Coencapsulation of Synbiotics as enhancer of Immunological activity: An \textit{in vivo} study

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ABSTRACT

Synbiotics are nutritional supplements that are combinations of probiotic bacteria and prebiotic food ingredients. The manipulation of composition of the gut microbiota in swiss albino mice through dietary supplementation is possible by the synbiotics. Probiotic products (dietary supplements containing potentially beneficial bacteria) and prebiotics (mainly lactulose) make up an important part of maintaining intestinal health. The probiotics use the prebiotics as a food source, which enables them to survive for a longer period of time inside the digestive system. In the present study synbiotics were encapsulated in the sodium alginate beads to improve their viability in the gut and were tested for their immunomodulator activity in mice. Synbiotics were administered in encapsulated and unencapsulated form in swiss albino mice. The animals were sacrificed after feeding them on the diet for 17 days, then immune status of the treated swiss albino mice was assessed by employing the tests for Humoral Immune Response and Cell Mediated immune response i.e Delayed Type Hypersensitivity, Nitroblue Reduction test, Inducible Nitric Oxide Synthase, Bactericidal activity and antibody titer. The results showed that the encapsulated form of synbiotics are a better immune enhancer than unencapsulated form and hence may be employed for improved bioactivity of probiotics.

Keywords: \textit{Lactobacillus casei} 17, Coencapsulation, Immunomodulatory activity, Humoral Immune Response, Cell Mediated Immune Response.

INTRODUCTION

Synbiotics show many benefits to health, which are improved by the prebiotics (non digestible food ingredients), that beneficially effect the host by increasing the bioactivity of the probiotics[1]. One of the important activity of probiotics is the immunomodulation. In the present time there is increase in the incidence of the infectious diseases and immunological disorders which raises a need to discover some alternative that boost host immune response[2]. 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 immunomodulators than the probiotics or prebiotics given separately. Probiotics in the gut are exposed to harsh environmental conditions which may affect their survival. Hence the present study has conducted to evaluate the improvement in immunological activity of the synbiotics by coencapsulation in sodium alginate beads in vivo. The probiotics employed was \textit{Lactobacillus casei} 17 and prebiotic was Lactulose.
MATERIALS AND METHODS

Strains of microorganisms
Strains 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. A concentration of $10^9$ cells/ml of *Lactobacillus casei* 17 was used in the experiment.

Animals
Swiss albino male mice (18-22 g) maintained on a standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water *ad libitum* were employed in the study. The animals were divided into 5 groups in the departmental animal house and were exposed to 12 hr cycles of light and darkness.

Experimental animal design: Animals were divided into five major groups:
- **Group I**: Control group (not subjected to any treatment i.e. kept only on diet)
- **Group II**: Positive control (mice subjected to immune enhancer i.e Levamisole (2.5 mg kg⁻¹) with normal diet)
- **Group III**: Antigen sensitized control (mice sensitized with Sheep Red Blood Cells (SRBC) and kept on normal diet).
- **Group IV**: Mice dosed with co-encapsulated synbiotics (2:2 ratio of prebiotic (lactulose) and probiotic at the rate of $10^8$ cells/day/mouse)
- **Group V**: Mice dosed with unencapsulated synbiotics (2:2 ratio of prebiotic (lactulose) and probiotic at the rate of $10^8$ cells/day/mouse)

Immunization
Sheep blood was collected in Alsever’s solution from the subjuglar vein in the ratio 1:2 and the solution was centrifuged at 400 x g for 10 min at 4 °C [3]. The erythrocyte pellet was obtained. The pellet was washed and suspended in PBS (0.1 M, pH 7.2). The saline suspension of the washed SRBC was prepared to approximately $10^7$ cells/ml. The intraperitoneal injection with a single dose (100µl/ml of $1 \times 10^7$ cells/ml) of sheep red blood cells (SRBC) was given to the animals to make them antigenically challenged.

Humoral Immune Response
The blood was withdrawn from retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8th and 13th (post immunization) to assess the humoral immune response. The serum was separated and assayed by direct hemagglutination [4]. The hemagglutination was performed by using the microtiter plate. Titer was described as highest dilution capable of visible agglutination. The results were expressed as mean ± S.E.M. log titer of individual animals.

Cell mediated immune response
Delayed Type Hypersensitivity assay
The SRBC specific DTH reactions were elicited in the mice by injecting all SRBC primed groups intradermally on day 15th with SRBC suspension ($1 \times 10^7 /100$µl saline-1) in the right hind footpad and equal volume of saline was given in the left hind footpad as control. Paw thickness was measured with vernier-caliper at 24h interval up to 72h. The difference in paw thickness was compared with control. It was taken as a measure of DTH and expressed in millimeter[5]. Results are expressed as mean ± S.E.M. of footpad thickness up to 72h.

Total lymphocyte isolation from the spleen
To isolate the lymphocyte, the teasing of the spleen was done aseptically in the MEM. Cells were centrifuged (400 x g for 10 min at 4 °C). The lyses of the cells was done by ACK lyses solution (0.5M NH₄Cl, 10mM KHCO₃ and 0.1 mM disodium EDTA, pH 7.2). Lymphocytes obtained were washed thrice in PBS, counted and adjusted to desired concentration in MEM for further use[6].

