The biological activity of fermented milk produced by *Lactobacillus casei* ATCC 393 during cold storage

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ABSTRACT

Lactobacillus casei ATCC 393 is an important probiotic strain widely known in dairy technology. However, its capability to produce bioactive peptides from milk proteins has not been studied. The viability of the *Lb. casei* ATCC 393 strain and some physicochemical properties in fermented milk throughout storage for 21 days at 4 °C was evaluated; biological activity, i.e., antioxidant, angiotensin converting enzyme inhibitory and anticancer activities of water soluble extract and its filtrate (< 2 kDa; F1) were determined. *Lb. casei* counts remained over 9 log cfu g^{-1} during the storage period in fermented milk. These bio-activities were increased significantly ($P < 0.01$) during storage. F1 of fermented milk after three weeks of storage showed the highest bioactivity impact. De novo sequencing assay for peptide identification was applied to the mass spectrum of F1. The promising capability of *Lb. casei* ATCC 393 to release bioactive peptides from milk proteins was demonstrated.

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1. Introduction

Over the last century, drugs have been massively used to treat, mitigate or avert diseases. However, increasing analytical capability at the molecular level and the discovery of nutrients have led to superior understanding of the complex biochemical relationships of the abundant chemicals that develop naturally in food and their functional influences and activities on the human health and well-being. Accordingly, consumption of functional foods has been rising in popularity and certainly appears to be more than just a trend, but has become a way of life (Banks, 2004). Fermented milk products are considered as the first functional foods (Sanders, 1998). Milk has potentially changed from being just a raw material for dairy products to being a source of a various bioactive compounds that are generally characterised with health promoting properties (Miller, Jarvis, & McBean, 2000). Extracting valuable and promoting health components from milk promises to be a significant added value to dairy products.

Probiotics are an important category of functional foods as they provide health benefits beyond the traditional nutrition value. Lactic acid bacteria (LAB) including *Lactobacillus* spp. are considered to be among the probiotic microorganisms. Many *Lactobacillus* strains display high proteolytic activity toward milk protein, which in turn provides the ability to potentially release the bioactive peptides. Bioactive peptides with antioxidant activity and ACE-inhibitory activity in food products were intensively studied; particularly the release of bioactive peptides during milk fermentation by lactobacilli (Gobbetti, Minervini, & Rizzello, 2004). Various antioxidant and ACE-inhibitory peptides have been separated from fermented milk types produced with different *Lactobacillus* spp (Elfahri, 2012).

Lactobacillus casei strains are generally utilised as starters in fermented milk, and have proven to display antioxidant and ACE inhibitory activities. It has been reported that the *Lb. casei* strains PRA205, YIT9029, LC26, LC279, FC113, Shirota, and Zhang exhibited ACE-inhibitory activity (Gonzalez-Gonzalez, Tuohy, & Jauregi, 2011; Nejati et al., 2013; Solieri, Rutella, & Tagliazucchi, 2015). Furthermore, *Lb. casei* strains 01, KCTC3260, SY13 and PRA205 showed antioxidant activity as reported by Kim et al. (2005); Lee, Hwang, Chung, Cho, and Park (2005); Solieri et al. (2015) and Zhang et al. (2011), respectively.

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Lb. casei ATCC 393 is well known probiotic strain with health promoting activities including, lowering cholesterol, anticancer activity and reducing the risk of osteoporosis. In addition, *Lb. casei* was successfully used to produce probiotic dairy products, i.e., probiotic cheese, probiotic yoghurt and fermented milk (Dimitrellou et al., 2016; Sah, Vasiljevic, McKechnie, & Donkor, 2014; Sidira et al., 2013). Moreover, *Lb. casei* ATCC 393 showed high proteolytic activity (Vukotić et al., 2016; Yeo & Liong, 2010). It is worth noting that only Sah et al. (2014) focused on the ability of this strain to produce bioactive peptides with antioxidant and anticancer activities when it was co-cultured with yoghurt starters for probiotic yoghurt production.

To the best of our knowledge, the antioxidant, ACE-inhibitory and anticancer activities in vitro for peptides released in milk fermented by only *Lb. casei* ATCC 393 strain has not been studied. Therefore, the aim of this work was to assess, in vitro, the antioxidant, ACE-inhibitory and antiproliferation activities of peptide fractions obtained from milk cultured with only *Lb.* casei ATCC 393 addition, during 21 days of storage at 4 °C.

2. Material and methods

2.1. Chemicals and media

Ferrozine™, *o*-phthalaldehyde (OPA), potassium persulphate and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Tokyo chemical industry (Tokyo, Japan). De Man Rogosa Sharpe (MRS-Oxoid) medium was purchased from Thermo Scientific™ (Dublin, Ireland). ACE kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All chemicals used in this study were analytical grade.

