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Phytosynbiotics as an Enhancer of Immunological Activity: An *In Vitro* Study

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ABSTRACT

In the present time, there is an increase in the incidence of the infectious diseases and immunological disorders, which raises a need to discover some alternative that boosts host immune response. It is assumed that the synergistic effect of the essential bioactive phytochemical and synbiotics will be higher than using them alone as the health product. Moreover, Synbiotics and piperine are safe and can be delivered easily as such or through food. Piperine, FOS and probiotic were used for the *in vitro* immunomodulatory study to find whether these components are Immunomodulatory in nature and their comparison in combined form as phytosynbiotics. These components were screened for *In vitro* immunomodulatory potential employing Nitro blue Tetrazolium Reduction test (NBT), Inducible Nitric Oxide Synthase test iNOS), Bactericidal activity of macrophages (Phagocytosis). Results showed a significant difference in the immunomodulatory activities according to the different concentration of piperine, probiotic and prebiotic. Among all the tested concentrations, the optimum effective concentration of piperine was 30 µg/ml for NBT, iNOS, and Phagocytosis, whereas the activity of FOS was increased with increase in concentration, and Probiotic, showed maximum potential at 10^9 cells/ml. Results, demonstrate the immunostimulatory effect of individual components in a concentration-dependent manner and these components could be used as an immunomodulator for further studies in combination as phytosynbiotics. Further, the *in vitro* experiments demonstrated that PSBs show significantly higher immunomodulatory activity as compared to individual components, which indicates that PSB could be explored as an immunomodulatory and alternative therapeutic agent for the treatment of different diseases.

INTRODUCTION

Since ages, plants or their products have been employed in medicine and health benefits. The increasing awareness about side effects and cost of synthetic medicines makes the interest of consumer towards alternative therapeutic agents. These agents support health beyond providing nutrition and act as an alternative source of medicine. Over a period, the experimental and clinical work revealed that probiotics (the microbes with GRAS status) and many medicinal plants are safe immunomodulator [1]. Moreover, the immunomodulatory effects of probiotics can be enhanced by prebiotics and that of medicinal plants by using their effective bioactive components than the whole extracts. Various plants and their purified compounds including Quercetin, Rutin, Daidzein, Genistein, Ellagic acid and Betulinic acid have been studied for their immunomodulatory efficacy *in vitro* and *in vivo* [2].

Probiotics, which are defined as viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects and possess immunomodulatory functions. Studies reveal the treatment of infectious diseases including viral, bacterial or antibiotic-associated diarrhea and altered intestinal microbiota with probiotics. Many studies have shown that probiotics can stimulate the immune system, synthesize vitamins, decrease serum cholesterol [3], alleviate lactose intolerance, act as antibiotics, suppress tumors and protect against colon cancer, improve intestinal microflora, decrease the oxidative stress, immune modulatory and antidiabetic effects.

On the other hand, Prebiotics are non-digestible food ingredients that stimulate the growth and activity of bacteria in the digestive system. The growth and properties of probiotics are enhanced by prebiotics such as fructooligosaccharides (FOS), inulin, lactulose, and galactooligosaccharides (GOS) and enhance the gastrointestinal functions and immunity [4]. Health effects of prebiotics include lowering the incidence of clinical allergy, infections, preventing cancers, infections, allergies, inflammatory bowel diseases. Combining probiotics with prebiotics could improve the survival of the bacteria crossing the upper part of the GI tract, thus enhancing their effects in the large bowel.

Piperine is one of the major alkaloidal constituents of black pepper (*Piper nigrum*) and long pepper (*Piper longum*) family *Piperaceae.* Piperine has been shown to be antioxidant, anti-inflammatory [5], hepatoprotective [6], immunomodulatory and antitumor properties [7].

Synbiotic is the combination of prebiotic and probiotic components that are beneficial for health. Synbiotics show many benefits to health, which are improved by the prebiotics. Probiotics have been shown to be one of the alternative agents, which strengthen the immune response of the body. Previous studies in our lab showed that synbiotics are better immunomodulator than the probiotics or prebiotics separately. Therefore, we have undertaken this study to evaluate the immunomodulatory activity of different components individually and in combination as phytosynbiotics.

