1 **Optimization of Cholesterol Removal, Growth and Fermentation Patterns of**

2 ***Lactobacillus acidophilus* ATCC 4962 in Presence of Mannitol, FOS and Inulin: A**

3 **Response Surface Methodology Approach**

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5 **M. T. Liong and N. P. Shah***

6 School of Molecular Sciences, Victoria University, Werribee Campus, PO Box 14428,

7 Melbourne City Mail Centre, Victoria 8001, Australia

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9 **Running headline:** Optimizing removal of cholesterol

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11 ***Corresponding author:**

12 Professor Nagendra P. Shah

13 Phone: +61-3-9216-8289

14 Fax: +61-3-9216-8284

15 Email: nagendra.shah@vu.edu.au

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26 **ABSTRACT**

27 **Aims:** To optimize cholesterol removal by *L. acidophilus* ATCC 4962 in the presence

28 of prebiotics, and study the growth and fermentation patterns of the prebiotics.

29 **Methods and Results:** *L. acidophilus* ATCC 4962 was screened in the presence of six

30 prebiotics, namely sorbitol, mannitol, maltodextrin, hi-amylose maize,

31 fructooligosaccharide (FOS) and inulin in order to determine the best combination for

32 highest level of cholesterol removal. The first-order model showed that the combination

33 of inoculum size, mannitol, FOS and inulin was best for removal of cholesterol. The

34 second-order polynomial regression model estimated the optimum condition of the

35 factors for cholesterol removal by *L. acidophilus* ATCC 4962 to be 2.64% w/v

36 inoculum size, 4.13% w/v mannitol, 3.29% w/v FOS and 5.81% w/v inulin. Analyses of

37 growth, mean doubling time and short-chain-fatty-acid (SCFA) production using

38 quadratic models indicated that cholesterol removal and the production of SCFA were

39 growth associated.

40 **Conclusions:** Optimum cholesterol removal was obtained from the fermentation of *L.*

41 *acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin. Cholesterol

42 removal and the production of SCFA appeared to be growth associated and highly

43 influenced by the prebiotics.

44 **Significance and impact of the study:** Response surface methodology (RSM) proved

45 reliable in developing the model, optimizing factors, and analyzing interaction effects.

46 The results provide better understanding on the interactions between probiotic and

47 prebiotics for the removal of cholesterol.

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49 Key words: optimization; cholesterol removal; inoculum size; prebiotic; RSM

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INTRODUCTION

Interest in the usage of probiotics for human health dated back to 1908 when Metcnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (O'Sullivan et al. 1992). More recently, probiotics have been defined as 'cultures of live microorganisms that, applied in animals or humans, benefit the host by improving properties of indigenous microflora' (Arihara and Itoh, 2000). They mainly consist of lactobacilli, streptococci, enterococci, lactococci and bifidobacteria. Over the years, lactobacilli have been associated with the improvement of lactose intolerance, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, improved digestion and reduction in serum cholesterol level (Gibson and Roberfroid, 1995). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks (Lourens-Hattingh and Viljoen, 2001). Various studies reported that lactobacilli could lower total cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Sanders, 2000).

Prebiotics are defined as nondigestable substances that exert biological effect on humans by selectively stimulating the growth or bioactivity of beneficial microorganisms either present, or therapeutically introduced to the intestine (Tomasik and Tomasik, 2003). Several non-starchy polysaccharides such as fructooligosaccharides, lactulose and β-cyclodextrin have been considered to have prebiotic properties. Recently, polyols such as mannitol, sorbitol and xylitol have been included to the prebiotics group (Klahorst, 2000). Prebiotics have been linked with cholesterol reducing effects. It was previously found that hepatocytes isolated from oligofructose-fed rats had a slightly lower capacity to synthesize triacylglycerol from radiolabeled acetate. This led to the hypothesis that decreased de novo lipogenesis in the

76 liver, through lipogenic enzymes, is the key to reduction of VLDL-triglyceride secretion

77 in rats fed with oligosaccharides (Robertfroid and Delzenne, 1998). Administration of

78 oligofructose was postulated to modify lipogenic enzyme gene expression, observed by

79 a 50% reduction of activity of acetyl-CoA carboxylase, malic enzyme and ATP citrate

80 lyase (Delzenne and Kok, 2001).

81   Probiotics and prebiotics simultaneously present in a product are called either

82 synbiotics or eubiotics. Such a combination aids survival of the administered probiotic

83 and facilitates its inoculation into the colon. Additionally, the prebiotic induces growth

84 and increases activity of positive endogenic intestinal flora (Tomasik and Tomasik,

85 2003). Experiments with rats showed that synbiotics protect the organism from

86 carcinogens significantly better than either prebiotics or probiotics individually

87 (Gallaher and Khil, 1999). However, there is little information on suitable combinations

88 of probiotics and prebiotics specifically targeting removal or lowering of cholesterol.

89   Response surface methodology (RSM) is a collection of statistical and

90 mathematical techniques useful for developing, improving and optimizing processes. It

91 also has important applications in design, development and formulation of new

92 products, as well as improvement of existing product designs (Myers and Montgomery,

93 1995). Response surface models may involve main effects and interactions or have

94 quadratic and possibly cubic terms to account for curvature. It has been successfully

95 utilized to optimize compositions of microbiological media (Oh et al. 1995), improving

96 fermentation processes (Lee and Chen, 1997) and product development (Gomes and

97 Malcata, 1998). Conventional methods (such as one factor at one time) have been

98 applied previously to evaluate the *in vitro* performance of probiotics and/or prebiotics to

99 remove cholesterol. However, these methods require a large number of experiments to

100 describe the effect of individual factors, were time consuming, and no statistical method

101   was established to distinguish the interaction effects from main effects. Thus, the aim of

102   this study was to optimize cholesterol removal by using *L. acidophilus* ATCC 4962 in

103   the presence of mannitol, FOS and inulin, through the approach of response surface.

104

105   **MATERIALS AND METHODS**

106   **Bacteria and media preparation**

107   *L. acidophilus* ATCC 4962 is a human derived strain that was obtained from the

108   Australia Starter Culture Collection Center (ATCC) (Werribee, Australia). All stock

109   cultures were stored in 40% glycerol at –80 °C, and transferred successively three times

110   in sterile de Mann, Rogosa, Sharpe (MRS) broth using 1% inoculum and 20 h

111   incubation at 37 °C prior to use. The culture was then centrifuged at 4 °C for 15 min at

112   2714 x g (Sorvall RT7, Newtown, Conn., U.S.A.). The supernatant was discarded while

113   the pellet was washed twice with sterile distilled water, resuspended by vortexing in 50

114   ml of 0.1 M phosphate buffer (pH 6.8), and recentrifuged at 2714 x g at 4 °C for 15 min.

115   After discarding the supernatant, 50 ml of 0.1 M phosphate buffer (pH 6.8) containing

116   2.0 % (w/v) of food grade cryoprotectant Unipectin^{TM} RS 150 (Savannah Bio Systems,

117   Balwyn East, Australia) was added to the pellet. The mixture was vortexed, poured into

118   large petri dishes and freeze-dried (Dynavac FD300, Airvac Engineering Pty. Ltd.,

119   Rowville, Australia) at -88 °C for 40 h for primary freezing and 8 h for secondary

120   freezing. After freeze-drying, the hygroscopic cultures were transferred into sterile

121   sealed bags and stored at -18 °C until used. Six types of commercially available

122   prebiotics were used, namely sorbitol (Sigma Chemical Co., St. Louis, MO, U.S.A.),

123   mannitol (Sigma), maltodextrin (Grain Processing Corp., Muscatine, IA, U.S.A.), hi-

124   amylose maize (Starch Australasia Ltd., Lane Cove, NSW, Australia), inulin (Orafti

125   Pty. Ltd., Tienen, Belgium) and FOS (Orafti). FOS used was Raftilose P95 that

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126 contained 5% of glucose, fructose and sucrose. It contained oligofructose with DP

127 ranging from 2 to 7, with an average DP of 4. Inulin used was Raftiline ST with a purity

128 of 92%, an average DP of 10. Hi-amylose maize contained > 70% amylose, and 32.5%

129 total dietary fiber.

