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Effects of cooking methods and *in-vitro* digestion on the digestibility and antioxidant properties of *ngari* (a fermented fish product of India)

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Ngari is a popular ethnic fermented fish product from North-Eastern India. It is consumed after heat process either as a part of regular meal or as a condiment. However, there is no adequate knowledge on the digestion of *ngari* and the bioactive potential of the digest. The present study was aimed to evaluate the effect of different heat processes *viz.*, air frying, roasting and *sautéing* on the chemical attributes and *in-vitro* digestion characteristics of *ngari* with reference to digestibility and antioxidant potential. The different heat processes significantly increased the dry matter content, thereby altering the proximate composition of the *ngari*. Nutritional analyses revealed that the highest value of protein is in air fried *ngari* (44.14%), while maximum fat in *sautéed ngari* (27.57%). Fatty acids analysis showed that palmitic, oleic and linoleic acid were the major fatty acids present in *ngari* irrespective of different heat processing. Antioxidant potential of *ngari* was significantly influenced by heating processes and digestion methods (pepsin or pepsin-cum-pancreatin). Heat processes significantly reduced DPPH (2,2-diphenyl-1-picrylhydrazyl) and metal chelating activity, while increasing the reducing power of the *nagri*. Study on peptide released during digestion by SDS-PAGE (Sodium lauryl sulphate-polyacrylamide gel electrophoresis) showed that pepsin digestion led to formation of low molecular weight peptides (14-66 kDa). Subsequently, the pepsin-cum-pancreatin digestion degraded the relatively larger peptides into further smaller ones. The different heat processes enhanced the sensory appeal of the product without exerting any negative influence on its digestibility and bioactive properties.

Keywords: Antioxidant properties, Digestibility, *in-vitro* digestion, *Ngari*

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Fermented fish products of various types and forms are consumed worldwide as condiment along with main course due to its unique flavour and aroma[1]. Contrary to other fermented fish products found in Southeast Asia (fish sauce, or fish paste) which uses salt or carbohydrate for fermentation[2], *ngari* is salt and carbohydrate free solid-state fermented fish product, widely popular among the masses of North-Eastern India. *Ngari* preparation involves age-old practices and detailed processing methods had been documented[3]. Briefly, sun-dried small (5-10 cm) freshwater fishes (undressed) are washed in water and then left overnight to drain the water. The moistened fishes are then tightly packed in the pre-oiled smeared earthen pot and sealed by pasting clay as lid. The sealed pot is kept undisturbed for 6-12 months for fermentation. The end product of fermentation is called "*Ngari*". The fermented product i.e. *ngari* is then removed from the pot and utilised for consumption. It is worth mentioning that *ngari* as such is non-palatable and requisite heat treatment before consumption even though the process of fermentation softens the fish due to endogenous enzymatic hydrolysis and bacterial activities. Usually, *ngari* is subjected to different heat treatment such as roasting and *sautéing* to extract its unique aroma before consuming either as a part of regular meal or as a condiment in various ethnic preparations *viz. Eromba, Kangsoi* of North Eastern India. Further, the consumption of fermented fish contributes to a reasonable proportion of daily protein requirement of the people from the region. Apart from providing its strong appetizing flavour, *ngari* is also believed to be antimalarial in nature[4].

The peptides released after protein breakdown during fermentation are shown to possess antioxidant

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aptitudes[2]. On the other hand, *in-vitro* digestion has been found to alter the bioactive properties of freeze and oven dried fish protein hydrolysate[5]. The information on the fermentation process, proximate composition, microbial diversity and other chemical and physical parameters of *ngari* has been reported by some authors[3,6]. *Ngari* is invariably heat processed before consumption and data regarding the same is not available. Study on effects of *in-vitro* digestion of heat processed *ngari* on the digestibility and antioxidant activity is not available. Therefore, the study was envisaged to evaluate the biochemical changes occurring in *ngari* upon heat processing *viz*., air frying, roasting and *sautéing*. Further, the protein digestibility characteristics of *in-vitro* digested *ngari*, along with antioxidant potential and peptide profile of the digest has been assessed.

Material and Methods

Raw material and cooking methods

Ngari (a fermented fish) was purchased from the Ima market in Manipur, India. Approximately, 100g of sample was taken and subjected to three cooking methods *viz* air frying, roasting and *sautéing* (Fig. 1). The air frying was carried out utilizing air fryer (HD9220/20, Philips Viva Collection Airfryer, Philips India Ltd.) at 160°C for 4 min. Roasting was carried out on hot pan at 160°C for 3 min. The *sautéing* was also done on hot pan with 5 ml of sunflower oil at 180°C for 3 min. The heat treating time was optimized based on the sensory score (data not shown). The uncooked sample was kept as control.