2.2. Culture propagation

Lb. casei subsp. *casei* ATCC 393 was obtained from Biological Resource Centre, National Institute of Technology and Evaluation, Japan (NBRC), as culture collection no. NBRC 15883. Frozen culture of the strain was activated by spreading on MRS agar and incubated under anaerobic conditions for 72 h at 37 °C. One colony was inoculated in 10 mL MRS broth and incubated for 24 h at 37 °C. After two successful transitions, bacterial cells were separated by centrifugation (CF15RXII, Hitachi koki co., Ltd, Hitachi, Japan) at $5000 \times g$ for 10 min at 4 °C, then washed twice with sterilised physiological saline (PS) (0.85% NaCl). Cell pellet was re-suspended in PS to obtain approximately 10^9 cfu mL^{-1} and used as a starter culture to produce fermented milk.

2.3. Milk fermentation

Cow milk was obtained from local farm in Ishikawa prefecture, Japan. Milk was heated for 15 min at 90 °C in water bath with agitation, cooled to 40 °C, inoculated with 2% (v/v) of *Lb. casei* ATCC 393 suspended in physiological saline, and then incubated at 37 °C for 24 h. The fermented milk was cooled to 4 °C and stored at this temperature for three weeks. Analysis was carried out at 1, 7, 14, and 21 days of storage.

2.4. Titratable acidity and pH analysis

Titratable acidity (TA), expressed as percent lactic acid, of fermented milk samples was determined according to AOAC (2000) using titration with 0.1 M NaOH and phenolphthalein as an indicator. The pH value was measured using a digital pH meter (WTW-InoLab® pH 7310, VWR, UK).

2.5. Viable cell counts

Viable cell count of *Lb. casei* in fermented milk was performed by counting the colony forming units (cfu) on MRS agar according to Sah et al. (2014). Plates were incubated under anaerobic conditions for 72 h at 37 °C. The number of viable cells per mL was counted and presented as log cfu mL^{-1}.

2.6. Determination of proteolytic activity

The ability of *Lb. casei* to hydrolyse milk protein was determined using the OPA method described by Elfahri, Vasiljevic, Yeager, and Donkor (2016). Two and a half millitres of fermented milk was mixed with 5 mL of trichloroacetic acid (TCA) (0.75%, w/v) then centrifuged at $3000 \times g$ for 15 min at 4 °C. The supernatant was filtrated through a 0.45 μm syringe filter (Advantec, Tokyo, Japan). Fifty microlitres of filtrate was mixed with 1 mL OPA reagent and incubated for 2 min at room temperature. A UV/Vis spectrophotometer (model v-630Bio, Jasco International Co., Ltd., Tokyo, Japan) was used to measure the absorbance at 340 nm. The proteolytic activity of *Lb. casei* was expressed as the absorbance of free NH_3 groups read at 340 nm.

2.7. Preparation of water-soluble extract

Water soluble peptide extract (WSE) was prepared according to Sah et al. (2014). Fermented milk was centrifuged at $20,000 \times g$ for 30 min, filtrated through 0.45 μm syringe filter. The obtained WSE was further fractionated using Vivaspin® 15R filter with 2 kDa cut off (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The filtrate (containing peptides < 2 kDa) was collected and named F1. WSE and F1 were collected, and stored at −20 °C until further analysis. The protein content of WSE and F1 was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

2.8. Antioxidant activity

2.8.1. ABTS radical scavenging activity

ABTS assay evaluates the ability of the antioxidant to scavenge the ABTS radical cation (ABTS$^{\bullet+}$) causing a reduction in absorbance at 405 nm. The ABTS method used was as adapted in De Gobba, Espejo-Carpio, Skibsted, and Otte (2014). ABTS$^{\bullet+}$ stock solution was prepared by the addition of 19.4 mM ABTS to 6.7 mM potassium persulphate (final concentration) and stored in the dark at ambient temperature for 16 h. ABTS$^{\bullet+}$ stock solution was diluted 400 times with distilled water to obtain absorbance of 0.6−0.7 at 405 nm before using. Fifty microlitres of WSE or F1 was mixed with 200 μL ABTS$^{\bullet+}$ and incubated at ambient temperature for 30 min in the dark. A plate reader (Benchmark Plus, Bio-Rad Laboratories, Hercules, CA) was used to monitor the absorbance at 405 nm. The ABTS radical scavenging activity (%) was calculated as follows:

$$\text{Radical scavenging (\%)} = 100 - \left[\frac{A \, sample}{A \, control} \times 100 \right] \quad (1)$$

where *A sample* is the absorbance of the WSE or F1 samples, and *A control* is the absorbance of the control with water.

The antioxidant activity was expressed as SC$_{50}$ (protein concentration required for 50% scavenging of ABTS radicals). SC$_{50}$ was calculated using a linear regression of curves of radical scavenging % versus sample concentration.

