Processing and sensory characteristics of a fermented low-fat skim milk drink containing bioactive antihypertensive peptides, a functional milk product

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Effects of processing and sensory characteristics of fermented low fat skim milk drink containing bioactive antihypertensive peptides as a functional milk product

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Sensory evaluation is an important task for manufacturers to improve product quality of new functional fermented milk drinks (FFMD) containing angiotensin converting enzyme inhibitory (ACE-I) bioactive peptides during milk processing by enzymatic/mechanical hydrolysis. Herein, fermented reconstituted skim milk (RSM) by L. helveticus supplemented with Flavorzyme® using bioreactor was able to improve cell viability and ACE-I bioactive peptides. Sensory evaluations of 3 different fermented RSM drink samples were tested by 20 trained panellists. There were no significant differences in flavor, bitterns, appearance and acceptance of FFMD compared to commercial Yakult. Addition of sucrose and flavor provided positive changes in terms of acceptability by consumers

Keywords ACE-inhibitory peptides, Bioreactor, Fermented milk drink, Sensory evaluation

INTRODUCTION

Fermented milk product is defined as a dairy product which, during the fermentation process, has its nutritional aspects as well as its physical and chemical sensory characteristics changed according to the Food and Agriculture Organization of the United Nations and World Health Organization). This process is a result of the activities of lactic acid bacteria (LAB) that use milk as substrate as their main carbon source and growth factors (Sodini *et al.* 2002). Numerous LAB used in the production of fermented milk products are considered as probiotics. Probiotic organisms are defined as 'live microorganisms which when administered in adequate amounts confer health benefits to the host' (Hill *et al*. 2014). The scientific understanding in the field of probiotic bacteria and the processes of bacterial fermentation are improving. The genera of bacteria and dairy yeasts commonly used as probiotics are able to hydrolyze dairy proteins and carbohydrates to produce different types of fermented dairy products (Ahtesh *et al*. 2017). Hydrolyzed dairy proteins have several benefits over non-hydrolyzed proteins as they have developed functionality in the food matrix and are rich sources of bioactive peptides. Bioactive compounds in foods provide physiological benefits including reduced blood pressure (Chen *et al.* 2014). Milk proteins have long been considered as an essential source of amino acid, and a potential media for the production of biologically active peptides (Shi *et al*. 2017; Ahtesh *et al*. 2017). There have been different processes employed to release bioactive peptides in short-time fermentation; some of which have used a stirred bioreactor system (Sodini *et al.* 1998; Yadav *et al.* 2014). Furthermore, various bioactive peptides have been isolated from hydrolysates of casein, which include opioid agonists and angiotensin-converting enzyme inhibitor (ACE-I) peptides (Ahtesh *et al.* 2016). Peptides derived from milk fermentation appear to survive gastrointestinal digestion and have been identified in faeces (Ganjam *et al.* 1997). Among LAB, *L. helveticus* (Lh) has been reported to have high proteolytic activity (Griffiths and

Tellez, 2013; Ahtesh *et al.* 2016) and has been used for milk fermentation, usually in cheese processing. These processes require long fermentation time to obtain the curd. One of these processes is cell bioreactor technology, and this has been proposed for the continuous inoculation and acidification of fermented milk products (Stressler *et al.* 2013; Yadav *et al.* 2014). An immobilized cell bioreactor may also be used to inoculate and acidify milk simultaneously because of the growing activity of the immobilized culture and the resulting cell release into the bulk media. Bioreactor technology has optimal microbiological stability and a massive inoculation of milk with the starter culture of > 10^8 cfu/ mL being observed. Continuous inoculation and milk acidification using four strains of mesophilic LAB that had been separately entrapped had very high productivity and good microbiological stability when operated with milk (Lacroix, 2005). Productivity increased further by 70 % when pH was controlled at 6.4 (by the addition of fresh milk) as compared to when pH was controlled at 6.2 (Sodini *et al.* 1998).

