

1 **Title**

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

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

51 **Introduction**

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

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;

101 Singh and others 2011; Lim and others 2011; Unal and Akalin 2012; Griffiths 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:

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

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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 $\text{Log}_{10} \text{cfu ml}^{-1}$, the *L. helveticus* 881188 showed highest growth (2) $\text{Log}_{10} \text{cfu ml}^{-1}$ 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 NH₃ 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 NH₃ 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

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

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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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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376

377

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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397 assistance with the paper.

398

399 **Author Contributions**

400 Fatah Ahtesh, Nagendra Shah and Vijay Mishra planned the study. Fatah Ahtash carried out
401 experimental work. Vijay Mishra helped in analysis and interpretation. Vijay Mishra and
402 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 | <i>L. helveticus</i> strains used without combination | Combination of <i>L. helveticus</i> 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

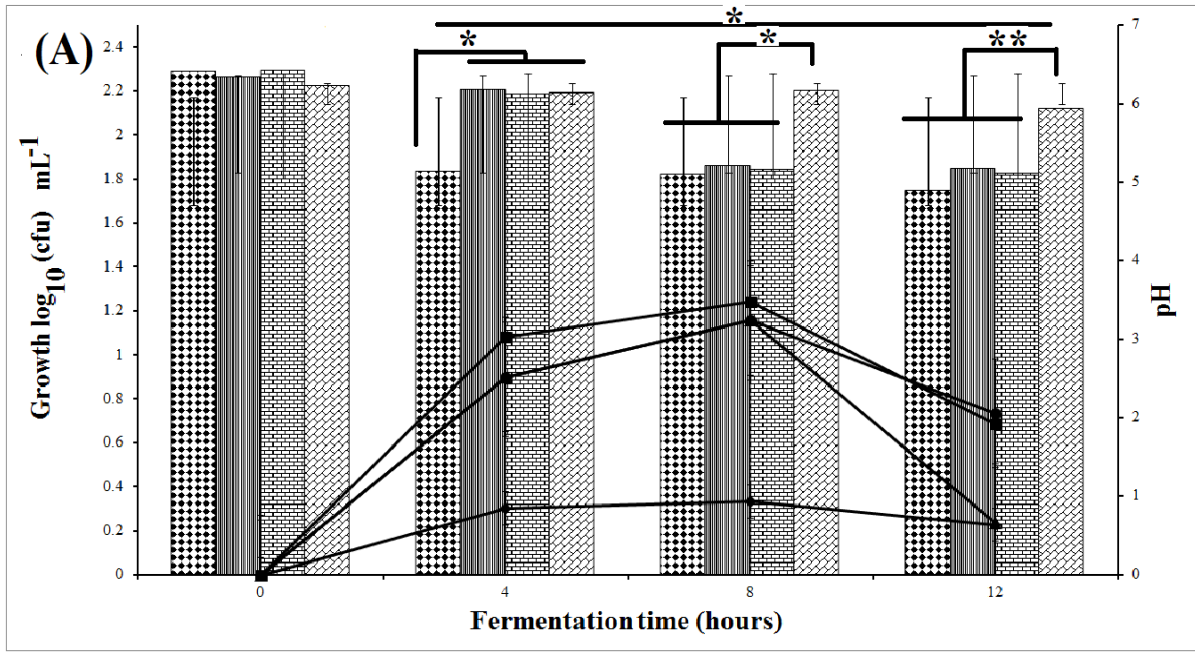
| <i>L. helveticus</i> strains | Variables | Without Flavourzyme® | | | With Flavourzyme® | | |
|------------------------------|-----------|----------------------|---------|---------|-------------------|---------|---------|
| | | OPA | ACE | CFU | OPA | ACE | CFU |
| 881315 | OPA | 1.000 | 0.988** | 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.

