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Original Article

Immunology Section

Correlation of the Probiotic Cell Number ml^{-1} and the Cell Mediated Immune Response: an *in vitro* Study

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ABSTRACT

Probiotics are live microorganisms which when administered in adequate amount confer a health benefit on the host.The beneficial effects of probiotics have been related to their survival number in the gut. Moreover, different strains differ with regard to their ability to colonize and proliferate in the GI tract due to which the efficiency of any therapeutic effect would also be expected to be strain dependant. It is expected that the minimum concentration of a probiotic microorganisms required demonstrating health promoting effects will also be dependent on many factors. Hence, this study was planned to evaluate the in vitro, cell mediated immune response of 6 probiotic strains by differing their cell number ml-1 viz. 1 x 10^6 cell ml^{-1} and 1 x 10^9 cell ml^{-1} by using pig splenocytes. Splenocytes which were incubated with the six strains separately at concentrations of 1 x 10^6 cell ml^{-1} and 1 x 10^9 cell ml^{-1} for 24 hours, were assayed to study the cell mediated immune response by employing the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test and by studying the bactericidal activity. The results demonstrated that a substantial increase in the stimulation of the cells occurred by the effect of all the probiotic strains at both the concentrations i.e., 1 x 10^6 cell ml^{-1} and 1 x 10^9 cell ml^{-1}.However, the stimulation of the splenocytes was invariably higher at the concentration of 1 x 10^9 cell ml^{-1}.The study suggests that higher number of cells should be employed during animal experimentation with probiotics to get observable effects.

Key Words: Cell mediated immune response, Lactobacillus, Bifidobacterium

INTRODUCTION

Probiotics are a live microbial food supplement which benefits the health consumers by maintaining or improving their intestinal microbial balance [1]. Probiotics such as *Lactobacilli* and *Bifidobacterium* are increasingly recognized as a means to prevent and /or treat intestinal disorders [2]. Other health benefits which are conferred by probiotics on the hosts include anticarcinogenic, antimutagenic, anti-infectious and immunomodulating activities [3, 4, 5].

The beneficial effects of probiotics are based on many mechanisms, out of which the most important ones are: inhibition of the intestinal pathogenic bacteria by the production of organic acids, reduction of the intestinal pH, production of bacteriocins [6], adherence to the intestinal surface and the subsequent colonization of the human gastrointestinal tract [7]. Moreover, probiotics exert their immunity enhancing effects by increasing both the non-specific (e.g. phagocyte function and natural killer cell activity) and the specific (e.g. Antibody production, cytokine production, lymphocyte proliferation and delayed type hypersensitivity) host immune responses [8].

Probiotic strains produce different amounts of metabolic products according to the temperature and the fermentation time, which illustrate the importance of controlling the parameters 9.However, it remains challenging to set up in vitro tests with a fair predictive value, that would allow us to narrow down the number of candidate strains which have to be tested in the animal models. There are varieties of parameters that may interfere in the systematic comparison of the strains, such as the bactericidal preparations which are used, like the viability, growth phase, dose and the timing of the administration [2].

The purpose of the current study was to compare the in vitro elicitation of the cell mediated immune response by different probiotic strains at concentrations of 1 x 10^6 cell ml^{-1} and 1 x 10^9 cell ml^{-1}, so that the further results of the *in vitro* studies can be applied to animal experimentation.

MATERIALS AND METHODS

2.1. The strains of microorganisms:

6 strains of probiotics, *Lactobacillus casei subsp. Casei 17* (LB 17), *Lactobacillus brevis 403 (LB 403), Lactobacillus delbrueckii* 405 (LB 405), *Bifidobacterium bifidium (BA3 233), Bifidobacterium bifidium (BD4 234) and Bifidobacterium bifidium (BD1 235)* were procured from the NDRI (National Dairy Research Institute), Karnal, Haryana. *The Lactobacillus spp.* were given two revival cycles in the de Man–Rogosa–Sharpe broth (MRS broth) at 37°C for 24 hours, whereas the *Bifidobacterium spp.* were given two revival cycles in the MRS broth at 37°C for 24 to 72 hours in anaerobic conditions.