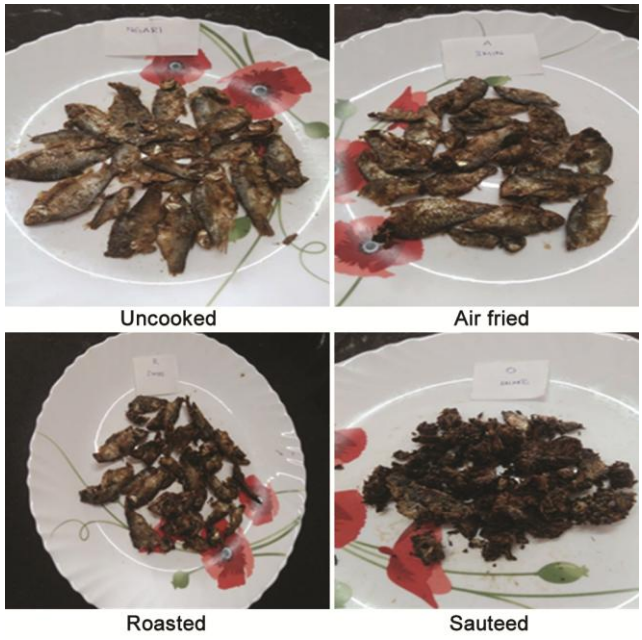

Fig. 1 — Heat processing of *ngari*

Proximate composition

The proximate composition of the *ngari* samples was determined according to AOAC[7]. Moisture content of the *ngari* samples was evaluated by drying the sample in a hot air oven (Rivotex, India) to a constant weight at 105±2°C. The crude protein was calculated by estimating nitrogen content using Kjeldahl distillation unit and multiplying with a conversion factor of 6.25. Crude fat was determined from moisture free *ngari* samples by using Soxhlet apparatus. Ash content was determined by subjecting the *ngari* samples to ash in a muffle furnance (Nabertherm, Germany).

Biochemical characteristics

Total volatile basic nitrogen (TVBN) was determined according to Conway[8]. Thiobarbituric acid (TBA) value was determined by distillation method as per Tarladgis *et al.*[9]. The pH value of *ngari* samples was determined utilizing pH meter (pHep tester, HANNA, USA). Water activity of *ngari* samples was measured employing water activity meter (Aqualab meter, Decagon Devices, U.S.A.) at 25°C.

Fatty acids composition

Lipid was extracted from five grams of *ngari* samples by Folch method using chloroform methanol mix (2:1) and fatty acids methyl esters were prepared using BF_3. The methyl esters of fatty acids present in the extracted oil were analysed using gas chromatography (Varian CP-3800, USA). Nitrogen was used as carrier gas (80 psi) at flow rate of 1.2 ml/min. GC temperature was gradually raised from an initial temperature of 120°C to 250°C at rate of 4°C/min. Fatty acids separated were identified by the comparison of retention time with those obtained by the separation of a mixture of standard fatty acid methyl esters [Supelco 37 component FAME (fatty acids methyl esters) mix, Analytical standard, Sigma-Aldrich Co., St.Louis, USA]. Each fatty acid was expressed as percentage of total area of the identified fatty acid methyl ester peaks.

In-vitro digestion of ngari

In-vitro digestion study was performed by following Lo *et al.*[10] with slight modifications. *Ngari* samples (25 g) were mixed with 50 ml distilled water until a smooth paste was obtained. The pH of the homogenate was adjusted to 2 using 1N HCl and

pepsin (Himedia, India) was added to the homogenate at a ratio of 4:100 (enzyme: protein). The reaction mixture was incubated at 37ºC for one hour. Later the reaction was terminated by raising the pH to 7.5 by 1N NaOH. Then pancreatin (Himedia, India) was added after pepsin digestion at the ratio of 2:100 (enzyme: protein) and re-incubated at 37 ºC for two hours. At the end, the reaction was inactivated by heating the mixture to 100ºC for 10 min. For the control sample, the same procedure was followed without adding enzymes. The undigested fraction after digestion procedure was removed by using qualitative filter paper (Himedia, Grade1) and the filtrate (digest) was used for further analysis.

Degree of hydrolysis

Degree of hydrolysis (DH %) was evaluated as proportion of α-amino nitrogen in the filtrate with respect to the total nitrogen content in the *ngari* samples. The α-amino nitrogen was determined by formol titration method[11] and DH% was calculated as follow:

$$DH(\%) = \frac{AANxTVS}{WMxTN} X\ 100$$

where, AAN: α-amino nitrogen (mg/ml of supernatant); TVS: total volume of supernatant (ml); WM: weight of *Ngari* taken for hydrolysis (g); TN: total nitrogen content (mg/g of *ngari*)

Antioxidant potential

The antioxidant properties of the digest were determined as a function of different concentrations of protein (1 mg/ml). The protein concentration in the digest was determined by Lowry's method.