130 All prebiotics were used at concentrations as per the experimental design (Table

131 1). Prebiotics were prepared in phosphate buffer (0.1 M, pH 6.0) containing ammonium

132 citrate (2.0 g L^{-1}), sodium acetate (5.0 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), manganese

133 sulfate (0.05 g L^{-1}), dipotassium phosphate (2.0 g L^{-1}) and Tween 80 (1.0 ml L^{-1}).

134 Freeze-dried cells of *L. acidophilus* ATCC 4962 were inoculated at appropriate levels as

135 described in the experimental design.

136

137 **Cholesterol removal**

138 Freshly prepared media containing prebiotics were added with water-soluble

139 filter-sterilized cholesterol (polyoxyethanyl-cholesteryl sebacate), at a final

140 concentration of 70-100 μg ml^{-1}, inoculated with appropriate levels of freeze-dried *L.*

141 *acidophilus* ATCC 4962 (Table 1), and incubated anaerobically at 37 °C for 48 h. After

142 the incubation period, cells were centrifuged and the remaining cholesterol

143 concentration in the spent broth was determined using the OPA colorimetric method as

144 described previously (Rudel and Morris, 1973).

145

146 **Growth of *L. acidophilus* ATCC 4962 in the presence of prebiotics**

147 The growth was determined using the plate count method. Bacilli generally

148 divide in one plane, and can produce chains of cells due to the failure to separate

149 completely. Thus, at the end of the fermentation time, fermentation broth containing

150 probiotic cultures sonicated for 5 s to disrupt clumps of lactobacilli (Bermudez et al.

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151 2001) before serial dilutions were performed. Subsequent serial dilution blanks were

152 vortexed for 30 s individually. One milliliter sample was taken after the incubation

153 period, and 10-fold serial dilutions were made using peptone water diluent. MRS agar

154 was used for plating and the plates were incubated anaerobically at 37 °C for 24 h in an

155 anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, U.S.A.) with a

156 Gas Generating Kit® (Oxoid, Ltd.). Growth was calculated as log10 colony forming

157 units (CFU ml^{-1}) and expressed as percentage difference between initial growth values

158 obtained at time = 0 and at the end of the incubation period.

159

160 **Mean doubling time**

161   Mean doubling time was calculated as described previously (Shin et al. 2000).

162 The specific growth rate (μ) of the cultures was obtained using the following equation:

163       $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$

164 where X_2 and X_1 are the cell density at time t_2 and t_1, respectively. Mean doubling time

165 (T_d) was calculated as:

166       $T_d = \ln2/\mu$, and expressed in min.

167

168 **Short chain fatty acids (SCFA) determination**

169   The fermentation of prebiotics was determined by measuring short chain fatty

170 acids as the end products of fermentation using high performance liquid

171 chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia). At the end of

172 the incubation period, fermentation broths containing *L. acidophilus* ATCC 4962 and

173 the prebiotics used were centrifuged at 2714 x g at 4 °C for 15 min, and the supernatant

174 was prepared for HPLC analysis using the method as described previously (Dubey and

175 Mistry, 1996). Briefly, 5 ml of supernatant was added to 100 μL of 15.5 N HNO_3 and 5

176 ml of 0.009 N H_2SO_4. The mixture was vortexed for 10 sec and recentrifuged at 14 000

177 x g for 10 min. The supernatant was filtered (0.20 μm) and stored at 4 °C until analysed.

178 SCFA was expressed as the total acetic, butyric and propionic acids.

179

180 **Experimental design and statistical analyses**

181 Screening experiments to select prebiotics were performed with seven

182 independent factors namely, inoculum size of *L. acidophilus* ATCC 4962 (X_1), sorbitol

183 (X_2), mannitol (X_3), maltodextrin (X_4), hi-amylose maize (X_5), inulin (X_6) and FOS

184 (X_7), using a two level partial factorial design 2^{7-2} resulting in 64 experimental runs

185 (including duplicates) and 5 middle point runs. The units and the coded levels of the

186 independent factors are shown in Table 1. First order empirical equation was used to

187 exclude insignificant factors and to generate steepest ascent. Optimization was

188 performed using a rotatable central composite design (CCD) with an alpha value of ±

189 2.00 for four factors. The treatment combinations of CCD were allocated in 2 blocks,

190 with the first block representing the first day of the experiment and contained all

191 factorial runs accompanied by 4 center runs. The second block, representing the second

192 day of the experiment, contained all axial runs accompanied by 2 center runs. These

193 modeling and statistical analyses were performed using the Design Expert version 5.07

194 software (Stat-Ease Corp., Minneapolis, MN, U.S.A.). All data presented are means of

195 triplicate experiments, n = 3.

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RESULTS

Screening of factors and steepest ascent

Results from the two-level partial factorial design are shown in Table 1, while analysis of variance (ANOVA) for the evaluation of the first-order model is shown in Table 2. ANOVA showed that the model used was suitable, lack-of-fit test was insignificant with only 9.58% total variation that was not explained by the model. Removal of cholesterol was significantly influenced by inoculum size of *L. acidophilus* ATCC 4962 (X_1), mannitol (X_3), FOS (X_6) and inulin (X_7), while the other prebiotics were found to have insignificant influence and were not included in the ANOVA. Thus, further optimization processes will only involve these four factors. A first-order equation (coded term) was generated from this first-degree order model, for response of cholesterol removal (Y), with the significant factors now redefined as inoculum size (X_1), mannitol (X_2), FOS (X_3) and inulin (X_4):

$$Y = 33.28 + 3.50X_1 + 1.17X_2 + 0.83X_3 + 1.17X_4.$$

From the equation and coefficient estimate, inoculum size (X_1) produced greatest effect and was used as the fundamental scale in the next step, steepest ascent. In this study, the steepest ascent design was based on the increase of 0.50 (% w/v) concentrations for X_1. This produced 5 design units (0.50/0.10 = 5). Thus, movement for X_2 was 1.67 design units [(1.17/3.50)(5) = 1.67], for X_3 was 1.19 design units [(0.83/3.50)(5) = 1.19] and for X_4 was 2.53 design units [(1.17/3.50)(5) = 2.53]. The following steepest ascent coordinates were generated as shown in Table 3. Steepest ascent coordinates showed that removal of cholesterol decreased after the fifth step, with highest value of 50.938 $\mu g\ ml^{-1}$, from the combination of inoculum size (2.20% w/v), mannitol (4.36% w/v), FOS (3.40% w/v) and inulin (6.08% w/v). This combination was used as the middle point for optimization experiments.

226 **Optimization of cholesterol removal**

227   Optimization was performed using CCD with fixed middle point of inoculum

228 size (2.20% w/v), mannitol (4.30% w/v), FOS (3.40% w/v) and inulin (6.00% w/v).

229 Design matrix for CCD and responses are shown in Table 4, while the adequacy and

230 fitness were evaluated by ANOVA and regression coefficients (Table 5). ANOVA

231 results indicated that the quadratic regression to produce the second-order model was

232 significant. Lack-of-fit test was insignificant and a good coefficient regression was

233 obtained. Inoculum size, mannitol, FOS and inulin significantly influenced cholesterol

234 removal.

235   The effect of each factors were further assessed using perturbation plots, to show

236 how the response changes as each factor moves from the chosen reference point, with

237 all other factors held constant at reference values (Oh et al. 1995). In this study, as one

238 particular chosen factor was assessed, the other factors were held constant at the

239 optimum point. Figure 1 shows the perturbation plot of the factors used in this study.

