Effect of Chitin-Chitosan Treatment on Fatty Liver in Rats with a High Fat Diet

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Summary  The present study was designed to investigate the hepatoprotective effect of 2% chitin-chitosan mixture on high fat diet-induced fatty liver, an experimental model for non-alcoholic fatty liver disease, in Wistar strain male rats. The hepatoprotective property was determined based on the following criteria: total lipid in liver, concentrations of total cholesterol, triglycerides, free fatty acids and phospholipids of serum and liver tissue; liver lipid/protein ratio; serum and tissue activities of aspartate amino transferase (AST) and alanine amino transferase (ALT); fecal fat determination and activity of pancreatic lipase. Chitin-chitosan fed animals gained less weight compared to the controls. A significant ($\rho < 0.001$) reduction in the liver lipid/protein ratio was observed in chitin-chitosan mixture supplemented rats as compared to that of control animals. Also the blood and liver fat, cholesterol, triglycerides, free fatty acids and phospholipid concentrations, decreased significantly by chitin-chitosan supplementation. Serum levels of aspartate aminotransferase and alanine aminotransferase showed significant ($\rho < 0.001$) reduction while tissue levels exhibited a concomitant increase upon chitin-chitosan treatment. Enhanced fat excretion ($\rho < 0.001$) was observed in the chitin-chitosan supplemented group. In the present study, supplementation of chitin-chitosan mixture did not induce any significant change in the activity of pancreatic lipase as compared to normal animals. This indicates that the antilipidaemic effect of chitin-chitosan mixture supplementation was mainly due to the interference in the gastrointestinal absorption of dietary fat rather than the mixture having any direct role on lipid metabolism. In conclusion, dietary supplementation of chitin-chitosan mixture can alleviate the high fat diet-induced aberrations related to lipid metabolism in fatty liver of experimental animals by their antilipidaemic property.

Key Words: high fat diet, fatty liver, chitin, chitosan, nonalcoholic fatty liver disease

Introduction

Diet-induced non-alcoholic fatty liver disease (NAFLD) is now a widespread condition in affluent societies, in which up to 24% of the general population has been estimated to have NAFLD. NAFLD is an increasingly recognized condition that may progress to end-stage liver disease. It is a major cause of liver-related morbidity and mortality [1]. It represents a spectrum of conditions characterized histologically by macrovesicular hepatic steatosis and occurs in those who do not consume alcohol in amounts generally considered to be harmful to the liver. Although NAFLD mostly remains asymptomatic, up to 20% of affected subjects may progress to cirrhosis and some of them require liver transplantation. NAFLD often is associated with type II diabe-

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tes, hyperlipidaemia, and other metabolic abnormalities [1].

There are no published controlled trials of treatment modalities, of proven efficacy for NAFLD and in their absence, therapy is directed towards correction of the risk factors for NAFLD (i.e., weight management, insulin resistance, decreasing delivery of fat and fatty acids to the liver, and use of drugs with potentially hepatoprotective effects) [2]. Although prolonged and consistent reduction of caloric intake may alleviate NAFLD, this rarely is achieved in real life. Under these circumstances an effective remedy would be useful. In the present study we attempt to address the possibility of decreasing the amount of fat reaching the liver by way of intestinal absorption by supplementing chitin-chitosan in the experimental feed.

Chitin is a naturally abundant mucopolysaccharide consisting of 2-acetamido-2-deoxy-β-D-glucose through a β(1→4) linkage. It forms the exoskeleton of crustaceans, insects, and is also produced extracellularly by fungal mycelia and brown algae. Chitosan is the N-deacetylated derivative of chitin, the deacetylation ranging from 100 to 20% [3, 4]. These polysaccharides have been extensively researched and many industrial, medical and biomedical applications have been identified [5, 6]. Chitin and chitosan are not hydrolyzed by human digestive enzymes and behave as dietary fiber. Animal studies have shown that chitosan can increase the amount of fat eliminated in the stools [7, 8]. This finding has resulted in chitosan being promoted as a non-prescribed dietary supplement and claims that chitosan may assist obese individuals to lose weight and lower elevated serum cholesterol levels have received clinical support [9, 10]. It has been reported earlier that chitin-chitosan (20-80%) treatment for 9 weeks reduced fat storage, plasma triacylglycerol, cholesterol and FFA as well as enhanced fat excretion [11]. In this study we attempt to study if 2% chitin and chitosan mixture in the ratio 1:1 is effective in reducing fat storage, and alleviating high-fat induced fatty liver in albino rats, an experimental model for non-alcoholic fatty liver disease.

