Preparation, characterization and hypocholesterolemic effect of sodium alginate encapsulated lab isolate

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ABSTRACT

Probiotics are live friendly bacteria which help in lowering cholesterol, reducing diabetes and have many other health improving functions which may be in part due to its CLA conversion potential. Out of a large number of probiotics studied Lactobacillus or Bifidobacteria genera are the most exploited ones. The present study was designed to study the hypocholesterolemic properties of CLA producing LAB isolate in unencapsulated and encapsulated form. Result and conclusion: In Vivo experiment showed that the percentage decrease in cholesterol level by probiotic bacteria is almost parallel to that of percentage decrease in cholesterol level of drug treated mice. However there was hardly any difference in cholesterol reduction by encapsulation in this 20 days experiment.

Keywords: CLA, Probiotic, hypocholesterolemic, Encapsulation, Cholesterol.

INTRODUCTION

One of the factors which may be responsible for health benefits by probiotics is conversion of Linoleic acid (LA) to CLA. CLA is Conjugated Linoleic Acid which has shown to have anticancerous [1] and anticholesterolemic action [2].

Elevated levels of blood and dietary cholesterol are considered to be a major risk factor for coronary heart diseases, and also a factor inducing colon cancer in addition to high dietary fat and low fibre [3], respectively. Reduction of serum cholesterol can lower the incidence of coronary thrombosis in hypercholesterolemic individuals [4]. Several investigators have suggested that certain lactic acid bacteria including strains of L. acidophilus are capable of reducing cholesterol [5-14]. On the other hand survival of bacteria in the intestine is affected by the gastric juices etc. Hence encapsulation of these bacteria can prolong their survival in the intestine and in turn the in vivo bioactivity of the bacteria. Hardly any literature is available where in vivo bioactivity has been checked of encapsulated bacteria. In the present study a CLA producing LAB isolate has been tested for its anti hypocholesterolemic activity in vivo in mice in the unencapsulated and encapsulated form and compared with that of a drug.

MATERIALS AND METHODS

Microorganism: Lactobacillus (LAB) isolated from healthy buffalo milk in de Man Rogosa Sharpe broth (MRS broth) at 37 °C containing polyoxyethylenecholesterol sebacate. The bacterial culture was grown and maintained on MRS broth for further use. A concentration of 10⁹ cells/ ml of LAB were used in the experiment.[15]

Growth pattern of LAB: Starter culture was grown in MRS Broth (pH 6.5±0.2 for 24hr) at 37 °C. Autoclaved MRS Broth pH 6.5±0.2 was taken and was inoculated 1% v/v culture and incubated at 37 °C along with control (Uninoculated MRS Broth). 2ml sample was taken out after every 3hr and absorbance at 600nm was measured.
CLA production and estimation: MRS broth with 1% Tween 80 and 0.1% cottonseed oil was autoclaved for CLA production. Production media was inoculated with 5% LAB inoculum and kept at 37 °C for 24 hr. 1 ml of culture media after 12hr and 24hr was taken in a micro centrifuge tube and was centrifuged at 5000 rpm for 5 min. Supernatant was collected in a test tube. To the supernatant 2ml of isopropanol was added and was then vortexed for 3 min. It was allowed to stand for 1min and then 1.5ml of hexane was added into it. It was vortexed for 1 min and was allowed to stand for 1 min. The upper layer was collected and O.D was taken at 233nm using hexane as blank. The CLA production profile was determined by plotting O.D233 VS Time interval [16].

Experimental Animals: Female and male mice aged 6-8 weeks were purchased from Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. All experiments were employed in accordance with Institutional Ethics Committee (IEC, ICMR). Animals were kept under normal room conditions and temperature maintained at 35 °C.

Preparation of Sodium alginate beads: Sodium alginate solutions at concentration of 2.5%, 3.0%, 3.5%, 4.0% were used and calcium chloride solution (0.5 M) were autoclaved at 121°C for 15 minutes. Sodium alginate was filled in syringe. Beads were formed by dropwise addition of sodium alginate into gently stirred chilled CaCl₂ solution with the help of magnetic stirrer. After membrane formation beads were removed, washed with distilled water and transferred into hardening solution CaCl₂ (100mM) at 4°C[17].

Characterization of beads:
Physical property of beads such as its diameter was noted with the help of vernier calliper, and also weight of beads were noted based on wet basis and dry basis [18].