240 Although all factor showed significant quadratic effect, the curve with the most

241 prominent change was the perturbation curve of inoculum size, compared to the other

242 factors that were fixed at their maximum levels. Thus, we believe that inoculum size

243 was the most significant factor that contributed to the removal of cholesterol with the

244 most obvious quadratic effect. Although the P-values of both FOS and inulin showed

245 similar levels of significance, it could be clearly seen from the perturbation plot that the

246 response curve of inulin was less prominent than that of FOS.

247   The best explanatory equation to fit the second-order model and subsequently

248 produce the response surface was expressed as:

249       $Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_4X_4 + c_{11}X_1{}^2 + c_{22}X_2{}^2 + c_{33}X_3{}^2 + c_{44}X_4{}^2 +$

250           $c_{12}X_1X_2 + c_{13}X_1X_3 + c_{14}X_1X_4 + c_{23}X_2X_3 + c_{24}X_2X_4 + c_{34}X_3X_4$

251   where $c \dots c_{23}$ are regression coefficients and X_1, X_2, X_3, X_4 are the coded independent

252   factors. Here, the second-order regression model involved four factors, thus producing

253   four linear, four quadratic and six interaction terms. Response surface was generated

254   (Figure 2) based on the second-order equation:

255         $Y_0 = \quad 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2$

256         $- 5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 -$

257         $1.01X_3X_4$

258   An optimum point was produced with optimum cholesterol removal obtained at 58.142

259     $\mu g\ ml^{-1}$. The combination that produced the optimum point was $(X_1, X_2, X_3, X_4) =$

260   (0.437, -0.082, -0.115, -0.092). The original levels that correlated with those coded

261   values were found to be inoculum size at 2.64% w/v, mannitol at 4.14% w/v, FOS at

262   3.28% w/v and inulin at 5.82% w/v.

263   All these predictions by the regression model were further ascertained by a

264   validation experiment. We compared the cholesterol removal patterns over a 24 h period

265   using four different media: the optimum medium (inoculum size: 2.60% w/v; mannitol:

266   4.10% w/v; FOS: 3.30% w/v; inulin: 5.80% w/v), the center-point medium (inoculum

267   size: 2.20% w/v; mannitol: 4.30% w/v; FOS: 3.40% w/v; inulin: 6.00% w/v), the high-

268   point medium (inoculum size: 3.20% w/v; mannitol: 6.30% w/v; FOS: 4.40% w/v;

269   inulin: 8.00% w/v) and the low-point medium (inoculum size: 1.20% w/v; mannitol:

270   2.30% w/v; FOS: 2.40% w/v; inulin: 4.00% w/v). The cholesterol removal curves are

271   shown in Figure 3. Although the exact cholesterol removal quantities were different

272   from the predictions, the patterns were in tandem with predictions by the model.

273   Highest cholesterol was removed from the optimum medium, and lower from the

274   center-point medium. Least cholesterol was removed from both high-point and low-

275   point media, as supported by the response surface of cholesterol removal (Figure 2).

Growth, mean doubling time and production of SCFA

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277   We further studied patterns of growth, mean doubling time and production of

278   SCFA from the fermentation of prebiotics, at the experimental regions used to obtain

279   optimum removal of cholesterol. The response obtained using the CCD is shown in

280   Table 6. The statistical analyses with coefficient estimates and the significance of each

281   response model are presented in Table 7.

282   The response surface of growth (Y_1) is shown in Figure 4, and was generated

283   based on the following coded factor equation:

284        $Y_1 = 41.97 + 2.49X_1 - 0.12X_2 - 1.49X_3 - 3.35X_4 - 3.90X_1^2 - 4.05X_2^2 - 2.77X_3^2$

285            $- 0.50X_4^2 - 0.22X_1X_2 + 1.66X_1X_3 + 1.63X_1X_4 + 0.89X_2X_3 - 0.08X_2X_4 +$

286            $0.53X_3X_4$

287   The response surface clearly indicated that an optimum point (45.21%) was produced

288   with X_1, X_2, X_3 and X_4 at 2.23% w/v, 4.21% w/v, 3.04% w/v and 4.00% w/v,

289   respectively. Growth increased with increasing inoculum size level from 1.20% w/v to

290   2.23% w/v. Further increase in concentrations of inoculum size beyond 1.69% w/v

291   generated a decrease in growth. Similarly, increasing concentrations of mannitol and

292   FOS from 2.30% w/v to 4.21% w/v and 2.40% w/v to 3.04% w/v, respectively,

293   increased growth, but further increase in the prebiotics concentration generated a

294   decrease in growth. Inulin produced highest growth at its lowest concentration of 4.00%

295   w/v, and produced lowest growth at its highest concentration of 8.00% w/v. It appeared

296   that growth of *L. acidophilus* ATCC 4962 was influenced by inulin in a linear manner,

297   while inoculum size, mannitol and FOS showed significant quadratic effects. Other than

298   main quadratic effects, interactions between inoculum size and FOS, and inoculum size

299   and inulin produced strongest influence towards growth, while the other interactions

300   were insignificant.

301   In this study, patterns of mean doubling time (Y_2) were studied using the

302   response surface (Figure 5) that was generated from the equation:

303        $Y_2 = 291.21 + 1.53X_1 + 0.32X_2 - 0.28X_3 - 1.31X_4 - 0.97X_1^2 + 0.095X_2^2 -$

304        $0.40X_3^2 - 0.60X_4^2 + 0.34X_1X_2 + 0.42X_1X_3 - 0.16X_1X_4 + 0.66X_2X_3 -$

305        $0.21X_2X_4 + 0.70X_3X_4$

306   Inoculum size, FOS and inulin showed significant quadratic effect, while mannitol did

307   not (Table 7). FOS mainly contributed to the interaction effects, with only interaction

308   terms involving FOS showed significant influence on mean doubling time. All these

309   significant interaction terms also showed positive regression coefficients, indicating that

310   either a decrease or increase in both factors will contribute to an increase in mean

311   doubling times.

312   The SCFA (Y_3) was obtained as a total of individual fatty acids, namely acetic,

313   butyric and propionic acids. A response surface (Figure 6) was generated from the

314   second-order equation:

315        $Y_3 = 60.03 + 6.67X_1 + 0.62X_2 + 2.30X_3 + 3.29X_4 - 6.08X_1^2 - 9.65X_2^2 -$

316        $10.69X_3^2 - 12.34X_4^2 + 0.66X_1X_2 + 3.80X_1X_3 + 4.84X_1X_4 + 1.45X_2X_3$

317        $+ 1.29X_2X_4 + 3.20X_3X_4$

318   All factors produced significant quadratic effects on production of SCFA. Response

319   surfaces produced showed that the production of SCFA appeared to be growth

320   associated.

321

322   **DISCUSSION**

323   Various factors normally affect the response surfaces that are produced. Thus,

324   screening experiments are needed to segregate important main effects from less

325   important ones (Montgomery, 1996). In this study, first degree order equation was

326 generated and significance of factors was tested using screening experiments. A

327 complete replication of the seven factors using a 2^x factorial design would need 128

328 experimental runs. However, only seven degree of freedoms would be needed to

329 estimate main effects, and 21 degree of freedoms would estimate two-factor interaction

330 effects, while the remaining 99 degree of freedoms would estimate error or/and three or

331 higher-factor interaction effects (Cox and Reid, 2000). Thus, a partial two-level factorial

332 design (2^{7-2}) was applied in this study. Partial factorial designs are capable of

333 identifying important factors using less number of experimental runs without loss of

334 information on main factor effects and their interactions (Li et al. 2002). Following the

335 screening of significant factors, design points were subjected to steepest ascent before

336 subsequent optimization steps. Steepest ascent or steepest descent involved the

337 generation of mathematical movements along an ascending or descending path until no

338 improvement occurred (Montgomery, 1996).

