Effect of Flavourzyme® on Angiotensin-Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by Lactobacillus helveticus

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4

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26 **Practical Application**

27 *Lactobacillus helveticus* in combination with Flavourzyme® was used for producing ACE-I

28 peptides from reconstituted skim milk and whey protein concentrate. Fermentation of skim

29 milk by *L. helveticus* in combination with Flavourzyme® resulted in >80% ACE-I after 8 h.

30 These conditions can be used for developing a functional drink with antihypertensive activity.

31

32 **Abstract**

33 Angiotensin converting enzyme inhibitory (ACE-I) activity as affected by *Lactobacillus*

34 *helveticus* strains (881315, 881188, 880474 and 880953), and supplementation with a

35 proteolytic enzyme was studied. Reconstituted skim milk (12 % RSM) or whey protein

36 concentrate (4 % WPC), with and without Flavourzyme® (0.14 % w/w), were fermented with

37 4 different *L. helveticus* strains at 37 °C for 0, 4, 8, 12 hours. Proteolytic and *in vitro* ACE-I

38 activities, and growth were significantly affected ($P < 0.05$) by strains, media and with

39 enzyme supplementation. RSM supported higher growth and produced higher proteolysis and

40 ACE-I than that WPC without enzyme supplementation. The strains *L. helveticus* 881315 and

41 881188 were able to increase ACE-I to > 80 % after 8 h of fermentation when combined with

42 Flavourzyme® in RSM compared to the same strains without enzyme supplementation.

43 Supplementation of media by Flavourzyme® was beneficial in increasing ACE-I peptides in

44 both media. The best media to release more ACE-I peptides was RSM with enzyme

45 supplementation. The *L. helveticus* 881315 outperformed all strains as indicated by highest

46 proteolytic and ACE-I activities.

47

48 **Keywords:** ACE Inhibition, Flavourzyme, *Lactobacillus helveticus*, skim milk

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50

Introduction

Hypertension is considered a risk factor for coronary heart disease such as, myocardial infarction and stroke (FitzGerald and others 2004). According to the World Health Organization nearly one billion people worldwide suffer from hypertension (World Health Organization 2013). Hypertension is usually controlled by a number of drugs, the most common being synthetic angiotensin converting enzyme inhibitory (ACE-I) drugs such as captopril and enalapril (Hansson and others 1999; Turner and Hooper 2002). ACE-I drugs decrease active angiotensin-II production from inactive angiotensin-I (Erdos 1975; FitzGerald and others 2004). Angiotensin-II receptor antagonists are agents used to modify the renin-angiotensin-aldosterone system through blocking angiotensin receptors, resulting in a decrease in blood pressure (Miura and others 2011). Also, ACE-I is a single polypeptide chain, composed of 2 separate and independent catalytic domains. Each domain contains the zinc-binding; these domains, called N- and C-domains have a high conservation of sequence and exon structure (Soubrier and others 1988). N- and C-terminal domains of ACE are similar in amino acid sequence, although both domains are sensitive to chloride. The C-domain requires a much higher chloride concentration for optimal activity than that the N-domain. The 2 catalytic domains exhibit different sensitivities to individual ACE inhibitors (Michaud and others 1997). Long term use of synthetic ACE-I drugs however, may result in side effects such as, cough, skin rash or development of impaired renal function (Sesoko and Kaneko 1985; Coulter and Edwards 1987). Peptides such as Val-Pro-Pro and Ile-Pro-Pro derived from milk proteins (FitzGerald and Meisel 2000; Nielsen and others 2009; Pihlanto and others 2010; Pihlanto-Leppälä 2000; Pan and others 2005; Phelan and Kerins 2011; Tsai and others 2008) have been identified to have similar effects of ACE-I action opening possibilities of replacing or complementing synthetic drugs (FitzGerald and Meisel 2000; Pan and others 2005; Tsai and others 2008; Nielsen and others 2009; Yamaguchi and others 2009;

3

76   Pihlanto and others 2010; Phelan and Kerins 2011). Lactic acid bacteria (LAB) used to

77   produce fermented dairy products (i.e. yoghurt, fermented milk, cheeses) have shown varied

78   but significant ACE-I activities during fermentation as reported in several studies (Korhonen

79   2009; Phelan and Kerins 2011; Korhonen and Pihlanto 2003, 2006, 2007; Hernández-

80   Ledesma and others 2011). The use of specific LAB or proteases in producing ACE-I

81   peptides from various milk media (yoghurt, cheese, sour milk) have been reported (van der

82   Ven and others 2002; Donkor and others 2005; Pan and others 2005; Kilpi and others 2007;

83   Meena and others 2008; Tsai and others 2008; Korhonen 2009; Hamme and others 2009;

84   Ramchandran and Shah 2010, 2011; Tellez and others 2011; Chaves-López and others 2012;

85   García-Tejedor and others 2013).The proteolytic activity and bioactivity of peptides is

86   influenced by a number of factors, such as, type of growth media, fermentation time,

87   temperature and pH type of LAB species and strain type used for fermentation (Ramesh and

88   others 2012). Several bioactive peptides which have ACE-I activity have been derived from

89   hydrolysis of proteins using skim milk and whey protein concentrate (Madureira and others

90   2010; Donkor and others 2007b). Such peptides have clinically documented effects in the

91   reduction of hypertension in humans (Aihara and others 2005).

92   *Lactobacillus helveticus* (*L. helveticus*) is homo fermentative thermophilic lactic acid bacteria

93   and known to possess strong proteolytic activity and is used in the production of Swiss

94   cheese and fermented milk beverages (Kenny and others 2003; Griffiths & Tellez 2013). Due

95   to its high proteolytic activity, *L. helveticus* is more effective than that other LAB such as *L.*

96   *delbrueckii* sp. *bulgaricus* and *L. acidophilus* in the production of ACE-I peptides (Korhonen

97   and Pihlanto 2006). Several studies have reported the use of *L. helveticus* for production of

98   ACE-I peptides and *L. helveticus* strains are specifically used for cheese production such as

99   Swiss cheese (Maeno and others 1996; Leclerc and others 2002; Kilpi and others 2007;

100   Nielsen and others 2009; Sun and others 2009; Pan and Guo 2010a; Otte and others 2011;

4

101 Singh and others 2011; Lim and others 2011; Unal and Akalin 2012; Grifiths and Tellez

102 2013). The effect of temperature, fermentation time and initial pH of fermented milk by *L.*

103 *helveticus* has been reported for sour milk production (Pan and Guo 2010b). Peptides can be

104 derived from enzymatic hydrolysis and considered to be safer (Pihlanto-Leppälä, 2000).

105 Moreover, enzymatic hydrolysates exert a variety of additional physiological properties such

106 as antioxidant and antimicrobial (Pihlanto-Leppälä 2000). Some of these peptides are present

107 within the parent protein structure and could be released through proteolysis (FitzGerald and

108 Murray 2006). However, proteinases such as (Alcalase, chymotrypsin pancreatin, pepsin,

109 enzymes from bacterial and fungal) have been utilized to generate bioactive peptides

110 (Pihlanto-Leppälä, 2000). In this study, we chose Flavourzyme® individually or in

111 combination, to increase the production of peptides from milk proteins hydrolyses. Since

112 there are no published reports on the production of ACE-I peptides from milk employing

113 proteases and LAB, we screened *L. helveticus* strains for production of ACE-I peptides from

114 12 % reconstituted skim milk (RSM) and 4 % whey protein concentrate (WPC) with or

115 without protease (Flavourzyme®) supplementation by measuring the bacterial growth,

116 proteolytic activity and *in vitro* ACE-I activity. Therefore, the present study was performed to

117 evaluate the hypothesis that combination of Flavourzyme® with *L. helveticus* significantly

118 increase ACE-I % in both media.