The DPPH free radical scavenging activity of the digest samples were determined by following the method described by Blois[12]. Briefly, two millilitres of the digest samples was mixed with 2 ml of 0.2 mM DPPH solution prepared in 95% ethanol. The mixture was then vortexed for 30 s and incubated in dark for 30 min before measuring the absorbance at 517 nm.

The metal chelating activity was determined by the method described by Razali *et al.*[13]. One millilitre of the digest samples was diluted with 3.7 ml distilled water and 0.1 ml of 2M ferrous chloride was added. The reaction was then initiated by adding 0.2 ml of 5 mM ferrozine. Then the mixture was vortexed for 30 s and incubated for 10 min before measuring the absorbance at 562 nm.

The reducing power of the filtrate samples was determined according to the method of Razali *et al.*[13]. One millilitres of the digest was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated for 30 min at 50°C. The reaction was terminated by adding 2.5 ml of 10% trichloroacetic acid. The reaction mixture was then centrifuged at 1650×g for 10 min. The supernatant (2.5 ml) was collected and diluted with distilled water (1:1). The mixture was vortexed for 30 s after adding 0.5 ml ferric chloride and then incubated for 10 min before measuring the absorbance at 700 nm.

Gel electrophoresis

SDS PAGE was performed according to the procedure of Laemmli[14] to characterize the protein profile of *ngari* before and after *in-vitro* digestion. Briefly, the filtrate obtained after *in-vitro* digestion was adjusted to protein concentration of 3 mg/ml and then diluted with the sample buffer (30% SDS, 10% glycerol, 5% β-Mercaptoethanol, 12.5% Tris HCl) at 1:1 ratio and centrifuged at 6500xg for 10 min after heating at 90ºC for 2 min. Ten microliters of the supernatant along with pre-stained high molecular weight standards (Sigma, St. Louis, USA) were loaded in the precast gel (10% loading gel and 4% stacking gel). The gels were run at 90 V until the protein reached the end. Protein bands were stained with Coomassie Blue R250 for 60 min. Later, the stained gel was destained with the destaining solution (5% methanol and 7.5% acetic acid) until the bands were visible.

Statistical analysis

One-way analysis of variance was used to test the statistically significant differences between the mean values for proximate composition, biochemical parameters and degree of hydrolysis. A three-factor analysis of variance was carried out to study effects of independent variables *viz*., heat treatments, digestion methods and protein/peptide concentration of the digest. Significant differences between the means of different factors were assessed at 95% confidence level using Duncan's post-hoc test. An estimated marginal mean values were used to express the results of antioxidant potential. All statistical tests were performed with the SPSS 20.1 (SPSS South-Asia, Bangalore, India).

Results

Effects of cooking methods on proximate, biochemical and fatty acids compositions of ngari

The proximate composition of *ngari* processed by different heating methods *viz*., air-frying, roasting and

sautéing are shown in Table 1. The moisture, protein, crude fat and ash contents in uncooked *ngari* samples were 26.74, 38.16, 18.14 and 17.30% respectively. The moisture loss was higher (10.98%) in *sautéing* and least in air frying (19.22%). Similar observation was made in a_w of the *ngari*, where least a_w was recorded (0.593) in *sautéed ngari*. The protein content increases significantly in all the studied heating methods. Protein content in uncooked, roasted, air-fried and *sautéed ngari* were 38.16, 42.02, 44.14 and 41.03 % respectively. Significantly higher lipid content was found in *sautéed Ngari* (27.57%). TVBN was found maximum in air fried (659 mg/100g) *ngari* and least in *sautéed ngari* (317 mg/100g). After different cooking methods employed, variation in the fatty acids compositions of the *ngari* were noticed. Higher content in the total polyunsaturated fatty acids (PUFA) was recorded in *sautéed ngari* (37.10%) as compared to uncooked *ngari* (18.57%).

Effects of cooking methods and *in-vitro* digestion on DH and antioxidant activities of the ngari

Table 2 shows the DH of *ngari* as influenced by different cooking methods and digestion methods

Table 1 — Effects of cooking methods on the biochemical and fatty acids composition of *ngari*

