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The effects of fish collagen on the proteolysis of milk proteins, ACE inhibitory activity and sensory evaluation of plain- and *Allium sativum*-yogurt

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ABSTRACT

The effects of fish collagen on the acidification, milk protein proteolysis and organoleptic properties of plain- and *Allium sativum*-yogurt were investigated. Titratable acidity (TA%) increased ($p < 0.05$) in the presence of fish collagen in plain-yogurt (0.87–0.95%) and in *A. sativum*-yogurt (0.77–0.95%) compared to in the absence of fish collagen (0.79–0.84% and 0.56–0.86%) for plain- and *A. sativum*-yogurt respectively. Free amino acids (FAAs) in all yogurts decreased ($p < 0.05$) by about 1 mM during yogurt fermentation. The presence of fish collagen in plain- and *A. sativum*-yogurts increased the FAAs by about 4.2 and 3.5 mM respectively compared to their respective control 0.14 and 0.16 mM for plain- and *A. sativum*-yogurts respectively. Effects of plain- and *A. sativum*-yogurts in presence or absence of fish collagen on the aggregation of milk proteins in yogurt was measured by SDS-PAGE. Some milk proteins, κ-caseins, β-lactoglobulin and α-lactalbumin were reduced as a result of fermentation both in plain- and *A. sativum*-yogurts. The presence of fish collagen showed further proteolysis in these proteins in plain- and *A. sativum*-yogurts during fermentation and refrigerated storage. However, there were little changes in the degradation of α- and β-caseins in all yogurts with or without fish collagen. The extent of proteolysis based upon OPA values was the highest on day 7 of refrigerated storage in *A. sativum*-fish collagen-yogurt (337.0 ± 5.3 μg/g) followed by fish collagen-yogurt (275.3 ± 2.0 μg/g), *A. sativum*-yogurt (245.8 ± 4.2 μg/g) and plain-yogurt (40.4 ± 1.2 μg/g). In addition, plain- and *A. sativum*-yogurts with or without fish collagen showed maximal inhibition of ACE-I on day 7 of storage which ranged between 55% and 80%. The addition of *A. sativum* reduced aroma and increased graininess of yogurt but these and overall consistency were improved in the presence of fish collagen. In conclusion, fish collagen increased FAAs content in yogurt and the enhanced proteolysis of certain milk proteins may be responsible for improving ACE inhibitory activity and organoleptic properties of *A. sativum*-yogurt.

1. Introduction

Yogurt is regarded as a nutritious food because of the action of viable yogurt bacteria and their metabolites [1,2]. It is recommended to be taken daily to boost our health and the bodies' general well-being [3] because it enhances HCl production which is important in food digestion [4,5] and assists in the absorption of vitamin and mineral.

The formation of smaller peptides and free amino acids results from the proteolytic activity of microbial enzymes produced by the starter culture, the secondary starter and the non-starter bacteria [6]. Proteolysis contribute to yogurt flavor directly, via the formation of peptides and free amino acids (FAAs) and indirectly through precursors such as amines, acids, thiols, aldehydes, ketones, lactones, methyl esters and secondary alcohols [7]. It is important to regulate the enzymatic hydrolysis of casein because the activity of proteases from psychrotrophic bacteria or by milk native plasmin is known to give yogurts with different firmness, viscosity and degree of syneresis [8]. This is because proteolysis of casein by native milk proteinases alone is an important factor linked to high moisture levels and low quality of yogurt produced [9].

Hypertension is interrelated metabolic disorders that strongly predispose an individual to atherosclerotic cardiovascular disease (CVD) and to renal failure [10]. One of the most important intermediary factors for controlling hypertension is the action of the angiotensin converting enzyme-I (ACE-I) [11]. ACE-I converts angiotensin I to angiotensin II, a potent vasoconstrictor and stimulator of aldosterone secretion by the adrenal gland. Inhibition of ACE-I is therefore considered a useful therapeutic approach in the treatment of high blood pressure [12]. Increase interest in this approach is reflected in the discovery of several functional foods

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capable of modulating the physiological effects [13,14] in the prevention and cure of diseases. Milk proteins are a rich source of bioactive peptides that possess drug-like activity. These peptides are capable to modulate physiological functions (ACE inhibitory activity) through binding interactions to specific receptors on target cells leading to induction of physiological responses [15].