Test Samples:

The above mentioned six strains i.e. LB 17, LB 403, LB 405, BA3 233, BD4 234 and BD1 235, 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 10ml PBS, counted and standardized as 1 x 10^6cells ml^{-1} and 1 x 10^9cells ml^{-1} for each strain.

2.2. Cell mediated immune response: 2.2.1. Total splenocyte

isolation from the 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 NH4Cl, 10mM KHCO3 and 0.1 mM disodium EDTA, pH 7.2). The splenocytes which were obtained were washed thrice in PBS, counted and adjusted for cell number (2 x 10^6cells/ml) in RPMI. They were incubated with the test strains for 24 hours and were thereafter assayed for their cell mediated immune response by employing the following tests: the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test, bactericidal activities.

2.2.2. The Nitroblue Tetrazolium Reduction assay:

The NBT reduction test was evaluated by employing the method which has been described [10]. Briefly, the splenocyte suspension was incubated with NBT and the formazon which was formed was extracted in dioxan. The reduction in the NBT was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as the blank. The results were expressed as mean ± S.E.M. of the percentage dye which was reduced to formazon.

2.2.3. Inducible Nitric Oxide Synthase activity:

The inducible nitric oxide synthase activity in the splenocyte suspension was evaluated by a procedure which was previously described [10] by using arginine. The colour which was developed (indicating the presence of citrulline) was measured spectrophotometrically at 540nm against RPMI and the Griess reagent as the blanks and the results were expressed as mean ± S.E.M. of the percentage enzyme which was produced.

2.2.4 Bactericidal activity:

The bactericidal activity was determined by the following procedure [11]. Briefly, 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 mean ± S.E.M. of the bactericidal activity.

2.3. Statistical Analysis:

All the results were expressed as mean ± S.E.M. The data of the tests were statistically analyzed by using one-way ANOVA, followed by Turkey's multiple range test, which were applied for the post hoc analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

RESULTS

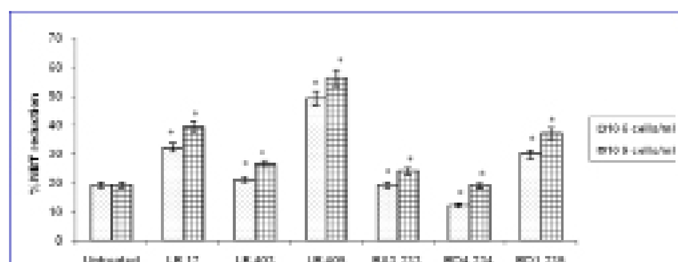
3.1. Cell mediated immune response:

3.1.1. NBT reduction:

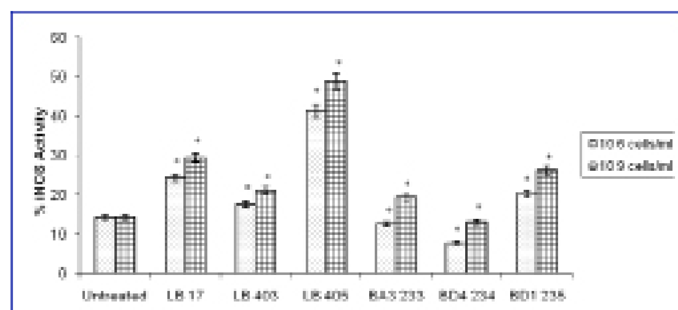
Strains of Lactobacilli and Bifidobacterium bifidium which had 1 x 10^6cells ml^{-1} and 1 x 10^9cells ml^{-1} significantly increased ($p< 0.001$) the NBT reduction as compared to the controls. However, the NBT reduction was more in the 1 x 10^9cells ml^{-1} as compared to that in the 1 x 10^6cells ml^{-1} in all the 6 strains (LB 17, LB 403, LB 405, BA3 233, BD4 234 and BD1 235). The maximum NBT reduction was seen in LB 405 at a concentration of 1 x 10^9cells ml^{-1} and it was 14.47 % higher than that which was seen at a cell concentration of 1 x 10^6cells ml^{-1} [Table/Fig-1].

