Activity[11]

The splenocyte suspension was incubated with the bacterial suspension (*Escherichia coli*) at 37°C for 60 minutes. The splenocytes were lysed with sterile distilled water and they were spread on an agar plate and incubated at 37°C for 24 hours. The bacterial suspension was spread on the control plate. The number of colony forming units (CFUs) which were developed in the control and test plates were counted and the results were expressed as percentage bactericidal activity.

Two strains of probiotics were selected for further studies on DNA: (i) *Lactobaciluus acidophilus* NCDC343 (on the basis of growth characteristics and *in vitro* immunomodulation) and (ii) isolated strain (LcS) of yakult. These were further subjected to DNA extraction procedure. For genomic DNA preparation, cells were grown in the corresponding medium containing 1.5 % glycine to facilitate cell lysis[12].

Isolation of Genomic DNA from Probiotic Strains[13]

Genomic DNA was isolated and purified with several modifications. An overnight culture (2ml) was pelleted at 8000 rev min^{-1} (microcentrifuge) 25°C for 8 minutes. Cell lysis was achieved using 1ml lysis buffer and incubation 2hr at 55°C with gentle shaking after every 10 minutes. Protein removal was carried out by adding equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant and centrifugation at 4000 rpm for 12 min at 4°C. DNA was precipitated by addition of 100µl of 5M sodium acetate and equal volume of isopropanol and incubation at 20°C overnight. To remove residual contamination, washing was done with absolute ethanol. DNA was then resuspended in 40-50µL of TE (Tris 10mM, EDTA 1mM, pH 8.0). The concentration and purity of DNA were analyzed spectrophotometrically (Shimadzu, UV-1650 PC spectrometer) by measuring OD_{260}/OD_{280}. Only the DNA with OD_{260}/OD_{280} ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1 % agarose gel (100V for 20-40 min) containing 0.5 µg ml^{-1} ethidium bromide.

Comparative *in vitro* Immunomodulatory Potential of Selected Probiotic Strains and their DNA

Splenocyte suspension was made in the same manner as mentioned above and was incubated for 1 hour with the respective test samples i.e. LA 343 and LcS (each suspension having 1 x 10^9cells ml^{-1}); 100 µg genomic DNA each of LA 343 (LA DNA) and LcS (LcS DNA). The splenocyte suspension was further subjected to NBT reduction[9], iNOS[10] and Bactericidal activity[11] tests.

RESULTS

Procured Probiotic Strain's Growth Study

The probiotic strains procured from NDRI, Karnal attain the maximum cell number within 18-27 hours. Variation in the strains was seen in the time to attain the maximum growth, in the order of as follows: LA 343 (18 hrs) > LA 600 (21 hrs) > LA 702 (24 hrs) > LA 291 (27 hrs).

Screening and Selection of Probiotic Strain with Maximum Immunomodulatory Potential *in Vitro*

The immunomodulatory potential of different strains is shown in Figure 1.

The Nitroblue Tetrazolium Reduction Assay

It was seen that all the probiotic strains significantly increased the NBT reduction as compared to the control. LA 291 and LA 343 have more potential of NBT reduction, 80.54% and 79.92% respectively. Other two probiotics i.e. LA 600 and LA 702 showed lesser NBT reduction in comparison to LA 291 and LA 343, with LA 600 being the lowest in NBT reduction. The decreasing order of NBT reduction test is as: LA 291> LA 343> LA 702> LA 600

Inducible Nitric Oxide Synthase Activity

Similar to NBT reduction, iNOS activity of LA 291 (51.68%) and LA 343 (48.17%) was maximum; whereas, LA 600 and LA 702 have lesser activity of 16.32 % and 10.66%

respectively as compared to control. The decreasing order of iNOS activity is as: LA 291> LA 343> LA 600> LA 702

Bactericidal Activity

The effect of probiotics on bactericidal activity was studied in terms of number of colony forming units (CFU). All the probiotic strains effectively enhanced the bactericidal activity of splenocytes. LA 291 and LA 343 reduced the number of colonies much more than LA 600 and LA 702. The percentage decrease in colonies is as: LA 291> LA 343> LA 702> LA 600