Component	Cooking methods			
	Uncooked	Roasted	Air fried	*Sautéed*
	*Biochemical composition**			
Moisture (%)	26.74±0.65d	16.82±0.97b	19.22±0.28c	10.98±1.04a
Protein (%)	38.16±0.15a	42.04±0.53c	44.14±0.57d	41.03±0.56b
Crude fat (%)	18.14±1.13a	20.20±0.13b	18.14±0.16a	27.57±0.12c
Ash (%)	17.30±0.22a	21.54±0.70c	18.83±0.71b	20.72±0.73c
pH	06.19±0.01d	05.86±0.01b	05.92±0.01c	05.81±0.01a
a_w	0.784±0.00d	0.645±0.00c	0.627±0.00b	0.593±0.00a
TVBN (mg/100g)	622.0±7b	429±8c	659±21d	317±8a
TBA(mg/kg)	01.77±0.01a	02.69±0.01d	02.44±0.03b	02.54±0.01c
	Fatty acids (%)			
Lauric	ND	0.20	ND	ND
Myristic	2.20	2.02	2.27	ND
Palmitic	24.15	22.64	23.22	14.78
Heptadecanoic	2.07	2.01	2.26	1.14
Stearic	7.94	8.35	8.34	6.37
Behenic	ND	ND	0.51	ND
∑ SFA	36.36	35.22	36.60	22.29
Palmitoleic	8.68	8.01	9.01	3.93
Oleic	29.52	30.85	29.59	31.07
cis -11- Eicosenoic	5.79	5.39	5.0	2.92
Erucic	ND	ND	ND	2.03
∑ MUFA	43.99	44.25	43.60	39.95
Linoleic	10.38	11.32	10.39	31.51
Linolenic	1.40	1.38	1.62	1.06
Linolenic	0.44	ND	0.45	ND
cis-11,14-Eicosadienoic	ND	ND	ND	0.31
Eicosatrienoic	0.61	ND	0.46	0.80
Eicosatrienoic	ND	ND	0.12	0.73
Arachidonic	3.10	2.87	2.35	1.27
Docosadienoic	ND	ND	1.27	ND
Eicosapentaenoic	0.54	1.55	0.14	0.49
Docosahexaenoic	2.10	2.36	1.77	0.93
∑ PUFA	18.57	19.48	18.57	37.1

*Values are expressed as mean± standard deviation and different letters in a column indicate significant difference ($p<0.05$) between the treatment

ND: Not detected

Table 2 — Effect of cooking on degree of hydrolysis (DH) during *in-vitro* digestion of *ngari*

Heating methods	Non-digested *ngari* (%)	Pepsin digested *ngari* (%)	Pepsin-pancreatin digested *ngari* (%)
Uncooked	20.46±0.20c	24.02±0.09b	30.51±0.04a
Air fried	19.40±0.12b	22.76±0.13a	33.71±0.14b
Roasted	18.02±0.08a	24.18±0.15c	36.17±3.51d
Sautéed	21.54±0.11d	25.65±0.13d	36.01±0.51c

Values are expressed as mean± standard deviation and different letters in a column indicate significant difference ($p<0.05$) between the treatment

(pepsin and pepsin-cum-pancreatin). The DH of the *ngari* was slightly decreased upon cooking. The mean DH value of uncooked, air-fried, roasted and *sautéed ngari* were 20.46, 19.40, 18.02 and 21.54% respectively. Upon *in-vitro* digestion using pepsin and pepsin-pancreatin the DH of the *ngari* (within the same cooking method) was found to be improved. The DH in *sautéed ngari* increased from 21.54% in non-digested to 36.01% in pepsin-pancreatin digested sample.

The effect of cooking and digestion methods on the antioxidant properties of *in-vitro* digested *ngari* were assessed by DPPH activity, metal chelating activity and reducing power. Figure 2 depict the DPPH activity of the digest of heat processed *ngari*. Non-digested samples of uncooked (73.93) and *sautéed* (71.09) *ngari* had the highest percentage of DPPH activity while those of pepsin-pancreatin digestion of uncooked and *sautéed ngari* had the lowest (26.67% and 35.91% respectively). The metal chelating activity of the heat processed digest is showed in Figure 3. The highest metal chelating activity (94.54%) was shown by the non-digested and uncooked *ngari* sample. The least metal chelating activity (18.21%) was reported by pepsin digested digest of the air fried *ngari* sample. Digestion of *ngari* decreased the metal chelating activity and the decreased was found to be dependent on digestion methods. For the entire *ngari* sample the trend of metal chelating activity is as follows: uncooked > pepsin digestion> pepsin-pancreatin digestion. The reducing power of the heat processed digest of *ngari* samples are presented in Figure 4. Indigested sample of air fried *ngari* was found to exhibit highest reducing power (0.34) while the least was showed by digest of roasted (0.11) sample of *ngari*. Similar trend of metal chelating activity was also found in reducing power of the digest.