Collagen is the protein that is present in the highest concentration (about 30%) in the living body including skin, blood vessels, viscera and bone tissues. Fish collagen peptide is a fine powder, white or pale yellow in color, obtained by extracting collagen from sources including the bones and scales of fish such as bonito, tuna, halibut, and sea bream [16]. It is also identical to human collagen and 100% absorbable through the skin [17]. The human body was found to lose about 1.5% of collagen every year from the 25 to 40, the age at which the body stops to manufacture collagen [18]. Therefore, fish collagen has been increasingly added into dairy food [17,19] as thicker agent and to increase dietary supply of collagen.

Recent study in vitro found that inclusion of *Allium sativum* in yogurt during fermentation increased the proteolysis of milk protein. In addition, the sensory evaluation of *A. sativum*-yogurt was lower than that in plain-yogurt [20]. Therefore, the objective of this research was to study the effects of fish collagen in *A. sativum*- and plain-yogurts on the changes of acidification, proteolysis of milk protein, ACE-I inhibitory activity and organoleptic properties of theses yogurts compared to their control yogurts (without fish collagen) during refrigerated storage.

2. Materials and methods

2.1. Materials and chemicals

Fish collagen and *A. sativum* powder (McCORMICK®) were purchased from local store. Pasteurized and homogenized full cream milk (Dutch Lady®) was purchased from the supermarket. The commercial yogurt powder with probiotic mixture (Chris-Hansen, Denmark) consists of *Lactobacillus acidophilus* LA-5, *Bifidobacterium* Bb-12, *Lactobacillus casei* LC-01, *Streptococcus thermophilus* Th-4 and *L. delbrueckii* ssp. *Bulgaricus* in the ratio of 4:4:1:1:1. All the chemicals used in the present study were purchased from (Sigma, USA).

2.2. Preparation of herbal-water extract

A. sativum (25 g) was added to 250 ml of distilled water in an Erlenmeyer flask (500 ml). The mixture was mixed well and placed in a water bath at 70 °C for 12–18 h. The mixture was then centrifuged at 2500 rpm for 10 min and the supernatant was harvested and used in the herbal-yogurt preparation.

2.3. Starter culture preparation

Pasteurized full cream milk (1 l) was heated to 41 °C. The yogurt powder with probiotic mixture was added to the pre-heated milk and the mixture was mixed thoroughly followed by incubation for 24 h at 41 °C. The yogurt formed was then refrigerated (4 °C) and used as starter culture within 3 days.

2.4. Yogurt preparation

Pasteurized fresh full cream milk (850 ml) was heated to 41 °C. Starter culture (50 g), *A. sativum* extract (100 ml) and 20 g of full cream milk powder to correct the solid content were added and the mixture was mixed thoroughly. The mixture was distributed into small containers of 100 ml each. These were placed in an incubator at 41 °C. The pH in one of the containers was monitored every 30 min and the fermentation process was stopped at pH 4.5 by placing the yogurts in ice-bath for 60 min. Yogurts were then kept in the refrigerator for predetermined period prior to analysis. The same procedures were carried out to prepare plain-yogurt except that 100 ml of distilled water was used in place of *A. sativum* water extract. *A. sativum*-fish collagen yogurt and plain-fish collagen yogurt were prepared by adding fish collagen (2.5%, w/v) to *A. sativum*- and plain-yogurt prior to incubation in 41 °C.

2.5. Determination of pH and titratable acidity (TA)

The pH of yogurt was determined using a digital pH meter (Mettler Toledo 320) at room temperature. The yogurt samples were mixed with distilled water (1:1 ml) before pH measurement. The titratable acidity percentage (TA%) was determined by titrating yogurt: distilled water (1:9) mixture using 0.1 N NaOH. TA% was calculated as follows:

$$\mathrm{TA}\% = \frac{10 \times V_{\mathrm{NaOH}} \times 0.1 \times 0.009}{W} \times 100\%$$

dilution factor (df) = 10; V_{NaOH} is the volume of NaOH used to neutralize the lactic acid; 0.1 normality of NaOH; 0.009 conversion factor whereby 1 ml NaOH (0.01 N) neutralizes 0.009 g of lactic acid; and W is the weight of yogurt sample for titration.