Generally, dairy products, particularly fermented milks, are the most popular vehicles for delivery of bioactive peptides to the body due to their good compatibility, pleasant and attractive sensory profiles as well as high consumption around the world (Granato *et al.* 2010; Shah *et al.* 2011). However, bitterness of enzymatic hydrolysate may limit the use of these products (Spellman *et al.* 2009). Sensory evaluation is a method that provides integrated direct measurements of perceived intensities of target attributes (Bleibaum *et al.* 2002). The traditional method of evaluating the bitterness of fermented milk products is by sensory analysis using a human taste panel (Newman *et al.* 2014). Physicochemical characteristics have been used previously as predictors for bitterness in fermented foods, such as measuring polyphenol content by HPLC analysis or by measuring peptide size and hydrophobicity using Urea-PAGE and RP-HPLC. The consumption of fermented milk is widely associated with the presence of LAB due to their desirable sensory characteristics promoted by these

microorganisms and the associated health benefits to the consumer. To the best of our knowledge, this work is the first to investigate the efficiency of agitation on ACE-I bioactive peptides by combination of *L. helveticus* and Flavorzyme® using bioreactor. Therefore, herein the objectives of this study were to evaluate the ability of consumers to accept the fermented milk drink containing bioactive peptides by adding flavor and sucrose at the end of the fermentation processes and, furthermore, to increase casein hydrolyses of fermented skim milk (SM) drink product in short fermentation time using a stirred bioreactor.

MATERIAL AND METHODS

Materials

Skim milk (SM) powder was obtained from (Murray Goulburn Co-operative Co. Ltd., Brunswick VIC Australia and United Milk Tasmania Ltd., TAS Australia), food acid, nature color and flavor (Natural Strawberry, Flavoring Essence) were purchased from a local supermarket (Werribee, Victoria Australia), while MRS broth and sucrose were purchased from Oxoid (West Heidelberg, Vic Australia). Flavorzyme® [Flavorzyme®1000 L (EC 3.4.11.1, an amino peptidase with an activity of 1000 Leucine Amino-peptidase (LAPU/ g) as quoted by Novozymes Australia] was purchased from Novozymes Australia, North Rocks, NSW, Australia, *Lactobacillus helveticus* ASCC 881315 strain was obtained from Dairy Innovation Australia Ltd. Bioreactor system was from (Bio-Stat® A plus, Germany). Bradford reagent and standard bovine serum albumin (BSA) were purchased from Sigma Chemical Company (St Louis, MO, USA).

Human ethics

The study was approved by the Human Research Ethics Committee (HREC) of Victoria University, under application ID number HRE 13-079, for the conduct of sensory evaluation. All participants signed consent forms before taking part in the sensory test.

Bacteria storage, culture conditions and propagation

Lactobacillus helveticus (*L. helveticus*) strain ASCC 881315 (Lh 881315) was stored at -80 °C. Sterile 10 mL aliquots of MRS broth were inoculated with 1 % culture and incubated at 37°C for 18 h. Lh 881315 was inoculated at 1 % (v/v) into 10 mL aliquots of reconstituted skim milk (RSM, 12 % w/w) supplemented with 0.14 % Flavorzyme®. Following two successive transfers the cultures were finally transferred into sterile RSM.

Preparation of Fermented skim milk drink

Reconstituted skim milk (12 %) was prepared by mixing skim milk powder (SMP; Murray Goulburn Co-operative Co. Ltd., Brunswick VIC., Australia) in distilled water 5 liters (L), and heated at 90°C for 20 min. The media was then inoculated with a combination of *L. helveticus*(1 % level) and Flavorzyme® (0.14 % w/w) (Novozymes Australia, North Rocks NSW Australia) and incubated at 37 °C for 12 h with agitation. Flavorzyme® was added to improve proteolysis in milk. After the fermentation process, the samples were heat treated at 85 °C for 20 min in water bath to kill and inactivate probiotic bacteria and enzyme activities. The product was cooled to room temperature, and strawberry flavor and sugar (5-15 %) were added.

Bioreactor assay of low fat skim milk to increase the % of ACE-I activity

Bioreactor (5 L) capacity (Bio-Stat® A plus, Germany) was employed to ferment 5 L of 12 % pasteurized RSM using a combination of Lh 881315 and Flavorzyme® at 37 °C for 12 h. A

jacketed thermostatic water bath bioreactor held at a constant temperature was used. Milk was continuously stirred by impellers (at 250 rpm). The pH during fermentation was measured using a sterile pH electrode (DPAS Ingold, Paris, France) connected to a transmitter (Demca 3B 1015; Alfortville, France). The pH electrode was calibrated before inoculating the medium. Bacterial growth, proteolytic and, ACE-I activities and pH were determined at 0, 2, 4, 6, 8 and 12 h of fermentation.

Measurement of bacterial growth

Lactobacillus helveticus strain 881315 co-cultured with Flavorzyme® were added to 12 % RSM. Bacterial growth was measured by pour-plate method. Appropriate serial dilutions were made using 0.1 % peptone solution and the strain were incubated at 37°C for 48 h using anaerobic jars with anaerobic kit. The colony enumeration system used was the Stuart colony. The growth of *L. helveticus* was examined every 2 h up to 12 h during the fermentation process at 37 °C. Plates showing 25 to 250 colonies were counted and expressed as colony forming units per mL (cfu/ mL) of sample.