MATERIALS AND METHODS

1. Strain of microorganism

The strain of *Lactobacillus casei subsp. casei* 17 was procured from National Dairy Research Institute (NDRI), Karnal, Haryana. The culture so obtained was revived in the de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. The bacterial culture was grown and maintained on MRS broth for further use.

2. Lymphocyte isolation from the spleen

To isolate the lymphocyte, the teasing of the spleen was done aseptically in the MEM. Cells were centrifuged at 400 × g for 10 min at 4°C. The lysis of the cells was done by ACK lysis solution (0.5M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.2). Lymphocytes obtained were washed thrice in PBS, counted and adjusted to the desired concentration in MEM for further use.

3. Immunomodulatory activity

3.1Nitroblue Tetrazolium Reduction assay [8]

NBT test was employed to measure the macrophage function. This assay is based on the reduction that the addition of a yellow colored NBT dye to splenocyte suspension results in the formation of the colored complex, which can be phagocytosed by macrophages. The yellow colored NBT is reduced to blue colored formazone and this can be measured spectra photometrically at 520 nm using dioxane as blank. The results were expressed as the mean ± S.E.M. of percentage dye reduced to formazon.

3.2Inducible Nitric Oxide Synthase activity [9]

Inducible nitric oxide synthase activity in splenocytes suspension was evaluated by using arginine. The color developed (indicating presence of citrulline) was measured spectrophotometrically at 540nm against MEM and Griess reagent as blank and the results were expressed as mean ± S.E.M. of percentage enzyme produced.

3.3 Phagocytic activity[10]

Phagocytic activity is one of the important parameters to measure the phagocytosis of splenocytes. The bacterial culture was incubated at 37°C for 24 hrs. Centrifuged the bacterial suspension and pellet was taken. The pellet was washed trice with KRP buffer, centrifuged and the supernatant was discarded. Took splenocytes and bacterial suspension and incubated at 37°C for 60 mins. The bacterial suspension was spread on the agar plate and incubated at 37°C for 24 hrs. The number of colony forming units (CFU) developed in control, test plates was counted, and results were expressed as mean ± S.E.M. of bactericidal activity.

RESULTS

3. IMMUNOMODULATORY ACTIVITY OF PIPERINE

In the cell-mediated immune response, it was seen that piperine showed maximum activity at the concentration of 30µg/ml in NBT as compared to other concentrations and control. In iNOS piperine had also showed maximum activity at 30µg/ml as compared to other concentrations and in phagocytosis the maximum no. of colonies were also reduced by the piperine at the conc of 30 µg/ml which was significantly higher as comparison to 10µg, 20µg, 40µg, 50µg, 70µg and 100µg/ml, control and levamisole.

Table 1: Immunomodulatory activity of piperine at different concentrations.

Concentration (ug/ml)	% reduction of NBT	% phagocytic activity	% I NOS Activity
Control	12± 1.07	15.24± 1.01	9± 1.11
10	61.90± 1.28	61.09± 1.2	55.43± 1.66
20	80.35± 2.1	75.33± 2.21	59.3±2.11
30	85.71±3.03	88.41±3.01	68.29±2.33
40	81.66± 1.05	68.83± 1.81	64.65± 1.41
50	48.1± 1.31	62.66± 0.59	59.8± 1.2
70	54.16± 1.01	50.81±1.06	56.38± 1.99
100	27.27±1.1	60.14± 1.23	58.84± 0.59
Levamisole	76.22±1.4	77.01±0.33	65.11±2.28

Data is represented as Mean ± S.E.M (n=6). p< 0.05 as compared to Normal Control.