2.8.2. Iron chelating activity

Iron chelating activity of WSE and F1 fraction was determined based on their potency to inhibit the formation of Ferrozine™

complex with Fe^{2+} as described by De Gobba et al. (2014). Twenty-five microlitres of WSE or F1 were mixed with 100 µL 75 µM $FeSO_4$ and incubated at room temperature for 10 min, followed by an addition of 100 µL 500 µM Ferrozine™ solution and then incubated at room temperature for a further 10 min. The absorbance was measured at 560 nm using the plate reader. Iron chelating activity was calculated as follows:

$$\text{Iron chelation (\%)} = 100 - \left[\frac{A\ sample}{A\ control} \times 100 \right] \quad (2)$$

where *A sample* is the absorbance of WSE or F1 fraction, and *A control* is the absorbance of the control with water. Iron chelating activity was expressed as IC_{50} (the protein concentration of WSE or F1 needed to inhibit 50% of the iron−Ferrozine complex formation). A curve was plotted of the iron chelation as a function of WSE or F1 concentrations, and the slope of the linear part around 50% activities was used to calculate the IC_{50}.

2.9. ACE-inhibitory activity

ACE inhibitory (ACE-I) activity of WSE and F1was determined by a colorimetric method using ACE kit−west (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the protocol provided by the manufacturer. The pH of WSE and F1 fractions were adjusted to pH 7.0 using 1 M NaOH then 20 µL WSE or F1 was placed in a 96-well microplate. Twenty microlitres of substrate buffer was added, followed by an addition of 20 µL enzyme mixtures. After incubation for 60 min at 37 °C, the indicator solution was added to each well, followed by incubation at room temperature for 10 min. The plate reader was used to read the absorbance at 450 nm. The ACE-I activity was calculated as follows:

$$\text{ACE inhibition (\%)} = \left[\frac{A\ blank\ 1 - A\ sample}{A\ blank\ 1 - A\ blank\ 2} \right] \times 100 \quad (3)$$

where *A blank 1* is the absorbance of positive control (without samples), *A blank 2* is the absorbance of reagent blank (without addition of enzyme mixtures), *A sample* is the absorbance in the presence of WSE or F1 fraction.

ACE-I activity was expressed as $IC_{50.}$ The concentration of the WSE or F1 required to inhibit 50% of ACE activity was defined as the IC_{50} and calculated as mentioned above in section 2.8.2.

2.10. Anticancer activity

Anticancer activity of WSE and F1 were measured against MCF-7 and Caco-2 carcinoma cell lines according to the method described by Elfahri et al. (2016). Caco-2 (RCB 0988) and MCF-7 (RCB1904) cells were purchased from RIKEN BRC (Tsukuba, Ibaraki, Japan). Caco-2 cells were cultured by Dulbecco's Modified Eagle's Medium (DMEM; Wako) containing 20% foetal bovine serum (FBS) and 1% nonessential amino acids (NEAA). MCF-7 cells were cultured in DMEM containing 10% FBS, 1% NEAA and 1 mM sodium pyruvate. Cells were seeded into a 96-well flat-bottom plate (Corning Japan K.K, Osaka, Japan) at 1×10^3 cells per well and incubated overnight at 37 °C and 5% CO_2. Thereafter, 25−50 µL sample was added to each well and incubated at 37 °C and 5% CO_2 for 72 h. The treated cells were assayed with the Cell Proliferation Kit (MTT assay: Roche Diagnostics Co. Ltd., Tokyo, Japan) following the protocol provided by the manufacturer.

The expression to calculate inhibition rate was as follows:

Antiproliferative activity (%) = [1 − (A − B)/(C − B)] × 100

where A is the sample absorbance; B is the background absorbance (non-cell control); C is the vehicle control absorbance (sterilised water).

2.11. Identification of peptide amino acid sequences

Peptides in F1 fraction were purified and concentrated using a Pierce C18 Spin Column (Thermo Fisher Scientific, UK) and were analysed using tandem mass spectrometry as described by Oyama, Van Hung, Yoda, He, and Suzuki (2017). The mass spectra were analysed using PEAKS software (Bioinformatics Solutions Inc., Ontario, Canada) for peptide sequences identification.

2.12. Statistical analysis

All tests were performed in triplicate and the results were presented by the mean values ± SD. Analysis of variance (ANOVA) was performed by Minitab® 18.1 (MINITAB Inc., Coventry, UK), using the general linear model (GLM) procedure and Tukey's test for pairwise comparison. Pearson correlation analysis was used to explore the relationship between variables.

3. Results and discussion

3.1. Fermented milk acidity

Titratable acidity percentage (TA%) and pH values of fermented milk manufactured by *Lb. casei* ATCC 393 during storage are given in Table 1. TA% increased significantly ($P < 0.01$) during 21 days storage at 4 °C. A similar trend was reported by Sah, Vasiljevic, McKechnie, and Donkor (2015), who used *Lb. casei* 393 to produce probiotic yoghurt. On the other hand, the pH values of the fermented milk were decreased significantly ($P < 0.01$) during storage. These results are in agreement with those of Dimitrellou et al. (2016) who found that TA% was increased from 0.7 to 0.9, whereas the pH values were reduced from 4.69 to 4.05 during 28 days storage at 4 °C of fermented milk produced by yoghurt culture enriched with *Lb. casei* ATCC 393 More recently, a reduction in pH values (pH 4.27 to 4.03) and an increase in TA% values (from 1.1% to 1.44%) during 60 days cold storage of probiotic yoghurt containing *Lb. casei* ATCC 393 were reported by Bosnea, Kopsahelis, Kokkali, Terpou, and Kanellaki (2017). These findings confirm the potential capability of *Lb. casei* ATCC 393 to produce organic acids during cold storage. In this context, Terpou et al. (2017) reported the ability of *Lb. casei* ATCC 393 to produce organic acids in sour milk.