2.6. Proteolysis activity

2.6.1. Preparation of soluble nitrogen extracts from yogurt

Yogurts (10 g) were added to 40 ml distilled water in a conical flask. The mixture was homogenized with a homogenizer (Polytron PT2100) followed by holding at 40 °C for 1 h. The homogenate was then centrifuged at 6000 rpm for 30 min and the supernatant obtained (water-soluble nitrogen extract) was harvested.

2.6.2. Determination of total free amino acids

The concentration of total free amino acids was determined by the Cd–ninhydrin method as described in Folkertsma and Fox [21] with some modifications. Water-soluble nitrogen (50 μl and 200 μl from yogurt with or without fish collagen respectively) was diluted with 1 ml of distilled water. The samples were mixed with 2 ml of cadmium–ninhydrin reagent (0.8 g ninhydrin was dissolved in a mixture of 80 ml 99.5% ethanol and 10 ml acetic acid) followed by addition of 1 g $CdCl_2$ dissolved in 1 ml of distilled water. The mixture was heated at 84 °C for 5 min and was then allowed to cool at room temperature before the absorbance at 507 nm was determined. A blank was prepared by making a mixture of 1 ml distilled water with 2 ml of Cd–ninhydrin. The absorbance of total free amino acids was read against leucine standard curve (0.0–0.1 mM) for yogurt without fish collagen and (0.1–0.4 mM) for yogurt with fish collagen.

2.6.3. Preparation of yogurt casein for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Yogurt casein extract was prepared as follows: 10 g of yogurt was added to 20 ml of 1 M ammonium-acetate buffer pH 4.3. The mixture was then homogenized (Polytron – PT2100) for 1 min followed by centrifugation at 6000 rpm for 15 min. The supernatant was discarded to remove fat and the pellet (casein) was re-suspended in 25 ml of ammonium-acetate 1 mM pH 4.3. The mixture was again centrifuged at 6000 rpm for 15 min at 4 °C. The supernatant was discarded and the washing procedure was repeated twice. The unhydrolyzed casein (pellet) was then washed with acetone (15 ml) to remove residual fat and the protein was allowed to precipitate. The upper layer of acetone was removed

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using pipettes and the unhydrolyzed casein was left to dry at room temperature. The dried unhydrolyzed casein was then ground into powder and kept in dry condition in the fridge (8 °C) until required for further analysis.

2.6.4. SDS-PAGE analyze

SDS-PAGE was used according to Kuchroo and Fox [22] to monitor proteolysis before fermentation and during refrigerated storage. A 15% acrylamide–bisacrylamide running gel and 10% acrylamide–bisacrylamide stacking gel were used. Prior to the electrophoresis, 50 mg of casein powder was suspended in a mixture of 1 ml of EDTA (1 mM)–Tris (10 mM) pH 8.00 buffer, 350 μl 10% SDS and 50 μl β-mercaptoethanol. The mixture was heated in boiling water for 5 min. An aliquot of 25 ml from each preparation was diluted with 200 μl sample buffer (0.01% (w/v) Bromophenol blue, 62.5 mM Tris–HCl pH 6.8, 10% (w/v) Glycerol and 2% (w/v) SDS) prior to loading (7 μl) into the wells. Detected protein bands were identified using the following standards of α-lactalbumin (14.2 kDa), β-lactoglobulin (18.4 kDa), κ-casein (19 kDa), α_s-casein (23.6 kDa), β-casein (24 kDa) and Bovine Serum Albumin (BSA, 69.3 kDa).

2.6.5. o-Phthaldialdehyde (OPA) method

The rapid, sensitive and convenient o-phthaldialdehyde (OPA) based spectrophotometric method was determined according to Church et al. [23]. The OPA solution was made by combining the following reagents: 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (wt/wt) sodium dodecyl sulfate (SDS); 40 mg of OPA and 100 μl of β-mercaptoethanol. The volume was made up to 50 ml by adding dH_2O. This reagent was prepared fresh and used within 2 h of preparation. Since OPA reagent is light-sensitive it was protected from light source during preparation and running of the assay. A small aliquot of standard solution or yogurt water extract (30 μl) was added directly into 1.0 ml of OPA reagent in a 1.5 ml cuvette. The solution was mixed briefly by inversion and incubated for 2 min at room temperature. The absorbance was read at 340 nm (Shimadzu spectrophotometer UV Mini 1240). The proteolytic activity of yogurt bacteria was expressed as the free amino group using a standard curve. The standard curve had been prepared using tryptone solutions of known concentrations (0.25–1.50 μg/ml).