119

120   **Material and methods**

121 **Material and chemical**

122

123 Four strains of *L. helveticus* 881315, 881188, 880474 and 880953 were obtained from

124 (Dairy Innovation Australia Ltd, Werribee, VIC, Australia) and stored in 40 % glycerol de

125 Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Ltd., West Heidelberg, VIC, Australia) at

126   −80 °C. Flavourzyme® was 1000 L (EC 3.4.11.1, an amino peptidase with an activity of

127   1000 Leucine Amino-peptidase (LAPU g^{-1}) as quoted by Novozymes Australia., NSW,

128   Australia). RSM was purchased from (Murray Goulburn Co-operative Co. Ltd., VIC.

129   Australia). WPC was obtained from (United Milk Tasmania Ltd., TAS Australia). MRS agar

130   was obtained from (Merck Pty. Ltd., VIC Australia). Anaerobic kit was purchased from (An

131   aero Gen™, Oxoid. Zweigniederlassung, Austria). The O-phthaldialdehyde (OPA) obtained

132   from (Sigma Aldrich., NSW Australia). Disodium tetra-borate was obtained from (Merck

133   Pty. Ltd., VIC Australia). Sodium dodecyl sulphate (SDS) was from (Merck Pty. Ltd). β-

134   mercaptoethanol (Sigma Aldrich). Trichloroacetic acid (TCA) was purchased from (Sigma

135   Aldrich). Advantech # 231 filter paper was from (Advantech Australia., NSW Australia). The

136   ACE enzyme and Hippuryl-histidyl-leucine (HHL) were obtained from (Sigma, St. Louis,

137   MO, USA). C-18 column Gemini® C18 110 Å (100 mm x 4.6 mm, 5 µm) was from

138   (Phenomenex, Pty Ltd., NSW Australia). Acetonitrile was from (Merck Pty. Ltd., VIC

139   Australia). RP- HPLC was from (Varian Analytical Instruments., CA USA). C-18

140   monomeric column (5 μm, 300 Å, 250 mm x 4.6 mm) was from (Grace Vydac, Hesperia CA,

141   USA). Freeze-dried was (Air vac Engineering Private Ltd., VIC, Australia, model FD-300).

142   For activation, an aliquot (100 µL) of each strain was individually transferred into MRS broth

143   and incubated at 37 °C for 24 hours (h). Weekly subculturing of bacteria into MRS broth was

144   performed to maintain the bacterial activity. Prior to each experiment, bacteria were

145   subcultured 3 times and fermented for 12 h in 12 % RSM or 14 % WPC.

146

147   **Media preparation and bacteria propagation** RSM (12 %) and WPC (4 %) were prepared

148   by dissolving appropriate quantities of skim milk powder (52 % lactose, 37 % protein, 8.6 %

149   ash and 1.2 % fat) and WPC (47.5 % lactose, 35 % protein, 9 % ash, 2.5 % fat) in distilled

150   water . Both media were heated to 90 °C for 30 minutes (min). The prepared media, with or

151   without 0.14 % (w/w) Flavourzyme®1000 L were inoculated with *L. helveticus* strains and

152   fermented at 37 °C for 4 h, 8 h and 12 h. Sixteen different combinations of bacterial strains,

153   Flavourzyme® (0.14%) and growth media (12 % RSM or 4% WPC) were used (Table 1).

154   Samples were collected and stored at -20 °C for analysis of bacterial growth, proteolytic

155   activity and ACE-I activities and peptide profiling by Reverse phase - High-performance

156   liquid chromatography (RP-HPLC).

157

158   **Methods**

159

160   **Measurement of bacterial growth**

161   Growth was assessed every 4 h up to 12 h during fermentation by pour plate method using

162   MRS agar following serial dilutions with 0.1 % peptone. The plates were incubated

163   anaerobically at 37 °C for 48 h using anaerobic jars with anaerobic kit. Plates having 25 - 250

164   colonies were counted and the growth was expressed as logarithm of colony forming unit

165   (cfu) per mL^{-1} of sample.

166

167   **Determination of proteolytic activity**

168   The O-phthaldialdehyde (OPA)   reagent was prepared by mixing 25 mL of 100 mM

169   disodium tetra-borate, 2.5 mL of 20 % (w/w) sodium dodecyl sulfate (SDS), 1 mL methanol

170   containing 40 mg of OPA and 100 µL of β-mercaptoethanol . The final volume was made to

171   50 mL with Deionized water. Briefly, the sample (3 mL) was mixed with equal volume of 1

172   % trichloroacetic acid (TCA) followed by filtration using Advantech # 231 filter paper .

173   Filtrate (150 µL) was placed into 4 mL cuvette and mixed with 3 mL OPA reagent, and

174   absorbance measured at 340 nm using UV-VIS spectrophotometer (LKB NOVASPEC II

175   Pharmacia, LKB Bio- Chrom, UK) after allowing 2 min of reaction time at room temperature

176 as a measure of proteolysis. The degree of proteolysis was determined as the difference

177 between proteolytic activities in fermented media to that of unfermented media (Donkor and

178 others 2007b).

179

180 **Determination of ACE-Inhibitory activity**

181 ACE-I activity was determined according to a Donkor and others (2007a). Briefly, fermented

182 media (WPC or RSM) (10 mL) was centrifuged at 4000 x g at 4 °C for 30 min and the

183 supernatant was freeze-dried for 72 h. The freeze-dried powder (40 mg) was dissolved in 2

184 mL Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride. ACE enzyme and

185 Hippuryl-histidyl-leucine (HHL) were prepared in Tris buffer. Fifty µL of 3.0 mM HHL, 50

186 µL of 1.25 MU ACE enzyme (from rabbit lung), and 50 µL of experimental samples were

187 placed in a glass tube and incubated for 1 h at 37 °C ensuring mixing for the first 30 min.

188 Glacial acetic acid (150 µL) was added to stop the reaction. The reaction mixture was stored

189 at -20 °C before further analysis of released hippuric acid (HA) by HPLC. An external

190 standard curve of hippuric acid was prepared to quantify the resultant hippuric acid in

191 fermented samples. An aliquot (20 µL) of the mixture was injected into Gemini® C18 110 Å

192 (100 mm x 4.6 mm, 5 µm) using Varian HPLC equipped with an auto sampler. The

193 separation was conducted at room temperature (~22 °C) at a mobile phase flow rate of 0.6

194 mL min^{-1}. The mobile phase consisting of 12.5 % (v/v) acetonitrile in Deionized -water, and

195 pH was adjusted to 3.0 using glacial acetic acid. Ultraviolet-visible detector was set at 228

196 nm. The % ACE-I was calculated as follows:

197
$$\text{ACEI \%} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100$$

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202

203 **RP-HPLC analysis of water-soluble peptides extract**

204 Reconstituted skim milk fermented with the highest ACE-I activity of strains; *L. helveticus*

205 881188 and 881315 (with or without combination of Flavourzyme®) were collected after 12 h

206 of fermentation (pH 4.6). From this, 50 mL sample was centrifuged at 4000 x g at 4 °C for 30

207 min to separate proteins. The supernatant containing soluble peptides was freeze-dried for 72

208 h. The powder (40 mg) was dissolved in 0.1 % trifluoroacetic acid (TFA). Water soluble

209 peptides were profiled by a RP- HPLC using C-18 monomeric column (5 μm, 300 Å, 250

210 mm x 4.6 mm) (Donkor and others 2007a).