339   A significant quadratic regression, insignificant lack-of-fit and a small total

340 variation (4.60%) that was not explained by the model, suggested that the model

341 accurately represented data in the experimental region. This also indicated that second-

342 order terms were sufficient and higher-order terms were not necessary (Oh et al. 1995).

343 It must also be noted that the *t* value of the quadratic term of inoculum size (X_1^2) was

344 higher than others (Table 5), indicating that the quadratic effect of inoculum size had

345 the strongest effect on cholesterol removal, which was also confirmed using the

346 perturbation plot. Validation experiments showed that the predicted value was 58.142

347 µg/ml while the actual experimental result was 52.941 µg/ml. However, it must be

348 noted that the conditions for both were slightly different. The predicted value was

349 obtained at the predicted 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v

350 FOS and 5.82% w/v inulin, while the actual experiments were conducted with 2.60%

351   w/v inoculum size, 4.10% w/v mannitol, 3.30% w/v FOS and 5.80% w/v inulin. Under

352   such dissimilarity, the difference between the prediction and actual data was only

353   8.95%. The obvious difference of cholesterol removal between the optimum, high-

354   point, low-point and center-point media proved the validity of the model and the

355   reproducibility of the prediction.

356   From Table 5, it must be noted that the coefficient estimates of the interaction

357   terms of (X_2, X_4) and (X_3, X_4) had negative signs $(X_{24} = -0.50, X_{34} = -1.01)$. These

358   negative signs may imply that for an increase of the response, the coded levels of $(X_2,$

359    $X_4)$ and (X_3, X_4) must have different signs, either one must be higher than zero and the

360   other lower than zero (Oh et al. 1995). However, it must be noted that the optimum was

361   achieved at $(X_2 = -0.082, X_4 = -0.092)$ and $(X_3 = -0.115, X_4 = -0.092)$, which would

362   produce a positive sign instead. This may be due to other terms that may dominate this

363   particular interaction term (Oh et al. 1995). Considering that the lack-of-fit test was

364   insignificant, other higher terms would not have contributed to this, thus, we postulate

365   that the linear term might have played a role.

366   The response surface of growth showed similar patterns with the response

367   surface of removal of cholesterol, indicating a strong correlation between removal of

368   cholesterol and growth. Previous studies also showed that cholesterol assimilation by

369   strains of *L. acidophilus* during refrigerated storage of nonfermented milk was

370   associated with bacterial growth and their viability, and was growth dependent (Piston

371   and Gilliland, 1994; Pereira and Gibson, 2002). This has led us to postulate that

372   cholesterol removal *in-vitro* was growth associated. Significant interaction terms of

373   inoculum size with FOS and inulin showed that these two prebiotics strongly

374   encouraged growth of *L. acidophilus* ATCC 4962. Comparing these two, a higher

375   coefficient of regression for X_1X_3 than X_1X_4 indicated that FOS was more preferred

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than inulin. Studies using bifidobacteria showed that the bifidogenic effects of inulin and FOS are independent of chain lengths or GF_n type. FOS of the GF_2 and GF_3 moiety were also found to be more rapidly consumed compared to GF_4 (Kaplan and Hutkins, 2000). All these may have contributed to the preference of *L. acidophilus* ATCC 4962 on FOS than on inulin, and the fact that linear decrease in concentration of inulin contributed to an increase in growth.

Mean doubling time was used as a measure of the effectiveness of a specific carbon source in modulating bacterial growth rate (Bruno et al. 2002). Of all factors, FOS contributed significantly in the interaction patterns of mean doubling time, and higher growth rates (lower mean doubling time) were obtained at lower concentration of FOS (Figure 5). It was previously reported that both the uptake and hydrolysis of FOS are induced by higher oligosaccharides but repressed by products of their hydrolysis (Kaplan and Hutkins, 2003). In this experiment, it appeared that at higher concentration of FOS, more product of hydrolysis were produced and repressed bacterial growth rate, producing a higher mean doubling time. It must also be noted that the interaction between FOS and inulin produced lower mean doubling times when one factor was at lower levels and the other at higher levels . This indicated that when FOS was at its lower level, *L. acidophilus* ATCC 4962 utilized a higher level of inulin for higher growth rate and vice versa. It appeared that although *L. acidophilus* ATCC 4962 preferred FOS over inulin, but under conditions of substrate limitation, inulin was beneficially utilized for the modulation of growth rate.

The major products of metabolism of prebiotics are short chain fatty acids (SCFA), carbon dioxide and hydrogen, and bacterial cell mass (Cummings et al. 2001). Although much work has been done on SCFA production and the significance of the individual acids, no particular pattern of SCFA production from prebiotic fermentation

401   has emerged as yet. Hence, in this study, we analyzed the SCFA production from

402   fermentation of mannitol, FOS and inulin by *L. acidophilus* ATCC 4962. Production of

403   SCFA appeared to be growth associated and correlated with the patterns of cholesterol

404   removal. Although all factors significantly affected the production of SCFA, mannitol

405   exhibited the strongest effect (Table 7). While the utilization of FOS and inulin has been

406   widely reported, the utilization of mannitol to produce high concentration of SCFA was

407   less studied and was also found to be strain dependent. Lactic acid bacteria that

408   produced NADH oxidase would have the alternative $NADH\text{-}H^+$-oxidizing mechanism,

409   resulting in higher ability to grow on substrates more chemically reduced than glucose,

410   such as mannitol (Stanton et al. 1999). This may contribute to the better growth of *L.*

411   *acidophilus* ATCC 4962 in the presence of mannitol and subsequently produced higher

412   amount of SCFA and higher cholesterol removal. Previous study showed that strains of

413   *L. acidophilu*s that utilized mannitol also exhibited capability of cholesterol uptake

414   (Gupta et al. 1996).

415   In conclusion, cholesterol removal was optimized after selecting a combination

416   of inoculum size and prebiotic, with the predicted optimum removal of 58.142 µg ml^{-1}

417   obtained at 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v FOS and 5.82%

418   w/v inulin. Validation experiment showed that RSM was reliable in developing a

419   model, optimization of factors, and analysis of interaction effects. Analysis of growth,

420   mean doubling time and production of SCFA showed that cholesterol removal and the

421   production of SCFA was growth associated.

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Table1. Treatment combinations and response for screening experiments.

Standard order	Factors*							Response
	Inoculum size (% w/v)	Sorbitol (% w/v)	Mannitol (% w/v)	FOS (% w/v)	Hi-maize (% w/v)	Inulin (% w/v)	Maltodextrin (% w/v)	Cholesterol assimilated ($\mu g\ ml^{-1}$)
1	-1	-1	-1	-1	-1	1	1	31.36
3	1	-1	-1	-1	-1	-1	-1	33.13
5	-1	1	-1	-1	-1	-1	-1	25.52
7	1	1	-1	-1	-1	1	1	36.09
9	-1	-1	1	-1	-1	-1	1	27.71
11	1	-1	1	-1	-1	1	-1	39.17
13	-1	1	1	-1	-1	1	-1	32.53
15	1	1	1	-1	-1	-1	1	36.15
17	-1	-1	-1	1	-1	-1	-1	27.50
19	1	-1	-1	1	-1	1	1	39.01
21	-1	1	-1	1	-1	1	1	31.51
23	1	1	-1	1	-1	-1	-1	34.90
25	-1	-1	1	1	-1	1	-1	34.58
27	1	-1	1	1	-1	-1	1	36.15
29	-1	1	1	1	-1	-1	1	30.64
31	1	1	1	1	-1	1	-1	39.58
33	-1	-1	-1	-1	1	1	-1	28.70
35	1	-1	-1	-1	1	-1	1	34.22
37	-1	1	-1	-1	1	-1	1	26.30
39	1	1	-1	-1	1	1	-1	36.20
41	-1	-1	1	-1	1	-1	-1	28.49
43	1	-1	1	-1	1	1	1	38.54
45	-1	1	1	-1	1	1	1	31.09
47	1	1	1	-1	1	-1	-1	34.01
49	-1	-1	-1	1	1	-1	1	25.25
51	1	-1	-1	1	1	1	-1	38.23
53	-1	1	-1	1	1	1	-1	30.16
55	1	1	-1	1	1	-1	1	35.73
57	-1	-1	1	1	1	1	1	33.59
59	1	-1	1	1	1	-1	-1	36.82
61	-1	1	1	1	1	-1	-1	31.61
63	1	1	1	1	1	1	1	40.52
65	0	0	0	0	0	0	0	32.81
66	0	0	0	0	0	0	0	31.98
67	0	0	0	0	0	0	0	33.02
68	0	0	0	0	0	0	0	31.88
69	0	0	0	0	0	0	0	33.96

*Inoculum size: 0.10-0.30% w/v; Sorbitol: 0.50-1.50% w/v; Mannitol: 0.50-1.50% w/v; Maltodextrin: 0.50-1.50% w/v; Hi-amylose maize: 0.50-1.50% w/v; FOS: 0.50-1.50% w/v; Inulin: 0.50-1.50% w/v.