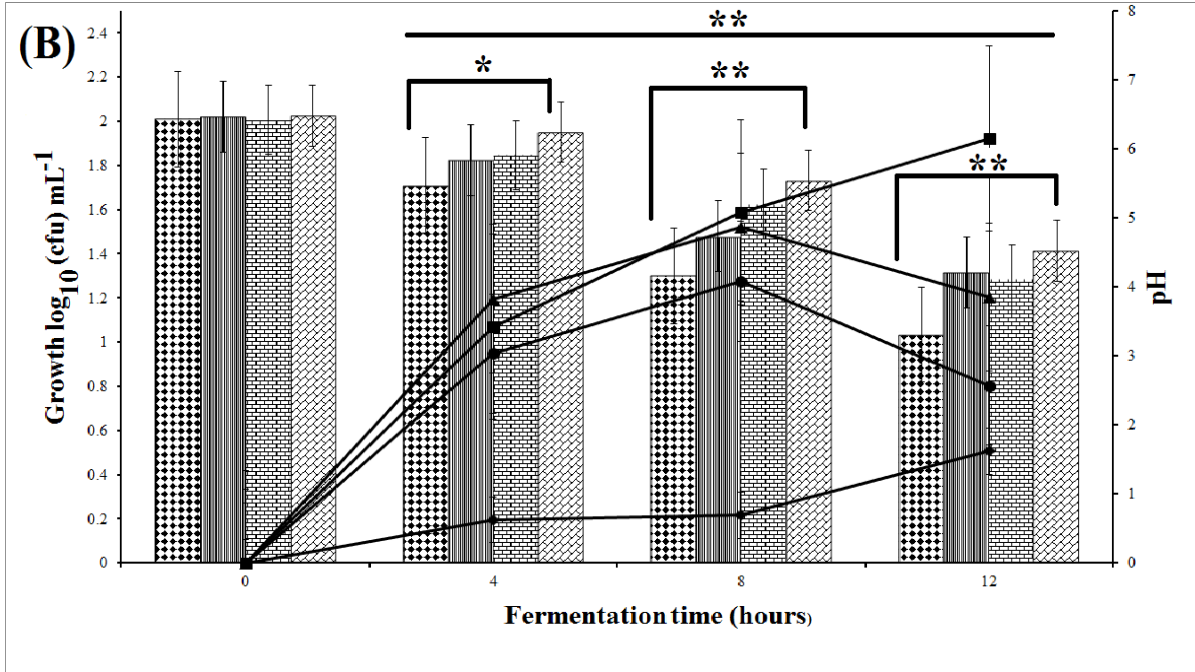
598 **Table 3-** Pearson coefficient correlations (r), proteolytic activity (OPA), ACE-inhibitory activity (ACE) and bacterial growth (CFU) of, *L.*
 599 *helveticus* 881315, *L. helveticus* 881188, *L. helveticus* 880474 and *L. helveticus* 880953 grown in 4 % WPC at 37 °C for 12 h with or without
 600 Flavourzyme® combination

| <i>L. helveticus</i> 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 |

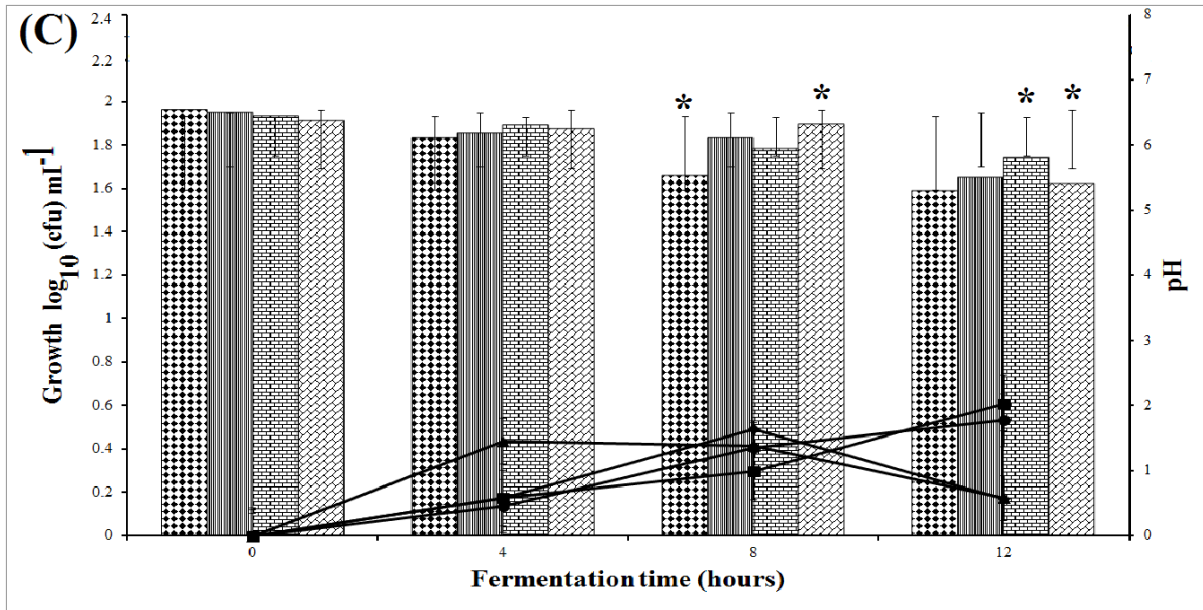
601 *P < 0.05, ** P < 0.01.