Nitroblue Tetrazolium Reduction assay
NBT test was employed to measure the macrophage function. This assay is based on the reduction that the addition of yellow coloured NBT dye to splenocyte suspension results in the formation of coloured complex which can be phagocytozed by macrophages[6]. The yellow coloured NBT is reduced to blue coloured formazone and this can be
measured spectro photometrically at 520 nm using dioxane as blank[6]. The results were expressed as mean ±
S.E.M. of percentage dye reduced to formazon.

**Inducible Nitric Oxide Synthase activity**

Inducible nitric oxide synthase activity in splenocytes suspension was evaluated by using arginine as described by
procedure [6]. The color developed (indicating presence of citrulline) was measured spectrophotometrically at
540 nm against MEM and Griess reagent as blank and the results were expressed as mean ± S.E.M. of percentage
enzyme produced.

**Bactericidal activity**

Bactericidal activity is one of the important parameter to measure the phagocytosis of splenocytes as described by
procedure [6]. The bacterial culture was incubated at 37 ºC for 24 hrs. Centrifuged the bacterial suspension and
pellet was taken. The pellet was washed trice with KRPM buffer, centrifuged and supernatant was discarded. Took
splencytes and bacterial suspension and incubated at 37 ºC for 60 minutes. Bacterial suspension was spread on the
agar plate and incubated at 37º C for 24 hrs. Number of colony forming units (CFU) developed in control and test
plates were counted and results were expressed as mean ± S.E.M. of bactericidal activity.

**RESULTS**

**Humoral Immune Response:**

In all the groups i.e. Control (normal diet), levamisole treated, antigen challenged group with sheep RBCS, test gp fed
on encapsulated synbiotics, test group fed on unencapsulated synbiotics, no anti SRBC antibody titer was observed
on day 0. Levamisol treated group had a significantly higher antibody titer as compared to encapsulated and
unencapsulated synbiotics treated group. The encapsulated synbiotics treated group had more antibody titer than
unencapsulated synbiotics treated group.

*Table 1: Antibody titer of different groups of swiss albino mice*

<table>
<thead>
<tr>
<th>Group</th>
<th>0th DAY</th>
<th>3rd DAY</th>
<th>12th DAY</th>
<th>No. of times ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1:2</td>
<td>1:16</td>
<td>1:64</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>1:2</td>
<td>1:56</td>
<td>1:1024</td>
<td>16</td>
</tr>
<tr>
<td>SRBC treated</td>
<td>1:2</td>
<td>1:32</td>
<td>1:128</td>
<td>2</td>
</tr>
<tr>
<td>Unencapsulated</td>
<td>1:2</td>
<td>1:64</td>
<td>1:128</td>
<td>2</td>
</tr>
<tr>
<td>Encapsulated</td>
<td>1:2</td>
<td>1:128</td>
<td>1:256</td>
<td>4</td>
</tr>
</tbody>
</table>

*↑ increase in antibody titer as compared to control.

**Cell mediated immune response:**

**Delayed type hypersensitivity response**

Effect of synbiotics on T-cell response was studied by assessing the footpad swelling as a measure of Delayed type
hypersensitivity. In untreated control group, no rise in footpad thickness was observed. However, encapsulated
synbiotics treated groups showed significant elicitation of the T-cells response as evident by an increase in foot pad
thickness as compared to antigen sensitized group, levamisole treated group and unencapsulated synbiotics treated
group. It was found that encapsulated synbiotics treated group showed 1.16% more, levamisole treated group
showed 1.11% and unencapsulated synbiotics treated group showed 1.09% more rise in footpad thickness as
compared to control group after 72 hours (Table 2).

*Table 2: Delayed type hypersensitivity response*

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Foot pad thickness (nm)</th>
<th>Time periods (h) after SRBC challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>24hr</td>
</tr>
<tr>
<td>Control</td>
<td>1.67±0.2</td>
<td>1.67±0.2</td>
</tr>
<tr>
<td>Levamisole</td>
<td>1.88±0.162</td>
<td>1.94±0.2</td>
</tr>
<tr>
<td>SRBC treated</td>
<td>1.68±0.12</td>
<td>1.71±0.12</td>
</tr>
<tr>
<td>Unencapsulated</td>
<td>1.85±0.18</td>
<td>1.89±1.2</td>
</tr>
<tr>
<td>Encapsulated</td>
<td>1.96±0.12</td>
<td>2.43±0.2</td>
</tr>
</tbody>
</table>

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iNOS activity
In the cell mediated immune response, it was seen that encapsulated synbiotics treated group showed maximum activity which was significantly higher in comparison to control, unencapsulated synbiotics treated, levamisole treated and SRBC sensitized group. In encapsulated synbiotics treated group, iNOS activity was 7% higher than unencapsulated synbiotics treated group. Similarly encapsulated synbiotics treated group showed 6% higher iNOS activity than levameisole treated groups.