3.2. Viable cell counts

Health promoting benefits of probiotic bacteria depend on live bacteria counts. *Lb. casei* ATCC 393 cell counts during storage are shown in Fig. 1. The storage period showed no significant ($P > 0.05$) influence on viable *Lb. casei* cells; counts remained very similar

Table 1
Physiochemical properties of milk fermented by *Lactobacillus casei* ATCC 393 during storage at 4 °C.[a]

Storage period (days)	pH	Acidity (%)	Proteolytic activity (Abs 340 nm)
1	$4.52^a \pm 0.02$	$0.73^d \pm 0.02$	$0.36^d \pm 0.01$
7	$4.33^b \pm 0.03$	$0.77^c \pm 0.02$	$0.44^c \pm 0.01$
14	$4.09^c \pm 0.02$	$0.85^b \pm 0.01$	$0.58^b \pm 0.01$
21	$3.95^d \pm 0.01$	$0.90^a \pm 0.01$	$0.67^a \pm 0.01$

[a] Data are means ± standard deviation (n = 3); values in the same column with different superscript letters are significantly different ($P < 0.05$).
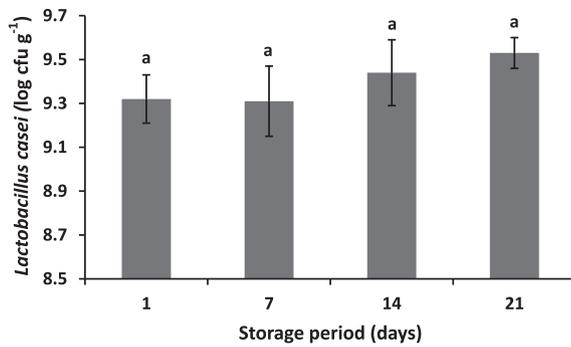
Fig. 1. Effect of cold storage on viability of *Lactobacillus casei* ATCC 393; different letters indicate significant differences ($P > 0.05$).

(over 9 log cfu g^{-1}) during storage time. In this context, Sidira et al. (2013) found a similar trend for *Lb. casei* ATCC 393 in probiotic yoghurt. In contrast, Bosnea et al. (2017), Dimitrellou et al. (2016) and Sah et al. (2015) reported a significant reduction of *Lb. casei* ATCC 393 during 60 days cold storage of probiotic yoghurts. Additionally, Terpou et al. (2017) reported a significant decline in *Lb. casei* ATCC 393 count during cold storage of sour milk for 30 days. These different trends in viability of *Lb. casei* ATCC 393 during storage may be due to the difference in the type of fermented milk, starter inoculation volume and incubation temperature and storage period. Counts of live probiotic bacteria should be ≥ 6 log cfu g^{-1} to obtain probiotic effects (Tripathi & Giri, 2014). As it can be seen in Fig. 1, the count of *Lb. casei* ATCC 393 during the storage period was high enough for health promoting benefits of probiotic bacteria. The high viability of *Lb. casei* obtained in this work can be attributed to the acid-resistant nature of the strain as reported by Kourkoutas, Xolias, Kallis, Bezirtzoglou, and Kanellaki (2005). Furthermore, the liberation of amino acids and small peptides as micro-nutrients may enhance the viability of the probiotic bacteria.

3.3. Proteolytic activity of Lb. casei ATCC 393

Proteolysis extent in fermented milk by *Lb. casei* ATCC 393 during cold storage was measured using OPA methods and data were expressed as the absorbance at 340 nm and given in Table 1. Proteolysis extent was significantly ($P < 0.01$) increased during cold storage, particularly throughout the last two weeks of storage. This is mainly attributed to the potential viable counts of *Lb. casei* until end of storage time with particularly respect to its extracellular and intracellular proteolytic enzymes that led to high proteolysis activity as stated by Vukotić et al. (2016). These authors indicated that whole cell of *Lb. casei* ATCC 393 caused complete hydrolysis of α$_S$-casein and β-casein and partial hydrolysis of k-casein after 4 h of digestion at 30 °C. Additionally, Arora and Lee (1990) reported that *Lb. casei* ATCC 393 produce highly specific activities peptidases and peptidases with broad substrates specificities. Likewise, Ramchandran (2009) reported that the release of proteolytic enzymes from cell wall of lysed bacterial cells accelerates the proteolysis progress.