2.7. Determination of ACE-I inhibitory activity

ACE was extracted from fresh rabbit lung by grinding rabbit lung using pestle and mortar with ice-cold 50 mmol/l Tris–HCl with 400 mmol/l NaCl, pH 8.3 [24]. ACE inhibitory activity was assayed by mixing ACE reagent (500 μl; 1.0 mmol/l Furanacryloyl-Phe-Gly-Gly (FAPGG) in 50 mmol/l Tris–HCl with 400 mmol/l NaCl, pH 8.3) with 300 μl of yogurt water extract in a cuvette. The mixture was mixed thoroughly and incubated in a water bath (37 °C) for 2 min. This was followed by addition of 300 μl of rabbit lung enzyme in 50 mmol/l Tris–HCl, pH 8.3 and the mixture was mixed carefully. Absorbance was measured at 340 nm and the decrease in the enzymatic reaction was monitored for a total period of 20 min with brief absorbance reading every 5 min. Inhibition effects on the enzyme activity by test samples were represented as % of inhibition which calculated as follows:

$$\text{ACE-inhibition } (\%) = \left[1 - \left(\frac{C-D}{A-B}\right)\right] \times 100$$

where A is the absorbance in the presence of ACE and without the sample, B is the absorbance without both ACE and the sample, C is the absorbance with ACE and the sample and D is the absorbance with the sample but without ACE.

2.8. Organoleptic evaluation

Organoleptic evaluation of yogurts was carried out during 21 days and was assessed by untrained panels (age between 20 and 23). Samples were presented in disposable plastic cups containing 100 g yogurt per cup. The panelists were required to evaluate all the samples on a 10 point scale. Aspects included overall aroma, overall consistency, grainy, lumpy, firmness, overall appearance, watery, overall taste, sourness and sweetness.

2.9. Statistic and analysis

Experiments were performed in three batches whereas; SDS-PAGE was performed using yogurt from two batches. Data are presented as mean ± SEM. The results were analyzed by ANOVA using the SPSS. Means were compared using Duncan's multiple range tests, and a significant deviation was recognized by ANOVA at $p < 0.05$.

3. Results and discussion

3.1. Acidification activity in yogurt

The pH and TA% of yogurts before fermentation and during storage are shown in Figs. 1 and 2 respectively. The initial pH of *A. sativum* milk mixture (6.9 ± 0.04) was not different from plain milk (6.8 ± 0.01). The presence of fish collagen reduced ($p > 0.05$) the initial pH of the milk mixture by 0.2–0.3 units. All pH of yogurts were not significantly different from controls throughout the storage periods. The addition of fish collagen increased the initial TA by about 0.2% lactic acid equivalent, this acid being the most organic acid present in fermented dairy products [2]. TA of plain yogurt increased ($p < 0.05$) by about 7 folds whereas the presence of *A. sativum* in yogurt increased TA formation by only about 5 folds compared to TA in milk. The presence of fish collagen in milk and *A. sativum* + milk increased ($p < 0.05$) the TA by about 3 folds compared to respective controls. Yogurt containing fish collagen showed higher TA% than those without fish collagen during storage (Fig. 2). The overall pH decrease during storage period can be attributed to the post-acidification of yogurt by the starter culture which is known to occur even at refrigerated temperature [25].

3.2. Total free amino acids in yogurt

The inclusion of fish collagen into milk increased ($p < 0.05$) the FAAs of the mixture (4.6–4.8 mM) compared to those without (~0.9 mM, Fig. 3). The level of FAAs in the milk (without fish collagen) decreased markedly ($p < 0.05$) during fermentation to

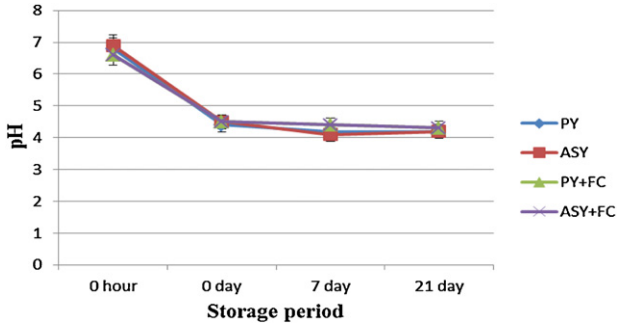

Fig. 1. Changes in pH values in yogurts before fermentation (0 h) and during 21 days of refrigerated storage (4 °C). PY, plain yogurt; ASY, *A. sativum*-yogurt; FC, fish collagen. PY and ASY presented as controls. Results are show as a mean ($n = 3$) ± standard error. The level of significance was present at $p = 0.05$ compared to control at the same storage period.