Determination of proteolytic activity

Proteolytic activity of *L. helveticus* strain 881315 was determined using the O-pathalaldehyde (OPA) method. Briefly, 3 mL of sample was mixed with equal volume of 1 % trichloroacetic acid followed by filtration using Advantech #231 filter paper. Filtrate 150 µL was mixed with 3 mL of OPA reagent and allowed to react at room temperature for 2 min. The OPA reagent was prepared by adding 25 mL of 100 mM di-sodium tetra-borate, 2.5 mL of 20 % (w/w) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL methanol and 100 µl of β-mercaptoethanol in 50 mL total volume of the reagent. Absorbance of the samples was measured at 340 nm using UV-VIS spectrophotometer (LKB NOVASPEC II Pharmacia,

LKB Bio- Chrom UK). The relative absorbance between the control and sample was used as an indication of proteolysis.

Determination of ACE-Inhibitory activity

A crude extract of the fermented sample (50 mL) was prepared by centrifugation at 4000 x g at 4 °C for 30 min using Beckman Coulter (Avanti J-26S XPI) and the supernatant was freeze-dried (Freeze-drier model ALPHA 1-4 LSC plus; John Morris Scientific Pty. Ltd. Deepdene Australia) for 72 h. The freeze dried extract (40 mg) was dissolved in 2 mL of Tris buffer (50 mM, pH 8.3) containing 300 mM Sodium chloride as we previously reported (Ahtesh *et al.* 2016). Fifty µL of 1.25 mU ACE enzyme from rabbit lung in Tris buffer and 50 µL of 3.0 mM Hippuryl-Histidyl-Leucine (HHL) in Tris were added to 50 µL of sample and incubated at 37 °C in a shaking water bath for 30 min. 150 µL of Glacial acetic acid was added to stop the reaction. The amount of Hippuric acid (HA) released was analysed by HPLC. The HPLC system consisted of a Varian 9012 solvent delivery, a Varian 9100 auto-sampler and a Varian 9050 variable wavelength ultraviolet-visible detector. An analysis was carried out using Gemini® C18 110 Å (100 mm x 4.60 mm, 3 µm) column (Phenomenex, NSW Australia) at room temperature (~22 °C) with a mobile phase consisting of 12.5 % (v/v) Acetonitrile (Merck) in distilled water, pH adjusted to 3.0 using glacial acetic acid. The flow rate was set at 0.6 mL/min and the compounds were detected at 228 nm. The percentage ACE-I was calculated as follows:

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

A standard curve of HA was constructed using 5 predetermined concentrations (0.5 %, 1.0 %, 1.5 %, 2.0 %, and 2.5 %) for quantification of HA in the samples. The activity of ACE-I were plotted against protein concentration in the sample in order to calculate IC_{50}

value, defined as the protein concentration (µg/mL) required to inhibit 50 % of ACE-I activity.

Micro-fluidic Lab-on-a- chip electrophoresis (Loa C)

The preparation of dye and samples on a chip were carried out according to the manufacturer's recomendation and as previously described with minor modifications (Nikolić *et al.* 2012). Briefly, 0.5 µL of reconstituted dye solution added to 5 µL of protein ladder (5 - 240 kD), 5 µL of sample in micro tubes respectively, vortexed and incubated for 30 min on ice. The samples and protein ladder in tubes were heated (95 °C, 5 min) all tubes were cooled for 15 s to recover the condensate of liquid and then briefly spun in a centrifuge (3000 x g) to ensure that the liquid sample and any condensate collected at the bottom of the tube. Distilled water (85 µL) added to the protein ladder and milk samples to give each a total volume of 90 µL. In a typical analysis, a new chip is primed with gel–matrix after which the protein ladder (6 µL) and samples (6 µL) are loaded and analysed.

