Title

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

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Practical Application

Lactobacillus helveticus in combination with Flavourzyme® was used for producing ACE-I peptides from reconstituted skim milk and whey protein concentrate. Fermentation of skim milk by *L. helveticus* in combination with Flavourzyme® resulted in >80% ACE-I after 8 h. These conditions can be used for developing a functional drink with antihypertensive activity.

Abstract

Angiotensin converting enzyme inhibitory (ACE-I) activity as affected by *Lactobacillus helveticus* strains (881315, 881188, 880474 and 880953), and supplementation with a proteolytic enzyme was studied. Reconstituted skim milk (12 % RSM) or whey protein concentrate (4 % WPC), with and without Flavourzyme® (0.14 % w/w), were fermented with 4 different *L. helveticus* strains at 37 °C for 0, 4, 8, 12 hours. Proteolytic and *in vitro* ACE-I activities, and growth were significantly affected ($P < 0.05$) by strains, media and with enzyme supplementation. RSM supported higher growth and produced higher proteolysis and ACE-I than that WPC without enzyme supplementation. The strains *L. helveticus* 881315 and 881188 were able to increase ACE-I to > 80 % after 8 h of fermentation when combined with Flavourzyme® in RSM compared to the same strains without enzyme supplementation. Supplementation of media by Flavourzyme® was beneficial in increasing ACE-I peptides in both media. The best media to release more ACE-I peptides was RSM with enzyme supplementation. The *L. helveticus* 881315 outperformed all strains as indicated by highest proteolytic and ACE-I activities.

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

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;

Pihlanto and others 2010; Phelan and Kerins 2011). Lactic acid bacteria (LAB) used to produce fermented dairy products (i.e. yoghurt, fermented milk, cheeses) have shown varied but significant ACE-I activities during fermentation as reported in several studies (Korhonen 2009; Phelan and Kerins 2011; Korhonen and Pihlanto 2003, 2006, 2007; Hernández-Ledesma and others 2011). The use of specific LAB or proteases in producing ACE-I peptides from various milk media (yoghurt, cheese, sour milk) have been reported (van der Ven and others 2002; Donkor and others 2005; Pan and others 2005; Kilpi and others 2007; Meena and others 2008; Tsai and others 2008; Korhonen 2009; Hamme and others 2009; Ramchandran and Shah 2010, 2011; Tellez and others 2011; Chaves-López and others 2012; García-Tejedor and others 2013).The proteolytic activity and bioactivity of peptides is influenced by a number of factors, such as, type of growth media, fermentation time, temperature and pH type of LAB species and strain type used for fermentation (Ramesh and others 2012). Several bioactive peptides which have ACE-I activity have been derived from hydrolysis of proteins using skim milk and whey protein concentrate (Madureira and others 2010; Donkor and others 2007b). Such peptides have clinically documented effects in the reduction of hypertension in humans (Aihara and others 2005).

Lactobacillus helveticus (*L. helveticus*) is homo fermentative thermophilic lactic acid bacteria and known to possess strong proteolytic activity and is used in the production of Swiss cheese and fermented milk beverages (Kenny and others 2003; Griffiths & Tellez 2013). Due to its high proteolytic activity, *L. helveticus* is more effective than that other LAB such as *L. delbrueckii* sp. *bulgaricus* and *L. acidophilus* in the production of ACE-I peptides (Korhonen and Pihlanto 2006). Several studies have reported the use of *L. helveticus* for production of ACE-I peptides and *L. helveticus* strains are specifically used for cheese production such as Swiss cheese (Maeno and others 1996; Leclerc and others 2002; Kilpi and others 2007; Nielsen and others 2009; Sun and others 2009; Pan and Guo 2010a; Otte and others 2011;

Singh and others 2011; Lim and others 2011; Unal and Akalin 2012; Grifiths and Tellez 2013). The effect of temperature, fermentation time and initial pH of fermented milk by *L. helveticus* has been reported for sour milk production (Pan and Guo 2010b). Peptides can be derived from enzymatic hydrolysis and considered to be safer (Pihlanto-Leppälä, 2000). Moreover, enzymatic hydrolysates exert a variety of additional physiological properties such as antioxidant and antimicrobial (Pihlanto-Leppälä 2000). Some of these peptides are present within the parent protein structure and could be released through proteolysis (FitzGerald and Murray 2006). However, proteinases such as (Alcalase, chymotrypsin pancreatin, pepsin, enzymes from bacterial and fungal) have been utilized to generate bioactive peptides (Pihlanto-Leppälä, 2000). In this study, we chose Flavourzyme® individually or in combination, to increase the production of peptides from milk proteins hydrolyses. Since there are no published reports on the production of ACE-I peptides from milk employing proteases and LAB, we screened *L. helveticus* strains for production of ACE-I peptides from 12 % reconstituted skim milk (RSM) and 4 % whey protein concentrate (WPC) with or without protease (Flavourzyme®) supplementation by measuring the bacterial growth, proteolytic activity and *in vitro* ACE-I activity. Therefore, the present study was performed to evaluate the hypothesis that combination of Flavourzyme® with *L. helveticus* significantly increase ACE-I % in both media.

Material and methods

Material and chemical

Four strains of *L. helveticus* 881315, 881188, 880474 and 880953 were obtained from (Dairy Innovation Australia Ltd, Werribee, VIC, Australia) and stored in 40 % glycerol de 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

without 0.14 % (w/w) Flavourzyme®1000 L were inoculated with *L. helveticus* strains and fermented at 37 °C for 4 h, 8 h and 12 h. Sixteen different combinations of bacterial strains, Flavourzyme® (0.14%) and growth media (12 % RSM or 4% WPC) were used (Table 1). Samples were collected and stored at -20 °C for analysis of bacterial growth, proteolytic activity and ACE-I activities and peptide profiling by Reverse phase - High-performance liquid chromatography (RP-HPLC).