Materials and Methods

Chemicals
Chitin (MW 1.08×10^6 kDa; purity 97.2%) and chitosan (MW 7.5×10^5 kDa; viscosity 8 cps; deacetylation rate 85–87%; purity 98.6%) was prepared from dried prawn shell in the Fish Processing Division of our Institute [12]. Briefly for chitin preparation, fresh or dry prawn shell waste was treated with 5% boiling sodium hydroxide for a few minutes. It was filtered using linen and the residue washed with water until free of alkali. Sufficient 1.2 N hydrochloric acid was added and stirred to demineralize. Demineralization may take one hour for completion. The product obtained was washed with water until free of acid and dried in hot air drier. For chitosan preparation chitin was treated with 40% sodium hydroxide at 90–95°C for 90–120 min to deacetylate. While heating, samples are drawn, washed free of alkali and solubility tested in 1% acetic acid to check the degree of deacetylation. When complete solubility is achieved the product obtained is chitosan. It is washed with water until free of alkali and dried in hot air drier. All chemicals used were of analytical grade.

Animals
Rats studied were 4-week old male Wistar rats weighing about 120 g, housed individually in polypropylene cages. The present study was implemented according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and authorized by the Animal Ethics Committee of Institute.

Diets
Regular rodent diet containing a maximum of 4 g% fat was used as control diet. Regular rodent diet mixed with chitin-chitosan at 2% level in the ratio 1:1 was used as the test diet. High-fat diet (HFD) was prepared by mixing the regular rodent diet with 10% lard and 2% cholesterol.

Experimental design
All animals were held in standard cages at an animal facility in our institute at constant room temperature (22°C), under a 12-h light/dark cycle. Water was given ad libitum and animals were weighed weekly. The control animals (12 Nos.) were fed the control diet. The test animals (12 Nos.) were fed the test diet. One half of the control animals (group I) and one half of the test animals (group IV) were supplemented with high-fat diet (HFD). After a period of 4 weeks, animals were sacrificed following chloroform anesthesia. The livers and pancreata were excised, weighed, cut into small pieces of approxi-
Table 1. Body weight and weight of liver of rats on normal diet, high-fat diet, chitin-chitosan mixture and high-fat diet supplemented with chitin-chitosan mixture after feeding for 4 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>131.6±4.7</td>
<td>153.1±8.7</td>
<td>114.4±6.3</td>
<td>139.2±5.4</td>
</tr>
<tr>
<td>Weight of liver (g)</td>
<td>3.10±0.08</td>
<td>4.49±0.84</td>
<td>3.04±0.06</td>
<td>3.23±0.69</td>
</tr>
</tbody>
</table>

Group I, rats fed on regular rodent diet; group II, rats fed on high fat diet; group III, rats supplemented with 2% chitin-chitosan; group IV, rats supplemented with 2% chitin-chitosan and high fat diet. Values expressed as mean±SD for 6 animals in each group. *p<0.001 significantly different as compared with group I and †p<0.001 significantly different as compared with group II, ‡p<0.001 significantly different when compared to group III.