Chemical Measurements

Protein content of samples, ash and moisture, were examined according to the Association of Official Agricultural Chemists methods. For protein concentration, the Bradford method was used. Three mL Bradford Reagent (Sigma) and 0.1 mL protein sample were added to a test-tube and vortexed to mix. The sample was then incubated at room temperature for 25 min and absorbance was measured at 595 nm using a Pharmacia spectrophotometer (LKB Novaspec II, LKB Biochrom St Albans U.K). Ash and total solids content were obtained using the muffle furnace method; approximately 5 g of fermented RSM was placed in a stainless steel crucible and evaporated to dryness in an oven at 100 °C. The dry sample was placed in a muffle furnace at 550 °C for 16 h, until it was free of carbon. Once ash temperature was the

same as room temperature, the crucible containing the ash was weighed and the results calculated using the equation below:

$$\text{Ash } \% = \frac{\text{weight of residue x 100}}{\text{weight of sample}}$$

All samples were in triplicate using the same equipment and conditions. For pH measurements, a calibrated digital pH meter (Meter Lab, Pacific Laboratory Products, and Blackburn Victoria Australia) was used.

The percentage moisture content was determined by the oven-drying method at 102 °C, using the following equation:

$$\text{Moisture } \% = \frac{\text{A} - \text{B} - \text{C X 100}}{\text{D}}$$

A = Sample and dish weight/g

B = Blank average/g

C = Empty dish weight

D = Sample weight/g

Sensory analyses of the fermented skim milk drink

Sensory properties of the fermented skim milk and control batches were assessed by 20 trained panellists recruited from staff members and students from the College of Health and Biomedicine at Werribee campus, Victoria University. Panellists were selected according to international standards (ISO 11035:1994 and ISO-8586:2012). The panellists were first trained for perception of flavor by giving them standard solutions of lactose 5 %, for

sweetness judgement (normal sweetness) and 0.19 g/dL L-leucine for bitterness (extreme bitterness) based on the ISO 11035:1994, ISO 8589:2007 and ISO-8586:2012. They were presented with samples coded as (A) fermented RSM drink containing peptides (FSMP); (B) final product of fermented RSM containing peptides and 5 % sucrose (FSMPC) (to mask the bitterness) and (C) 15 % sucrose with 5 % strawberry flavor and aroma, FSMPCF; (D) Reference fermented milk commercially available-Yakult as control 1; and (E) unfermented Reconstituted skim milk (UNFSM) as control 2. The lighting and environmental conditions for the test were in accordance with international standards (ISO 8589:2007). Samples in 30 mL white plastic cups coded with three digits at room temperature (~25 °C), were presented to each panellist. Water and crackers were given to panellists for palate cleansing between samples allowing 15-min breaks between sessions. Panellists were advised not to swallow the product. Each panellist evaluated four samples for flavor (bitterness), texture, color, and appearance, using a 10-point hedonic scale (1 = dislike extremely to 10 = like extremely) and compared them to the two controls.

Three sensory evaluation sessions repeated in 3 weeks were performed by the same group of panellists in order to assess the acceptability of the products compared to the controls as affected by supplementation with sucrose and/or peptides. All sensors were conducted in triplicate and in a standardized room according tointernational standards 1988.

Statistical analyses

All data were expressed as mean values of three replicates with standard deviation. One-way ANOVA was performed to investigate the significant differences in the treatments; by Minitab 16 software. The level of significance was tested at $P < 0.01$. The test was used to investigate significant differences among the treatment means.

RESULTS AND DICUSSION

Efficiency of mechanical agitation on ACE-I peptides activity, proteolytic activities, growth and pH

Several studies on *Lactobacillus species* have focused on the enhancement of lactic acid and biomass production using bioreactor rather than on bioactive antihypertensive peptides production. This study, reports on the development of an efficient fermentation process, with respect to effect of agitation, along with ACE-inhibition (ACE-I) peptide activity production during the 12 h fermentation and strategies like fed-batch and semi-continuous fermentation in the 5 L bioreactor.

The effect of using a bioreactor on RSM fermentation with the combination of Lh 881315 and Flavorzyme® was compared to a traditional fermentation presented in (Figure 1, 2 and 3). There were sharp increases ($P < 0.01$) in ACE-I activity using stirred bioreactor between 0 and 2 h initial fermentation (~ 60 %) correlated to the protein hydrolysates (Figures 1, 3) and compared to the traditional fermentation method in the same fermentation time (~10 %). This may be due to the mechanical process of the bioreactor that has led to the improvement at the casein hydrolysis. ACE-I activity between 4 h and 12 h fermentation increased further from 60 % to 95 % at pH 3.5 using stirred bioreactor, whereas ACE-I activity using traditional fermentation was 82 % at 12 h fermentation (Figure 1) and the pH was 4.9 (Figure 2). This could be attributed to self-digestion of the enzyme, although it has been reported that casein may act as a protecting agent against self-digestion and subsequent loss of enzyme activity. Results show that mechanical treatment in the bioreactor actually aided membrane damage to bacteria and resulted in greater accessibility of enzyme hydrolyses of substrates and consequently yielded higher peptide production compared to the fermented RSM traditional fermentation and untreated RSM (Figures 1B, 1C). Previously we used same strains with Flavorzyme® and reported that peptide mixtures showed a