Methods

Measurement of bacterial growth

Growth was assessed every 4 h up to 12 h during fermentation by pour plate method using MRS agar following serial dilutions with 0.1 % peptone. The plates were incubated anaerobically at 37 °C for 48 h using anaerobic jars with anaerobic kit. Plates having 25 - 250 colonies were counted and the growth was expressed as logarithm of colony forming unit (cfu) per mL^{-1} of sample.

Determination of proteolytic activity

The O-phthaldialdehyde (OPA) reagent was prepared by mixing 25 mL of 100 mM disodium tetra-borate, 2.5 mL of 20 % (w/w) sodium dodecyl sulfate (SDS), 1 mL methanol containing 40 mg of OPA and 100 µL of β-mercaptoethanol . The final volume was made to 50 mL with Deionized water. Briefly, the sample (3 mL) was mixed with equal volume of 1 % trichloroacetic acid (TCA) followed by filtration using Advantech # 231 filter paper . Filtrate (150 µL) was placed into 4 mL cuvette and mixed with 3 mL OPA reagent, and absorbance measured at 340 nm using UV-VIS spectrophotometer (LKB NOVASPEC II Pharmacia, LKB Bio- Chrom, UK) after allowing 2 min of reaction time at room temperature

as a measure of proteolysis. The degree of proteolysis was determined as the difference between proteolytic activities in fermented media to that of unfermented media (Donkor and others 2007b).

Determination of ACE-Inhibitory activity

ACE-I activity was determined according to a Donkor and others (2007a). Briefly, fermented media (WPC or RSM) (10 mL) was centrifuged at 4000 x g at 4 °C for 30 min and the supernatant was freeze-dried for 72 h. The freeze-dried powder (40 mg) was dissolved in 2 mL Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride. ACE enzyme and Hippuryl-histidyl-leucine (HHL) were prepared in Tris buffer. Fifty µL of 3.0 mM HHL, 50 µL of 1.25 MU ACE enzyme (from rabbit lung), and 50 µL of experimental samples were placed in a glass tube and incubated for 1 h at 37 °C ensuring mixing for the first 30 min. Glacial acetic acid (150 µL) was added to stop the reaction. The reaction mixture was stored at -20 °C before further analysis of released hippuric acid (HA) by HPLC. An external standard curve of hippuric acid was prepared to quantify the resultant hippuric acid in fermented samples. An aliquot (20 µL) of the mixture was injected into Gemini® C18 110 Å (100 mm x 4.6 mm, 5 µm) using Varian HPLC equipped with an auto sampler. The separation was conducted at room temperature (~22 °C) at a mobile phase flow rate of 0.6 mL min^{-1}. The mobile phase consisting of 12.5 % (v/v) acetonitrile in Deionized -water, and pH was adjusted to 3.0 using glacial acetic acid. Ultraviolet-visible detector was set at 228 nm. The % ACE-I was calculated as follows:

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

RP-HPLC analysis of water-soluble peptides extract

Reconstituted skim milk fermented with the highest ACE-I activity of strains; *L. helveticus* 881188 and 881315 (with or without combination of Flavourzyme®) were collected after 12 h of fermentation (pH 4.6). From this, 50 mL sample was centrifuged at 4000 x g at 4 °C for 30 min to separate proteins. The supernatant containing soluble peptides was freeze-dried for 72 h. The powder (40 mg) was dissolved in 0.1 % trifluoroacetic acid (TFA). Water soluble peptides were profiled by a RP- HPLC using C-18 monomeric column (5 μm, 300 Å, 250 mm x 4.6 mm) (Donkor and others 2007a).

Statistical analysis

All results are expressed as mean values of 3 replicates with standard deviation of the mean. One way ANOVA was performed to differentiate the significant differences in the treatments which were strains, growth media, presence or absence of Flavourzyme®, and fermentation time. The level of significance was tested at $P < 0.05$. Fisher`s (least significant difference; LSD) test was used to investigate significant differences among the treatment means. Correlation analysis was carried out between variables for same bacteria strain, growth media and presence or absence of Flavourzyme®. The degree of correlation between these variables was expressed as Pearson coefficient (r) and corresponding *P* values. All statistical analyses were carried out using SAS Version 9.0 software (SAS Institute Inc., Cary, NC, USA)

Results and discussion

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

Figure 1 shows the microbial growth and pH in RSM and WPC during fermentation with *L. helveticus*. All *L. helveticus* strains were able to grow in both media (Figure 1). Analysis of variance showed that bacterial growth was significantly ($P < 0.05$) affected by media, media supplementation with protease (Flavourzyme®), fermentation time and strain type. Higher growth was significantly noted ($P < 0.05$) in RSM. This may be attributed to superior nutrient profile of RSM (Kilpi and others 2007; Leclerc and others 2002) and higher specificity to caseins than whey proteins. Media supplemented with Flavourzyme® led to increase growth owing to enhanced number of released more peptides and amino acids required for bacterial growth in log and early stationary phases, associated to no supplementation in both media types (Kenny and others 2003). While *L. helveticus* 881315 showed the least growth (0.6) Log_{10} cfu ml^{-1}, the *L. helveticus* 881188 showed highest growth (2) Log_{10} cfu ml^{-1} at 12 h compared to other strains in RSM containing Flavourzyme® for the entire duration of fermentation. *L. helveticus* strains 880474 and 880953 also showed the higher growth compared to 881315 in RSM. It appears that Flavourzyme® supplementation prolonged the log phase in 881188 whereas 880474 and 880953 strains went into a decline phase after 8 h. In general, WPC showed a weak growth for all strains without the combination of Flavourzyme® compared to same strains in combination with Flavourzyme® (Figure 1C and D). However, growth for all strains in WPC with Flavourzyme® were increased significantly at 8 h and declined after 8 h of fermentation at pH (3.4) possibly, due to the effect of pH and heat treatment on WPC's nutrient contents as previously reported (Zisu and Shah 2003 ; Dissanayake and others 2013). Furthermore the decrease in bacterial growth observed after 8 h of fermentation can be attributed to the production of lactic acid in media by growing lactic acid bacteria which can inhibit bacterial growth at low pH concentrations. Similar growth characteristics were noted with LAB at different temperatures and fermentation time in RSM using *L. sakei* CTCstrains which showed inhibition of *L. sakei* growth due to lactic acid

production (Leroy and de Vuyst, 2001).However, different results were noted that *L. helveticus* strain's cell counts were increased during fermentation 12 % RSM from (0 – 9) h followed by a slight decrease in viable counts until (24 h) of the fermentation (Leclerc and others 2002). It is clear that the difference observed in bacterial growth in both media could be related to the different nature of proteins present (Leclerc and others 2002).