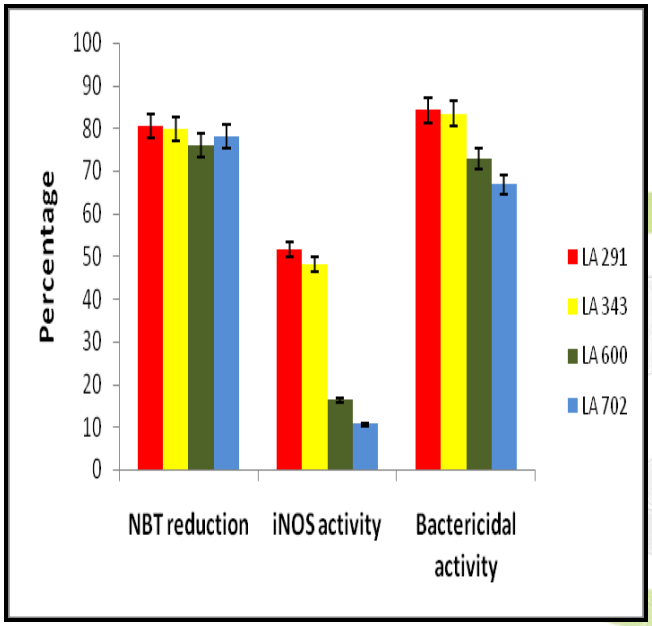

Figure 1: Influence of different probiotic strains on NBT reduction, iNOS and Phagocytic activity. The above data is represented as Mean ± S.E.M (n=3)

After the analysis of results, LA 343 was selected based on the growth characteristics (less time to reach maximum growth) and immune enhancing activity (higher NBT reduction, iNOS and phagocytic activity).

Isolation of Genomic DNA from Probiotic Strains

Genomic DNA was extracted from LA 343 and LcS. The concentration and purity of DNA was checked spectrophotometrically by measuring $O.D_{260}$ / $O.D_{280}$. The DNA with $O.D_{260}$ / $O.D_{280}$ ratio ranging between 1.8 and 2.0 were further subjected to agarose gel electrophoresis before testing there in vivo immunomodulation efficacy. The results of agarose gel electrophoresis are given in Figure 2.

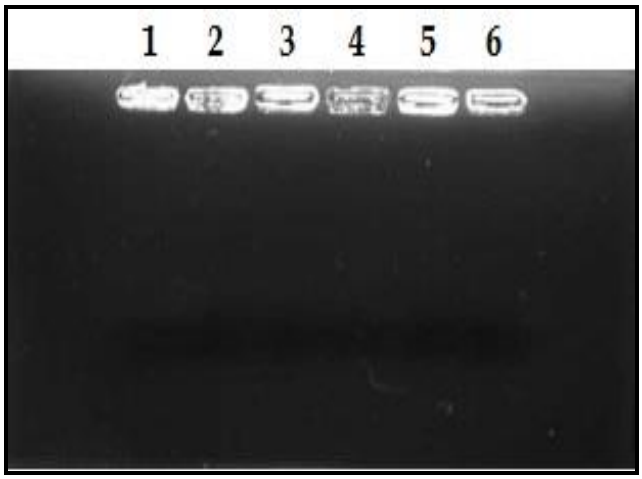

Figure 2: Agarose gel electrophoresis (0.8% agarose gel) analysis of the genomic DNA. 1-3 wells = Genomic DNA of LcS, 4-6 wells = Genomic DNA of LA 343

This result also showed that there is no trailing in the DNA electrophoresed, which indicated that there was no contamination of any protein or RNA.

Comparative *in vitro* Immunomodulatory Potential of Probiotic Strains and their DNA

The result of immunomodulatory activity of whole cells and their isolated DNA are given in Figure 3.

The Nitroblue Tetrazolium Reduction Assay

LcS DNA showed greater NBT reduction than LcS culture. LcS DNA showed 86% NBT reduction similarly the LA343 DNA also showed greater NBT reduction (84.09%) than its whole cells (70.06%). This showed that in both strains of *Lactobacillus* their DNA was more bioactive than whole cells. The comparison of LcS DNA and LA 343 DNA shows that the former was better than the later.

Inducible Nitric Oxide Synthase Activity

The pattern of iNOS activity in various groups was similar to those observed in NBT reduction. LcS DNA showed maximum iNOS activity (62.85%) than LcS culture. Similarly the LA343

DNA also showed greater iNOS activity (59.13%) than its whole cells (46.78%). The comparison of LcS DNA and LA 343 DNA showed that LcS DNA was better than LA 343 DNA.