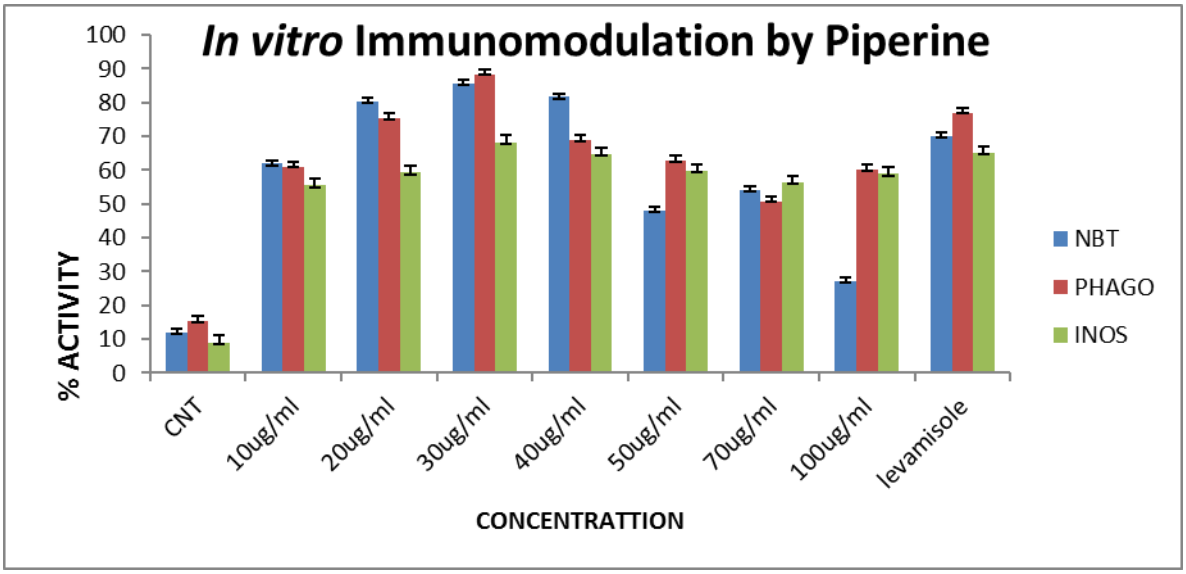

Fig: 1 *In vitro* immunomodulation by piperine at different concentrations. represented as Mean ± S.E.M (n=6). p< 0.05 as compared to Normal Control.

As showed in Table :1 and Fig: 1, the NBT reduction activity is 73.71% more in 30µg/ ml group as compared to the control group. The % phagocytic activity in 30µg/ml group is 73.17 % more than the control group.The % iNOS Activity was 59.29 % more in 30 µg/ml as compared to the control group.

3. IMMUNOMODULATORY ACTIVITY OF PROBIOTIC (*L.CASEI* 17)

3.1 NBT reduction:

NBT reduction was more in the 1 x 10 ^{9} cells ml-1 as compared to that in the 1 x 10^{6} , 1 x 10^{7} , 1 x 10^{8} , 1 x 10^{12} cells ml-1 in LB 17. The maximum NBT reduction was seen at a concentration of 1 x 10 ^{9} cells ml-1 and it was 14.47 % higher than that which was seen at a cell concentration of 1 x 10^{6} cells ml-1.

3.2 iNOS activity:

The iNOS activity at a cell concentration of 1 x 10^{9}cells/ ml was maximum as compared to cell concentration 1 x 10^{6}, 1 x 10^{7}, 1 x 10^{8}, 1 x 10^{12} cells ml-1.

3.3 Phagocytic activity:

The effect of the test materials on the bactericidal activity was studied in terms of the number of colonies forming units (CFU). A concentration of 1 x 10^{9}cells ml^{-1} reduced the number of colonies and thus enhanced the bactericidal activity as compared to the other concentration.