532   Table 2. Analysis of variance and coefficient estimates for the evaluation of the first-

533   order model.

Source of variation	Sum of squares	DF[*]	Mean square	F-value	P-value
Model†	1115.371	4	278.84	148.73	< 0.0001
Curvature	1.41	1	1.41	0.75	0.3890
Residual	118.11	63	1.87		
Lack-of-fit	49.73	27	1.84	0.97	0.5269
Pure error	68.38	36	1.90		
Correlation total	1234.90	68			

Factor:	Coefficient estimate	DF	Standard error	t-value	P-value
Inoculum size (X_1)	3.50	1	0.17	20.43	0.0001‡
Mannitol (X_3)	1.17	1	0.17	6.83	0.0001‡
FOS (X_6)	0.83	1	0.17	4.85	0.0001‡
Inulin (X_7)	1.77	1	0.17	10.36	0.0001‡

534   [*]DF: degree of freedom.
535   †$R^2 = 0.9042$.
536   ‡Significant at alpha 0.05.
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559   Table 3: Coordination path of steepest ascent for all chosen factors in coded and natural levels.

Step	Coded factors*				Natural factors†				Cholesterol removed (µg ml^{-1})
	ξ_1	ξ_3	ξ_6	ξ_7	X_1	X_2	X_3	X_4	
1) Base	0	0	0	0	0.20	1.00	1.00	1.00	16.478
Δ	5	1.67	1.19	2.53	(5)(0.1) = 0.5	(1.67)(0.50) = 0.84	(1.19)(0.50) = 0.60	(2.53)(0.50) = 1.27	
2) Base + Δ	5	1.67	1.19	2.53	0.70	1.84	1.60	2.27	36.563
3) Base + 2Δ	10	3.34	2.38	5.06	1.20	2.68	2.20	3.54	44.375
4) Base + 3Δ	15	5.01	3.57	7.59	1.70	3.52	2.80	4.81	50.781
5) Base + 4Δ	20	6.68	4.76	10.12	2.20	4.36	3.40	6.08	50.938
6) Base + 5Δ	25	8.35	5.95	12.65	2.70	5.20	4.00	7.35	48.813
7) Base + 6Δ	30	10.02	7.14	15.18	3.20	6.04	4.60	8.62	47.497

560   *ξ_1: inoculum size (% w/v), ξ_3: mannitol (% w/v), ξ_6: FOS (% w/v); ξ_7: inulin (% w/v).

561   †X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v); X_4: inulin (% w/v).

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576   Table 4. Combination matrix of the central composite design (CCD) using coded levels

577   for the response of cholesterol removal.

Standard run	Block*	Factors				Cholesterol removal ($\mu g\ ml^{-1}$)†
		Inoculum size (X_1)	Mannitol (X_2)	FOS (X_3)	Inulin (X_4)	
1	1	-1	-1	-1	-1	30.367
2	1	1	-1	-1	-1	46.304
3	1	-1	1	-1	-1	29.586
4	1	1	1	-1	-1	41.461
5	1	-1	-1	1	-1	26.461
6	1	1	-1	1	-1	42.086
7	1	-1	1	1	-1	31.929
8	1	1	1	1	-1	47.086
9	1	-1	-1	-1	1	28.023
10	1	1	-1	-1	1	40.367
11	1	-1	1	-1	1	23.648
12	1	1	1	-1	1	39.117
13	1	-1	-1	1	1	18.023
14	1	1	-1	1	1	38.179
15	1	-1	1	1	1	24.273
16	1	1	1	1	1	34.351
17	1	0	0	0	0	53.179
18	1	0	0	0	0	63.648
19	1	0	0	0	0	56.304
20	1	0	0	0	0	60.054
21	2	-2	0	0	0	15.211
22	2	2	0	0	0	33.414
23	2	0	-2	0	0	32.164
24	2	0	2	0	0	23.804
25	2	0	0	-2	0	34.586
26	2	0	0	2	0	24.976
27	2	0	0	0	-2	25.523
28	2	0	0	0	2	35.836
29	2	0	0	0	0	60.836
30	2	0	0	0	0	50.523

578   *1, first day of experiment; 2, second day of experiment.
579   †All factorial and axial points are means of duplicates.
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584   Table 5. Analysis of variance of the second-order model* and coefficient estimates for

585   the response Y_0 and factors X_1, X_2, X_3 and X_4.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model†	4302.42	14	307.32	10.78	0.0001
Residual	399.17	14	28.51		
Lack-of-Fit	284.11	10	28.41	0.99	0.5541
Pure error	115.07	4	28.77		
Total	4870.60	29			

Factor‡	Coefficient estimate	DF	Standard error	t-value	P-value
Intercept	$c = 56.58$	1	2.21		
X_1	$c_1 = 6.38$	1	1.09	5.85	0.0001§
X_2	$c_2 = -0.63$	1	1.09	-0.58	0.5735
X_3	$c_3 = -1.49$	1	1.09	-1.36	0.1938
X_4	$C_4 = -1.19$	1	1.09	-1.10	0.2915
X_1^2	$c_{11} = -7.34$	1	1.02	-7.20	0.0001§
X_2^2	$c_{22} = -6.42$	1	1.02	-6.30	0.0001§
X_3^2	$c_{33} = -5.97$	1	1.02	-5.86	0.0001§
X_4^2	$C_{44} = -5.75$	1	1.02	-5.64	0.0001§
X_1X_2	$c_{12} = -0.72$	1	1.33	-0.54	0.5993
X_1X_3	$c_{13} = 0.34$	1	1.33	0.250	0.8044
X_1X_4	$C_{14} = -0.034$	1	1.33	-0.026	0.9799
X_2X_3	$C_{23} = 1.51$	1	1.33	1.13	0.2774
X_2X_4	$C_{24} = -0.50$	1	1.33	-0.38	0.7120
X_3X_4	$C_{34} = -1.01$	1	1.33	-0.76	0.4615

586   *$Y_0 = 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2 -$
587    $5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 - 1.01X_3X_4$
588   †$R^2 = 0.9540$.
589   ‡X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v), X_4: inulin (%
590   w/v).
591   §Significant at alpha 0.05.

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Table 6. Combination matrix of the central composite design (CCD) using coded levels for the factors and five responses.