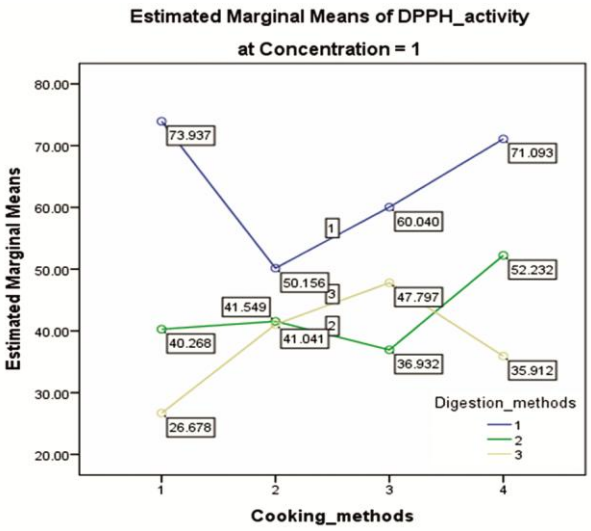

Fig. 2 —Estimated marginal means of DPPH activity (%) in the *ngari* in different cooking methods (1-uncooked;2-air-frying; 3-roasting and 4- *sautéing*), digestion methods (1: non-digestion; 2:pepsin digestion; 3:pepsin-pancreatin digestion) and protein concentration of the digest (1 mg/ml)

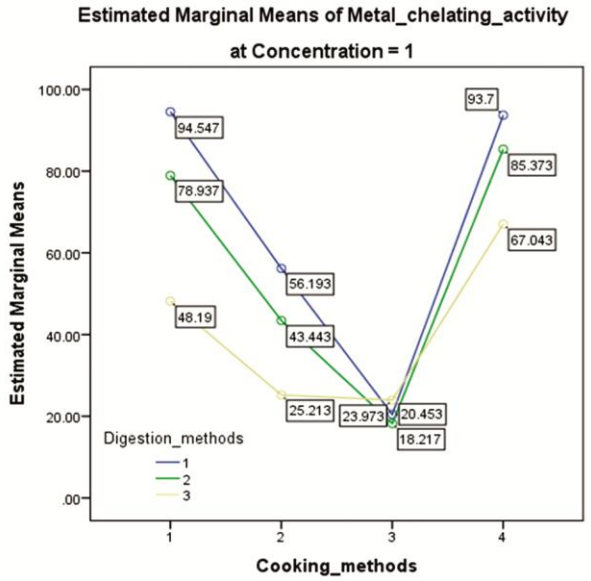

Fig. 3 — Estimated marginal means of metal chelating activity (%) in the *ngari* in different cooking methods (1-uncooked;2-air-frying;3-roasting and 4- *sautéing*), digestion methods (1: non-digestion;2: pepsin digestion; 3: pepsin-pancreatin digestion) and protein concentration of the digest (1mg/ml)

Effect of cooking methods and *in-vitro* digestion on protein pattern of the ngari digest

SDS-PAGE profile of uncooked and cooked samples is shown in Figure 5. The digest obtained from uncooked *ngari* showed no sharp peptide band

in the range of 200 -14 kDa. However, there were smeared bands around 20 kDa and below. However, all the samples after cooking treatments without any enzymatic digestion identified to have the multiple peptide units with the approximated molecular weight in the range of > 200 to 66 kDa and fainted bands below this region. Pepsin digest obtained from the entire cooked as well as uncooked sample found to have multiple peptide units with the approximated molecular weight range of 66 to 14 kDa with concomitant disappearance of peptide units found in control samples. Further digestion of peptic digest with pancreatin resulted in complete digestion of relatively larger peptide units into smaller peptides.

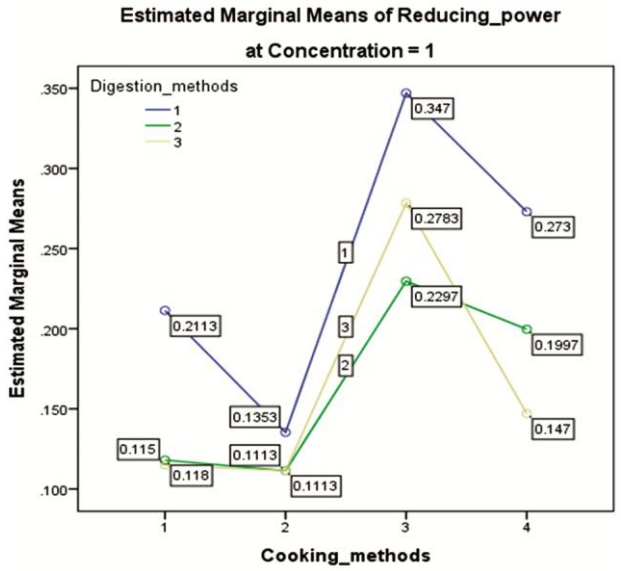

Fig. 4 — Estimated marginal means of reducing power (absorbance at 700 nm) in the *ngari* in different cooking methods (1:uncooked;2: air-frying;3:roasting and 4: *sautéing*), digestion methods (1: non-digestion; 2: pepsin digestion; 3: pepsin-pancreatin digestion) and protein concentration of the digest (1 mg/ml)