211

212 **Statistical analysis**

213 All results are expressed as mean values of 3 replicates with standard deviation of the mean.

214 One way ANOVA was performed to differentiate the significant differences in the treatments

215 which were strains, growth media, presence or absence of Flavourzyme®, and fermentation

216 time. The level of significance was tested at $P < 0.05$. Fisher`s (least significant difference;

217 LSD) test was used to investigate significant differences among the treatment means.

218 Correlation analysis was carried out between variables for same bacteria strain, growth media

219 and presence or absence of Flavourzyme®. The degree of correlation between these variables

220 was expressed as Pearson coefficient (r) and corresponding *P* values. All statistical analyses

221 were carried out using SAS Version 9.0 software (SAS Institute Inc., Cary, NC, USA)

222

223 **Results and discussion**

224 **Preferential growth of *L. helveticus* in RSM media with Flavourzyme® compared to**

225 **WPC**

226   Figure 1 shows the microbial growth and pH in RSM and WPC during fermentation with *L.*

227   *helveticus*. All *L. helveticus* strains were able to grow in both media (Figure 1). Analysis of

228   variance showed that bacterial growth was significantly ($P < 0.05$) affected by media, media

229   supplementation with protease (Flavourzyme®), fermentation time and strain type. Higher

230   growth was significantly noted ($P < 0.05$) in RSM. This may be attributed to superior nutrient

231   profile of RSM (Kilpi and others 2007; Leclerc and others 2002) and higher specificity to

232   caseins than whey proteins. Media supplemented with Flavourzyme® led to increase growth

233   owing to enhanced number of released more peptides and amino acids required for bacterial

234   growth in log and early stationary phases, associated to no supplementation in both media

235   types (Kenny and others 2003). While *L. helveticus* 881315 showed the least growth (0.6)

236   Log_{10} cfu ml^{-1}, the *L. helveticus* 881188 showed highest growth (2) Log_{10} cfu ml^{-1} at 12 h

237   compared to other strains in RSM containing Flavourzyme® for the entire duration of

238   fermentation. *L. helveticus* strains 880474 and 880953 also showed the higher growth

239   compared to 881315 in RSM. It appears that Flavourzyme® supplementation prolonged the

240   log phase in 881188 whereas 880474 and 880953 strains went into a decline phase after 8 h.

241   In general, WPC showed a weak growth for all strains without the combination of

242   Flavourzyme® compared to same strains in combination with Flavourzyme® (Figure 1C and

243   D). However, growth for all strains in WPC with Flavourzyme® were increased significantly

244   at 8 h and declined after 8 h of fermentation at pH (3.4) possibly, due to the effect of pH and

245   heat treatment on WPC's nutrient contents as previously reported (Zisu and Shah 2003 ;

246   Dissanayake and others 2013). Furthermore the decrease in bacterial growth observed after 8

247   h of fermentation can be attributed to the production of lactic acid in media by growing lactic

248   acid bacteria which can inhibit bacterial growth at low pH concentrations. Similar growth

249   characteristics were noted with LAB at different temperatures and fermentation time in RSM

250   using *L. sakei* CTCstrains which showed inhibition of *L. sakei* growth due to lactic acid

251   production (Leroy and de Vuyst, 2001).However, different results were noted that *L.*

252   *helveticus* strain's cell counts were increased during fermentation 12 % RSM from (0 – 9) h

253   followed by a slight decrease in viable counts until (24 h) of the fermentation (Leclerc and

254   others 2002). It is clear that the difference observed in bacterial growth in both media could

255   be related to the different nature of proteins present (Leclerc and others 2002).

256

257   **Proteolytic activity is higher in RSM media with Flavourzyme®**

258   Milk proteins were hydrolysed by *L. helveticus* strains (881315, 881188, 880474 and

259   880953), resulting in an increase in the amount of free NH_3 groups as quantified by the OPA

260   method (Figure 2). The proteolytic activity of *L. helveticus* strains grown in RSM or WPC

261   with or without Flavourzyme® supplementation at 37 °C for 0-12 h increased with

262   fermentation time (Figure 2). All strains preferred RSM over WPC with or without

263   supplementation with Flavourzyme® as indicated by higher proteolysis. The activity

264   remained significantly lower (≤ 0.5) in WPC compared to RSM (> 0.78), indicating that

265   proteins of RSM particularly, casein were the preferred substrate by enzymes of *L. helveticus*

266   strains. This correlated to a similar trend in the growth pattern (Figure 1). The order of

267   proteolytic activity of *L. helveticus* strains in RSM was 881315 > 881188 > 880474 >

268   880953. Supplementation of RSM with Flavourzyme® increased the proteolytic activity of all

269   strains significantly (P < 0.05), reaching a maximum absorbance > 1.8 in 12 h by *L.*

270   *helveticus* 881315, 880474 and 881188 (Figure 2). Interestingly, the proteolytic activity of

271   strain 881315 was high during 12 h (Figure 2). However, the growth was weak in both media

272   (Figure 1). The activity in RSM with Flavourzyme® was approximately higher by 45-60 %

273   than that without Flavourzyme® even after 4 h of fermentation and was sustained over the 12

274   h duration of fermentation. However, except for *L. helveticus* 880953, the response to

275   Flavourzyme® in increasing proteolysis was similar after 8 h of fermentation. Flavourzyme®

276   appears to have hydrolysed large proteins present in RSM to intermediate peptides, which

277   were used by *L. helveticus* to produce small peptides and free amino acids (Leclerc and others

278   2002). Co-fermentation of RSM with Flavourzyme® supplementation with *L. helveticus*

279   strains reduced the time required for a given degree of proteolysis. These results suggest that

280   proteolysis were enhanced in the higher protein content was in media supplemented with

281   Flavourzyme® and that casein was a better substrate than that whey proteins for *L. helveticus*

282   strains. Similar results have been noted that proteolytic activity was enhanced in the higher

283   protein content medium and that casein fraction was a better substrate than whey proteins for

284   the extracellular proteinases of lactic acid bacteria (Leclerc and others 2002). In addition, the

285   amount of free NH_3 groups in the media increased sharply until 12 h except for media

286   without Flavourzyme® for which a slightly slower than that sharply increase was observed

287   after 8 h (Figure 2). The different proteolytic activities between strains could also be

288   explained by the higher proteolysis noted by Matar and others (1996) for *L. helveticus* L89,

289   compared to those measured for strains 881315, 881188, 880474, and 880953 in this study.

290

291   **ACE-Inhibitory activity is influenced by strain type, media and Flavourzyme®**

292   **combination**

293   The amount and type of peptides produced during hydrolysis of proteins present in RSM or

294   WPC influenced ACE-I activity which was measured using an ACE-I method according to

295   Donkor et al., (2007a). The % of ACE-I activity of *L. helveticus* strains (881315, 881188,

296   880474 and 880953) in RSM or WPC with or without Flavourzyme® at 37 °C for 12 h are

297   presented in Figure 2. Flavourzyme® alone was used as a control. ACE-I activity for all

298   strains in both media increased significantly during fermentation period (P < 0.05). However,

299   differences existed between strains and media used when compared at the same time of

300   fermentation. Media type, strains, supplementation of Flavourzyme® and fermentation time

12

301   had significant (P < 0.05) effects on ACE-I activity. As with proteolytic activity, ACE-I

302   activity increased as fermentation time increased for all strains. Supplementation of RSM

303   with Flavourzyme® significantly (P < 0.05) increased ACE-I activity of *L. helveticus* strains.