Few studies have been conducted on the proteolytic activity of *Lb. casei* ATCC 393. It has been reported that probiotic yoghurt produced with *Lb. casei* ATCC 393 had a significantly higher degree of hydrolysis compared with the control yoghurt without *Lb. casei* ATCC 393 or probiotic yoghurt with *Lactobacillus acidophilus* ATCC 4356 (Sah et al., 2014). Additionally, Sah et al. (2015) reported a significant increase in the degree of hydrolysis percentage (% DH) in probiotic yoghurt made by mixed strains (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lb. acidophilus*, *Lb. casei* ATCC 393

and *Lactobacillus paracasei*) during 28 days of cold storage. In a fermented soymilk study, Yeo and Liong (2010) stated that the treatment containing *Lb. casei* ATCC 393 had higher proteolysis degree (0.43, absorbance at 340 nm) compared with treatments containing *Lactobacillus sp.* FTDC 2113, *Lb. acidophilus* FTDC 8033, *Bifidobacterium* FTDC 8943 and *Bifidobacterium longum* FTDC 8643 strains.

3.4. ACE-inhibitory (ACE-I) activity

Lactobacillus strains such as *Lb. casei* are known to produce low molecular mass peptides during hydrolysis of milk protein to fulfil their amino acid requirements. Extracellular proteinases hydrolyse milk protein to oligopeptides, which are then transported into the cell and further hydrolysed by intracellular peptidases into small peptides and amino acids (Gonzalez-Gonzalez et al., 2011).

ACE-I activity of WSE and F1 was determined and results are given in Table 2 as IC$_{50}$ (μg protein mL^{-1}). ACE-I activity of WSE increased gradually but significantly ($P < 0.01$) during cold storage. WSE of 21 days storage exhibited the highest ACE-I activity. It could be due to the accumulation of active peptides during the storage period that resulted from protein hydrolysis by *Lb. casei* cells. This result is in agreement with Nielsen, Martinussen, Flambard, Sørensen, and Otte (2009), who reported that the ACE-I activity increased during cold storage of fermented milk. As seen from data presented in Table 2, F1 exhibited higher ACE-I activity compared with WSE during the storage period. This finding is probably attributed to the high content of small peptides in F1 and their higher purity in this fraction. These results are in agreement with those of Abdel-Hamid, Otte, De Gobba, Osman, and Hamad (2017) who found that ACE-inhibitory peptides were smaller than 3 kDa. Furthermore, Moreno-Montoro et al. (2017) stated that permeate (<3 kDa) of probiotic fermented goat milk had the highest ACE-I activity. It is worth noting that significant ($P < 0.01$) inverse correlations were obtained between the proteolysis extent and ACE-I activity (IC$_{50}$) of WSE (r = -0.945) and F1 (r = -0.824). A similar trend in correlation between ACE-I activity % and proteolysis degree was reported by Pihlanto, Virtanen, and Korhonen (2010) for fermented milk produced by *Lactobacillus* strains. Additionally, Gonzalez-Gonzalez et al. (2011) stated a good correlation between the degree of hydrolysis and ACE-I activity in fermented milk containing the *Lb. casei* YIT 9029 strain.

With respect to ACE-I activity obtained by lactic acid strains, *Lb. casei* ATCC 393 showed the highest ACE-I activity (IC$_{50}$ = 0.09 mg protein mL^{-1}) compared with *Lactobacillus sp.* FTDC 2113, *Lb. acidophilus* FTDC 8033 & ATCC 4356, *Bifidobacterium* FTDC 8943 and *B. longum* FTDC 8643 strains as indicated by Yeo and Liong (2010) in fermented soymilk product. However, Gonzalez-Gonzalez et al. (2011) have reported that among all tested *Lactobacillus* spp. and *Bifidobacterium bifidum MF 20/5* strains in a fermented milk product, *Lb. casei* YIT 9029 showed the highest AC-I activity after 24 and

Table 2
ACE-inhibitory activity (IC$_{50}$) of water-soluble extract (WSE) and filtrate < 2 kDa (F1) of milk fermented by *Lactobacillus casei* ATCC 393 during storage at 4 °C.[a]

Storage period (days)	ACE (IC$_{50}$)	
	WSE	F1
1	52.28[a] ± 0.29	19.72[a] ± 0.46
7	49.38[b] ± 0.27	14.08[b] ± 0.18
14	47.74[c] ± 0.63	13.57[b] ± 0.16
21	42.78[d] ± 0.85	12.69[c] ± 0.07

[a] IC$_{50}$ is defined as the protein concentration (μg mL^{-1}) required to inhibit 50% of ACE activity. Data (in μg mL^{-1}) are means ± standard deviation (n = 3); values in the same column with different superscript letters are significantly different ($P < 0.05$).

48 h of fermentation with IC_{50} of 0.8 and 0.47 mg mL^{-1}, respectively. Solieri et al. (2015) have concluded that *Lb. casei* PRA 205 fermented milk exhibited stronger ACE-I activity (IC_{50} = 54.57 μg mL^{-1}) than that with *Lb. rhamnosus* PRA331. Furthermore, Donkor, Henriksson, Vasiljevic, and Shah (2007) reported an ACE-I activity of IC_{50} of 0.196 and 0.344 mg mL^{-1} in milk fermented by *Lb. casei* L26 and LC279, respectively. Also, milk fermented by *Lb. casei* FC113 showed significantly higher ACE-I Activity (IC_{50} = 0.25 mg mL^{-1}) than other tested probiotic strains (Nejati et al., 2013).