Standard run	Block*	Factors†				Responses‡		
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	1	-1	-1	-1	-1	39.629	288.677	6.308
2	1	1	-1	-1	-1	35.996	290.797	13.064
3	1	-1	1	-1	-1	38.381	288.303	8.220
4	1	1	1	-1	-1	33.925	290.649	16.503
5	1	-1	-1	1	-1	28.365	284.406	5.992
6	1	1	-1	1	-1	35.774	288.435	16.711
7	1	-1	1	1	-1	30.550	286.989	8.915
8	1	1	1	1	-1	36.249	290.791	15.324
9	1	-1	-1	-1	1	28.398	287.901	5.131
10	1	1	-1	-1	1	32.935	288.418	24.531
11	1	-1	1	-1	1	23.948	285.813	11.966
12	1	1	1	-1	1	32.318	288.530	17.959
13	1	-1	-1	1	1	20.730	286.911	7.239
14	1	1	-1	1	1	32.278	288.579	35.922
15	1	-1	1	1	1	24.742	286.840	7.448
16	1	1	1	1	1	31.398	291.750	62.947
17	1	0	0	0	0	38.706	290.243	67.026
18	1	0	0	0	0	48.981	291.175	53.419
19	1	0	0	0	0	38.739	290.372	46.826
20	1	0	0	0	0	42.216	291.505	67.139
21	2	-2	0	0	0	19.677	284.734	36.543
22	2	2	0	0	0	31.106	292.091	45.701
23	2	0	-2	0	0	24.825	292.169	31.714
24	2	0	2	0	0	24.734	293.195	22.015
25	2	0	0	-2	0	32.519	291.310	23.119
26	2	0	0	2	0	27.326	290.102	22.252
27	2	0	0	0	-2	46.054	290.716	16.866
28	2	0	0	0	2	31.942	289.108	15.285
29	2	0	0	0	0	45.946	290.791	44.787
30	2	0	0	0	0	38.688	291.465	72.814

*1, first day of experiment; 2, second day of experiment.
†X_1 = inoculum size, X_2 = mannitol, X_3 = FOS, X_3 = inulin.
‡Y_1 = growth (%), Y_2 = mean doubling time (min), Y_3 = SCFA (mmol l^{-1}).

609 Table 7. Regression coefficients of the second-order equation* for the five responses†.

Coefficient	Y_1	Y_2	Y_3
c	41.97	291.21	60.03
c_1	2.46‡	1.53‡	6.67‡
c_2	-0.12	0.32	0.62
c_3	-1.49‡	-0.28	2.30
c_4	-3.35‡	-0.31	3.29
c_{11}	-3.90‡	-0.97‡	-6.08‡
c_{22}	-4.05‡	0.095	-9.65‡
c_{33}	-2.77‡	-0.40‡	-10.69‡
c_{44}	-0.50	-0.60‡	-12.34‡
c_{12}	-0.22	0.34	0.66
c_{13}	1.66‡	0.42‡	3.80
c_{14}	1.63‡	-0.16	4.84
c_{23}	0.89	0.66‡	1.45
c_{24}	-0.08	-0.21	1.29
c_{34}	0.53	0.70‡	3.20
R^2	0.9173	0.9377	0.8448
P-value	0.0001	0.0001	0.0016

610 *$Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 +$
611 $c_{23}X_2X_3.$
612 †Y_1 = growth (%), Y_2 = mean doubling time (min), Y_5 = SCFA (mmol l^{-1}).
613 ‡Significant at alpha = 0.05.

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627 **Figure 1.** Perturbation plot of inoculum size (A), mannitol (B), FOS (C) and inulin (D).

628

629 **Figure 2.** Response surface for cholesterol removal (μg ml^{-1}) from the effects of (A)

630 FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in

631 the axes were fixed at their respective optimum levels.

632

633 **Figure 3.** Cholesterol removal by *L. acidophilus* ATCC 4962 in the optimum (■),

634 center-point (●), high-point (▲) and low-point (♦) media, for the validation

635 experiments. Factors combination for optimum medium were: inoculum size 2.60%

636 w/v, mannitol 4.10% w/v, FOS 3.30% w/v and inulin 5.80% w/v. Center-point medium

637 were: inoculum size 2.20% w/v, mannitol 4.30% w/v, FOS 3.400% w/v and inulin

638 6.00% w/v. High-point medium were: inoculum size 3.20% w/v, mannitol 6.30% w/v,

639 FOS 4.40% w/v and inulin 8.00% w/v, and low-point medium were inoculum size

640 1.20% w/v, mannitol 2.30% w/v, FOS 2.40% w/v and inulin 4.00% w/v. Error bars

641 represent standard error of means; n = 3.

642

643 **Figure 4.** Response surface for growth (%) from the effects of (A) FOS and mannitol,

644 and (B) inoculum size and inulin. Factors that were not included in the axes were fixed

645 at their respective optimum levels.

646

647 **Figure 5.** Response surface for mean doubling time (min) from the effects of (A)

648 inoculum size and FOS, and (B) FOS and inulin. Factors that were not included in the

649 axes were fixed at their respective optimum levels.

650

651   **Figure 6.** Response surface for the production of SCFA (mmol l^{-1}) from the effects of

652   (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included

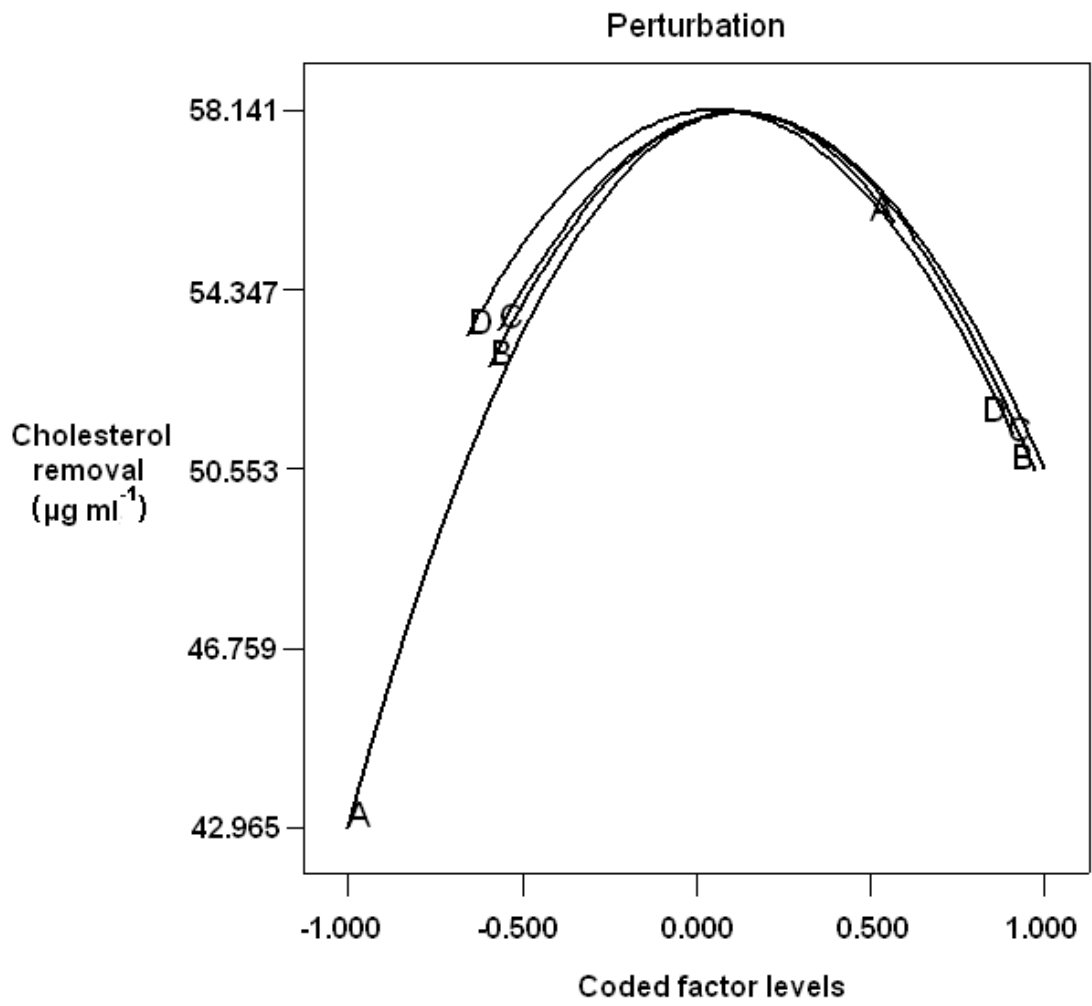653   in the axes were fixed at their respective optimum levels.
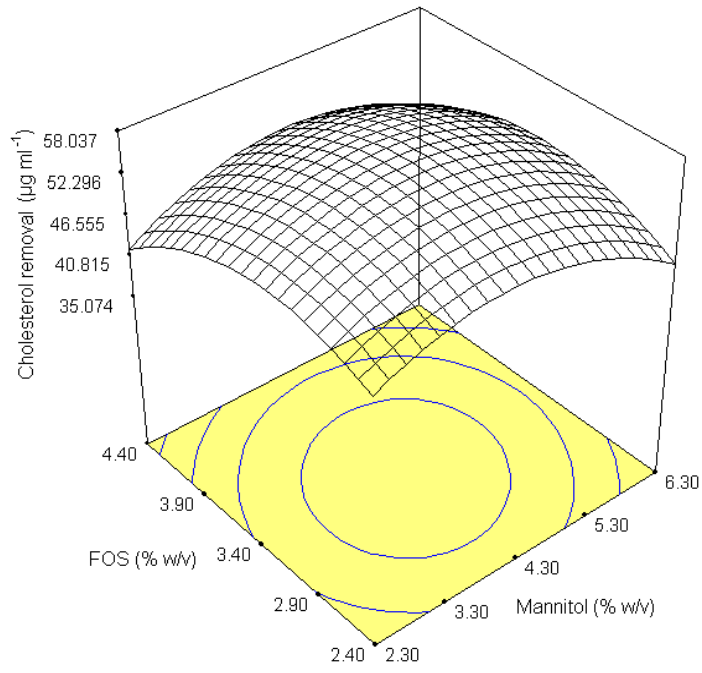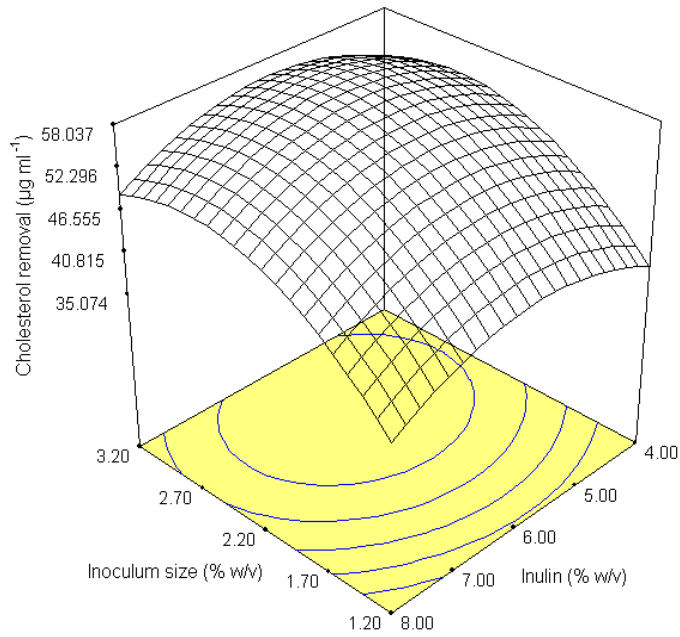
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Perturbation