304   Except for *L. helveticus* 880474, ACE-I increased from 40-60 % to ≥ 85 % in RSM with

305   Flavourzyme® supplementation after 8 h of fermentation. The inhibition increased during

306   fermentation when *L. helveticus* 881315 and 881188 were used from 10 % to 89.82 % and

307   from 5 % to 85 % in RSM with supplementation, respectively (Figure 2B). While the same

308   strains in WPC with Flavourzyme® supplementation, the ACE-I % increased from 10 % to 65

309   % and 5 % to 60 % during 12 h, respectively (Figure 2D). Since both of these strains

310   demonstrated high proteolysis, co-fermentation with enzyme appeared to have produced

311   higher amounts of ACE-I peptides as evident in increased number of peaks (Figure 3). The

312   inhibitory activity remained high at 12 h for all strains except *L. helveticus* 880474 which

313   showed a significant drop in ACE-I after 4 h of fermentation. There was no significant

314   difference (P < 0.05) in ACE-I between hydrolysates produced from WPC with or without

315   Flavourzyme® at 4 h fermentation. Thereafter, ACE-I increased differentially among the

316   strains and a maximum of 89.82 % was observed for *L. helveticus* 881315 with

317   Flavourzyme® in RSM at 12 h. However, the growth Log_{10} of same strain was weak during

318   12 h (Figure 1), that means this strain has high proteolytic activity. This supported by study

319   reported that "the declining number of live bacteria alterations in ACE-inhibitory activity in

320   the cultures of peptidase-negative mutants could be detected during the course of cultivation,

321   which indicates that the proteolytic enzymes released from cells of *L. helveticus* play an

322   important role in the conversion of bioactive peptides" (Kilpi and others 2007). Data also

323   suggest a delayed effect with addition of Flavourzyme® in WPC. The increase in ACE-I was

324   significantly (P < 0.05) higher in RSM compared to WPC due to the addition of

325   Flavourzyme® and may be due to the sensitivity of whey protein to heat treatments (Banks

326   and others 1995; Patel and Creamer 2008). The ACE-I activity differed significantly between

327   strains. *L. helveticus* 881315 and 881188 showed higher ACE-I activity compared to other

328   strains in RSM (Figure 2). This implies that Flavourzyme® enhanced the production of ACE-

329   I peptides as previously reported and supported by the proteolysis pattern observed (Tsai and

330   others 2008). Since ACE-I almost doubled in the first 8 h of fermentation, Flavourzyme®

331   supplementation can be used to reduce time of hydrolysis required for production of ACE-I

332   peptides. The differences observed between RSM and WPC may be attributed to differences

333   in the type of proteins present and therefore the variety of peptides present in the hydrolysates

334   (Matar and others 1996; Pan and Guo 2010b). Preference to casein by proteinases has been

335   reported (Lim and others 2011; Cheison and others 2007; Matar and Goulet 1996). It was

336   possible to achieve maximum ACE-I of 60 % and 70 % without supplementation of RSM or

337   WPC by *L. helveticus* strain 881315 after 8 h of fermentation, respectively. On the other

338   hand, with supplementation of RSM, ACE-I % was achieved to 89.82 %. However,

339   fermentation process using Flavourzyme® alone as control have a small effective in

340   increasing ACE-I activity as indicated by the low ACE-I (20 - 38%) for hydrolysates

341   produced.

342   **RP-HPLC analysis of water-soluble peptide extracts suggests that *L. helveticus* 881315**

343   **with Flavourzyme® is most optimal**

344   The profiles of water-soluble peptide extracts of 12 h fermented skim milk hydrolysates by

345   best performer strains (as measured by proteolytic and ACE-I activities) *L. helveticus*

346   (881315 and 881188) with or without supplementation of Flavourzyme® are shown in Figure

347   3. The RP-HPLC elution profile of the hydrolysates was based on the hydrophobicity of the

348   peptides. In the control unfermented RSM only one peak appeared at 10 min (not shown)

349   only. The chromatograms (Figure 3A, 3C) show that 881315 and 881188 strains without

350   supplementation hydrolysed proteins resulting in peptides in the retention time range of 10-40

14

351 min and 10-45 min by strains respectively. Supplement with Flavourzyme® (Figure 3B, 3D)

352 generally aided both strains to increase proteolysis as evident by the presence of more

353 peptides appearing in the range of 10-65 min (881315) and 10-45 min (881188). Strain

354 881315 combined with Flavourzyme® was most optimal in terms of providing peptides by

355 facilitating proteolysis of caseins present in RSM. This corroborated to the high ACE-I

356 activity (Figure 2). However, supplementation was more beneficial to strain 881315 than that

357 881188.

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360

361 **Correlation between proteolytic activity, ACE-Inhibition and bacteria growth**

362 The correlation between proteolytic activity and anti-hypertensive properties expressed as

363 ACE-I and bacterial growth for the same bacterial strain, growth media and with or without

364 Flavourzyme® are presented in (Tables 2 and 3) for RSM and WPC respectively. A

365 significant correlation in growth with all measurements for each strain in RSM was evident

366 for except *L. helveticus* 880953 which did not grow well in both media ($P < 0.05$). Even

367 stronger correlations between the same measurements were observed for RSM supplemented

368 with Flavourzyme® (Table 2). This suggests that Flavourzyme® enhanced the proteolytic and

369 ACE-I activities of *L. helveticus* in RSM. ACE-I activity positively and strongly correlated

370 with proteolytic activity for each strain, both with or without Flavourzyme® ($P < 0.05$) (Table

371 2) implying that increased proteolytic activity increased the production of ACE-I peptides.

372 ACE-I activity had positive and strong correlation with bacterial growth (cfu) in RSM (with

373 or without Flavourzyme®) for all strains except *L. helveticus* 880953. This suggests that

374 proteolytic and ACE-I activities were growth dependent. Similar trend was observed in WPC

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378 **Conclusion**

379 Production of ACE-I peptides by *L. helveticus* varied between the strains due to differences

380 in proteolytic activity. Casein rich RSM supported higher growth, higher proteolytic activity

381 and produced higher ACE-I activities by all *L. helveticus* strains. Therefore, RSM is superior

382 to WPC as a medium for production of ACE-I peptides irrespective of supplementation with

383 protease, which generally increased hydrolysis of proteins to produce more ACE-I peptides.

384 Beneficial effects of protease supplementation were more pronounced in the first 8 h of

385 fermentation and also sustained thereafter. However, *L. helveticus* 881315 showed the lowest

386 growth. The highest ACE-I activity was observed in 12% RSM supplemented with

387 Flavourzyme® and up to 12 h fermentation by *L. helveticus* 881315 and 881188 at 37 °C,

388 respectively. These conditions will aid in the production of a functional fermented drink with

389 high ACE-I activity.

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Author Contributions

Fatah Ahtesh, Nagendra Shah and Vijay Mishra planned the study. Fatah Ahtash carried out

experimental work. Vijay Mishra helped in analysis and interpretation. Vijay Mishra and

Lily Stojanovska helped in preparing the manuscript.