Proteolytic activity is higher in RSM media with Flavourzyme®

Milk proteins were hydrolysed by *L. helveticus* strains (881315, 881188, 880474 and 880953), resulting in an increase in the amount of free NH_3 groups as quantified by the OPA method (Figure 2). The proteolytic activity of *L. helveticus* strains grown in RSM or WPC with or without Flavourzyme® supplementation at 37 °C for 0-12 h increased with fermentation time (Figure 2). All strains preferred RSM over WPC with or without supplementation with Flavourzyme® as indicated by higher proteolysis. The activity remained significantly lower ($\leq$ 0.5) in WPC compared to RSM (> 0.78), indicating that proteins of RSM particularly, casein were the preferred substrate by enzymes of *L. helveticus* strains. This correlated to a similar trend in the growth pattern (Figure 1). The order of proteolytic activity of *L. helveticus* strains in RSM was 881315 > 881188 > 880474 > 880953. Supplementation of RSM with Flavourzyme® increased the proteolytic activity of all strains significantly ($P < 0.05$), reaching a maximum absorbance > 1.8 in 12 h by *L. helveticus* 881315, 880474 and 881188 (Figure 2). Interestingly, the proteolytic activity of strain 881315 was high during 12 h (Figure 2). However, the growth was weak in both media (Figure 1). The activity in RSM with Flavourzyme® was approximately higher by 45-60 % than that without Flavourzyme® even after 4 h of fermentation and was sustained over the 12 h duration of fermentation. However, except for *L. helveticus* 880953, the response to Flavourzyme® in increasing proteolysis was similar after 8 h of fermentation. Flavourzyme®

appears to have hydrolysed large proteins present in RSM to intermediate peptides, which were used by *L. helveticus* to produce small peptides and free amino acids (Leclerc and others 2002). Co-fermentation of RSM with Flavourzyme® supplementation with *L. helveticus* strains reduced the time required for a given degree of proteolysis. These results suggest that proteolysis were enhanced in the higher protein content was in media supplemented with Flavourzyme® and that casein was a better substrate than that whey proteins for *L. helveticus* strains. Similar results have been noted that proteolytic activity was enhanced in the higher protein content medium and that casein fraction was a better substrate than whey proteins for the extracellular proteinases of lactic acid bacteria (Leclerc and others 2002). In addition, the amount of free NH_3 groups in the media increased sharply until 12 h except for media without Flavourzyme® for which a slightly slower than that sharply increase was observed after 8 h (Figure 2). The different proteolytic activities between strains could also be explained by the higher proteolysis noted by Matar and others (1996) for *L. helveticus* L89, compared to those measured for strains 881315, 881188, 880474, and 880953 in this study.

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

The amount and type of peptides produced during hydrolysis of proteins present in RSM or WPC influenced ACE-I activity which was measured using an ACE-I method according to Donkor et al., (2007a). The % of ACE-I activity of *L. helveticus* strains (881315, 881188, 880474 and 880953) in RSM or WPC with or without Flavourzyme® at 37 °C for 12 h are presented in Figure 2. Flavourzyme® alone was used as a control. ACE-I activity for all strains in both media increased significantly during fermentation period ($P < 0.05$). However, differences existed between strains and media used when compared at the same time of fermentation. Media type, strains, supplementation of Flavourzyme® and fermentation time

had significant ($P < 0.05$) effects on ACE-I activity. As with proteolytic activity, ACE-I activity increased as fermentation time increased for all strains. Supplementation of RSM with Flavourzyme® significantly ($P < 0.05$) increased ACE-I activity of *L. helveticus* strains. Except for *L. helveticus* 880474, ACE-I increased from 40-60 % to ≥ 85 % in RSM with Flavourzyme® supplementation after 8 h of fermentation. The inhibition increased during fermentation when *L. helveticus* 881315 and 881188 were used from 10 % to 89.82 % and from 5 % to 85 % in RSM with supplementation, respectively (Figure 2B). While the same strains in WPC with Flavourzyme® supplementation, the ACE-I % increased from 10 % to 65 % and 5 % to 60 % during 12 h, respectively (Figure 2D). Since both of these strains demonstrated high proteolysis, co-fermentation with enzyme appeared to have produced higher amounts of ACE-I peptides as evident in increased number of peaks (Figure 3). The inhibitory activity remained high at 12 h for all strains except *L. helveticus* 880474 which showed a significant drop in ACE-I after 4 h of fermentation. There was no significant difference ($P < 0.05$) in ACE-I between hydrolysates produced from WPC with or without Flavourzyme® at 4 h fermentation. Thereafter, ACE-I increased differentially among the strains and a maximum of 89.82 % was observed for *L. helveticus* 881315 with Flavourzyme® in RSM at 12 h. However, the growth Log_{10} of same strain was weak during 12 h (Figure 1), that means this strain has high proteolytic activity. This supported by study reported that "the declining number of live bacteria alterations in ACE-inhibitory activity in the cultures of peptidase-negative mutants could be detected during the course of cultivation, which indicates that the proteolytic enzymes released from cells of *L. helveticus* play an important role in the conversion of bioactive peptides" (Kilpi and others 2007). Data also suggest a delayed effect with addition of Flavourzyme® in WPC. The increase in ACE-I was significantly ($P < 0.05$) higher in RSM compared to WPC due to the addition of Flavourzyme® and may be due to the sensitivity of whey protein to heat treatments (Banks