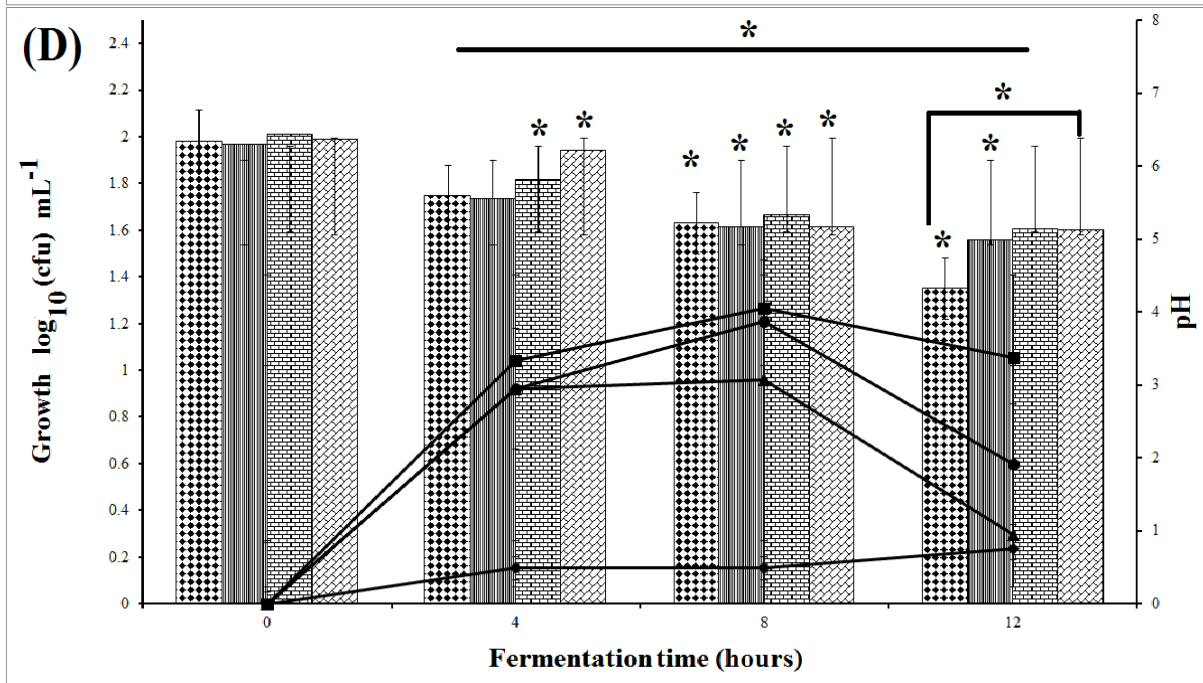
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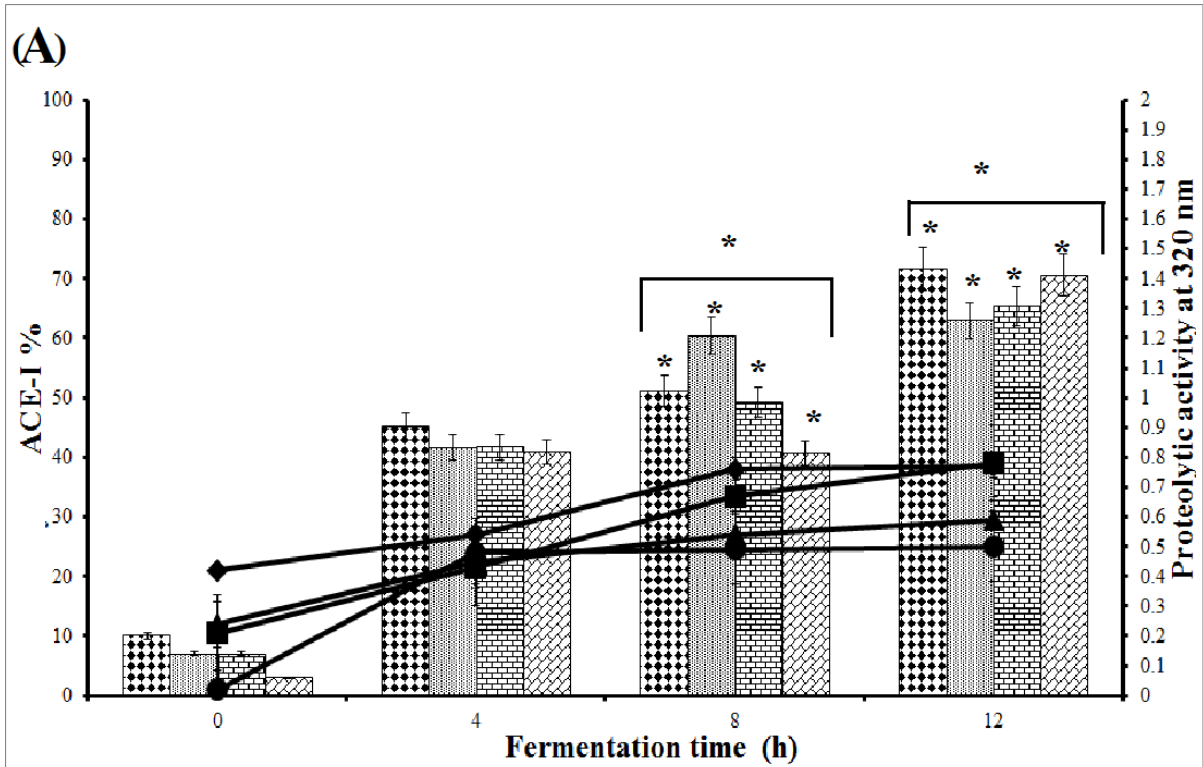
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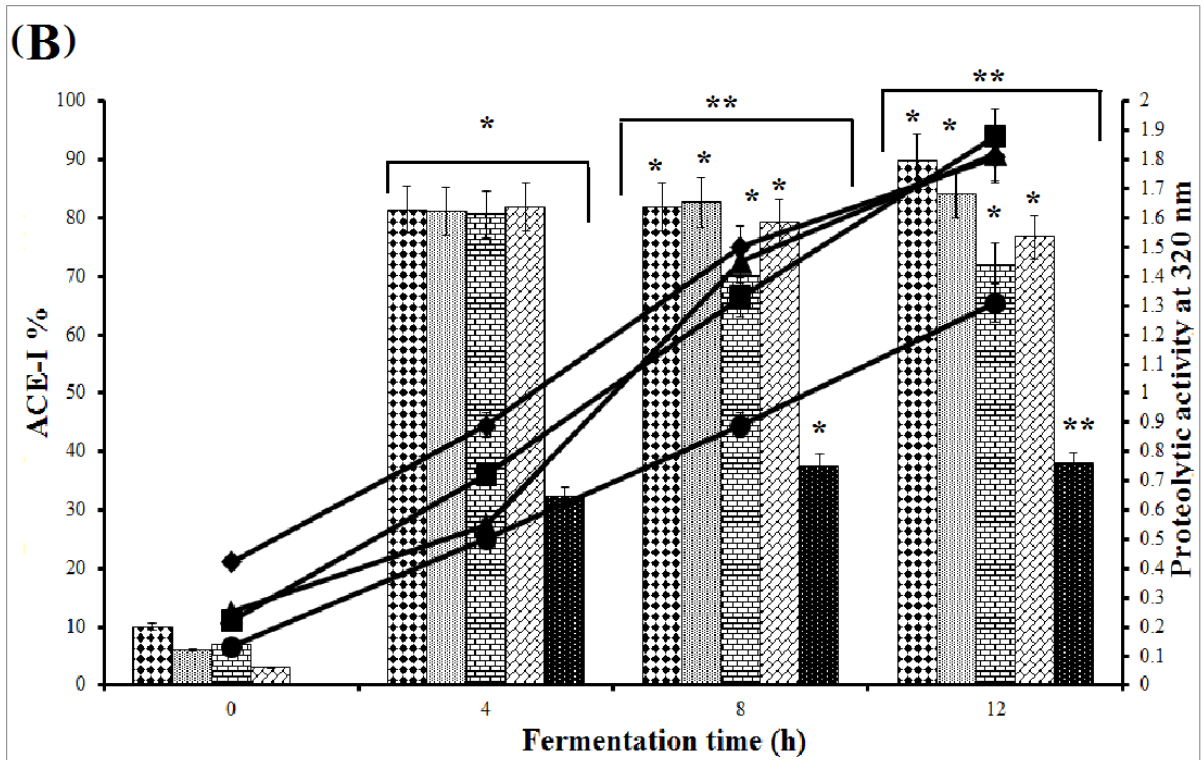
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Figure 1- Growth \log_{10} (cfu) mL^{-1} (line) and pH (bars) of *L. helveticus* strains (—●— 881315), (—■— 881188), (—▲— 880474) and (—◆— 880953) at 37 °C for 12 h in (A) RSM, (B) RSM with Flavourzyme®, (C) WPC and (D) WPC with Flavourzyme® (Error bars depict standard error of the means) and lines above signify differences at (*) $P < 0.05$ and (**) $P < 0.01$.