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575 **Table 1-** Experimental design to analyse and measure the pH, growth, proteolytic activity

576 and % of ACE-inhibitory activities during (0, 4, 8 and 12 h) fermentation of *L. helveticus*

577 strains in 12 % RSM or 4 % WPC and with or without combination of Flavourzyme®

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Media used		*L. helveticus* strains used without combination	Combination of *L. helveticus* strains with Flavourzyme® (1 % v/v each)
RSM		881315	881315+Flavourzyme®
		881188	881188+Flavourzyme®
		880474	880474+Flavourzyme®
		880953	880953+Flavourzyme®
	control	Flavourzyme®	
WPC		881315	881315+Flavourzyme®
		881188	881188+Flavourzyme®
		880474	880474+Flavourzyme®
		880953	880953+Flavourzyme®
	control	Flavourzyme®	

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594   **Table 2-** Pearson coefficient correlations (r), proteolytic activity (OPA), ACE-inhibitory activity (ACE) and bacterial growth (CFU) for strain, *L.*

595   *helveticus* 881315, *L. helveticus* 881188, *L. helveticus* 880474 and *L. helveticus* 880953 grown in 12 % RSM at 37 °C for 12 h with or without

596   Flavourzyme® combination

| *L. helveticus* strains	Variables	Without Flavourzyme®			With Flavourzyme®		
		OPA	ACE	CFU	OPA	ACE	CFU
881315	OPA	1.000	0.988**	0.918**	1.000	0.979**	0.946**
	ACE		1.000	0.945**		1.000	0.864**
	CFU			1.000			1.000
881188	OPA	1.000	0.991*	0.874*	1.000	0.966*	0.852*
	ACE		1.000	0.882*		1.000	0.694**
	CFU			1.000			1.000
880474	OPA	1.000	0.962**	0.805*	1.000	0.978*	0.835*
	ACE		1.000	0.825*		1.000	0.928*
	CFU			1.000			1.000
880953	OPA	1.000	0.989*	0.690**	1.000	0.891*	0.863*
	ACE		1.000	0.588**		1.000	0.597*
	CFU			1.000			1.000

597   * $P < 0.05$, **$P < 0.01$.

598 **Table 3-** Pearson coefficient correlations (r), proteolytic activity (OPA), ACE-inhibitory activity (ACE) and bacterial growth (CFU) of, *L.*

599 *helveticus* 881315, *L. helveticus* 881188, *L. helveticus* 880474 and *L. helveticus* 880953 grown in 4 % WPC at 37 °C for 12 h with or without

600 Flavourzyme® combination

L. helveticus strains	Variables	Without Flavourzyme®			With Flavourzyme®		
		OPA	ACE	CFU	OPA	ACE	CFU
881315	OPA	1.000	0.615*	0.713*	1.000*	0.530*	0.580*
	ACE		1.000	0.927**		1.000	0.949**
	CFU			1.000			1.000
881188	OPA	1.000	0.946*	0.866*	1.000	0.626*	0.562*
	ACE		1.000	0.947*		1.000	0.978**
	CFU			1.000			1.000
880474	OPA	1.000	0.615**	0.666*	1.000	0.867*	0.907*
	ACE		1.000	0.894*		1.000	0.971*
	CFU			1.000			1.000
880953	OPA	1.000	0.716**	0.567*	1.000	0.686*	0.608*
	ACE		1.000	0.827*		1.000	0.634**
	CFU			1.000			1.000