Table 2. Concentration of lipid fractions in the liver of normal and experimental groups of rats.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total liver fat</td>
<td>56.34±1.44</td>
<td>192.7±11.26</td>
<td>57.34±6.22</td>
<td>119.2±8.4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6.36±2.14</td>
<td>31.57±5.49</td>
<td>8.03±1.79</td>
<td>18.91±2.9</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>6.23±2.28</td>
<td>52.59±4.06</td>
<td>7.06±1.57</td>
<td>22.44±3.2</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>25.12±2.17</td>
<td>47.71±5.21</td>
<td>24.32±5.24</td>
<td>31.53±2.0</td>
</tr>
<tr>
<td>FFA</td>
<td>13.58±1.83</td>
<td>44.43±3.62</td>
<td>15.27±3.33</td>
<td>20.46±0.9</td>
</tr>
</tbody>
</table>

Group designations are same as in Table 1. FFA: free fatty acids. Values expressed as mg/g liver tissue. Values expressed as mean±SD for 6 animals in each group. *p<0.001 significantly different as compared with group I and †p<0.001 significantly different as compared with group II, ‡p<0.001 significantly different when compared to group III.

Analytical methods

The liver samples were weighed and homogenized by standard procedures. Lipids were extracted from an aliquot of the liver homogenate according to the procedure of Folch [13]. The total amount was calculated after evaporation of aliquot to constant weight. Serum and liver total cholesterol [14], triglycerides [15], and free fatty acids [16] were determined. Phospholipid in serum and liver was estimated by method of Fiske and Subba Row [17] as inorganic phosphorus liberated after Bartlett’s perchloric acid digestion [18]. Protein was determined using the Bradford method [19]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by the method Mohur and Cook [20]. Accurate measurements of feed consumed and feces excreted were made for each group and total fat was determined in all the samples of feed and excreta [13]. Pancreata were homogenized individually for two minutes at 0°C in nine times its volume of tris buffer pH 7.4 and the activity of pancreatic lipase was determined by the method of Schmidt [21].

Statistical analysis

Results are expressed as mean±SD. Each sample was tested in duplicate. One-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Tukey's test using a statistical package program (SPSS 10.0 for Windows). A p-value of less than 0.05 was considered significant.

Results

Animal weight

The weights of the animals at the end of the feeding trials are shown in (Table 1). There was a notable reduction in the weight of animals fed with 2% chitin-chitosan. Further the chitin-chitosan fed rats supplemented with a high fat diet gained significantly less weight as compared to the control rats supplemented with HFD. The weights of livers of the animals showed a similar trend (Table 1). The mean food consumption per week per rat was significantly (*p<0.05) different between group I control animals and high-fat diet groups II and IV being 1,465.4±41.3 kJ in the control group and 1,675.8±64.1 kJ in group II and 1,604.7±65.2 kJ in group IV. There was no significant difference in the average energy consumption between the two high-fat diet fed groups. The mean food utilization per week per rat in group III is 1,433.6±47.5 kJ.

Fat content in the liver

The amounts of fat per gram of liver tissue, the lipid fractions and the lipid protein ratio in the liver...
at the end of trials in rats on control diet, HFD, chitin-chitosan diet with and without HFD supplementation are shown in Table 2 and Fig. 1 respectively. In animals receiving a HFD, the liver total lipid concentration, the levels of cholesterol, triglycerides free fatty acids and phospholipid and the lipid/protein ratio were higher when compared to the controls. The group that received HFD with chitin-chitosan supplementation showed a significant decrease in both the total lipid content as well as the lipid protein ratio in the liver. In rats fed a HFD together with chitin-chitosan the fat concentration in liver tissue decreased from 192.7±11.26 to 119.2±8.40 mg/g liver (p<0.001) (Table 2) and lipid/protein ratio was lowered from 1.43±0.44 to 0.66±0.04 (p<0.001) (Fig. 1). Chitin-chitosan feeding also decreased the free fatty acids (FFA), phospholipids and cholesterol concentrations in the liver of experimental rats (Table 2).