homogenos pattern in HPLC-UV analysis and exhibited ACE-I activity (Ahtesh *et al.* 2016). The ACE inhibition of the peptide mixtures was dependent on the MWs of the membranes (Eisele *et al.* 2013). Results showing the migration pattern for proteins in the molecular weight ladder, control RSM and traditional fermented RSM and fermented RSM with agitation by bioreactor, respectively (Figure 1B, C). Untreated RSM proteins presented non peaks indicating non-hydrolysed proteins. The migration pattern and profile of fermented milk shows a number of peaks ranging from under 5 kDa (small size of molecular weight (MW) peptides) to above 240 kDa, compared to the migration pattern and profile of fermented milk with agitation shows an increase a number of peaks ranging from under 5 kDa (small size of MW peptides) to ~150 kDa. This provides evidence for casein and other milk protein hydrolyses with varying MW of peptides. We have identified these peptides and showed that they are able to normalize high blood pressure of spontaneously hypertensive rats following 10 weeks of oral administration (Shi *et al.* 2017; Ahtesh *et al.* manuscript submitted). The suggested concentration for probiotic bacteria providing health benefits was at least $\log_6$ CFU/ mL of a product during its shelf life. Probiotic reconstituted fermented skim milk drink revealed populations of Lh of $\log_6$ cfu/ mL using bioreactor fermentation at 37 °C during 12 h, whilst $\log_{5.6}$ cfu/ mL during 12 h with a traditional fermentation (Figure 2). There was an increase in growth during 12 h fermentation in both methods (agitation and non- agitation) with significantly higher growth in the agitated system ($P < 0.01$) (Figure 2). The growth correlated with a drop in pH measured during fermentation and was due to the lactic acid production which increased between 2 to 12 h fermentation in the bioreactor (pH dropped from ~ 6.5 to ~ 3.9 at 12 h) (Figure 2). There was no significant decrease in pH in the fermentation without agitation during the first 6 h; however, there was a significant decrease in pH between 6 - 12 h fermentation time ($P< 0.01$) (Figure 2). Similar results have been reported previously using the same strain with Flavorzyme® under same conditions of

traditional fermentation (Ahtesh *et al.* 2016). Overall, the bioreactor system hydrolyses with improved ACE-I activity correlated with growth in a shorter fermentation time compared to the traditional fermentation.

Chemical measurements

Thenutritional ingredients of low-fat fermented RSM drink compared to unfermented RSM and commercial dairy product (Yakult) are shown in (Table 1). The ash content,sugar,moisture and protein were not significantly different between fermented RSM containing peptides compared to Yakult, whereas results of fermented RSM drink were significantly different ($P < 0.05$) from untreated RSM. Similarly, the use of *Lactobacillus plantarum* to ferment 10 % SM for 8 h at 37 °C were reported (Souza *et al.* 2013). Most analysis involving the development of milk-based fermented beverages, like fermented milk, yoghurts and milk drink, have reduced content or even absence of fat (Venturoso *et al.* 2007). Overall, there is a similarity between commercial dairy products (Yakult) compared to fermented RSM drink; however, the protein content was less in fermented RSM drink (0.1/100 g) compared to Yakult (1.9/100 g) due to the protein hydrolyses during the fermentation process (Table 1). In general, most of the nutrition ingredients especially total minerals of fermented drink have declined about half compared to the untreated SM powder and RSM due to the bacterial strains consumed for growth.

Sensory analyses

In these studies, sensory evaluation was fundamental to observe the behaviour of different types of RSM drinks before and after the fermentation process using 5 sensory descriptors (Figure 5). As such, the addition of sucrose that characterises the resulting product in relation to appearance, flavor, taste and /or texture, during their shelf-life, beyond verifying the acceptability by consumers was key to the evaluation. Comparisons of samples were

conducted by means of sensory evaluation using the hedonic scale (Lawless, 2010). Sensory evaluations were carried out after 12 h fermentation time at 37 °C and cooled to 5°C. Twenty trained panellists were asked to taste and compare 3 different fermented RSM drink samples; (1) fermented RSM drink containing peptides (FSMP), (2) fermented RSM drink containing peptides and 5 % sucrose (FSMPC), and (3) fermented RSM drink containing peptides and 15 % sucrose with 5 % flavor (FSMPCF). Commercial product (Yakult) and unfermented RSM (UNFSM) were used as controls. The attributes described were bitterness, flavor, appearance, texture and overall acceptability compared to the control samples (Figure 4, 5). Comparison amongst groups and controls showed significant differences in terms of color and appearance ($P < 0.05$), acceptability ($P < 0.05$), flavor, ($P < 0.05$) and texture ($P < 0.001$).