601 *$P < 0.05$, ** $P < 0.01$.
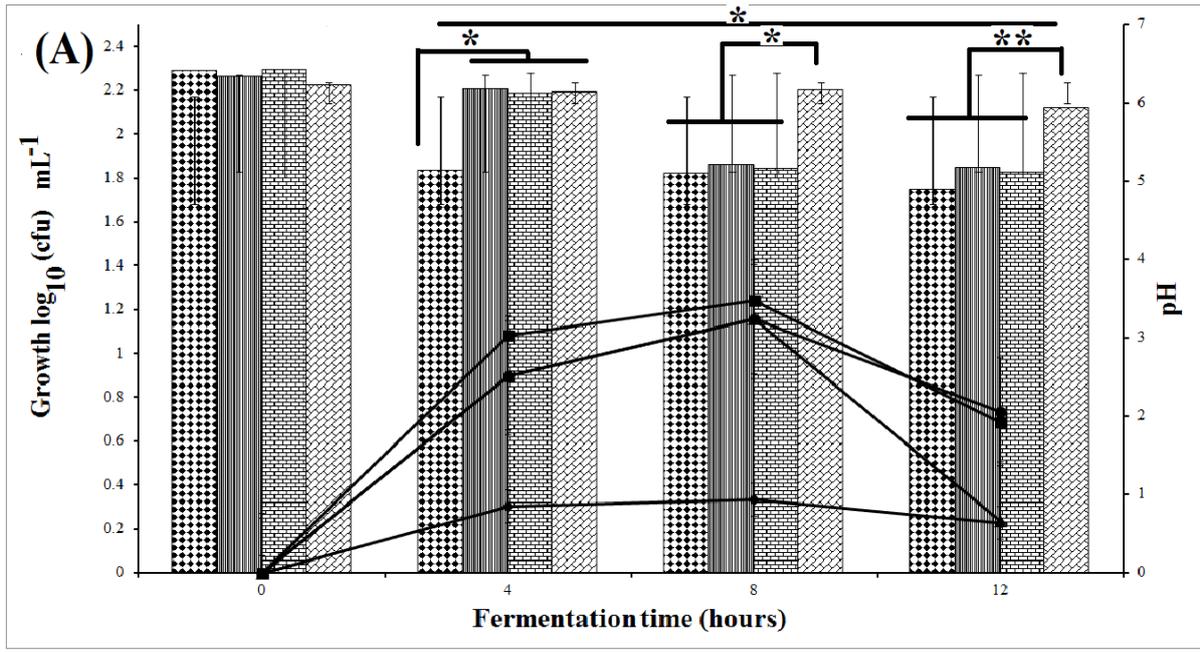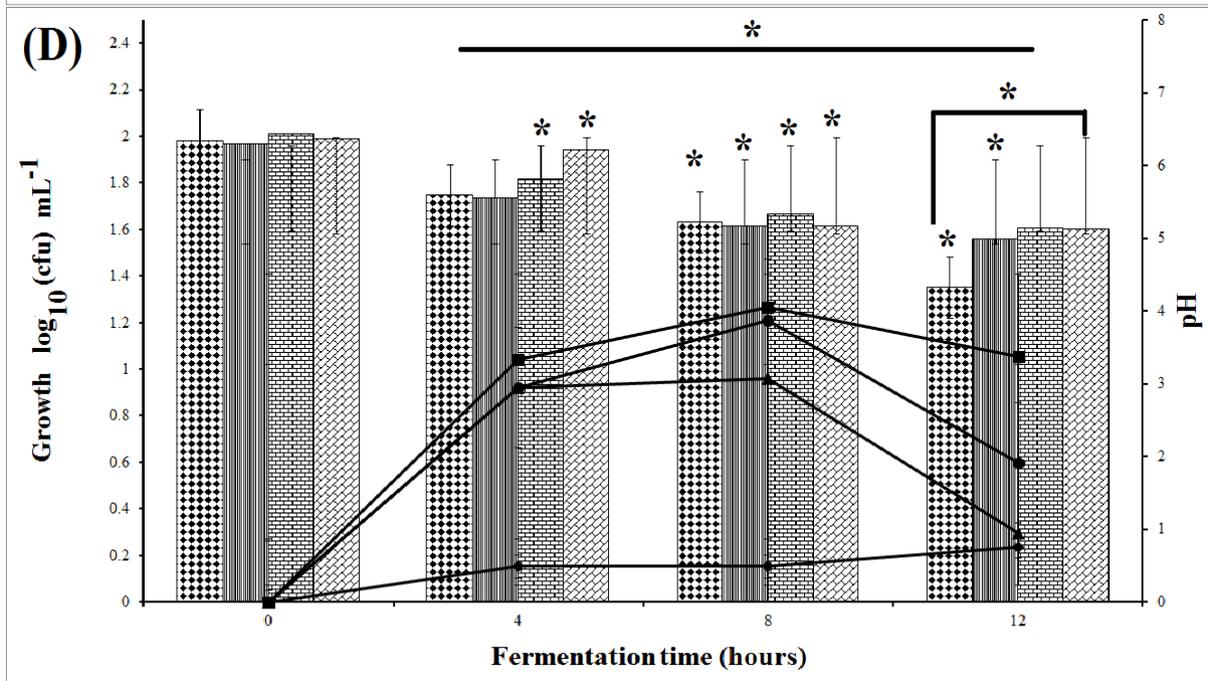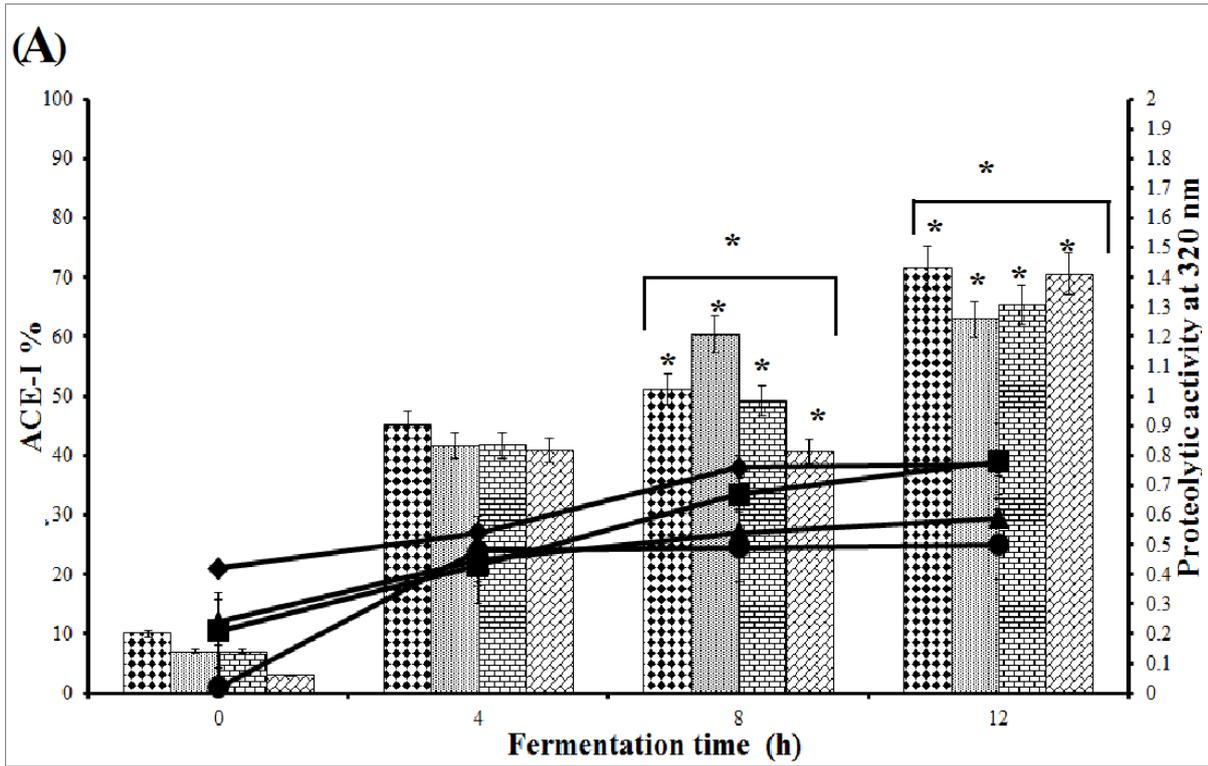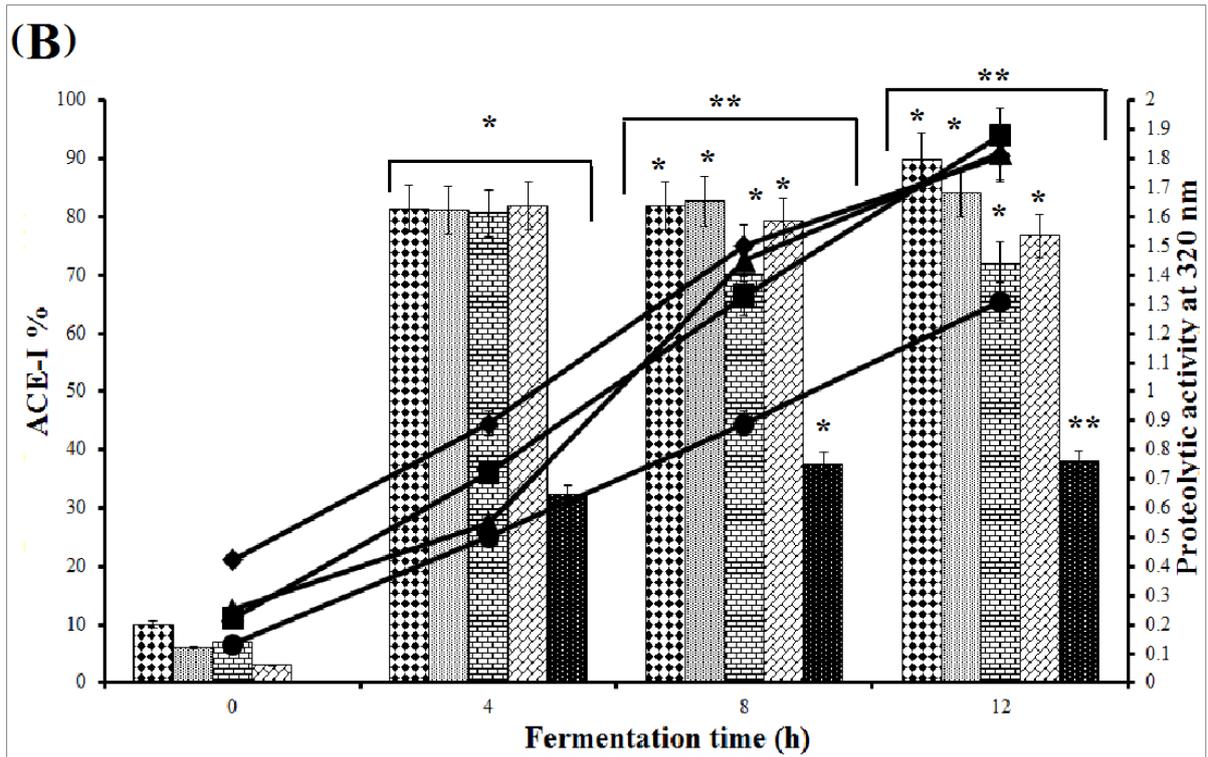
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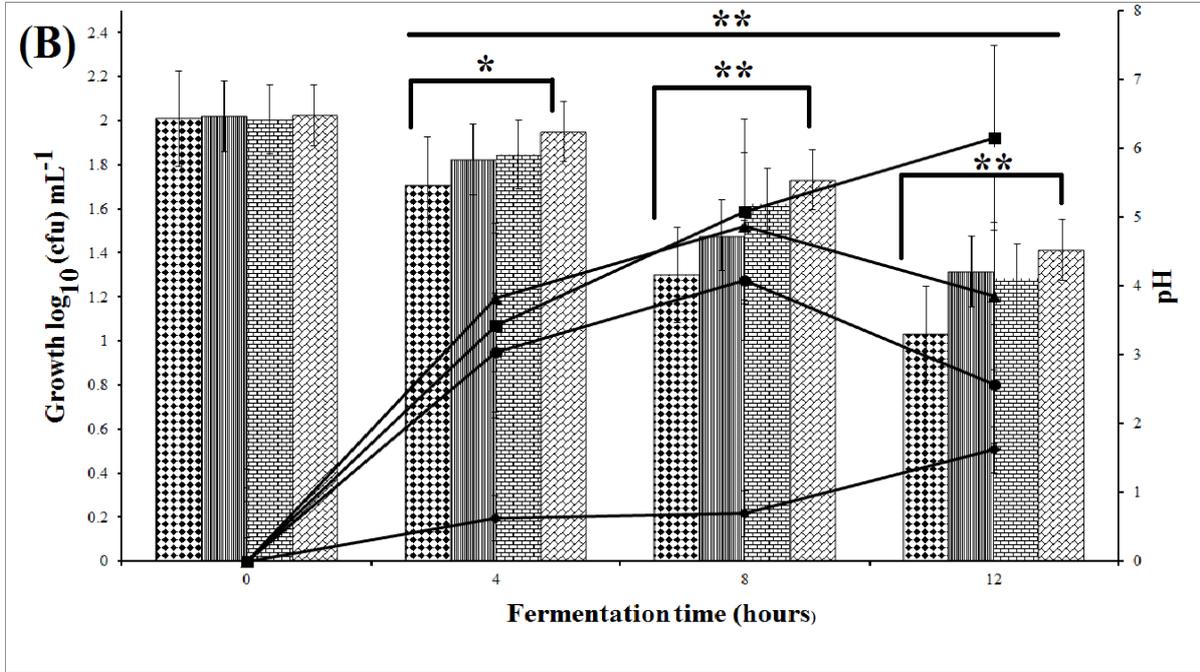