and others 1995; Patel and Creamer 2008). The ACE-I activity differed significantly between strains. *L. helveticus* 881315 and 881188 showed higher ACE-I activity compared to other strains in RSM (Figure 2). This implies that Flavourzyme® enhanced the production of ACE-I peptides as previously reported and supported by the proteolysis pattern observed (Tsai and others 2008). Since ACE-I almost doubled in the first 8 h of fermentation, Flavourzyme® supplementation can be used to reduce time of hydrolysis required for production of ACE-I peptides. The differences observed between RSM and WPC may be attributed to differences in the type of proteins present and therefore the variety of peptides present in the hydrolysates (Matar and others 1996; Pan and Guo 2010b). Preference to casein by proteinases has been reported (Lim and others 2011; Cheison and others 2007; Matar and Goulet 1996). It was possible to achieve maximum ACE-I of 60 % and 70 % without supplementation of RSM or WPC by *L. helveticus* strain 881315 after 8 h of fermentation, respectively. On the other hand, with supplementation of RSM, ACE-I % was achieved to 89.82 %. However, fermentation process using Flavourzyme® alone as control have a small effective in increasing ACE-I activity as indicated by the low ACE-I (20 - 38%) for hydrolysates produced.

RP-HPLC analysis of water-soluble peptide extracts suggests that *L. helveticus* 881315 with Flavourzyme® is most optimal

The profiles of water-soluble peptide extracts of 12 h fermented skim milk hydrolysates by best performer strains (as measured by proteolytic and ACE-I activities) *L. helveticus* (881315 and 881188) with or without supplementation of Flavourzyme® are shown in Figure 3. The RP-HPLC elution profile of the hydrolysates was based on the hydrophobicity of the peptides. In the control unfermented RSM only one peak appeared at 10 min (not shown) only. The chromatograms (Figure 3A, 3C) show that 881315 and 881188 strains without supplementation hydrolysed proteins resulting in peptides in the retention time range of 10-40

min and 10-45 min by strains respectively. Supplement with Flavourzyme® (Figure 3B, 3D) generally aided both strains to increase proteolysis as evident by the presence of more peptides appearing in the range of 10-65 min (881315) and 10-45 min (881188). Strain 881315 combined with Flavourzyme® was most optimal in terms of providing peptides by facilitating proteolysis of caseins present in RSM. This corroborated to the high ACE-I activity (Figure 2). However, supplementation was more beneficial to strain 881315 than that 881188.

Correlation between proteolytic activity, ACE-Inhibition and bacteria growth

The correlation between proteolytic activity and anti-hypertensive properties expressed as ACE-I and bacterial growth for the same bacterial strain, growth media and with or without Flavourzyme® are presented in (Tables 2 and 3) for RSM and WPC respectively. A significant correlation in growth with all measurements for each strain in RSM was evident for except *L. helveticus* 880953 which did not grow well in both media ($P < 0.05$). Even stronger correlations between the same measurements were observed for RSM supplemented with Flavourzyme® (Table 2). This suggests that Flavourzyme® enhanced the proteolytic and ACE-I activities of *L. helveticus* in RSM. ACE-I activity positively and strongly correlated with proteolytic activity for each strain, both with or without Flavourzyme® ($P < 0.05$) (Table 2) implying that increased proteolytic activity increased the production of ACE-I peptides. ACE-I activity had positive and strong correlation with bacterial growth (cfu) in RSM (with

or without Flavourzyme®) for all strains except *L. helveticus* 880953. This suggests that proteolytic and ACE-I activities were growth dependent. Similar trend was observed in WPC

Conclusion

Production of ACE-I peptides by *L. helveticus* varied between the strains due to differences in proteolytic activity. Casein rich RSM supported higher growth, higher proteolytic activity and produced higher ACE-I activities by all *L. helveticus* strains. Therefore, RSM is superior to WPC as a medium for production of ACE-I peptides irrespective of supplementation with protease, which generally increased hydrolysis of proteins to produce more ACE-I peptides. Beneficial effects of protease supplementation were more pronounced in the first 8 h of fermentation and also sustained thereafter. However, *L. helveticus* 881315 showed the lowest growth. The highest ACE-I activity was observed in 12% RSM supplemented with Flavourzyme® and up to 12 h fermentation by *L. helveticus* 881315 and 881188 at 37 °C, respectively. These conditions will aid in the production of a functional fermented drink with 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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Table 1- Experimental design to analyse and measure the pH, growth, proteolytic activity and % of ACE-inhibitory activities during (0, 4, 8 and 12 h) fermentation of *L. helveticus* strains in 12 % RSM or 4 % WPC and with or without combination of Flavourzyme®