Initially, appearance was notably significant different between groups (Figure 5A). This attribute presented a large variability in fermented drink groups due to the strawberry flavor used as ingredients in their preparation. Fermented RSM containing peptides with 5 % strawberry flavor presented a higher score of appearance than those prepared without strawberry. This significant increase in appearance is in accordance with previous results (Andrés, Villanueva *et al.* 2014), which correlated the chromatic parameter with β-carotene content. Only FSMCPF was noted as having small visible differences in appearance and texture compared to untreated RSM.

Bitterness score was significantly higher and homogeneous in beverages from fermented groups compared to controls, due to the influence on the amino acids taste of the peptides contained in the formulation of these beverages (Figure 5A, 5B). It has been noted that the production of quality of fermented milk products depends on the proteolytic activity of the strains used, since the amino acids and peptides formed have a direct impact on flavor. In addition, a study reported that bitterness was generated by peptides containing

phenylalanine (Akira Kawakami, 1995). There was a significant difference ($P < 0.05$) verified between the initial and final mean values of the attributes: flavor, bitterness, appearance and overall acceptance of the product (Figure 5A).No differences between groups FSMPC and Yakult as positive control were found because the sweetness added (Figure 5C).

Increasing sucrose concentration into 15% in strawberry-flavored fermented RSM containing peptides as was previously shown to increase perceived retronasal fruitiness (King *et al.* 2006). Panellists did not detect any significant difference in the strawberry aroma and flavor between FSMCPF groups and Yakult (Figure 5C).

In relation of the overall acceptability, significant differences were detected in group FSMPC containing 5 % sucrose (Figure 5B) in comparison with group FSMPCF containing 15 % sucrose (Figure 5C) because it had better acceptation by panellists compared to Yakult . This pattern agrees with that noted by Kale *et al.* (2012), where blends made with the highest concentration of orange were those that achieved overall best acceptability. Interestingly, the mixed beverages with 5 % strawberry-flavored, the overall acceptability were those with 15 % sucrose perhaps due to the smooth texture being associated with a more natural drink and therefore, perceived as healthier.

In summary, adding flavor and aroma are considered to be important parameters for consumer acceptance. In fact, the addition of fruit flavor and sugar can mask the sour taste in the formulations of fermented dairy products (Koksoy and Kilic, 2004). Four from the 20 panellists were able to accept the bitterness taste of fermented RSM containing peptides without flavor. Eighteen panellists preferred Yakult and FSMCPF, with no differences between them being reported. Hence, the addition of 15 % sucrose and 5 % flavor to fermented RSM positively affected the product and masked the bitter taste. The acceptability of 5 samples were significantly different, whilst there were no significant differences of the acceptability between 15 % sucrose FSMPC and Yakult as control ($P < 0.05$) (Figure 5C).

However, there were significant differences ($P < 0.05$) in acceptance, bitterness, texture and appearance of FSMPC compared to Yakult (Figure 5A,B). The UNFSM and un-flavored fermented RSM were not preferred. Similarly, what was noted in the present study for the fermented RSM drink was the addition of a sweetner (ie. sucrose) which was related to improvement of sensory behavior. However, Ranadheeraa concluded that, lower sensory acceptability was recorded for the samples of fermented drinking milk were made from goats' milk using various culture compositions of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalissubsp. lactis* BB-12 stored for 3 weeks than the respective fresh products may due to the increase of acidity (Ranadheera *et al.* 2016).

Generally, markets for functional dairy products have reached a significant level and are expected to grow in the future. Several dairy products have been tested as vehicles of probiotic cultures, which show functionally and sensorial appropriateness (Sodini *et al.* 2002). According to these results, the acceptable sensory quality and the nutritional and health claims may be used for the promotion of the products and increasing the marketing appeal of functional dairy products.

CONCLUSION

The efficiency of a bioreactor was improved with mechanical agitation during fermentation and resulted in increased cell viability and ACE-I activity from 90.3 % to 95.5 %, using *L. helveticus* 8801315 and Flavorzyme®. Fermented skim milk containing bioactive peptides was developed with acceptable sensory characteristics. However, increased acidity, as well as bioactive peptides, led to increased bitterness of the fermented skim milk drink. The addition of 15 % sucrose and 5 % strawberry flavor provided positive changes to the fermented product in terms of being accepted by consumers.