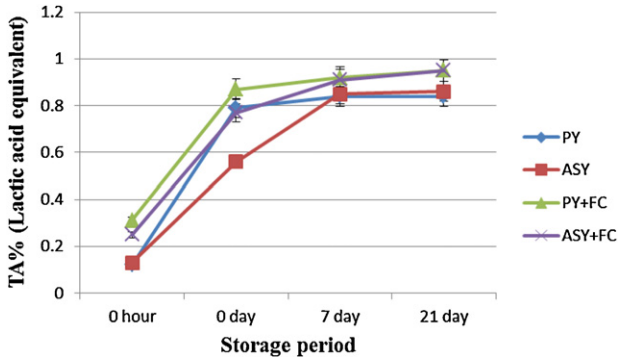

Fig. 2. Changes in titratable acidity (TA%) in yogurts before fermentation (0 h) and during 21 days of refrigerated storage (4 °C). PY, plain-yogurt; ASY, *A. sativum*-yogurt; FC, fish collagen. PY and ASY presented as controls. Results are show as a mean ($n = 3$) ± standard error. The level of significance was present at $p = 0.05$ compared to control at the same storage period.

about 0.15 mM. Refrigerated storage to 21 days tended to increase the FAAs (0.28 ± 0.10 and 0.24 ± 0.05 mM; $p > 0.05$) for plain- and *A. sativum*-yogurt respectively. The presence of fish collagen in milk caused less reduction ($p < 0.05$) in FAAs during yogurt fermentation compared to milk without fish collagen (0.4 mM vs. 0.7 mM respectively). The addition of fish collagen in milk with *A. sativum* resulted in greater reduction in FAAs (1.3 mM; $p < 0.05$) during yogurt fermentation. However, presence of fish collagen in plain- and *A. sativum*-yogurts showed no significant differences in FAAs on day 7 and 21 of storage. The changes of FAAs content in yogurt during fermentation or storage indicate the extent of these nitrogenous compounds are being utilized or produced during yogurt bacteria growth [26]. *A. sativum* appeared to increase the utilization of FAAs by lactic acid bacteria (LAB) in presence of fish collagen in yogurt but this may not be associated with faster growth of yogurt bacteria during fermentation, because both fish collagen yogurts (with or without *A. sativum*) had similar increase in TA during the fermentation (Fig. 2). The addition of fish collagen in yogurt could be a good source of available protein for LAB present in yogurt. The activity of LAB in all yogurts of study is needed to be verifying in future studies.

3.3. Proteolysis of milk protein in yogurt by SDS-PAGE

The SDS gel electrophoretic patterns of plain- and *A. sativum*-yogurts in the presence or absence of fish collagen are as shown in Fig. 4. There were little changes in the degradation of α_s- and β-caseins in plain- and *A. sativum*-yogurts both in the absence or presence of fish collagen. This is in agreement with other study in ewe milk that found no significant differences in α_s- and β-caseins degradation during fermentation and storage [27,28]. In contrast, the bands representing κ- caseins, β-lactoglobulin and α-lactalbumin were reduced as a result of fermentation both in plain- and *A. sativum*-yogurts. It is interesting to note that α-lactalbumin was degraded to a slightly bigger extent than β-lactoglobulin which is in line with those observed by Zahar et al. [27,28]. The presence of fish collagen was found to enhance further proteolysis in these proteins in plain- and *A. sativum*-yogurts during fermentation and refrigerated storage (Fig. 4b). Fresh *A. sativum*- yogurt in presence of fish collagen (0 day) showed higher degradation of milk proteins than fresh fish collagen-yogurt. However, refrigerated storage of *A. sativum*-fish collagen-yogurt