Table 2: Immunomodulatory activity of probiotic (*L.casei* 17)

Concentration (cells/ml)	% NBT REDUCTION	% INOS Activity	% Phagocytic activity
Control	12	14	15
10^{6}	33.18±1.04	25.61±1.28	26.31±1.11
10^{7}	35.1±1.09	27.65±1.28	30.32±1.21
10^{8}	37.16±1.30	29.41±1.32	35.1±1.32
10^{9}	40.1±1.11	30.11±1.44	36.2±0.28
10^{12}	38.18±1.04	28.61±1.28	27.31±0.27

Data are Mean value ± S.D (n=3)

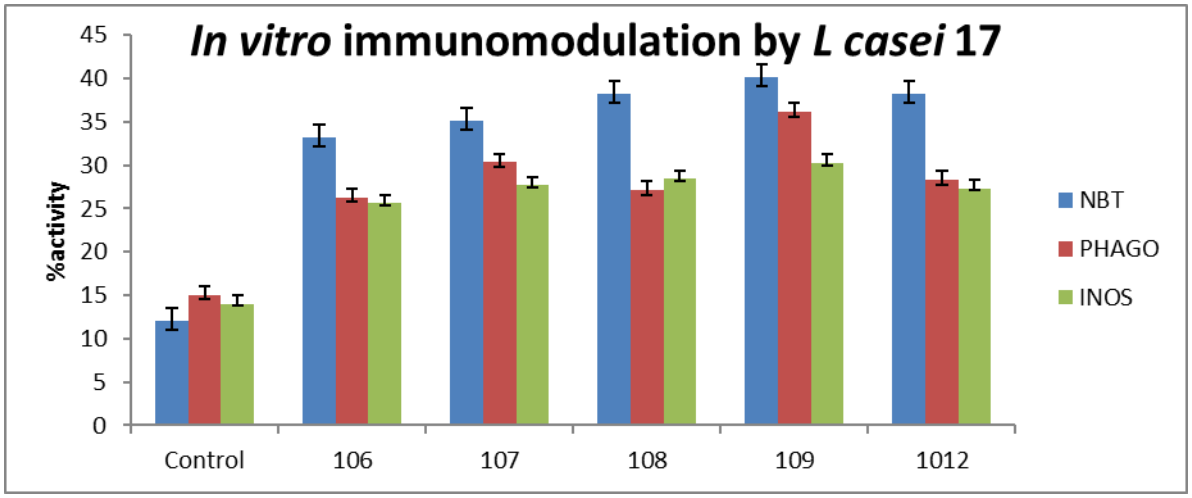

Fig 2: *In vitro* immunomodulation by *L.casei* 17. Data is represented as Mean ± S.E.M (n=6). p< 0.05 as compared to Normal Control.

Like, NBT and iNOS activities, LB 17(1 x 10^{9}cells ml-1) was found to have the maximum bactericidal activity as showed in the Table 2 and Fig 2.

IMMUNOMODULATORY ACTIVITY OF PREBIOTIC (FOS)

In the cell-mediated immune response, it was seen that FOS showed maximum activity at the concentration of 1000ug/ml in NBT as compared to other concentrations 100ug/ml, 250 ug/ml, 500ug/ml, and control.

Table 3: Immunomodulation by prebiotic (FOS)

Concentration ug/ml	% NBT REDUCTION	% iNOS activity	% Phagocytic activity
Control	12	9	15
100	14±1.2	11±1.04	16±1.22
250	19±1.02	14±1.44	22±1.05
500	24±1.30	16±1.03	24±1.11
750	25±2.01	17±1.33	26±1.29
1000	27±1.05	19±1.5	28±1.04

Data is represented as Mean ± S.D (n=6).

In iNOS FOS had also shown maximum activity at 1000ug/ ml as compared to other concentrations and in phagocytosis, the maximum no. of colonies were also reduced by the FOS at the conc of 1000 ug/ml which was significantly higher as compared to other concentrations and control as showed in Table: 3 and Fig: 3.