Liver marker enzymes

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum and liver tissue of the four experimental groups of rats are shown in Table 3. The group fed with a high fat diet had significantly (p<0.001) higher activities of the aminotransferases in their serum when compared to the control animals. In addition, a parallel decrease in their activities was noted in the liver tissue of these animals. Supplementing HFD with chitin-chitosan mixture reversed this trend, with the result that serum activities of the enzymes were lowered while the tissue exhibited an increase in activity of the enzymes.

Table 3. Activities of aspartate amino transferase and alanine amino transferase in serum and liver tissue of normal and experimental rats.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST*</td>
<td>A</td>
<td>0.72±0.04</td>
<td>0.36±0.03</td>
<td>0.75±0.01</td>
</tr>
<tr>
<td>B</td>
<td>1.04±0.02</td>
<td>0.26±0.02</td>
<td>0.99±0.06</td>
<td>0.92±0.07</td>
</tr>
<tr>
<td>ALT*</td>
<td>A</td>
<td>0.72±0.04</td>
<td>0.36±0.03</td>
<td>0.75±0.01</td>
</tr>
<tr>
<td>B</td>
<td>1.04±0.02</td>
<td>0.26±0.02</td>
<td>0.99±0.06</td>
<td>0.92±0.07</td>
</tr>
</tbody>
</table>

Group designations are the same as in Table 1. Serum; B, Liver. Values expressed as mean±SD for 6 animals in each group.
*Values expressed: AST, ALT µmol of pyruvate liberated/µg protein for liver tissue and µmol of pyruvate liberated/µl for serum. p<0.001 significantly different as compared with group I and p<0.001 significantly different as compared with group II, p<0.001 and p<0.05 significantly different when compared to group III.

Table 4. Concentration of lipid fractions in the serum of normal and experimental groups of rats.

<table>
<thead>
<tr>
<th>Lipid Fraction</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>85.7±8.1</td>
<td>205.1±10.4</td>
<td>82.6±7.4</td>
<td>134.4±8.3</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>110.5±11.8</td>
<td>246.3±15.6</td>
<td>114.2±11.8</td>
<td>195.2±7.4</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>108.4±12.1</td>
<td>197.8±9.5</td>
<td>111.7±5.6</td>
<td>152.6±6.9</td>
</tr>
<tr>
<td>FFA</td>
<td>46.4±4.2</td>
<td>89.7±4.6</td>
<td>48.4±4.3</td>
<td>69.43±5.7</td>
</tr>
</tbody>
</table>

Group designations are the same as in Table 1. FFA, free fatty acids. Values expressed as mg/dl serum. Values expressed as mean±SD for 6 animals in each group. p<0.001 significantly different as compared with group I and p<0.001 significantly different as compared with group II, p<0.001 significantly different when compared to group III.
Table 5. Percentage of fat consumed and excreted in feces of normal control and experimental groups of rats.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Fat in feed consumed</td>
<td>3.50±0.09</td>
<td>15.47±0.56a</td>
<td>3.99±0.39b</td>
<td>15.74±0.66bc</td>
</tr>
<tr>
<td>% Fat in fecal matter</td>
<td>1.34±0.09</td>
<td>3.34±0.05a</td>
<td>1.72±0.06b</td>
<td>14.05±0.15bc</td>
</tr>
</tbody>
</table>

Group designations are same as in Table 1. Values expressed as mean±SD for 6 animals in each group. *p<0.001 significantly different as compared with group I and †p<0.001 significantly different as compared with Group II, ‡p<0.001 significantly different when compared to Group III.

Table 6. Activity of pancreatic lipase in control and experimental groups of rats.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic lipase</td>
<td>143.16±11.64</td>
<td>145.12±9.07</td>
<td>143.22±9.98</td>
<td>144.36±14.45</td>
</tr>
</tbody>
</table>

Group designations are same as in Table 1. Values expressed as mean±SD for six animals. Pancreatic lipase measured as units/mg protein.