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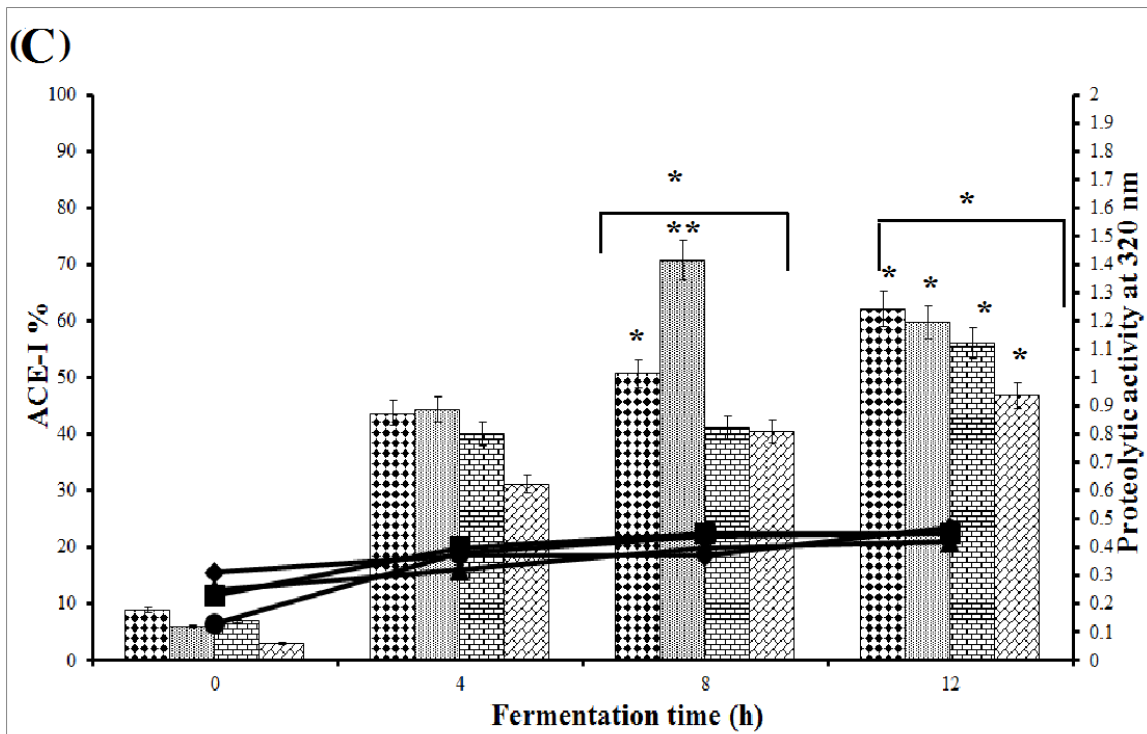