Discussion

Proximate, biochemical and fatty acids composition of heat processed ngari

The proximate composition of *ngari* was significantly ($p<0.05$) affected by the cooking methods. Compared to control, a significant increase in the protein content and a significant decrease in the moisture content were noticed in the *ngari* samples prepared by different methods of cooking ($P < 0.05$). The moisture loss was maximum in *sautéing* process and minimum in air-frying compared to uncooked sample. The loss of moisture during thermal processing is due to evaporation of water molecules. It should be mentioned that the temperature is higher in *sautéing* process (180°C) than the air-frying process (160°C). Further, the heating process also favours the hydrophobic interaction of proteins which ultimately leads to the exudation of water[15]. The decrease in moisture content results in increased solids content, mainly protein on weight basis. Similar observation with reference to increase in protein content has been reported for salmon and Chilean jack mackerel cooked fillets[16]. The similar explanation holds good for changes observed in fat and ash content. However, the roasting showed a slight increase in the ash content. The literature available mentioned similar observations in the changes of ash content with reference to cooking methods. Ersoy and Ozeren[15] reported an increase in ash content of cooked African catfish. Whereas, Bastias *et al.*[16] found no significant differences in ash content of salmon or Chilean jack mackerel prepared by

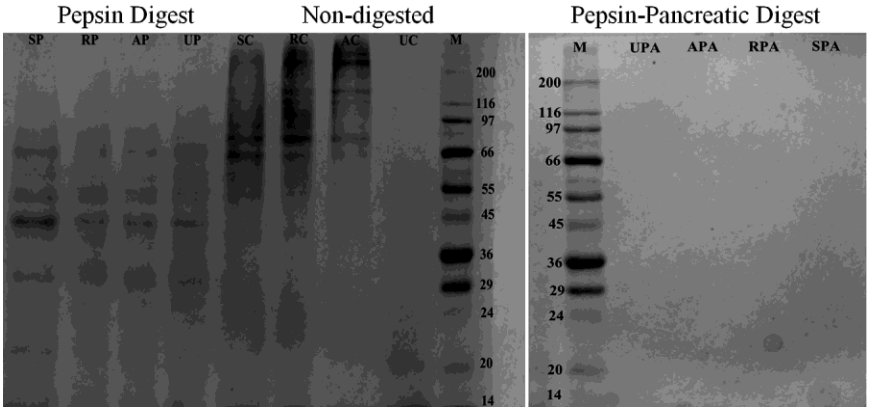

Fig. 5 — SDS-PAGE of *ngari* (M:marker; UC: uncooked control; AC: air fried control; RC: roasted control; SC: *sautéed* control; UP: uncooked pepsin; AP: air fried pepsin; RP: roasted pepsin; SP: *sautéed* pepsin; UPA: uncooked pepsin-pancreatin; APA: air fried pepsin-pancreatin; RPA: roasted pepsin-pancreatin; SPA: *sautéed* pepsin- pancreatin

different cooking methods. Higher fat content was found in the *sautéed ngari* as a consequence of absorption of oil used during the process. The passive absorption of oil could be attributed to the porous structure developed upon evaporation of water.

Cooking significantly reduced the pH of *ngari*. The pH of an uncooked *ngari* was 6.19 while the pH of cooked sample showed a slight drop. The reduction in pH could be attributed to the elimination of volatile substance by heating and the formation of free fatty acids due to hydrolytic rancidity. Similar results have been reported in the literature[17-19].

Water activity (a_w) was found correspondingly lesser in lower moisture samples. Among the samples, the lowest a_w of 0.59 was found in sautéed sample which also had the lowest moisture (10.98%) content.

Compared to uncooked samples, both the processing methods, *sautéing* and roasting found to reduce the TVBN significantly ($P<0.05$) whereas the air-frying methods increased the TVBN content significantly ($P<0.05$). TVBN consists of group of biogenic amines formed in fermented and non-fermented food products during storage[20]. Majumdar *et al.*[21] indicated that the production of volatile bases due to the breakdown of proteins by action of microbes is believed to be responsible for the generation of typical flavour and aroma of the fermented fish. These results also agree with Dhanapal *et al.*[22] on tilapia fish steak.

Cooking by different methods resulted in significant ($P<0.05$) increase in TBA values. The lowest value was found in uncooked followed by air-fried, sautéd and roasted *ngari*. Heat generated during cooking act as a pro-oxidant and increases the lipid oxidation[23]. Uran and Gokoglu[24] reported that lipid oxidation increased in anchovy due to different cooking methods and cooking temperature. Farag[25] also reported significant influence of cooking on the TBA content of fish.