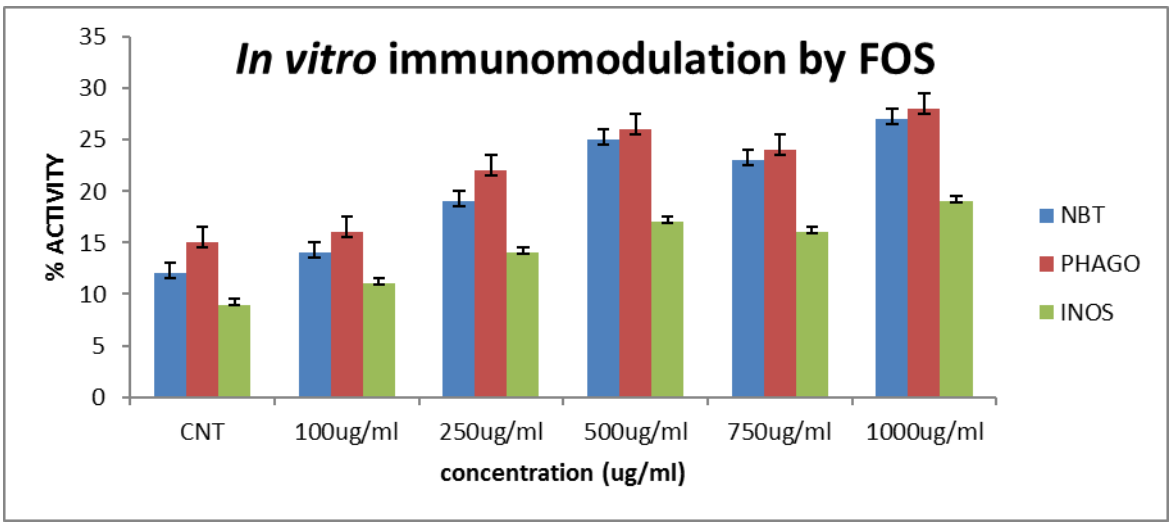

Fig: 3 In vitro immunomodulation by FOS at different concentrations.

IN VITRO IMMUNOMODULATION BY PHYTOSYNBIOTICS

In the immunomodulatory study, when phytosynbiotics at the concentration of 30ug/ml piperine, 1000mg/ml FOS and 10 ^{9} cells of probiotic were tested. The results showed an increase in NBT, i NOS and phagocytosis percentage as compared to the percentage of individual components.

Table: 4 Immunomodulation by phytosynbiotics.

Groups	NBT reduction (Mean%)	iNOS activity (Mean%)	Phagocytic activity (Mean%)
Control	12.51 ± 0.49	10.70 ± 0.38	15.60 ± 0.97
Levamisole	76.57 ± 0.70	65.13 ± 0.27	75.35 ± 0.61
PSBs	95.19 ± 0.89	90.97 ± 0.63	92.58 ± 0.35

Data are Mean value ± S.D (n=3).

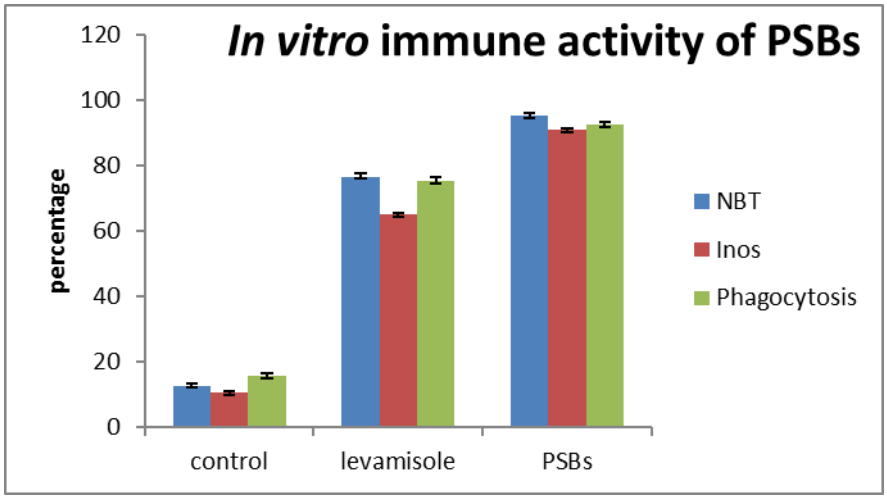

Fig 4: Immunomodulation by PSBs

The *in vitro* combination study showed in Table: 4 and Fig: 4 indicated that the PSB has more potential to inhance the immune response as compared to individual components (prebiotic, probiotic and phytochemical).