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®	

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

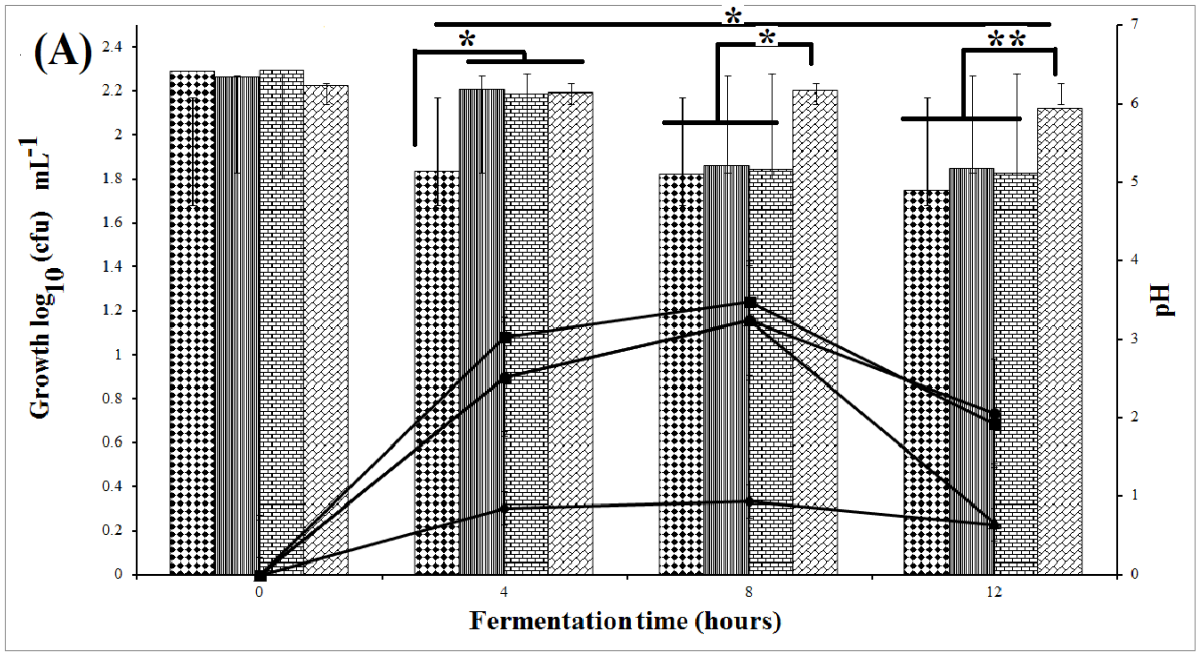
***L. helveticus* strains**	**Variables**	**Without Flavourzyme®**			**With Flavourzyme®**		
		OPA	ACE	CFU	OPA	ACE	CFU
881315	OPA	1.000	0.988**	0.918**	1.000	0.979**	0.946**
	ACE		1.000	0.945**		1.000	0.864**
	CFU			1.000			1.000
881188	OPA	1.000	0.991*	0.874*	1.000	0.966*	0.852*
	ACE		1.000	0.882*		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

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

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.


(A)
Growth log10 (cfu) mL-1
pH
Fermentation time (hours)
0
4
8
12
*
*
*
**

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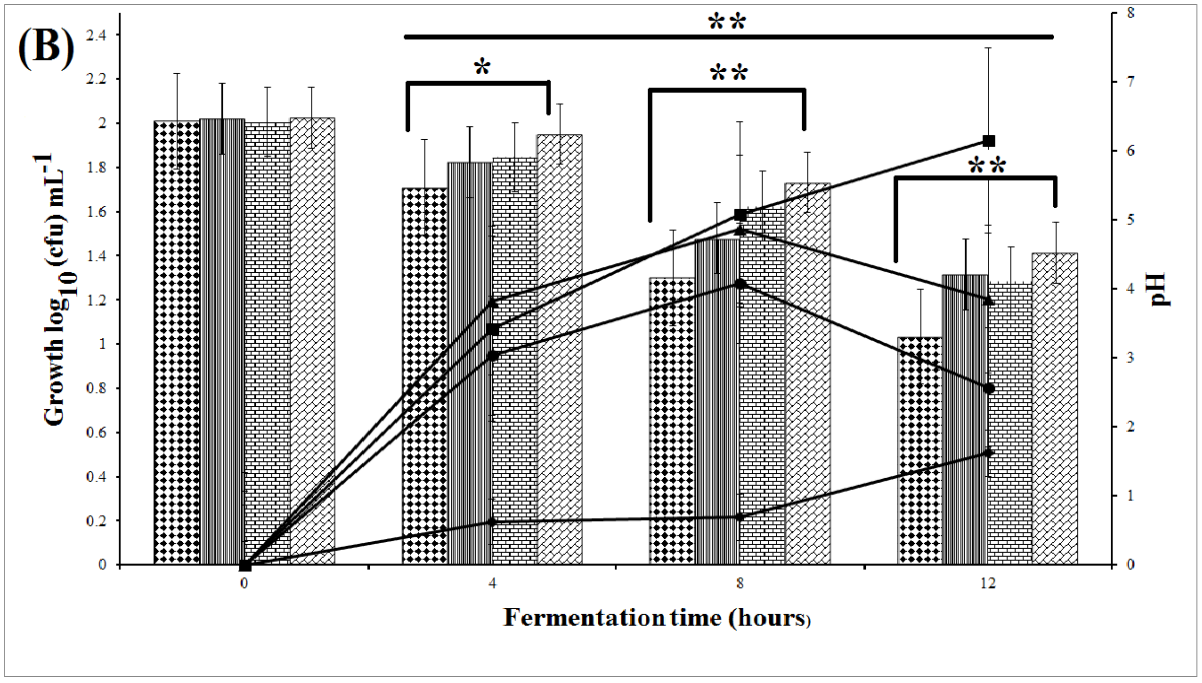
(B)
Growth log10 (cfu) mL-1
pH
Fermentation time (hours)
0
4
8
12
**
*
**
**