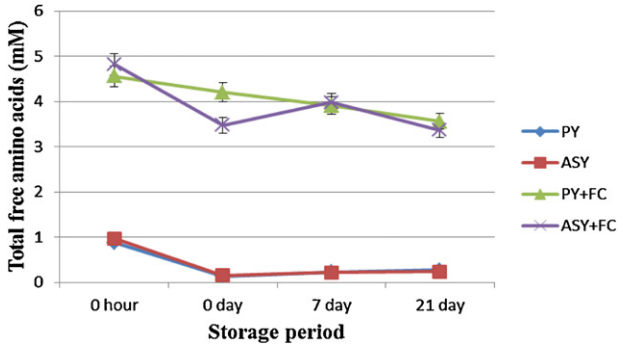

Fig. 3. Changes in total free amino acids (leucine equivalent; mM) in yogurts before fermentation (0 h) and during 21 days of refrigerated storage (4 °C). PY, plain-yogurt; ASY, *A. sativum*-yogurt; FC, fish collagen. PY and ASY presented as controls. Results are show as a mean ($n = 3$) ± standard error. The level of significance was present at $p = 0.05$ compared to control at the same storage period.

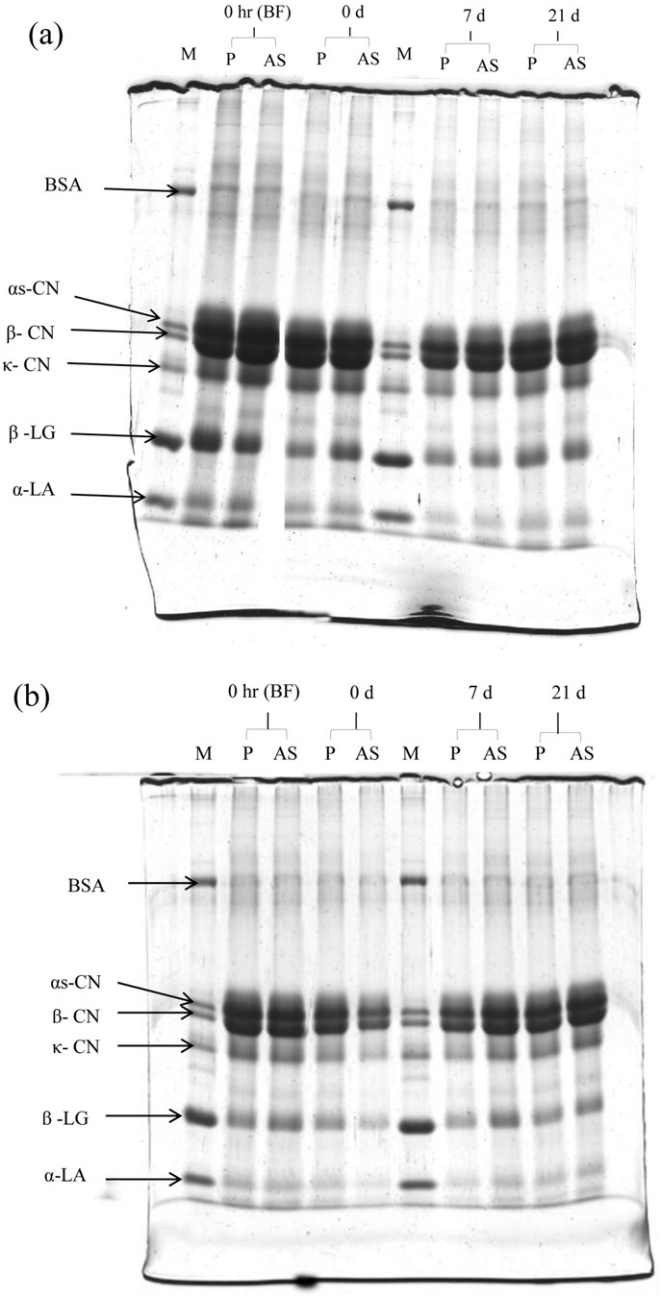

Fig. 4. Protein profiles evolution in yogurt before fermentation (0 h) and during 21 days refrigerated storage (4 °C). (a) Protein profiles in plain- and *A. sativum*-yogurt (as control). (b) Protein profiles in plain- and *A. sativum*-yogurt in the presence of fish collagen. M, markers for caseins, *i.e.* α_s-CN, β-CN, κ-CN, α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and bovine serum albumin (BSA). P, plain-yogurt; AS, *A. sativum*-yogurt; and BF, before fermentation.

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showed less degradation of milk proteins on day 21 of storage. The presence of fish collagen could have provided additional free amino acids which stimulate the growth of starter culture and subsequently release more proteolytic enzymes [29,30] leading to more extensive milk protein degradation during yogurt fermentation. Caseins play an important role in milk coagulation [31] thus, even small differences in proteolysis activity may lead to the improvement in production and formulation of yogurts differing in their physico-chemical and rheological properties.