Fat determination in blood

Blood lipid parameters showed a similar pattern of change as that of liver tissue (Table 4). Total cholesterol, triglycerides, free fatty acids, and phospholipid levels were significantly higher in high-fat diet fed rats when compared to that of normal rats and there was a substantial decrease in their levels when HFD was supplemented with chitin-chitosan mixture.

Fat excretion

A significant increase was observed in the amount of fat excreted in the chitin-chitosan supplemented high-fat diet fed rats when compared to the normal control rats (*p<0.001) (Table 5). Groups I, II and III recorded fat excretion in the normal range.

Pancreatic lipase

No significant changes were observed in the activity of pancreatic lipase among all the groups of rats (Table 6).

Discussion

The diets used in the experiment to induce the formation of fatty liver in the rats were high-fat diets (HFDs) containing 2-fold larger fat than the regular diet. Expectedly, most of the experimental animals developed fatty livers. There are a number of studies describing high-fat diet-induced obesity [22–24]. Our data show that chitin-chitosan prevent/reduce diet induced fatty liver. They produce this effect even though the animals continue to feed on a HFD. This fat reduction in the liver was confirmed by chemical analysis of liver fat, blood fat, by measurement of the lipid/protein ratio in the liver, and by determination of liver specific marker enzymes. Chitin-chitosan fed animals showed reduced weight, liver lipid/protein ratio and liver and blood fat, in the process improving the fatty liver condition. The energy consumed over the experimental period did not vary significantly among the high fat-diet fed groups II and IV. This signifies that chitin-chitosan prevents the high-fat-diet induced gain in body weight by affecting food absorption. Because the test animals gained less weight than the controls, and because chitin-chitosan cannot be digested in the intestine, this would suggest that the mechanism of this effect might be due to a decline in the absorption of fat in this group. This fact has been substantiated by enhanced fat excretion observed in the chitin-chitosan supplemented high-fat diet fed rats. Dietary supplementation of chitosan has been shown to limit the increase in blood cholesterol in cholesterol fed rabbits [25] and rats [26–29], but did not reduce blood cholesterol levels in rabbits with preexisting hypercholesterolemia [25]. This suggests that the hypcholesterolemic effect of chitosan is limited to gastrointestinal tract. The present study demonstrates the beneficial effect of chitin-chitosan as a dietary supplement as it decreases plasma and tissue lipids due to its ability to bind dietary lipids, thereby reducing intestinal lipid absorption. Also the decrease in the cholesterol in blood and liver tissue that was observed in this study can be explained by binding of bile acids by chitin-chitosan, reducing the amount of cholesterol-containing bile acids available for re-absorption by enterohepatic circulation in the lower intestine. This would result in more endogenous cholesterol being used to make bile [30], thus lowering the blood and tissue cholesterol.

There are reports that both adipocyte size and number increase in animals with obesity caused by
high fat diet [31]. Bael lipolysis in the absence of lipolytic hormones was increased in enlarged fat cells in aged and/or obese animals [32]. An increase in plasma FFA occurs following basal lipolysis, which are then converted to lipids in liver and secreted into blood as very low density lipoprotein [11]. From the present study it can be deduced that chitin-chitosan reduced the high fat diet induced alterations in lipid metabolism and in so doing may have lowered basal lipolysis in fat cells which in turn resulted in decreased levels of plasma FFA. That the mechanism of lowering of blood and tissue fat by chitin-chitosan is limited to gastrointestinal tract is further confirmed by the measurements of pancreatic lipase whose activity was found to be similar in all the groups of rats involved in the study. It is widely accepted that dietary fat when influences the activity of pancreatic lipase [33]. In a study involving supplementation of diet with chitin-chitosan, it was observed that hydrolysis of triolein emulsified with lecithin was inhibited but not that of triolein emulsified with gum arabic and Triton X-100. This suggests that the site of inhibitory action of chitin chitosan is not the enzyme but its substrate [11]. The inhibition by chitosan of hydrolysis of dietary fat may cause a decrease in intestinal absorption of fat and reduce blood chylomicron, an excess of which is known to induce hyperlipidemia, obesity and fatty liver [11].