603
604

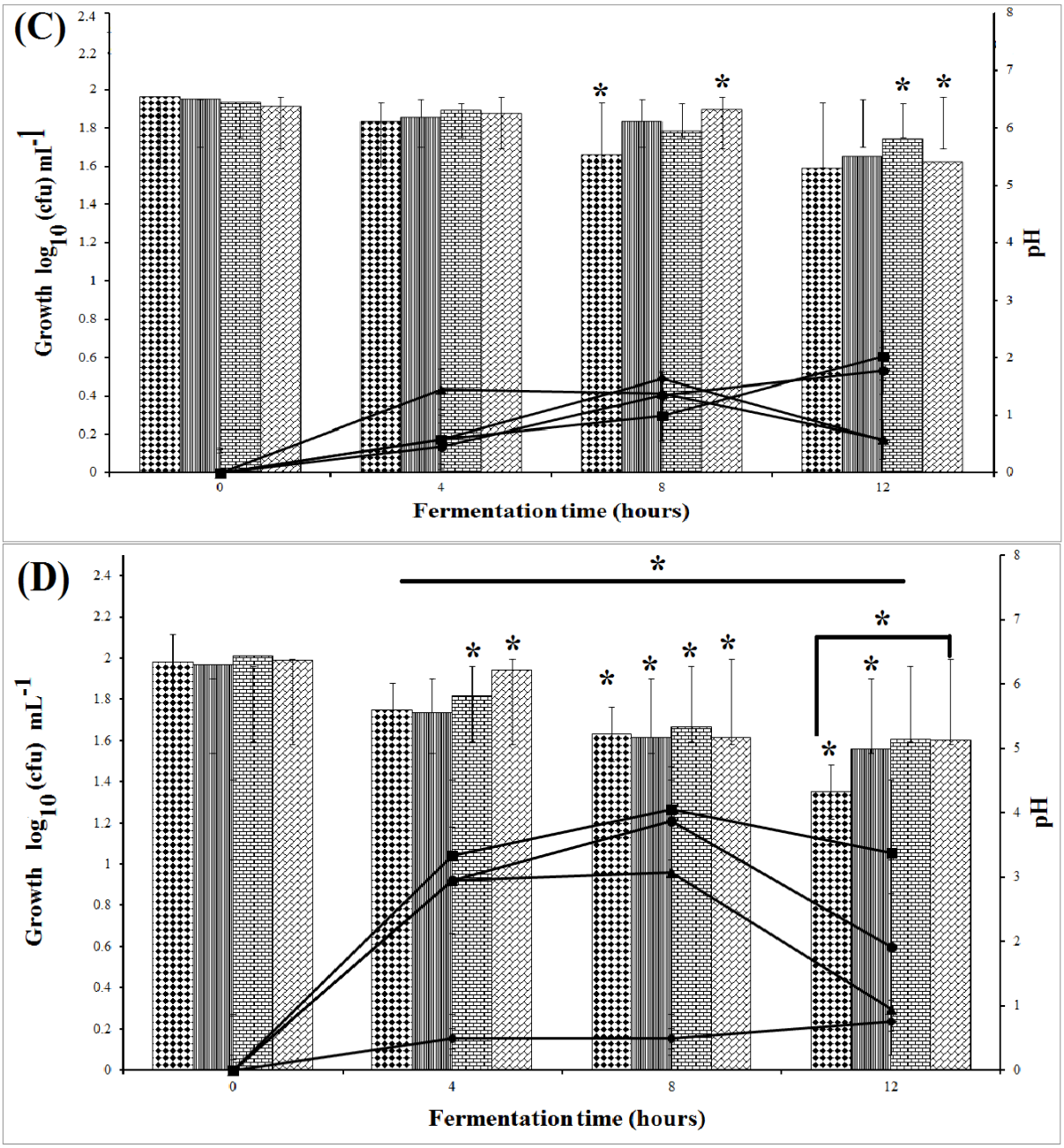

Figure 1- Growth $\log_{10}$ (cfu) mL^{-1} (line) and pH (bars) of *L. helveticus* strains (881315), (881188), (880474) and (880953) at 37 °C for 12 h in (**A**) RSM, (**B**) RSM with Flavourzyme®, (**C**) WPC and (**D**) WPC with Flavourzyme® (Error bars depict standard error of the means) and lines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

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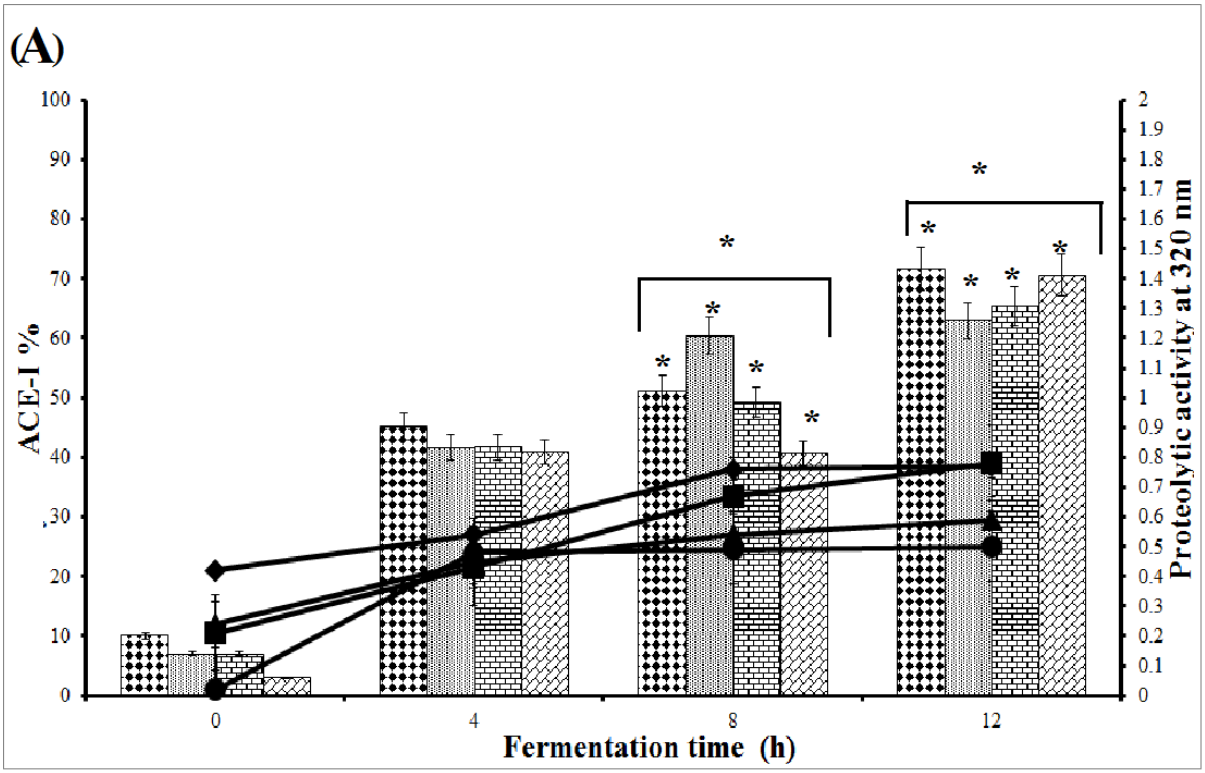