3.4. Proteolytic activity

During fermentation, LAB produced extracellular proteinases that hydrolyzed milk proteins resulting in an increase in the amount of free amino groups as quantified by the OPA method [32]. The extent of proteolysis in fresh plain-yogurt (0 day) was 23.55 ± 1.05 μg/g (Table 1). Refrigerated storage to 7 and 14 days showed no significant increase in proteolytic activity. However, the presence of fish collagen increased ($p < 0.05$) proteolytic activity in yogurt on day 7 of storage (275.3 ± 2.0 μg/g) compared to fresh fish collagen-yogurt (181.2 ± 4.5 μg/g). Prolonged refrigerated storage to 14 days reduced ($p < 0.05$) proteolytic activity in fish collagen-yogurt to 199.8 ± 9.8 μg/g. The presence of *A. sativum* increased ($p < 0.05$) proteolytic activity in yogurt from 33.90 ± 0.90 μg/g (0 day) to 245.8 ± 4.17 μg/g (7 day). On the other hand, there was no significant increase in proteolytic activity in presence of fish collagen in *A. sativum*-yogurt (337.0 ± 5.3 μg/g) on day 7 of storage compared to 0 day (303.3 ± 3.3 μg/g). Prolonged refrigerated storage of *A. sativum*-yogurt both in presence and absence of fish collagen decreased ($p < 0.05$) proteolytic activity to 290.8 ± 0.8 μg/g and 34.27 ± 0.10 μg/g respectively on day 14 of storage (Table 1). The growth of yogurt bacteria and acidification of yogurt are related to the proteolytic activities in yogurt [33]. Higher proteolytic activity in presence of fish collagen in yogurt than in absence indicated that fish collagen strongly contributed with amount of amino acids to yogurt bacteria. However, the decrease in proteolytic activity on day 14 of storage may be resulted in decrease the viability of these bacteria. Several studies reported that viable cell counts of *Lactobacillus* spp. significantly decrease by the 14th day of refrigerated storage [34–36,32]. There is a great deal of interest in the use of fish collagen as functional food ingredients to manipulate the growth of yogurt bacteria in order to improve bioactive peptides release with functional properties. These functions can be relate to general health conditions or reduce risk of certain chronic diseases of the nervous, cardiovascular, digestive and immune systems [33].

3.5. ACE-I inhibitory activity

Proteolytic activity of the starter cultures during milk fermentation can be generating peptides with various biological activities. Thus, end-products of fermented milk can be yield a wide range of peptides with functional properties such as peptides

Table 1
Proteolytic activity by OPA method (μg/g) in plain- and *A. sativum*-yogurts both in presence and absence of fish collagen.

Sample	Proteolytic activity (μg/g)		
	0 day	7 day	14 day
PY	23.55 ± 1.05	40.42 ± 1.25	23.76 ± 0.84
ASY	33.90 ± 0.90	245.8 ± 4.17	34.27 ± 0.10
PY + FC	181.2 ± 4.5*	275.3 ± 2.0*	199.8 ± 9.8*
ASY + FC	303.3 ± 3.3*	337.0 ± 5.3*	290.8 ± 0.8*

PY, plain-yogurt; ASY, *A. sativum*-yogurt; and FC, fish collagen. PY and ASY presented as controls. Results are show as a mean ($n = 3$) ± standard error.
* The level of significance was present at $p = 0.05$ compared to control at the same storage period.

Table 2
ACE-I inhibitory activity (%) in plain- and *A. sativum*-yogurts both in presence and absence of fish collagen.

	ACE-I inhibitory activity (%)		
	0 day	7 day	14 day
PY	40.12 ± 8.67	68.35 ± 2.62	50.81 ± 4.04
ASY	53.43 ± 1.82	77.82 ± 2.01	65.73 ± 2.02
PY + FC	53.23 ± 2.83*	55.85 ± 7.46	51.61 ± 10.08
ASY + FC	50.2 ± 0.20	82.46 ± 1.41	59.88 ± 7.86

PY, plain-yogurt; ASY, *A. sativum*-yogurt; and FC, fish collagen. PY and ASY presented as controls. Results are show as a mean ($n = 3$) ± standard error.
* The level of significance was present at $p = 0.05$ compared to control at the same storage period.