Cholesterol removal ($\mu g\ ml^{-1}$)

Coded factor levels

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667 **Figure 1.**
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679 (A)
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682 (B)
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685 **Figure 2.**

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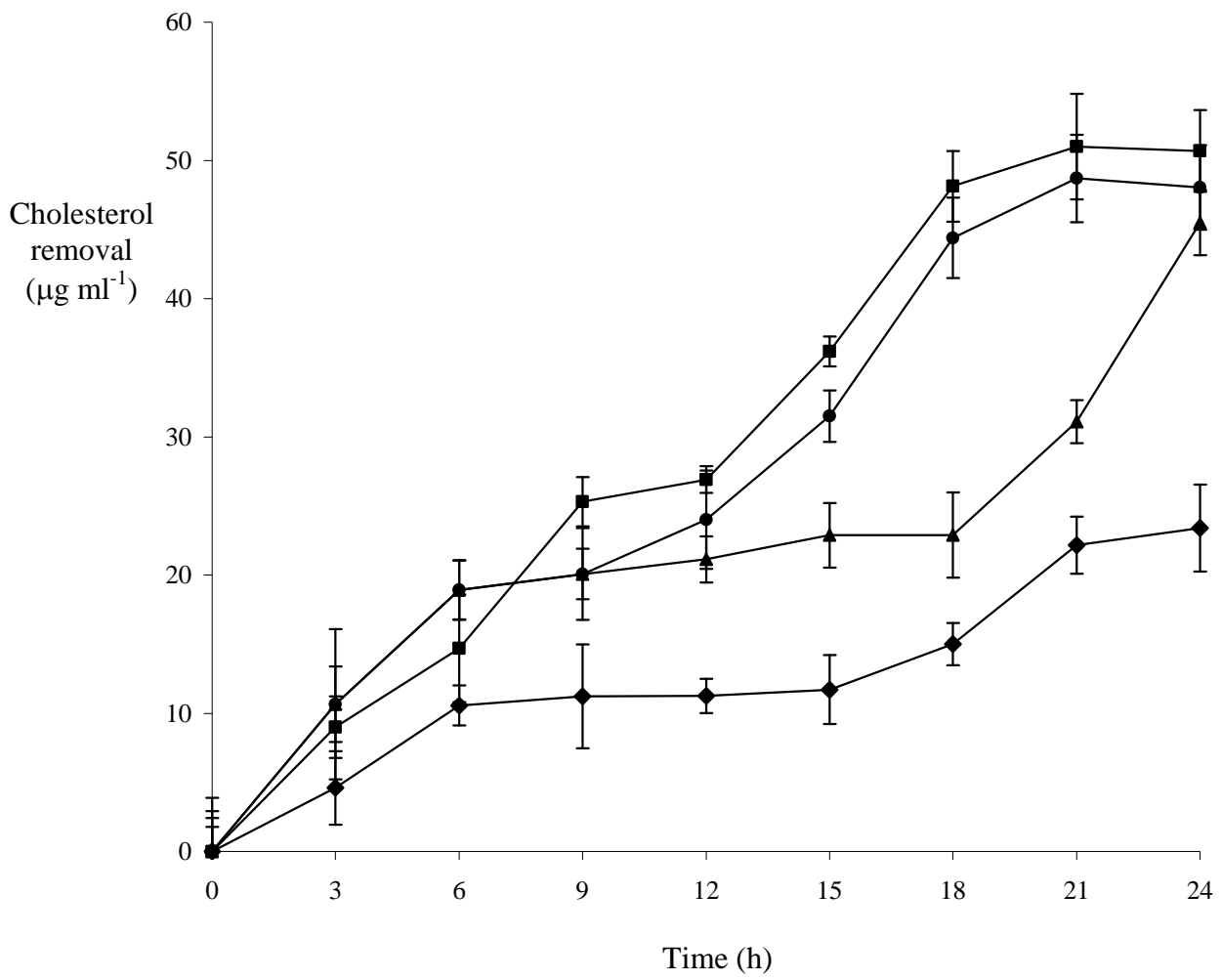
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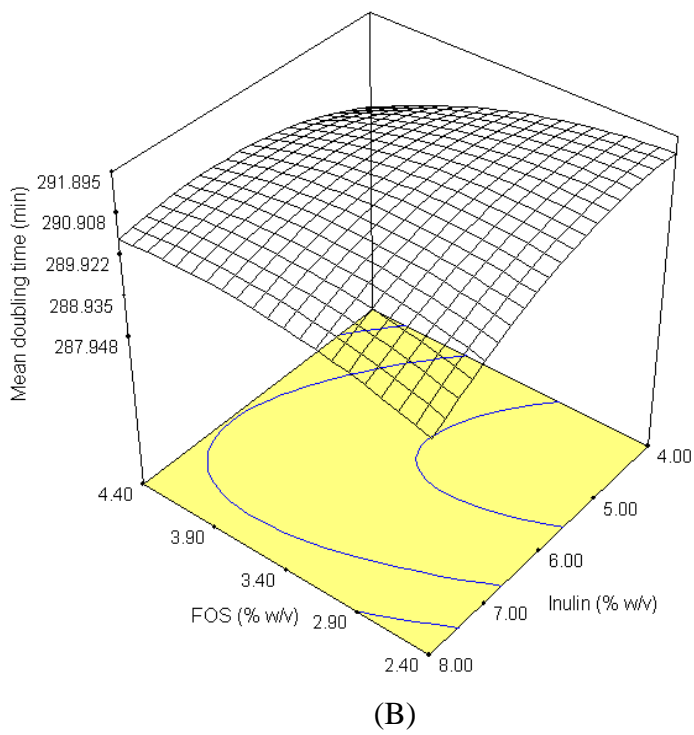688   **Figure 3.**