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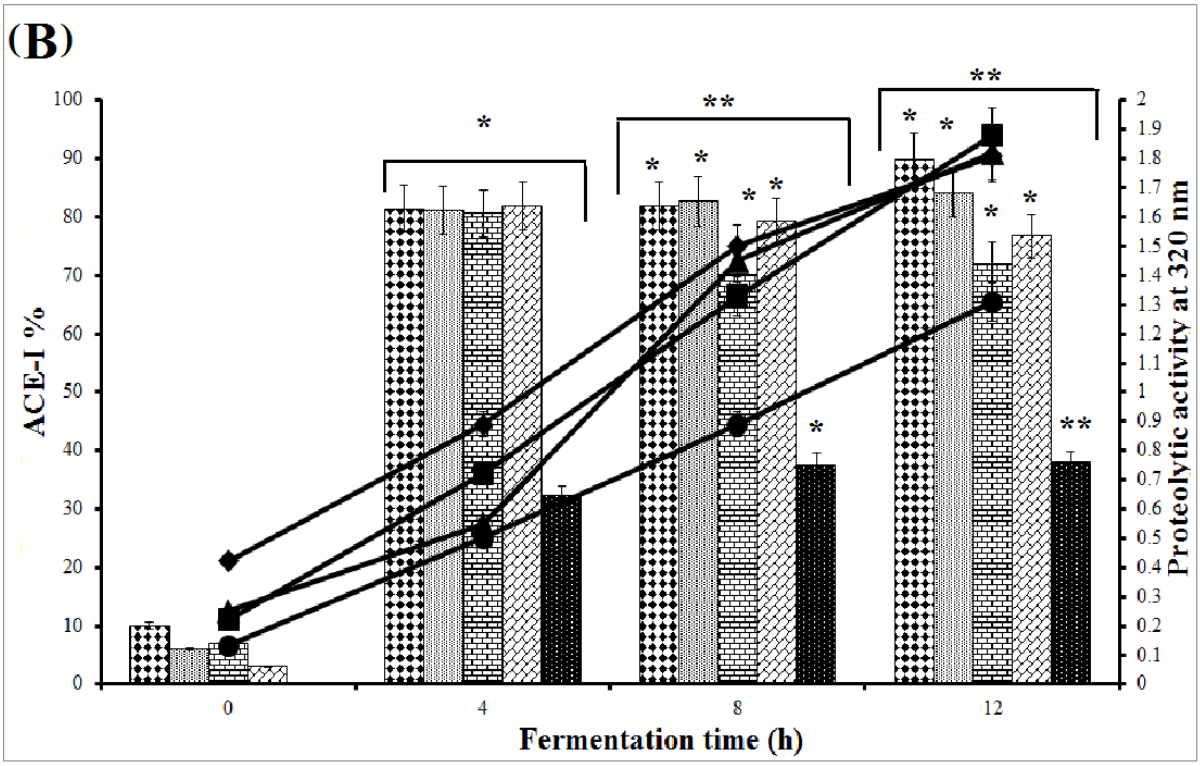

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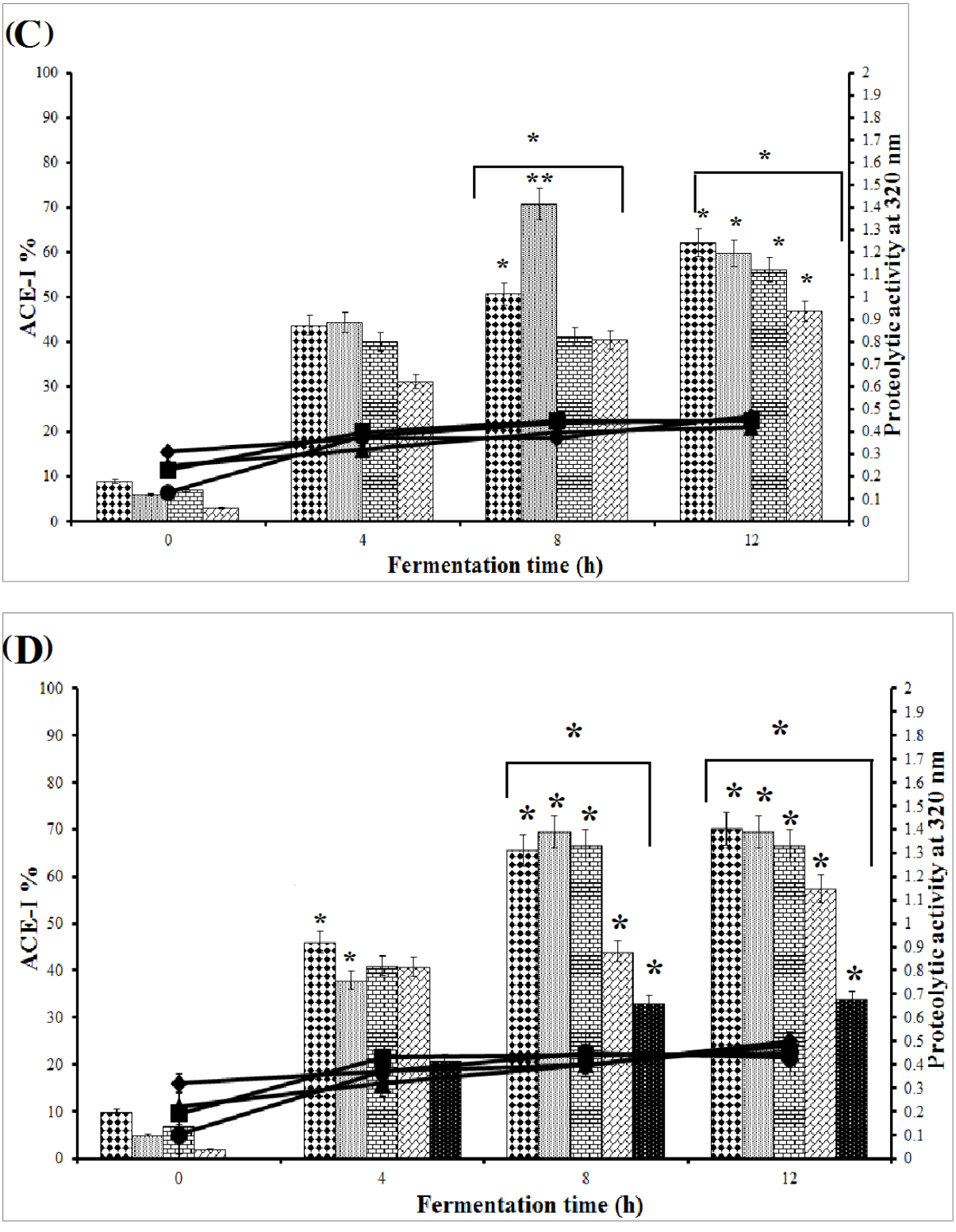