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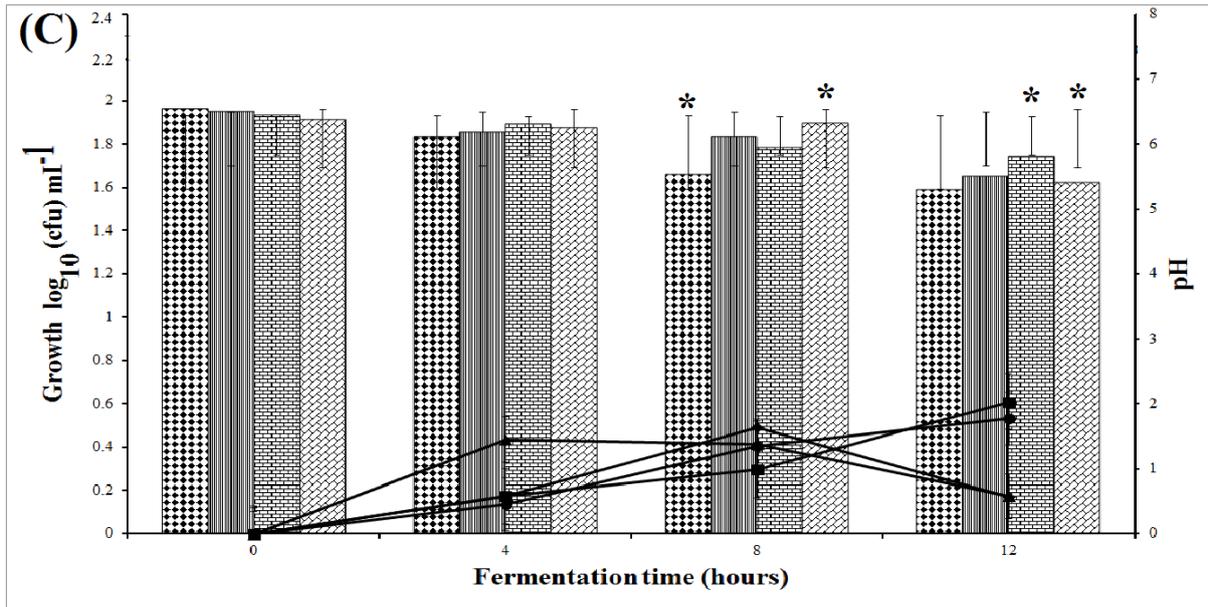
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611 **Figure 1-** Growth $\log_{10}$ (cfu) mL^{-1} (line) and pH (bars) of *L. helveticus* strains (