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AUTHOR CONTRIBUTIONS

FA designed the study, collected and analyzed the data. FA carried out experimental work, interpretation, recruited and communicated with the study participants. VA and LS helped in data interpretation and discussion and helped prepare and edited the manuscript.

REFERENCES

Ahtesh F B, Apostolopoulos V, Stojanovska L, Shah N and Mishra V K (2017) Effects of fermented skim milk drink by *Kluyveromyces marxianus* LAF4 co-cultured with lactic acid bacteria to release angiotensin-converting enzyme inhibitory activities. *International Journal of Dairy Technology* In Press.

Ahtesh F B, Stojanovska L, Shah N and Mishra V K (2016) Effect of Flavorzyme® on angiotensin-converting enzyme inhibitory peptides formed in skim milk and whey protein concentrate during fermentation by *Lactobacillus helveticus*. *Journal of Food Science* **81** M135-M143.

Kawakami A (1995) Taste evaluations of angiotensin I converting enzyme inhibitors, Leu-Lys-Tyr analogues. *Bioscience, Biotechnology and Biochemistry* **59** 709–710.

Bleibaum R N, Stone H, Tan T, Labreche S, Saint-Martin E and Isz S (2002) Comparison of sensory and consumer results with electronic nose and tongue sensors for apple juices. *Food Quality and Preference* **13** 409-422.

Chen Y, Liu W, Xue J, Yang J, Chen X, Shao Y, Kwok L, Bilige M, Mang L and Zhang H (2014) Angiotensin-converting enzyme inhibitory activity of *Lactobacillus helveticus* strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9. *Journal of Dairy Science* **97** 6680-6692.

Eisele T, Stressler T, Kranz B and Fischer L (2013) Bioactive peptides generated in an enzyme membrane reactor using Bacillus lentus alkaline peptidase. *European Food Research and Technology* **236** 483-490.

Ganjam L S, Thornton Jr W H, Marshall R T and Macdonald R S (1997) Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *Journal of Dairy Science* **80** 2325-2329.

Granato D, Branco G F, Cruz A G, Faria J D and Shah N P (2010) Probiotic dairy products as functional foods. *Comprehensive Reviews in Food Science and Food Safety* **9** 455-470.

Griffiths M W and Tellez A M (2013) *Lactobacillus helveticus*: the proteolytic system. *Frontiers in Microbiology* **4** 1-9.

Hill C, Guarner F, Reid G, Gibson G R, Merenstein D J, Pot B, Morelli L, Canani R B, Flint H J, Salminen S, Calder P C and Sanders M E (2014) Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology* **11** 506-514.

Koksoy A and Kilic M (2004) Use of hydrocolloids in textural stabilization of a yoghurt drink, ayran. *Food Hydrocolloids* **18** 593-600.

Lawless H T and Hildegarde H (2010) *Sensory evaluation of food: principles and practices*. Springer Science & Business Media.

Newman J, Egan T, Harbourne N, O′Riordan D, Jacquier J C and O′Sullivan M (2014) Correlation of sensory bitterness in dairy protein hydrolysates: Comparison of prediction models built using sensory, chromatographic and electronic tongue data. *Talanta* **126** 46-53.

Ranadheera C S, Evans C A, Adams M and Baines S K (2016) Co-culturing of probiotics influences the microbial and physico-chemical properties but not sensory quality of fermented dairy drink made from goats' milk. *Small Ruminant Research* **136** 104-108.

Shah N P, de Cruz A G and Faria JdA (2011) In: *Probiotic and Prebiotic Foods: Technology, Stability and Benefits to the human health*, Nova Science Publishers. 1-518

Shi M, Ahtesh F B, Mathai M, McAinch A J and Su X Q (2017) Effects of fermentation conditions on the potential anti-hypertensive peptides released from yogurt fermented by *Lactobacillus helveticus* and Flavorzyme®. *International Journal of Food Science & Technology* **52** 137-145.

Sodini I, Lagace L, Lacroix C and Corrieu G (1998) Effect of continuous prefermentation of milk with an immobilized cell bioreactor on fermentation kinetics and curd properties. *Journal of Dairy Science* **81** 631-638.

Sodini I, Lucas A, Oliveira M N, Remeuf F and Corrieu G (2002) Effect of Milk Base and Starter Culture on Acidification, Texture, and Probiotic Cell Counts in Fermented Milk Processing. *Journal of Dairy Science* **85** 2479-2488.