The fatty acids compositions of all samples are given in Table 1. The fatty acids composition of roasted and air-fried *ngari* was similar to the uncooked sample. However, the fatty acid composition of *ngari* was greatly changed by *sautéing*. In all the *ngari* samples, the major saturated, monounsaturated and polyunsaturated fatty acids were palmetic, oleic and linoleic, respectively. *Sautéed ngari* had relatively lower content of saturated and monounsaturated fatty acids and higher level of polyunsaturated fatty acids (PUFA) when compared to other *ngari* samples. The increase in PUFA content of *sautéed ngari* may be attributed to the use of sunflower oil for sautéing purpose. Sunflower oil is considered to be rich in PUFA[26]. The absorption of lipids during *sautéing* might have altered the fatty acid composition of major fatty acids originally present in the *ngari*. Similar observation was reported by Gall *et al.*[27] where large amounts of the major fatty acids present in the soybean oil cooking medium were found to be absorbed by the low fat grouper fillet deep fried in the soybean oil. Hence, composition of cooking oil influences the fatty acids composition of *ngari*. The result also shows that *ngari* contain important polyunsaturated fatty acids such as EPA (eicosapentaenoic acid) and DHA (Docosahexaenoic acid). The polyunsaturated fatty acids such as EPA and DHA were also retained in all the cooked *ngari*. Hence, roasting and air-frying affected the PUFA content of *ngari* minimally. However, the fatty acids composition of *sautéed ngari* will vary according to the oil utilised for *sautéing* process.

Effects of heat processing methods and *in-vitro* digestion on DH and antioxidant activities of the ngari

DH represents the proportion of peptide bonds cleaved during the hydrolytic process. Uncooked *ngari* sample showed 20% DH. This is quite evident because *ngari* being a fermented fish product undergoes extensive breakdown of muscle proteins by endogenous enzymes, during fermentation period. Further perusal of the Table 2 indicates a significant effect of cooking methods on DH of *ngari*. Among three cooking processes followed, *sautéing* made the product more susceptible to peptide cleavage, followed by roasting and air frying. Similar trend was noticed for both pepsin (alone) and pepsin-cum-pancreatin digested *ngari*.

DH also showed significant difference among different digestion methods. DH in pepsin-pancreatin digestion method is substantially higher than other digestion methods. Further, roasting cooking method has showed higher degree of hydrolysis in pepsin-pancreatin digested samples among the cooking methods. Generally, proteins are hydrolysed into large peptides by pepsin while pancreatin degrades the large peptides resulting in the release of various forms of small peptides and free amino groups[28]. Overall, cooking enhanced the DH of *ngari*.

The main and interaction effects of cooking processes and digestion methods influence the DPPH

radical scavenging activity, metal chelating activity and reducing power of *ngari*. DPPH activity among the different digestion methods showed significance ($p<0.05$) difference. Overall, the undigested sample exhibited higher DPPH activity when compared to sequentially digested samples of each cooking methods. Highest DPPH activity (73.9%) was shown by uncooked undigested *ngari*. However, activity reduced to 40.2% and 26.6% at the end of pepsin and pepsin-pancreatic digestion, respectively. Upon cooking, significant increase in the DPPH activity was found at the end of pepsin-pancreatin digestion; roasted *ngari* displayed maximum anti-oxidative characteristics followed by air fried *sautéed* and uncooked *ngari* samples. Results implied that peptides present in the *ngari* reduced DPPH free radical by donating the hydrogen atom. Generally, peptides of hydrolysed protein possess good antioxidant properties; this further depends upon amino acid compositions and their sequence[29-30]. Peptides rich in aromatic amino acids such as tyrosine and phenylalanine are known to act positively as direct radical scavengers[31]. The observed differences in DPPH activity upon cooking may be due to differences in the amino acids composition/sequence of peptides released. The amino acid composition and sequence of digest will vary with the peptides released. The release of peptides affected by the nature of changes brought in the native protein structure by various processing methods which in turn affect the accessibility of sites available for cleavage by digestive enzymes. As depicted in Figure 2, reduction in DPPH activity was noticed upon pepsin digestion. Contrarily, pepsin-cum-pancreatin digestion increased the antioxidative nature of the *ngari*.

Reducing power assay measures the reduction ability of Fe^{3+} to Fe^{2+} in term of increasing absorbance at 700 nm[32]. The reducing ability was found to increase significantly with rising concentration of protein (1-4 mg/ml) in the digest. The increase in reducing ability may be due to the presence of more electron donor due to higher protein concentration (Fig. 3). Increasing reducing power with increasing amount of sample has been reported in Zhu[33] and Bougatef *et al.*[32]. Digestion methods significantly ($p<0.01$) influenced the reducing power of *ngari*. The higher reducing power was found in undigested *ngari* followed by pepsin-pancreatin and pepsin digested samples. Sheriff *et al.*[34] observed similar trend in reducing power of pepsin digested backbones of Indian mackerel as compared to papain digested ones. Among the cooking methods, roasting and *sautéing* yielded higher reducing activity while air-frying possessed lowest reducing activity.