Diffusion properties of beads were also checked. For diffusion experiments, 2 ml of 4 % Coomassie blue dye was added in sodium alginate solution and beads were made. The so far made beads were washed with distilled water and transferred into hardening solution (CaCl₂ 100 Mm) for 15 minutes and were incubated in 50 ml of distilled water for half an hour. Samples of supernatant were taken periodically to analyse the released dye measuring its absorbance at 600 nm. A standard solution of dye was prepared. O.D. was taken at 600 nm and % dye release was calculated by employing the formula given below [19].

% dye diffusion = (O.D. of sample)/(O.D. of standard)  x 100

Determination of entrapment efficiency of viable cells: Bacterial cultures were mixed at concentration of 2% with 4% sodium alginate. Beads were formed by the method described earlier [16]. The encapsulated beads were tested for entrapment efficiency by the method of Sandrine et. al [19]. Four beads were taken and were added into 6 ml MRS broth. It was incubated for 3.5 hrs (diffusion time of beads). Serial dilution was done from 10⁻¹ to 10⁻⁸ .10 µl of the each dilution was then spreaded on the petri plates (labelled 10⁻¹ to 10⁻⁸ ) containing MRS. Then it was incubated for 24 hrs at 37 °C. The viable number of cells in the solution was determined by using plate count method [20-21].

Evaluation of cholesterol reduction activity (In Vivo): Cholesterolemia was induced in mice by feeding them with 1 % cholesterol which was mixed in the normal diet. A total dose of 200mg/kg body wt. was given over a period of 7 days. After seven days, the animals showing serum cholesterol levels above 120 mg/dl were selected for further experiments. The day on which the microbial diet was started, was considered as day 0.

Groups of Animals:
Animals were divided into five groups:
• Group 1 (Control) i.e. animals were given normal diet and kept as control.
• Group 2 (Positive control) i.e. hypecholesterolemic mice
• Group 3 (Drug treated) Atorvastatin was given at the rate of 200 mg per kg body weight per animal.
• Group 4 (Unencapsulated LAB) Probiotic was given at the rate of 10⁸ cells/ day/ mouse)
• Group 5 (Encapsulated LAB) Probiotic Beads were given at the rate of 10⁸ cells/ day/ mouse)

Follow up of the study:
The blood sample of each animal was taken from retro-orbit plexus and serum was separated. The blood cholesterol level was checked by using a commercial diagnostic reagent kit by Avecon Healthcare Pvt. Ltd.
RESULTS AND DISCUSSION

Growth and CLA production pattern of *Lactobacillus* isolate LAB:
Growth and CLA production was checked spectrophotometrically by taking O.D. at 600nm and 233 nm respectively. As depicted in Figure 1 maximum CLA production was observed at 24 hr which corresponds to maximum growth. MRS broth with 1% Tween 80 and 0.1% cottonseed oil was used as standard which showed absorbance at 233 nm.

Red bars indicate CLA production (233nm) mean ± S.E.M. and Blue line indicates Growth Curve (600nm)

![Figure 1](image-url)

Preparation of Sodium Alginate Beads: Sodium alginate solutions at concentration of 2.5%, 3.0%, 3.5%, 4.0% were used and CaCl₂ were used for the preparation of beads but the best concentration for the formation of beads was 4% using 0.5M CaCl₂.

Characterization of beads:
Physical properties of beads were studied. Diameter and weight of beads on wet basis were more than the diameter and weight of beads on dry basis as showed in table 1.

Diffusion properties of beads were also studied. As depicted in Figure 2, with increase in time period from 0 hours to 3 hours, absorbance increased for 3.5 hours. Dye diffusion was maximum at 3 hours and thereafter remained constant.

Entrapment efficiency of viable cells in bead was calculated to be 2.5 x 10⁷ CFU/bead.

Diameter and Weight of sodium alginate beads:

<table>
<thead>
<tr>
<th>Table 1: Diameter and Weight of sodium alginate beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter(cm)</td>
</tr>
<tr>
<td>Dry Bead</td>
</tr>
<tr>
<td>Wet Bead</td>
</tr>
</tbody>
</table>

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Anticholesterolemic activity *(In Vivo study)*: The amount of residual cholesterol after treatment of hypercholesterolemic mice with probiotic is shown in Table 2 and Figure 3. The results showed that both the unencapsulated & encapsulated probiotics reduced the serum cholesterol level. The initial serum cholesterol in unencapsulated and encapsulated probiotic fed mice were 403.58 ± 1.57 and 463.4 ± 1.68 at day 0 and it reduced to 128.66 ± 1.68 and 167.6 ± 1.68 at day 20 respectively as shown in the Table 3. The encapsulated probiotics reduce the serum cholesterol level almost equivalent to drug.