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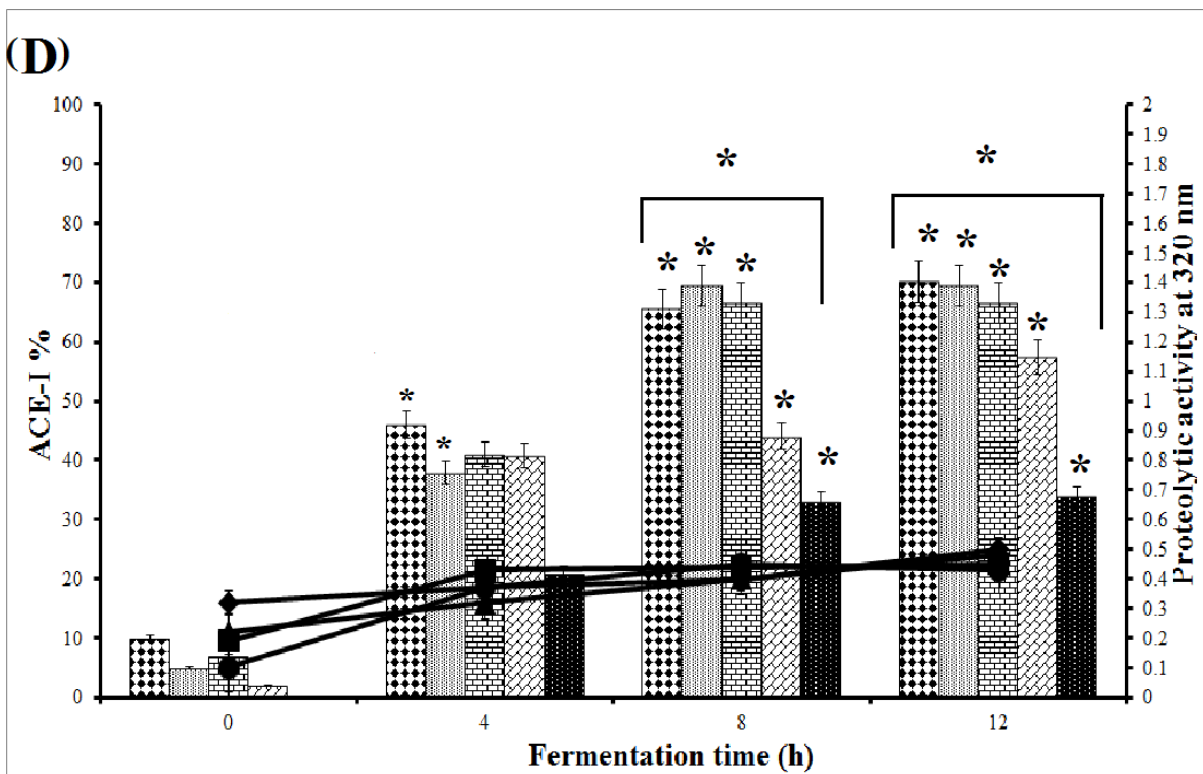
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624 **Figure 2-** Proteolytic activity at 320 nm (line) and ACE-I % (bars) of *L. helveticus* strains (