DISCUSSION

In the present study, the effect of the piperine, probiotic and prebiotic 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 compounds used are immunomodulatory in nature. Piperine is a phytochemical from *Piper longum* has the traditional uses include analgesic, antipyretic, CNS depressant, anti-inflammatory, antioxidant, anticonvulsant, anti-bacterial, anti-tumor and hepatoprotective activities[11]. According to literature, piperine has many health effects, but no study is available about the immunomodulation by piperine by NBT, iNOS and phagocytosis method. Synbiotics have the ability to affect innate, humoral and cellular immunity [12]. The present results indicate that phytochemical, probiotic and prebiotic are capable of stimulating the immune function of macrophages, as evidenced by an increase in NBT reduction and in the bactericidal activity in all the treated groups. The functional ability of the macrophages was evident from the increased expression of iNOS that oxidizes L-arginine to citrulline and nitric oxide. The iNOS activity is correlated to the bactericidal activity of the macrophages and has been documented as a measure of the immunomodulatory potential [6]. 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. The functional ability of the macrophages was evident from the increased expression of iNOS that oxidized L-arginine to citrulline and nitric oxide. The piperine had shown the maximum immunomodulatory potential at 30ug/ml concentration, FOS had immunomodulation at 1000ug/ ml and probiotic had maximum immunomodulation at the concentration of 10^9 cells when used individually. When we tested the immunomodulatory activity of these components in combination as phytosynbiotic, results were much better than individual components, indicated that Immunomodulatory activity by phytosynbiotics is higher than the prebiotic, probiotic and piperine. Synbiotics improve immune function and stimulation of appropriate immunomodulatory cells. As the Prebiotics fermented by the probiotic bacteria and other bacteria that reside in the colon, butyrate and other SCFAs are formed resulting in butyrate influence histone deacetylation which is responsible for decrease the proinflammatory cytokine secretion[13]. Synbiotics, because of their prebiotic content, may raise the levels of calcium and magnesium in the colon and may enhance the growth of *Bifidobacteria* and *Lactobacilli* in the large intestine[14]. Synbiotics stimulate the host immune response by increasing phagocytic activity; the synthesis of IgA and activation of T and B lymphocytes; alteration of physicochemical conditions of the colon with decreasing pH. the overall result suggests that piperine enhanced humoral immunity through the differentiation of B cells rather than proliferation, and its use in traditional medicine for the prevention of infection and enhancement of immunity is justified. However, further investigations are recommended.

Many healthy phytochemicals occur in food in the form of esters, glycoconjugates, or polymers, which are not directly bioavailable. Probiotic lactobacilli and bifidobacteria, which have evolved within the colonic ecosystem where indigestible oligo- and polysaccharides are their sole carbon sources, bear several glycosyl-hydrolases and can contribute to release the a glycones from glycoconjugated phytochemicals[15]. Hence, all the cell mediated tests showed that PSB is better immunopotentiator than prebiotic, probiotic and phytochemical alone.

CONCLUSION

Results showed that Piperine, FOS and *L casei* 17 are immunomodulatory in nature. Based upon *in vitro* study, Probiotic- *Lactobacillus casei* at conc 10^9, Prebiotic (FOS) at conc

1000ug/ml and Phytochemical (Piperine) at conc 30ug/ml was the best immunomodulatory and hence this conc is selected for further studies. Individually these compounds are immunomodulatory in nature. So further study is conducted on the combinatorial form of the prebiotic, probiotic and phytochemical as phytosynbiotics. The prebiotic is well known for enhancing the activity of probiotics. It was Hypothesized that the synergistic effect of the essential bioactive phytochemical and synbiotics will be higher than using them alone as a therapeutic agent. So the results revealed that phytosynbiotics (phytochemical + synbiotics) are better as compared to individual component. The present study makes us to conclude that PSB enhances immune efficacy and can be applied as immunotherapeutic agent to manage the immune related disorders.

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