Souza A H P D, Costa G N, Miglioranza L H D S, Furlaneto-Maia L and Oliveira A F D (2013) Microbiological, physical, chemical and sensory characteristics of milk fermented with Lactobacillus plantarum. *Acta Scientiarum* **35** 125-131.

Spellman D, O'cuinn G and Fitzgerald R J (2009) Bitterness in Bacillus proteinase hydrolysates of whey proteins. *Food Chemistry* **114** 440-446.

Stressler T, Eisele T, Schlayer M, Lutz-Wahl S and Fischer L (2013) Characterization of the recombinant exopeptidases PepX and PepN from *Lactobacillus helveticus*ATCC 12046 important for food protein hydrolysis. *PLoS ONE* **8** e70055.

Venturoso R C, Almeida K E D, Rodrigues A M, Damin M R and Oliveira M N D (2007) Determinação da composição físico-química de produtos lácteos: estudo exploratório de comparação dos resultados obtidos por metodologia oficial e por ultra-som. *Revista Brasileira de Ciências Farmacêuticas* **43** 607-613.

Yadav J S S, Bezawada J, Ajila C M, Yan S, Tyagi R D and Surampalli R Y (2014) Mixed culture of *Kluyveromyces marxianus* and *Candida krusei* for single-cell protein production and organic load removal from whey. *Bioresource Technology* **164** 119-127.

ISO 8589:2007 (2007) Sensory analysis: General guidance for the design of test rooms. *International Organization for Standardization* **2** 1-16

ISO 11035:1994 and ISO-8586:2012 (2012) Sensory analysis – General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors. Geneva, Switzerland: *International Organization for Standardization* **1** 1-28

Figure Legends

Figure 1 (A) Comparison between Bio-reactor fermentation system and traditional fermentation (without agitation) of 12 % RSM to produce ACE-I peptides activity by combination of L. helveticus 8801315 and Flavorzyme® at 37 °C for 12 h. (B) Micro-fluidic chip capillary electrophoresis and (C) elution profiles with molecular weight (MW). (1) Migration pattern for protein MW of the ladder, migration pattern for untreated reconstituted skim milk proteins as control, (2) migration pattern for fermented skim milk protein without agitation, and (3) with agitation by Bio-reactor.Error bars indicate the standard deviation of the measurement $P < 0.05$.

Figure 2 Effect of Bio-reactor fermentation system (solid line) on pH value and Bacterial growth of 12 % RSM by combination of *L. helveticus* 8801315 and Flavorzyme® at 37 °C, compared with traditional fermentation (without agitation; dotted line).Error bars indicate the standard deviation of the measurement $P < 0.01$.

Figure 3 The proteolytic activity of *L. helveticus* 8801314 combined with Flavorzyme® at 37 °C using Bioreactor system compared with traditional fermentation without agitation.Error bars indicate the standard deviation of the measurement $P < 0.05$.

Figure4 Effects of fermentation process between un-fermented RSM drink and fermented RSM drink with different color. RSM before fermentation process (1) and RSM after fermentation process (yield anti-hypertension peptides) (2).

Figure 5 A graphic representation of the mean of sensory evaluation by quantitative descriptive analysis (QDA) of unfermented skim milk (UNFSM ■) as control, commercial product Yakult (♦) as control 2 and (A) fermented low fat skim milk drink containing 95.5 % peptides, FSMP (▲), (B) fermented low fat skim milk drink containing peptides and 5 % sucrose FSMPC (▲), and (C) fermented low fat skim milk drink containing peptides and 15 % sucrose with 5 % flavour, FSMPCF (▲).

Table 1. Nutrition ingredients of the developed low fat fermented skim milk drink by combination of *L. helveticus* 8801315 and Flavorzyme® compared with skim milk powder, reconstituted skim milk and Yakult as commercial products containing probiotic strains.

Ingredients	**SM powder**	**(12 %) (RSM)**	**Fermented RSM (final product)**	**Yakult (control)**
Protein / 100 g	3.5±0.01	3.2±0.14	0.1±0.29	1.9±0.75
Fat / 100 g	0.1±0.06	0.01±0.05	0.01±0.09	0.1±0.96
Moisture (%)	3.00±0.21	88±0.54	89.9±0.01	82.4±0.02
Total minerals (%) ash	0.8±0.28	0.8±0.43	0.4±0.03	0.3±0.04
Sugar (%) / 100 g	5.3±0.043	0.62±0.07	15±0.17	16±0.01

RSM, reconstituted skim milk; SM, skim milk