with ACE-I inhibitory activity. In the presence study, fresh plain-yogurt showed ACE-I inhibitory activity about 40% whereas fresh fish collagen-yogurt and *A. sativum*-yogurt with/without fish collagen showed almost 50% of ACE-I inhibitory activity (Table 2). The presence of fish collagen in yogurt showed lower ACE-I inhibitory activity (55.85 ± 7.46%; $p < 0.05$) than the absence (68.35 ± 2.62%) on day 7 of storage. On the other hand, there were no significant differences in ACE-I inhibitory activities between *A. sativum*-yogurts in presence and absence of fish collagen (82.46 ± 1.41% and 77.82 ± 2.01% respectively) on day 7 of storage. Prolonged refrigerated storage to 14 days decrease ($p < 0.05$) ACE-I inhibitory activity in all yogurts with no significant differences between plain- and *A. sativum*-yogurts in presence or absence of fish collagen (Table 2). Regardless of the presence of fish collagen increased the free amino groups in plain- and *A. sativum*-yogurts, it can be suggesting that higher amount of peptides/amino acids in yogurt may not subsequently exert high ACE-I inhibitory activity. Amino acid composition as well as size and sequence of these amino acids found to play a key role in inhibition of ACE activity [37,23].

3.6. Sensory evaluation

The presence of *A. sativum* resulted in lower score for aroma ($p > 0.05$), wateriness ($p < 0.05$) and taste (sourness, $p > 0.05$) compared to plain yogurt during storage (Fig. 5). High graininess score for yogurt in the presence of *A. sativum* than in the absence (4.74 ± 0.40 and 3.32 ± 0.34 respectively; $p < 0.05$ on 0 day) was reduced by day 7 whereas other sensory attributes for both yogurts were not significantly different during the 21 days of storage. Thus, both plain- and *A. sativum*-yogurts should be consumed within 7 days of storage. The addition of fish collagen caused small differences ($p > 0.05$) in sensory attributes of plain- and *A. sativum*-yogurts (Fig. 5). There was a reduction ($p < 0.05$) in overall aroma in *A.*

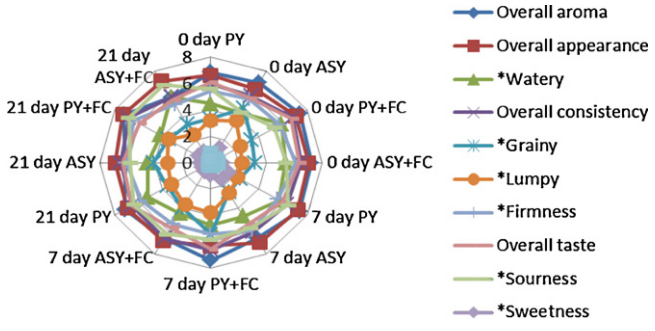

Fig. 5. Changes in sensory evaluation of yogurt during 21 days refrigerated storage (4 °C). PY, plain-yogurt; ASY, *A. sativum*-yogurt; FC, fish collagen. PY and ASY presented as controls. For each criterion, yogurt was ranked from 1 to 10 (1–2 = low, 5–6 = moderate, 9–10 = high). *$p < 0.05$ compared to control at the same storage period.

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sativum-fish collagen yogurt (5.67 ± 0.44) compared to fish collagen yogurt (7.22 ± 0.06) on day 21 of storage. *A. sativum*-fish collagen yogurt showed lower score in overall consistency (5.72 ± 0.34) than fish collagen yogurt (6.78 ± 0.20). Fish collagen has beneficial effects in increasing the organoleptic properties of yogurt as flavor and texture [38]. The contribution of milk proteins to sensory evaluation may be improved by the addition of supplement proteins such as fish collagen (Figs. 3 and 4 and Table 1) which limit the milk proteins proteolysis by LAB. In addition, fish collagen may also act as stabilizer to enhance the overall consistency of yogurt.

4. Conclusion

In this study, there were no significant changes on pH throughout the fermentation and storage period in *A. sativum*-yogurt with or without fish collagen. However, fish collagen has effect to increase TA% in yogurt. Besides, it found to increase the concentration of free amino acid in treated yogurt. Milk proteins proteolysis in yogurt samples in presence of fish collagen could contribute to the ACE-I inhibitory activity and organoleptic properties.

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