<table>
<thead>
<tr>
<th>Groups</th>
<th>-7 day</th>
<th>0 day</th>
<th>4 day</th>
<th>8 day</th>
<th>12 day</th>
<th>16 day</th>
<th>20 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (untreated control i.e. mice fed)</td>
<td>131.64 ± 1.53</td>
<td>127.75 ± 2.45</td>
<td>125.14 ± 3.92</td>
<td>124.74 ± 2.25</td>
<td>119.56 ± 1.98</td>
<td>114.46 ± 3.12</td>
<td>105.72 ± 3.21</td>
</tr>
<tr>
<td>Group II (positive control i.e. hypercholesterolemic mice)</td>
<td>130.7 ± 1.39</td>
<td>452.5 ± 1.78</td>
<td>443.8 ± 1.59</td>
<td>435.5 ± 1.59</td>
<td>416.5 ± 1.50</td>
<td>402.72 ± 1.50</td>
<td>381.7 ± 1.52</td>
</tr>
<tr>
<td>Group III (drug treated hypercholesterolemic mice)</td>
<td>139.36 ± 1.42</td>
<td>412.38 ± 1.55</td>
<td>342.34 ± 1.40</td>
<td>262.6 ± 1.51</td>
<td>214.7 ± 1.56</td>
<td>185.6 ± 1.67</td>
<td>125.58 ± 1.62</td>
</tr>
<tr>
<td>Group IV (hypercholesterolemic mice dosed with unencapsulated probiotic)</td>
<td>131.62 ± 1.06</td>
<td>403.58 ± 1.57</td>
<td>327.78 ± 1.53</td>
<td>276.74 ± 1.61</td>
<td>232.66 ± 1.56</td>
<td>192.64 ± 1.67</td>
<td>128.66 ± 1.68</td>
</tr>
<tr>
<td>Group V (hypercholesterolemic mice dosed with Encapsulated probiotic)</td>
<td>137.74 ± 1.61</td>
<td>463.4 ± 1.68</td>
<td>404.5 ± 1.50</td>
<td>350.62 ± 1.35</td>
<td>278.64 ± 1.43</td>
<td>231.7 ± 1.44</td>
<td>167.6 ± 1.68</td>
</tr>
</tbody>
</table>

Constant decrease was observed from 0th day till 20th day with encapsulated and unencapsulated probiotics (Figure 3).
Percentage decrease in the cholesterol level: In group V, unencapsulated probiotic, there was 68.12% decrease in the cholesterol levels and in group IV, which was treated with encapsulated probiotic, there was 63.8% decrease in the cholesterol levels which is 4.32% lower than unencapsulated probiotic. In group III, which was drug treated, the decrease in the cholesterol levels was 69.5% as is almost parallel to that of percentage decrease in cholesterol levels as compared to unencapsulated (1.38% ↓) probiotic than encapsulated (5.7% ↓) probiotic as shown in Figure 4. Cholesterol

![Figure 4: Percentage decrease in cholesterol level In Vivo Results are expressed as %mean ± S.E.M.](image)

Previously authors have reported that survival of LAB cells in encapsulated form is better than that of unencapsulated form. Coating of cells provides best protection in bile salt solution because ion exchange takes place when beads absorb bile salts [22]. Study with encapsulated *L. acidophilus* and *Bifidobacterium spp* in yoghurt showed better survival over an 8 week storage period compared to survival of free cells [23]. But none of the authors observed bioactivity of bacteria in encapsulated form *In vivo*.

It is clear from our results that encapsulated and unencapsulated probiotics as well as drug (Atorvastin) reduced the cholesterol level. Undoubtedly, drug reduced the cholesterol level more than encapsulated and unencapsulated probiotics but the percentage decrease in cholesterol level is almost parallel to that of percentage decrease in cholesterol level of drug treated mice. In our study encapsulated form show non significantly less percentage decrease of cholesterol level than unencapsulated form. This can be because of the reason that *In vivo* mice would have not taken the complete dose of beads or encapsulated bacteria may be less available as compared to unencapsulated bacteria. Moreover, if the experiment would have been carried out for the longer time, may be the results would have been better with encapsulated bacteria than unencapsulated bacteria because the slower release of bacteria from the capsule may have retarded the effect.

**CONCLUSION**

*In vivo* treatment of encapsulated and unencapsulated probiotic revealed significant decreasing effect on total cholesterol. It is concluded that CLA producing probiotic reduces the cholesterol similar to that of standard drug. Release of bacteria or CLA in encapsulated form and their bioactivity *in vivo* can be further exploited for alternative therapeutic strategies.

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**REFERENCES**


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