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624 **Figure 2-** Proteolytic activity at 320 nm (line) and ACE-I % (bars) of *L. helveticus* strains (

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

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

(**C**) WPC and (**D**) WPC with Flavourzyme® (Error bars depict standard error of the means) lines above signify differences at (*) $P < 0.05$ and (**) $P < 0.01$.

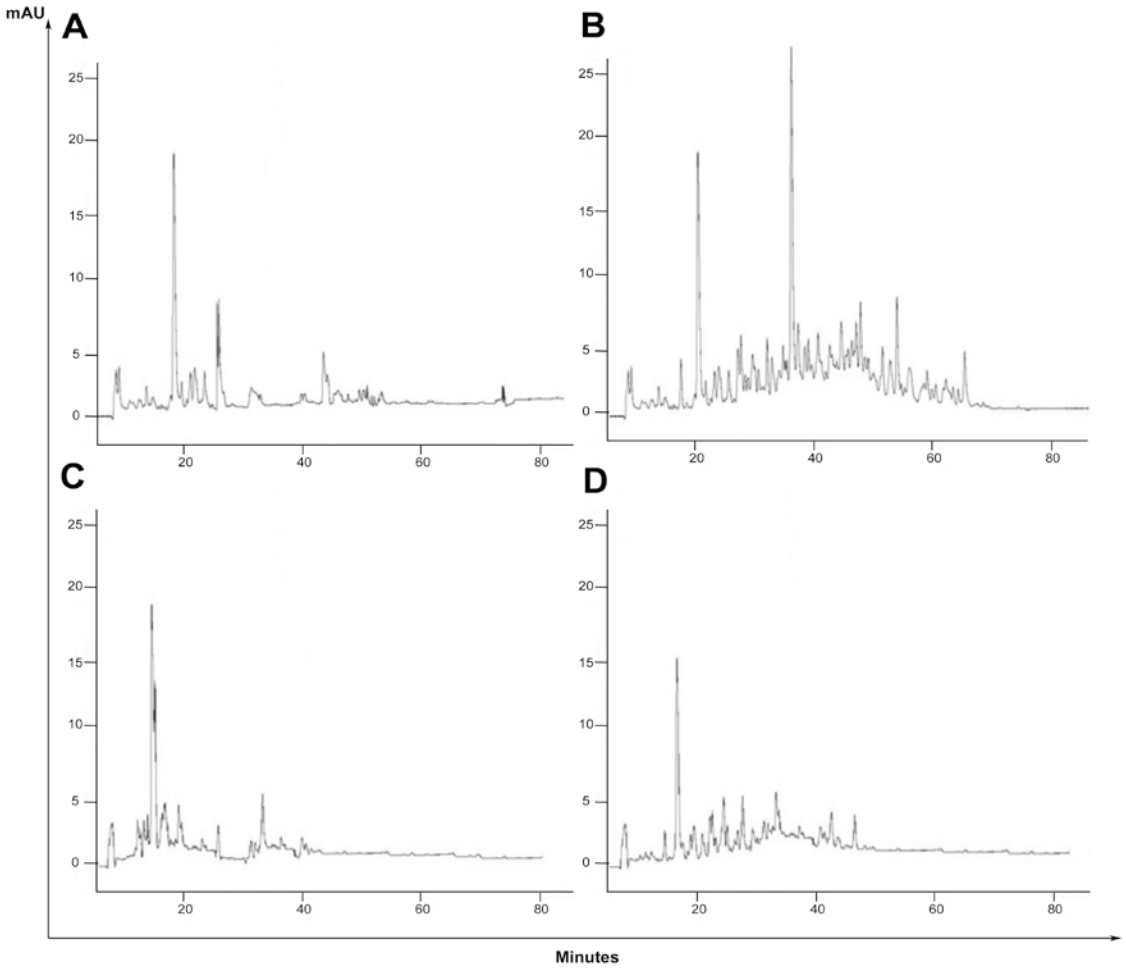

Figure 3- RP-HPLC peptide profile of water soluble extracts obtained from fermented skim milk made with a combination of *L. helveticus* strains and Flavourzyme® ; 881315(**A**), 881315 with Flavourzyme® (**B**), 881188 (**C**) and 881188 with Flavourzyme® (**D**) after 12 h fermentation at 37 °C.