The intent of our study was to devise treatment module for NAFLD by chitin-chitosan supplementation, by addressing the issue of excess fat being delivered to the liver through absorption in the gastrointestinal tract. Increased fatty acid delivery to the liver have complex effects within the hepatocytes, including interference with insulin function, [34, 35] preferential utilization of fatty acids for mitochondrial oxidation [36, 37] and the pro-apoptotic mitochondrial uncoupling protein 2 expression [38, 39]. These, along with other potential intrahepatic abnormalities, culminate in the development of steatohepatitis. In many cases of NAFLD insulin resistance is a common denominator. NAFLD is associated with decreased insulin-mediated suppression of lipolysis [35]. Consequently, subjects with NAFLD have high serum free fatty acid concentrations, allowing greater hepatic fatty acid uptake and oxidation. From the present study it is clear that supplementing chitin-chitosan in the feed can prevent the development of steatohepatitis as the amount of fat and thus the free fatty acid delivered to the liver can be restrained.

The serum alanine aminotransferase value has long been used as a surrogate marker of liver injury. Serum levels of aspartate aminotransferase and alanine aminotransferase in high-fat diet fed rats in our study are reflective of those typically found in NAFLD, the concentration of alanine aminotransferase being more than that of aspartate aminotransferase [40] unlike in alcoholic fatty liver disease where the reverse is true. Unexplained aminotransferase elevation was significantly associated with higher body mass index, waist circumference, and triglycerides, fasting insulin, and lower HDL, adiposity and other features of the metabolic syndrome and thus may represent nonalcoholic fatty liver disease [41]. Chitin-chitosan mixture supplementation has aided in reducing the liver injury induced by high fat diet as reflected by the reduction in the serum levels of aminotransferases.

Chitin and chitosan considered to have low toxicity, has been well-tolerated in clinical studies producing only mild and transitory nausea and constipation in 2.6–5.4% of subjects. Some research has shown that vitamin and mineral levels remain within normal range with chitosan supplementation; however other studies have shown that chitosan acts by forming gels in the intestinal tract, which entrap lipids and other nutrients, including fat-soluble vitamins and minerals, thus interfering with their absorption. The resulting undesirable effects reported are a marked decrease in vitamin E levels, reduction in bone mineral content and growth retardation, requiring twice the amount of calcium supplementation in rats [42]. The findings correlate with those of an earlier study in which dietary supplementation with 10% chitosan depressed growth in cholesterol fed rats [7]. The association between chitin-chitosan intake and reduced weight gain in our study is of significant concern. Whereas it can be reasoned that reduced weight gain may be attributed to the lipid lowering effect of chitin-chitosan, it may also be due to its reported adverse effects. Interestingly, a correlation between chitosan intake and increased growth; has also been reported [43]. The reported toxicity-related side effects can be to a large extent circumvented by supplementing chitin-chitosan containing diet with the necessary nutrients.

In this study, we have shown that chitin-chitosan is effective in preventing dietary fatty liver in rat model. Further studies of treating pre-established fatty liver with chitin-chitosan have to be done. At
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present, there is no unanimity and not enough experimental data in relation to therapy for NALFD. Based on presently available data chitin-chitosan would seem to offer a potentially nontoxic therapy. A comprehensive study of the metabolic effects of long-term dietary supplementary use of chitin-chitosan needs to be performed.

In summary, our studies show that chitin-chitosan reduces and prevents diet-induced fatty liver in the experimental rats tested. This effect was shown by chemical analysis of liver and blood fat. The presumed mechanism of action is a non-metabolic one as indicated by the fat absorption data in our study. Chitin-chitosan may have potential for therapy of human fatty liver due to dietary and other causes. The fact that chitin-chitosan may have growth retarding effects warrants further studies to attain a complete interpretation of the potentially adverse side effects of chitin-chitosan on growth and digestion.

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References