The massive variations in IC_{50} values reported in these studies might reflect the different peptide profiles and/or concentration of peptides produced by different *Lb. casei* strains. Overall, The IC_{50} values obtained in this study is lower than those of relevant fermented milk products with other *Lb. casei* strains, which could be due to the type and specificity of cell envelope proteinases and peptidases of *Lb. casei* ATCC 393.

3.5. Antioxidant activity

ABTS radical scavenging activity and iron chelating methods were used to measure the antioxidant activity of WSE and F1. It is clear from the data in Table 3 that the radical scavenging activity of WSE and F1 increased significantly ($P < 0.01$) during cold storage. In agreement with this finding, Sah et al. (2015) reported that radical scavenging activity of probiotic yoghurt produced by mixed LAB cultures (yoghurt cultures with *Lb. casei* ATCC 393, *Lb. paracasei*, and *Lb. acidophilus*) was increased during cold storage. Additionally, Sah et al. (2014) reported that probiotic yoghurt contained *Lb. casei* ATCC 393 showed significantly higher antioxidant activity (SC_{50} of 1.63 mg mL^{-1}) compared with the control yoghurt and probiotic yoghurt containing *Lb. acidophilus*. The radical scavenging activity (SC_{50}) of this study after 1 day of cold storage (Table 3) was higher than that reported by Sah et al. (2014). This difference is most probably due to the mixed cultures of lactic acid bacteria that are used by these authors to produce probiotic yoghurt. In another study, Solieri et al. (2015) reported that fermented milk containing *Lb. casei* PRA205 exhibited higher radical scavenging activity compared with that containing *Lb. rhamnosus* PRA331.

Clearly, F1 showed higher antioxidant activity than that of WSE during the storage period (Table 3). This finding suggests that small peptides have higher antioxidant activity. Similar findings were obtained by Moreno-Montoro et al. (2017) and Sah, Vasiljevic, McKechnie, and Donkor (2016a), who reported that the permeate (<3 kDa) of probiotic fermented goat milk and probiotic yoghurt exhibited significantly higher ABTS radical scavenging activity than the retentate (>3 kDa). Additionally, Abdel-Hamid et al. (2017) and De Gobba et al. (2014) found that the peptide fraction < 1 kDa of milk protein hydrolysed with different proteolytic enzymes showed the highest antioxidant activity.

Iron chelating activity of WSE and F1 of milk fermented by *Lb. casei* 393 are presented in Table 3 as IC_{50} values. It is obvious that the iron chelating activity of WSE is significantly ($P < 0.01$) increased during cold storage. WSE of 21 days cold storage showed the highest chelating activity. Iron chelating activity of the *Lb. casei* specie has also been demonstrated by other studies; i.e., Zhang et al. (2011) for *Lb. casei* SY13 strain and Kim et al. (2005) for *Lb. casei*-01 strain. Additionally, Lee et al. (2005) reported that *Lb. casei* KCTC 3260 showed a significantly high chelating activity for iron ions at concentration of 10.6 ppm.

Moreover, it is clear from the data in Table 3 that F1 exhibited higher iron chelating activity than WSE with a significantly ($P < 0.01$) increased activity trend during storage. F1 contained low molecular mass peptides that can chelate iron ions. A similar trend in significant iron chelating activity of low molecular mass fraction (<3 kDa) compared with higher molecular mass fraction has been reported by Sabeena Farvin, Baron, Nielsen, and Jacobsen (2010) in a yoghurt product. It is worth noting that a significant ($P < 0.01$) inverse correlation was obtained between the proteolytic activity and both ABTS scavenging activity and iron chelating activity of either WSE (r = −0.908 and −0.97, respectively) or F1 (−0.851 and −0.891, respectively) fractions. Similarly, Sah et al. (2014) reported a strong negative correlation (r = −0.827) between the degree of hydrolysis and ABTS scavenging activity as IC_{50} values.

3.6. Anticancer activity

The anticancer activity of WSE and F1 of milk fermented by *Lb. casei* ATCC 393 was determined by measuring the ability of WSE and F1 to inhibit the proliferation of MCF-7 and Caco-2 cell lines. It can be seen from the data presented in Table 4, WSE and F1 showed a significant ($P < 0.01$) antiproliferative effect against MCF-7 and Caco-2 cell lines. A closer observation of the data revealed that F1 showed more potent antiproliferative activity than WSE against both cell lines (Table 4), reflecting the substantial effect of short length and low molecular mass peptides in F1 to inhibit the proliferation of the cell lines. These findings are in line with Kannan, Hettiarachchy, Johnson, and Nannapaneni (2008) who reported that fractions <5 and 5−10 kDa of rice bran peptide hydrolysates had the highest anticancer activity and inhibited growth of Caco-2 cells by 80%. The authors also reported that the length of the anticancer peptides is ranged from 3 to 10 peptides.