612 881315), (881188), (880474) and (880953) at 37 °C for 12 h in

613 (**A**) RSM, (**B**) RSM with Flavourzyme®, (**C**) WPC and (**D**) WPC with Flavourzyme® (Error

614 bars depict standard error of the means) and lines above signify differences at (*) $P < 0.05$
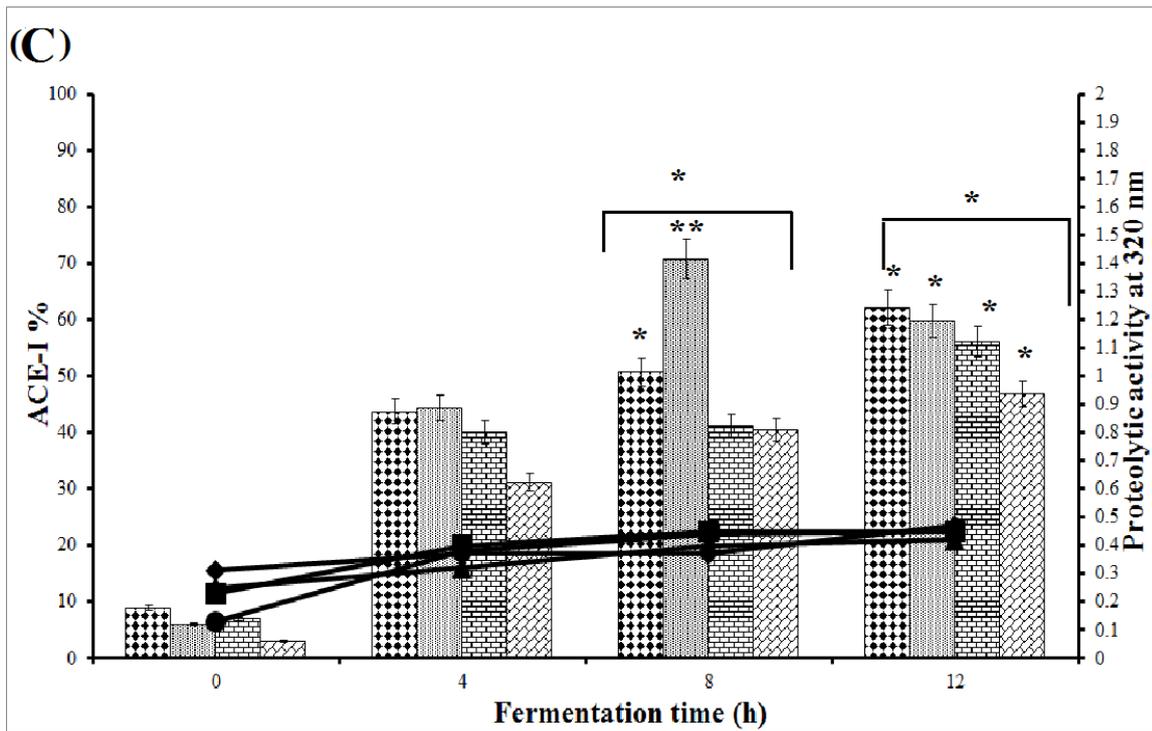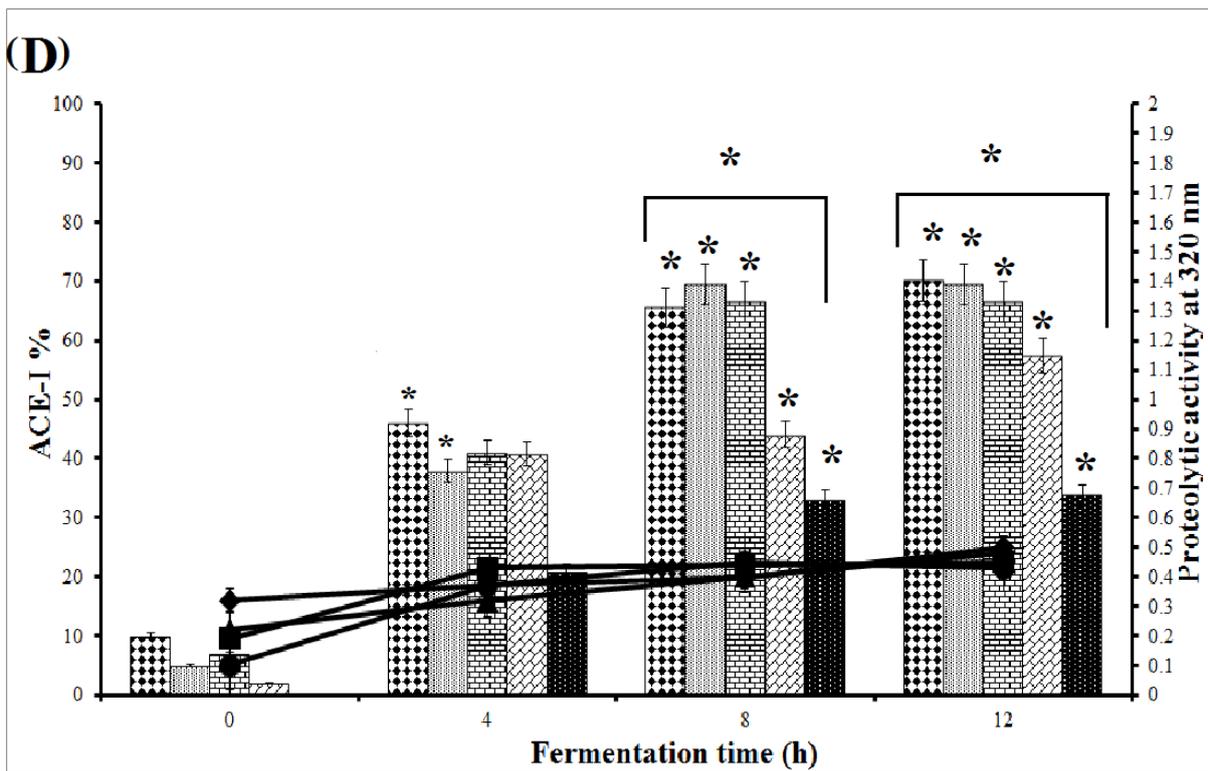
615 and (**) $P < 0.01$.

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(C)

(D)

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Figure 2- Proteolytic activity at 320 nm (line) and ACE-I % (bars) of *L. helveticus* strains (

—◆— 881315), (—■— 881188), (—▲— 880474), (—●— 880953) and (

Flavourzyme®)as control at 37 °C for 12 h in (**A**) RSM, (**B**) RSM with Flavourzyme®,

627   (**C**) WPC and (**D**) WPC with Flavourzyme® (Error bars depict standard error of the means)

628   lines above signify differences at (*) P < 0.05 and (**) P < 0.01.
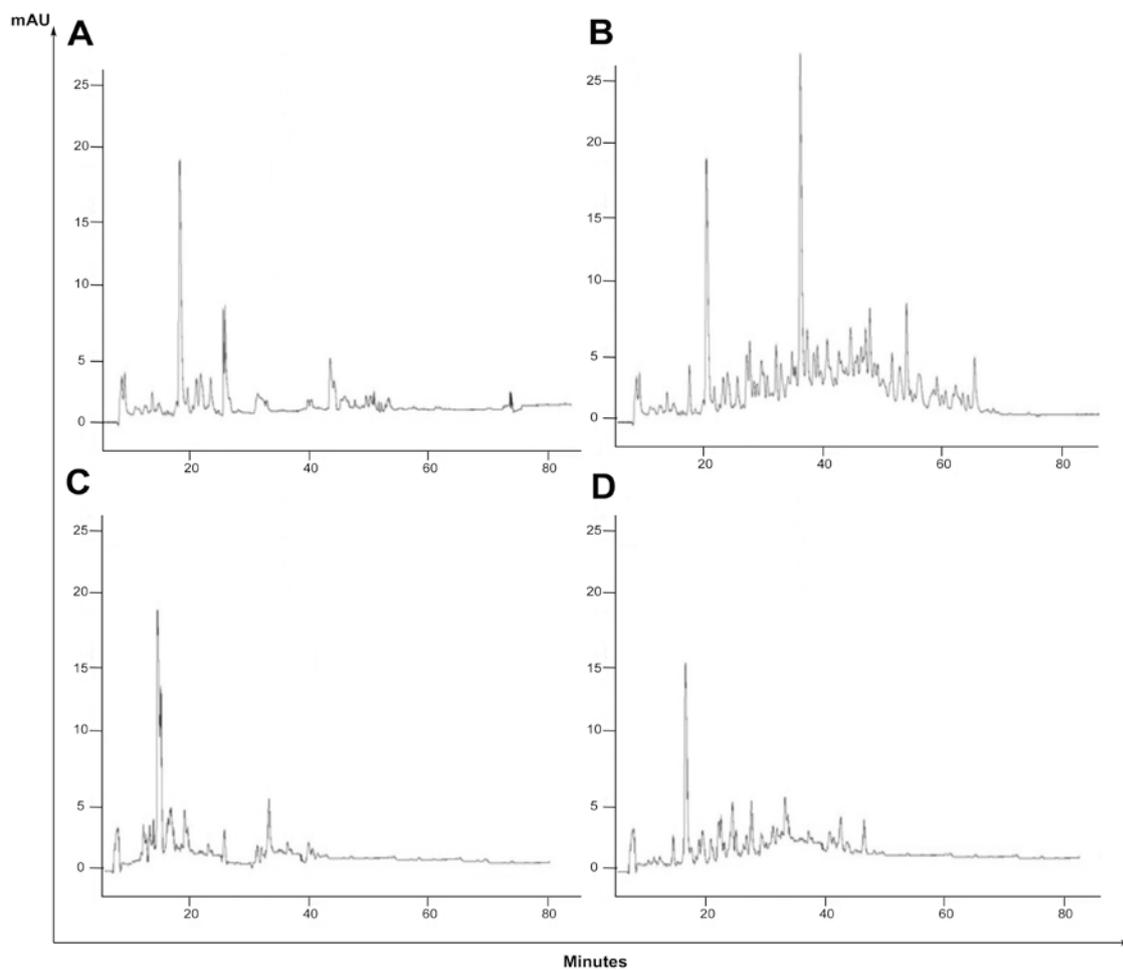
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636   **Figure 3-** RP-HPLC peptide profile of water soluble extracts obtained from fermented skim

637   milk made with a combination of *L. helveticus* strains and Flavourzyme® ; 881315(**A**),

638   881315 with Flavourzyme® (**B**), 881188 (**C**) and 881188 with Flavourzyme® (**D**) after 12 h

639   fermentation at 37 °C.

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