Table 3: iNOS activity of encapsulate, unencapsulated, levamisole, SRBC treated groups in Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>iNOS (% activity)</th>
<th>↑ % in activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.14</td>
<td>0</td>
</tr>
<tr>
<td>Levamisole</td>
<td>50.13</td>
<td>17.99</td>
</tr>
<tr>
<td>SRBC treated</td>
<td>35.1</td>
<td>2.96</td>
</tr>
<tr>
<td>Unencapsulated</td>
<td>49.26</td>
<td>17.12</td>
</tr>
<tr>
<td>Encapsulated</td>
<td>56.83</td>
<td>24.69</td>
</tr>
</tbody>
</table>

* increase in iNOS activity as compared to control.

NBT reduction
Encapsulated synbiotic treated group and levamisole treated group had significantly increased NBT reduction as compared to SRBC immunized and control group.

Table 4: % of NBT Reduction in encapsulated, unencapsulated, levamisole, SRBC treated groups in Swiss albino mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% reduction of NBT</th>
<th>↑ % in activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.6</td>
<td>0</td>
</tr>
<tr>
<td>Levamisole</td>
<td>62.3</td>
<td>26</td>
</tr>
<tr>
<td>SRBC treated</td>
<td>42</td>
<td>5.4</td>
</tr>
<tr>
<td>Unencapsulated</td>
<td>52</td>
<td>15.4</td>
</tr>
<tr>
<td>Encapsulated</td>
<td>63.1</td>
<td>26.5</td>
</tr>
</tbody>
</table>

* increase in NBT reduction activity as compared to control.

The NBT reduction activity is 11.1% more in the encapsulated synbiotics treated group as compared to unencapsulated synbiotics treated group.

Bactericidal activity
The effect of synbiotics on bactericidal activity was studied in terms of number of colony forming units (CFU). The no. of colonies were reduced in the levamisole treated and encapsulated synbiotics treated groups, thus enhanced the bactericidal activity as compared to control, unencapsulated and SRBC immunized groups.

Table 5: % phagocytic activity in different groups of swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Phagocytic activity</th>
<th>↑ % in activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.4±2.2</td>
<td>0</td>
</tr>
<tr>
<td>Levamisole</td>
<td>57.01±0.33</td>
<td>21.61</td>
</tr>
<tr>
<td>SRBC treated</td>
<td>51.2±11.2</td>
<td>15.8</td>
</tr>
<tr>
<td>Unencapsulated</td>
<td>51.26±1.31</td>
<td>15.86</td>
</tr>
<tr>
<td>Encapsulated</td>
<td>60.12±2.2</td>
<td>24.7</td>
</tr>
</tbody>
</table>

* increase in % phagocytic activity as compared to control.

The phagocytic activity is 9% more in encapsulated synbiotics treated group as compared to unencapsulated synbiotics treated group, where in Levamisole treated group it was 6% more than the unencapsulated synbiotics treated group.
The iNOS, NBT reduction and bactericidal activity shows that encapsulated synbiotics shows more activity than other forms.

DISCUSSION

In the present project, the effect of Coencapsulated Synbiotics was studied on the immune response. The results revealed that the synbiotics enhance the immune response as compared to the standard drug treatment. Levamisole, the active levo-form of tetramisole, is used as an anthelmintic in the treatment of many nematodes particularly in veterinary applications. It is also an immunomodulator as an adjunct with fluorouracil to make it work better against cancer of the colon following surgical resection of the primary tumor[7]. It is also a stimulant of B cells, T cells monocytes and macrophages. Levamisole was used as positive control while studying the effect of synbiotics on the immune response.

The immune response was evaluated employing various parameters to assess the function of various immunocytes viz; B cells in the form of anti SRBC antibody development and functions of the macrophages by their capacity to reduce NBT, iNO synthase expression and phagocytic activity[8]. The CMI study was assessed by employing DTH by foot pad swelling method. The maximum antibody titer was observed in the group treated with encapsulated synbiotics as compared to other groups. The DTH, NBT, iNOS and bactericidal activities were also maximum in the encapsulated synbiotic treated group as compared to control, unencapsulated synbiotics treated, levamisole treated, and SRBC immunized groups.

The present results indicate that synbiotics 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[8]. The iNOS activity is correlated to the bactericidal activity of the macrophages and has been documented as a measure of the immunomodulatory potential[9]. Our results concluded the findings of Uma Rani, it was observed that immunomodulation by synbiotics were more effective[9]. The coencapsulated synbiotics containing both the prebiotics and probiotics show more beneficial health effects than the prebiotics and probiotics individually[10][11]. The coencapsulated synbiotics are more effective because the coencapsulation protect the probiotics from the harsh environmental conditions and prebiotics provides the nutrition[12].

CONCLUSION

It is concluded that encapsulated form of synbiotics is a better immune enhancer than unencapsulated form and should be exploited for therapeutic potential in treatment of variety of diseases including infection, allergy effect and...
modulating immune responses. Encapsulated synbiotics is more effective for cell mediated immune response than the humoral immune response but the synbiotics had the ability to affect both cell mediated and humoral immunity.

REFERENCES