It is worth noting that the Caco-2 cell line was more sensitive than MCF-7 cell toward WSE and F1. In contrast, Ayyash, Al-Nuaimi, Al-Mahadin, and Liu (2018) reported that the bovine fermented milk fraction (< 3 kDa) showed higher antiproliferative effects on MCF-7 than Caco-2 cells. In this context, antiproliferative activity action may follow one of the following hypotheses: (i) competing reaction between the milk peptides and cancer growth factors for the receptors of cancer cell-membrane, or (ii) cytotoxicity of milk peptide on cancer cells that stimulate apoptosis (Chalamaiah, Yu, & Wu, 2018).

Furthermore, the anticancer activity of WSE and F1 was increased significantly ($P < 0.05$) during cold storage (Table 4). F1 of

Table 3
Antioxidant activity [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) scavenging activity (ABTS) and iron chelating activity (Fe chelating)] of water-soluble extract (WSE) and filtrate < 2 kDa (F1) of milk fermented by *Lactobacillus casei* ATCC 393 during storage at 4 °C.[a]

Storage period (days)	ABTS (SC_{50})		Fe chelating (IC_{50})	
	WSE	F1	WSE	F1
1	77.82[a] ± 1.6	32.24[a] ± 0.57	256.92[a] ± 07.92	50.66[a] ± 1.13
7	43.05[b] ± 0.39	19.52[b] ± 0.30	152.33[b] ± 4.59	39.93[b] ± 1.45
14	37.09[c] ± 0.27	5.15[c] ± 0.25	132.05[c] ± 1.99	38.44[b] ± 0.44
21	21.49[d] ± 0.39	2.30[d] ± 0.01	118.97[d] ± 2.09	12.92[c] ± 0.24

[a] SC_{50} is defined as the protein concentration needed to scavenge 50% of the ABTS$^{\bullet+}$; IC_{50} is defined as the protein concentration needed to inhibit 50% of the iron−Ferrozine complex formation. Data (in μg mL^{-1}) are means ± standard deviation (n = 3); values in the same column with different superscripts letters are significantly different ($P < 0.05$).

Table 4
Antiproliferative activity (%) of water-soluble extract (WSE) and filtrate < 2 kDa (F1) of milk fermented by *Lactobacillus casei* ATCC 393 during storage at 4 °C against Caco-2 and MCF-7 cell lines.[a]

Storage period (days)	Antiproliferative activity (%)			
	Caco-2		MCF-7	
	WSE	F1	WSE	F1
1	61.42[c] ± 1.7	83.68[b] ± 1.11	30.93[c] ± 4.33	64.25[b] ± 1.84
7	65.39[b] ± 0.65	79.96[c] ± 0.56	44.08[b] ± 1.14	66.52[b] ± 1.23
14	70.65[a] ± 1.52	84.62[b] ± 1.11	57.16[a] ± 2.15	78.98[a] ± 1.01
21	68.29[ab] ± 1.03	87.07[a] ± 0.61	56.81[a] ± 2.53	78.90[a] ± 1.04

[a] Data are means ± standard deviation (n = 3); values in the same column with different superscripts letters are significantly different ($p < 0.05$).

fermented milk after 21 days of storage had the highest antiproliferative effect against Caco-2 and MCF-7 cells (Table 4). These results suggest that the early stage of hydrolysis releases some peptides that may further degrade due to the secondary hydrolysis into new peptides during storage of fermented milk. In agreement with these findings, Sah, Vasiljevic, McKechnie, and Donkor (2016b) reported that the antiproliferative activity of water soluble peptide extract of probiotic yoghurt containing *Lb. casei* ATCC 393 against HT29 colon cancer cell increased significantly during 4 weeks of cold storage.

3.7. Identification of peptides sequence

F1 of fermented milk after 21 days cold storage showed the highest ACE-I, antioxidant and anticancer activities (Tables 2–4). This finding led us to investigate the peptide identification in F1 fraction using de novo peptide sequencing method. Peptides with high confidence score (ALC > 90%) are presented in Table 4. β-Casein is the main source of the identified peptides, which may be due to the specificity of most lactic acid bacterial cell wall proteinases for many cleavage sites in β-casein (Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005); only five peptides from α_{S1}-casein and two peptides from κ-casein were identified. It is worth noting that Sah et al. (2014) investigated only the anticancer and antioxidant activities of crude peptides of probiotic yoghurt produced by *Lb. casei* ATCC 393, while no peptide sequence was identified. The amino acid composition of the identified peptides confirms their biological activities. Previous work owes the antioxidant activity of the bioactive peptides to their content of His and Pro residues (Padghan, 2012). It has been reported that the peptide containing Pro, His, Met, Tyr, Val, Lys, Cys, and Gln showed high radical scavenging activity (Rajapakse, Mendis, Jung, Je, & Kim, 2005). The same authors indicated that Phe plays an important role as direct radical scavenger. Other studies suggested that basic and/or acidic amino acids play a crucial role in the chelation of metal ions by the side chain amino and carboxyl groups (Zhang et al., 2009).