625 881315), (881188), (880474), (880953) and (

626 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.

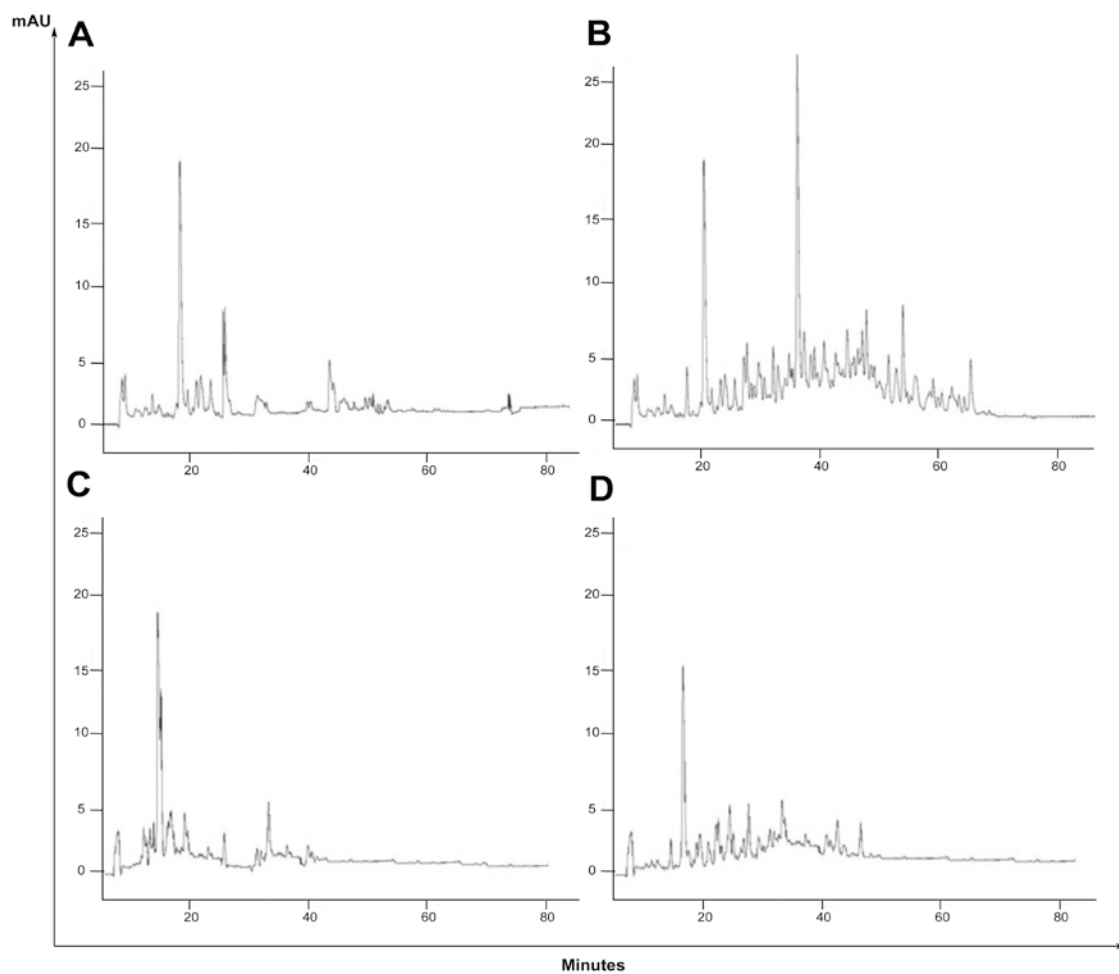
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636 **Figure 3-** RP-HPLC peptide profile of water soluble extracts obtained from fermented skim

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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