Bactericidal Activity

Like other two parameters i.e NBT and iNOS, the bactericidal activity against *E.coli* also followed the same trend as seen in Figure 7. The effect of probiotic strains and their DNA on bactericidal activity was studied in terms of decrease in number of colony forming units (CFU) Of E.coli (1×10^6cells ml^{-1}) LcS DNA showed maximum bactericidal activity (75%).

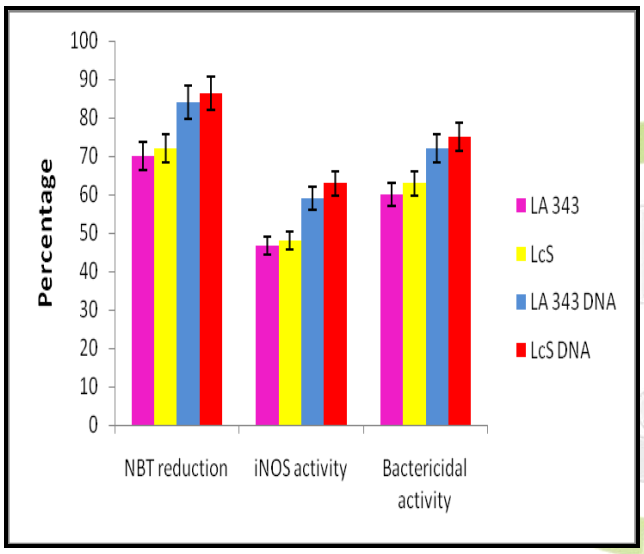

Figure 3: Comparative *in vitro* immunomodulatory evaluation of LA 343, LcS and their DNA

The above data is represented as Mean ± S.E.M (n=3).

The decreasing order of immunomodulatory activity is as follows:

LcS DNA (100 µg) > LA343 DNA (100 µg) > LcS (1×10^9cells/ml) > LA343 (1×10^9 cells/ml).

DISCUSSION

The study was conducted to find out the immunomodulatory potential of probiotic cells and their DNA in *in vitro* conditions. The DNA was isolated from two probiotic strains namely *Lactobacillus acidophilus* 343 and *Lactobacillus casei* strain Shirota (LcS, isolated from yakult). The experiment was conducted to find whether the genomic DNA of bacterial strains can be more efficient than its whole cell or it can be used as immunomodulator. This study was carried out by keeping in mind that whole cell probiotics may have ill effects in certain immunocompromised or extremely ill persons[14]. Moreover, the bacterial DNA has been shown to be more bioactive than the whole cells[8]. Previously, in our own lab, the results with other probiotics show that the whole cells and their isolated genomic DNA have immunomodulatory potential.

Our study corraborates the earlier finding that probiotic DNA is more efficient than its whole cell and employed as immunomodulators for complementary and alternative medicine[1,15]. In the present study, attempts were made to compare the immunomodulatory potential (*in vitro*) of a probiotic strain obtained from NDRI, Karnal (LA 343); its isolated DNA (LA DNA); as well as a commonly used drink commercial drink (Yakult**)** containing live bacteria (LcS) and the DNA of the isolated bacteria (LcS DNA). The results showed that the genomic DNA of LA343 was more efficient immunopotentiator than its whole cells and the genomic DNA of LcS was also showed much efficient bioactivity than its pure culture cells. But the immunomodulatory activity of these two probiotic strain LA343 and LcS was comparatively similar. The maximum immunomodulatory activity was shown by LcS DNA. Not only this, the immune enhancing activity of the probiotic DNA varies with the strain and species of the probiotics.

It is clear from the result that the probiotic DNA is good immunopotentiator. The proposed mechanism underlying the immune activity of DNA could be due to presence of unmethylated CpG motifs leading to the activation of T mediated B cells. Immunostimulatory CpG and non CpG Oligodeoxynucleotides (ODNs) have been identified from the genomic DNA of probiotics[16,17]. Similar to present results, it was reported that the foreign plasmid DNA can induce humoral and cell mediated immne response in mice after administration via

gastrointestinal tract[18]. Later on similar results showed that genomic DNA was more efficient immunomodulator than the whole cell from which it is isolated, in *in vivo* studies[1].

CONCLUSION

It is concluded that probiotic DNA is potent and safe immunopotentiator and can replace or act in synergism with standard drug available in the market. It can also be given in the form of vaccine adjuvant to enhance the effectiveness of vaccine and to induce immunity.

FUTURE RECOMMENDATION

The *in vivo* experiments on these lines, including the side effects of whole cells and DNA should be conducted.

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