Various studies have demonstrated the relation between the peptide structure and the ACE-I activity. Byun and Kim (2002) reported that peptides with potent inhibitory activity have hydrophobic amino acid at the C-terminal, as well as in their sequence. The positive charge of basic amino acid residue (Lys and Arg) at the C-terminal provides an ACE-I activity (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011).

Concerning the anticancer activity, different studies on anticancer peptides owes this activity to peptide hydrophobicity and net charge (Chalamaiah et al., 2018).

In light of the relationship between the amino acid sequence of peptides and their biological activities, the amino acid sequences of the identified peptides in this study contain one or more of the above amino acids which could explain their higher ACE-I, antioxidant and anticancer activities. By searching on the databases, all of the identified peptides in our study have previously been described as ACE inhibitory, antioxidant, anticancer peptide or multi-function peptide and their biological activities are summarised in Table 5.

Table 5
Peptides identified in filtrate < 2 kDa (F1) of milk fermented by *Lactobacillus casei* ATCC 393 after 21 days storage at 4 °C.[a]

Peptide	Protein fragment	Observed mass	Charge (z)	Activity	References
DKIHPF	β-CN	755.397	2	Antioxidant/ACE-I	Gobbetti, Ferranti, Smacchi, Goffredi, and Addeo (2000)
VVPPFLQPE	β-CN	1024.56	2	ACE-I	Contreras, Carrón, Montero, Ramos, and Recio (2009)
LLYQEPVLGPVRGPFPIIV	β-CN	2106.22	2	Anticancer/antioxidant/ACE-I	
LLYQEPVLGPVRGPFPII	β-CN	2007.16	2	ACE-I	
ELQDKIHPF	β-CN	1125.58	2	ACE-I	Gobbetti et al. (2000)
LYQEPVLGPVRGPFPIIV	β-CN	1993.14	2	ACE-I	Contreras et al. (2009)
LHLPLPLLQSW	β-CN	1315.77	2	ACE-I	
NLHLPLPLLQSW	β-CN	1430.79	2	ACE-I	
DVENLHLPLPLLQSW	β-CN	1773.93	2	ACE-I	Padghan (2012)
LYQEPVLGPVRGPFP	β-CN	1667.9	2	Antioxidant/ACE-I	Yamamoto, Akino, and Takano (1994)
MPFPKYPVEPF	β-CN	1350.67	2	ACE-I	Perpetuo, Juliano, and Lebrun (2003)
YQEPVLGPVRGPFPIIV	β-CN	1880.06	2	Anticancer/antioxidant/ACE-I	Contreras et al. (2009)
LLYQEPVLGPVRGPFP	β-CN	1780.99	2	Antioxidant/ACE-I	
NLHLPLPLL	β-CN	1028.64	2	ACE-I	Quirós et al. (2007)
NENLLRFF	α_{S1}-CN	1051.54	2	Immunomodulation	Juillerat-Jeanneret, Robert, and Juillerat (2011)
NLLRFF	α_{S1}-CN	808.46	2	Immunomodulation	
FVAPFPEVFGKE	α_{S1}-CN	1365.7	2	Antioxidant/ACE-I	Contreras et al. (2009)
ENLLRFF	α_{S1}-CN	937.502	2	ACE-I	Juillerat-Jeanneret et al. (2011)
RFFVAPFPEVFGKE	α_{S1}-CN	1668.87	3	Antioxidant/ACE-I	Contreras et al. (2009)
ALINNQFLPYPYYAKPA	κ-CN	1982.03	2	ACE-I	
KYIPIQYVL	κ-CN	1135.66	2	Antioxidant	Rana (2014)

[a] Abbreviations are: β-CN, β-casein; α_{S1}-CN, α_{S1}-casein; κ-CN, κ-casein.

4. Conclusion

This study demonstrated the utilisation of the proteolytic strain of *Lb. casei* ATCC 393 as a potential functional dairy starter culture. *Lb. casei* ATCC 393 was proven to be able to release a wide range of bioactive peptides from milk protein. Moreover, *Lb. casei* ATCC 393 grew well in milk under acidic conditions with viable counts that were above the minimum count required for health benefits even after 21 days of cold storage. The growth of *Lb. casei* ATCC 393 in fermented milk released ACE-I, antioxidant and anticancer peptides from milk protein. It is worth noting that the proteolysis extent, the concentration of released bioactive components and the storage period are key factors in determining the level of bioactivities in fermented milk. In comparison with other *Lb casei* strains, *Lb. casei* ATCC 393 has proven to have the potential to produce vigorous bioactive peptides with ACE-I, antioxidant and anticancer activities. Further in vivo work is needed to confirm the biological activities of the specified fermented milk, and to determine the dose/activity correlation.

Acknowledgements

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