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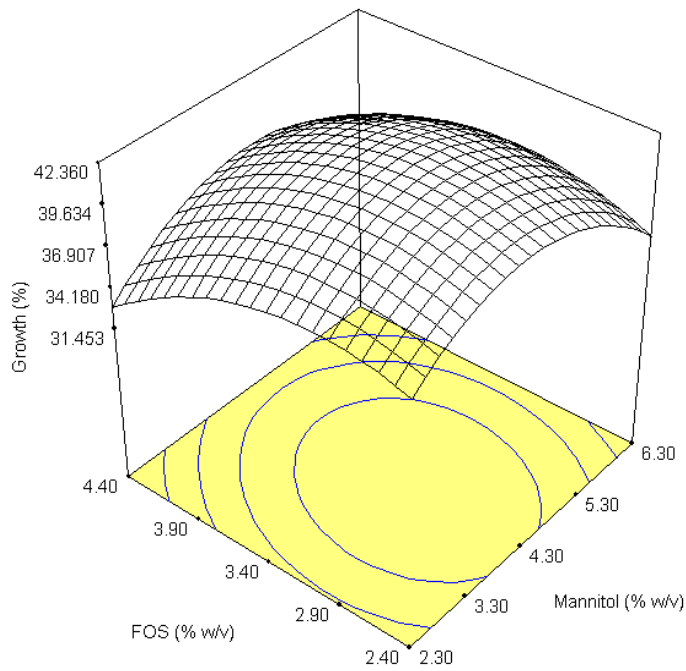
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697 (A)
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700 (B)
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704

705 **Figure 4.**

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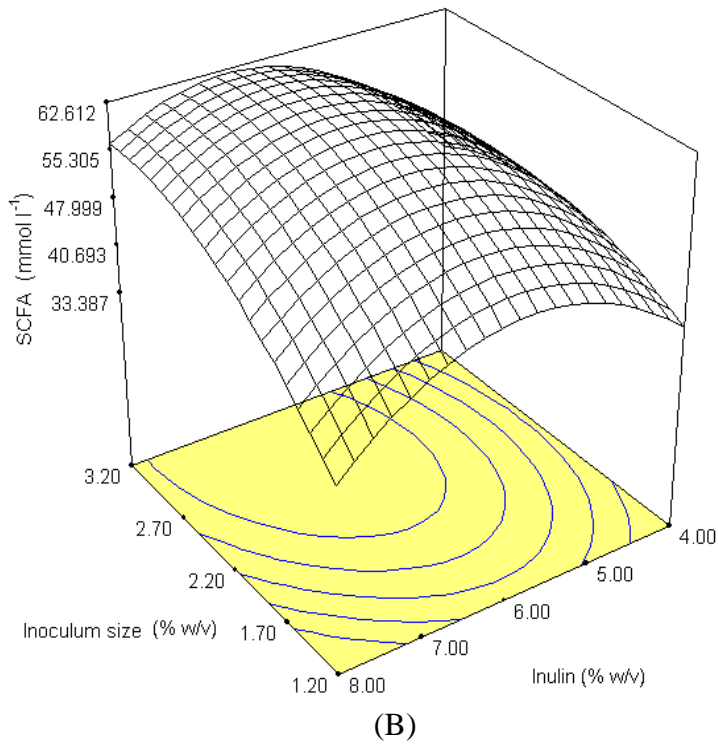
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711 (A)
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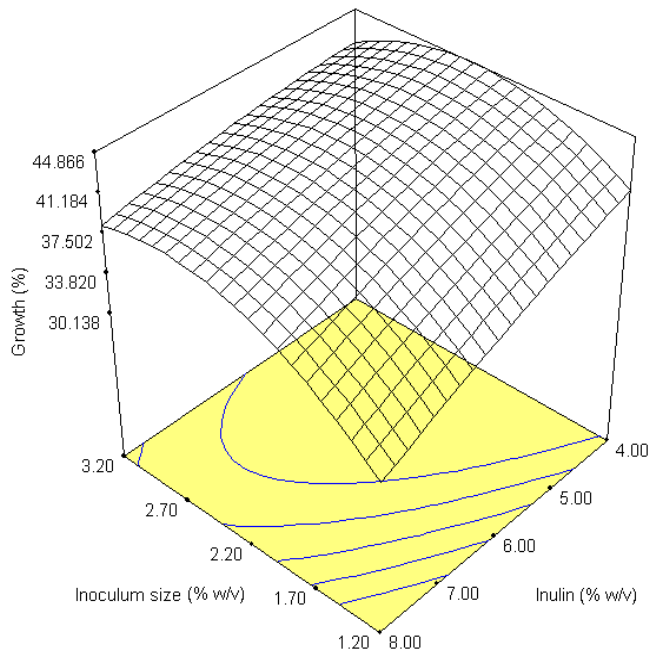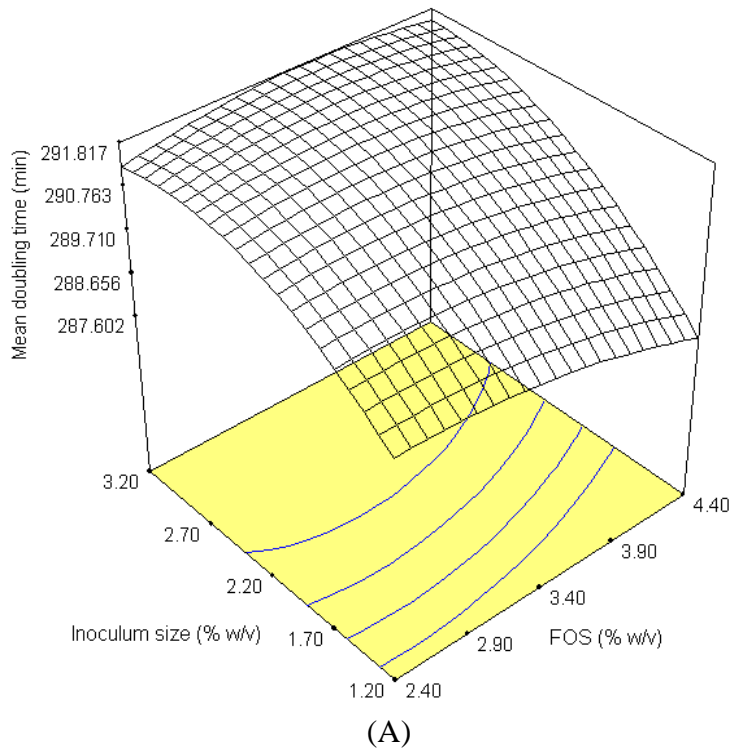
713
714 (B)
715

716 **Figure 5.**

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718
719 (A)
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721
722 (B)
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726 **Figure 6.**