3.1.2. iNOS activity:

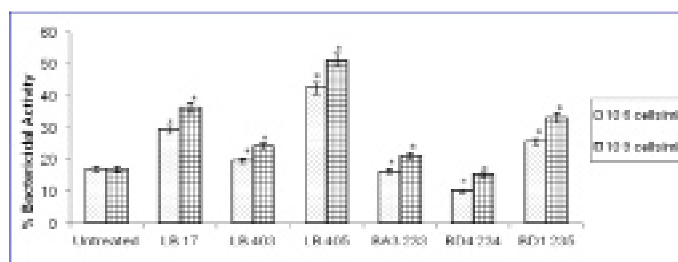
[Table/Fig-2] indicates the *Lactobacilli and Bifidobacterium bifidium* modulated cell mediated immune response. All the six strains (LB 17, LB 403, LB 405, BA3 233, BD4 234 and BD1 235) showed the maximum iNOS activity at a cell concentration of 1 x 10^9cells ml^{-1} as compared to that which was seen at a cell concentration of 1 x 10^6cells ml^{-1}. The maximum activity was observed in LB 405, followed by LB 17 with 1 x 10^9cells ml^{-1}, as compared to their respective 1 x 10^6cells ml^{-1}.



[Table/Fig-1]: Comparison of NBT reduction between 106 cells/ml and 109 cells/ml The results are presents as mean ± S.E.M
* p< 0.001 in comparison to untreated control



[Table/Fig-2]: Comparison of iNOS Activity between 106 cells/ml and 109 cells/ml The results are presents as mean ± S.E.M
* p< 0.001 in comparison to untreated control



[Table/Fig-3]: Comparison of Bactericidal Activity between 106 cells/ml and 109 cells/ml The results are presents as mean ± S.E.M * p< 0.001 in comparison to untreated control

3.1.3. Phagocytic activity:

The effect of the test materials on the bactericidal activity was studied in terms of the number of colony forming units (CFU). A concentration of 1 x 10^9cells ml^{-1} reduced the number of colonies and thus enhanced the bactericidal activity as compared to the concentration of 1 x 10^6cells ml^{-1}. As with the NBT and iNOS activities, LB 405 (1 x 10^9cells ml^{-1}) was found to have the maximum bactericidal activity [Table/Fig-3].

DISCUSSION

In the present study, the effect of the cell number ml^{-1} on the cell mediated immune response was evaluated *in vitro* by employing the Nitroblue Tetrazolium Reduction test and the Inducible Nitric Oxide Synthase test and by checking for the bactericidal activity. The results revealed that in all the tests, the bioactivity correlated directly with the number of cells, as it was higher with 1 x 10^9cells ml^{-1} in all the 6 strains than with 1 x 10^6cells

ml^{-1}. The possible mechanism for this could be that most of the host immunity which was produced by probiotics stimulated the immune cells, which in turn, produced cytokines such as IL-20 (p 70), IFN-c and TNF-a [8]. In addition to this, the cell walls and the cytoplasm of some *Bifidobacterium* strains stimulated murine lymphocyte proliferation and cytokine secretion [12,13].

As had been reported [14] earlier, the probiotic inclusion level had a significant effect on the broiler growth responses, the nutrient apparent digestibility coefficient (ADC) and the cecal micro-flora composition. However, the strains differed with regards to their ability to colonize and proliferate in the GI tract. The efficiency of any therapeutic effect depends on a minimum concentration of >1 x 106 CFU/ml or gram and a total of some 10^8 to 10^9 probiotic microorganisms should be consumed daily [15].

The NBT reduction test is an indirect marker for the oxygen dependent bactericidal activity of the phagocytes and the metabolic activity of granulocytes or monocytes [16,17]. The present results indicate that 1 x 10^9cells ml^{-1} were capable of stimulating the immune function of the macrophages, as was evidenced by an increase in the NBT reduction and the bactericidal activity with all the strains, as compared to 1 x 10^6cells ml^{-1}. The functional ability of the macrophages was evident from the increased expression of iNOS that oxidized L-arginine to citrulline and nitric oxide. The iNOS activity was correlated with the bactericidal activity of the macrophages and this was documented as a measure of the immunomodulatory potential [18].

CONCLUSION

It was concluded that a minimum concentration of >1 x 10^6 CFU ml-1 and that a total concentration of 1 x 10^9 CFU ml^{-1} probiotic microorganisms should be used *in vivo* if therapeutic effects are to be realized, so as to compensate for the reduction in the probiotic passage through the gut.

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