Metal chelating activity of *ngari* was significantly ($P<0.01$) affected by the cooking methods, digestion methods and protein concentration (Fig. 4). The chelating activity of *ngari* increased with increasing protein concentration in the filtrate. Significant ($p<0.01$) reduction in metal chelating activity were observed in all the cooking methods as compared to uncooked *ngari*, however, none of the cooking methods impaired the metal chelating activity to the extent of complete reduction. Least metal chelating activity was found in *sautéed ngari*. Metal chelating activity of undigested and pepsin had no significant difference ($p>0.05$) while pepsin-pancreatic digestion reduced the activity, significantly. Many authors have demonstrated the presence of antioxidant properties in fermented fish products such as *Kapi*[35] and Miso[36]. Further, peptides present in the *ngari* could chelate pro-oxidants such as iron, thereby giving antioxidant characteristics to *ngari*. Metal ions can also be chelated by the carboxyl and amino groups of the amino acids by formic ionic bond with oxygen of –COOH group and covalent bond with nitrogen of $–NH_2$ group[37].

Effects of heat processing methods and *in-vitro* digestion on protein pattern of the ngari digest

In order to understand the pattern of release of peptides from *ngari* during sequential digestion by pepsin and pancreatin as influenced by the method of cooking, SDS-PAGE analysis of samples drawn after pepsin and pepsin-cum-pancreatin digestion was studied using SDS-PAGE techniques. The absence of peptide band in the range of 200-14 kDa and presence of smeared bands around 20 kDa and below may indicate that incubation of uncooked sample at pH 2 and 37°C did not have any notable autolytic degradation of protein. It should be mentioned that the *ngari* preparation involves a pre-drying (under open sun) treatment which reduces the moisture level to certain extent which indirectly favours the formation of protein aggregates via hydrophobic as well as oxidation of amino acids and such aggregates might not have been dissociated by SDS. The presence of multiple peptide units in the range of > 200 to 66 kDa in all the samples after cooking treatments without any enzymatic digestion bands may be assigned to the thermally denatured collagen sub units during heat

treatment. The observed peptide aggregates with the molecular weight of above 200 kDa can be assigned to γ-component of collagen subunits. This explanation is very logical due to the reason that the raw material used for *ngari* production is small whole fish includes skin, bones and scale which are rich in collagen. Collagen is easily extractable in the form of gelatin at high temperature (>45°C) which allows the disruption of a number of intra- and intermolecular covalent crosslinks present in parent protein i.e collagen[38]. This was also supported by the observation that the keeping of supernatant obtained from cooked samples (after treating at the pH of 2 and the temperature of 37°C for 1 h without enzyme) in a refrigerator for an overnight showed formation of weak gel-like material (data not shown). This finding shows that *ngari* can be a good source of connective tissue protein like gelatin in addition to muscle protein and gelatin/collagen peptides have attracted the consumers for their potential bioactive properties. Pepsin digest obtained from the entire cooked as well as uncooked sample were found to have multiple peptide units with the approximated molecular weight range of 66 to 14 kDa with concomitant disappearance of peptide units found in control samples. It should be mentioned the peptide units in above mentioned range for the uncooked control sample formed as a result of pepsin digestion which were not observed in undigested-uncooked sample. The pattern of peptide units formed was found to be more or less similar in all the peptic digests. This indicates that the site of action available on *ngari* protein molecules for pepsin was not altered significantly by the cooking processes like air frying, roasting and *sautéing*. Pepsin digested the fish proteins aggregates and formed the peptides in the range of 66-14 kDa. In undigested samples the peptide bands in the above mentioned region were not prominent. Further digestion of peptic digest with pancreatin resulted in complete digestion of relatively larger peptide units into smaller peptides as evidenced by disappearance of the corresponding peptides identified in the 66-014 kDa region. Results clearly indicated that in terms of digestion of *ngari* proteins by pepsin and pepsin-pancreatin, the cooking processes employed do not have any negative influence qualitatively.

Conclusion

Heat treatments are usually applied to *ngari* prior to consumption. Heat is applied to make it palatable while allowing to extract the typical *ngari* aroma and flavour and also to lessen microbial concerns. However, heat can induce different changes to the nutritional value of *ngari*. In our study, we found that irrespective of cooking methods such as roasting, air-frying and *sautéing*, cooking increases the protein contents and reduce the moisture content of *ngari*. The protein content was highest in air-fried sample (44.14%). Fatty acids composition was also affected by the cooking methods. *Sautéing* greatly changed the fatty acids composition of *ngari*. However, overall cooking does not affect the two important polyunsaturated fatty acids such as EPA and DHA content of *ngari*. In general cooking enhances DPPH radical scavenging activity while drastically reduces metal chelating activities. However, it did not totally reduce the activity. The reducing power was not affected by the cooking methods. Peptides profile as studied by SDS-PAGE clearly indicated that in terms of digestion of *ngari* proteins by pepsin and pepsin-pancreatin, the cooking processes employed do not have any negative influence qualitatively.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

MDH conceived and design the experiment. EK performed data analysis and interpretation. AK carried out the experiment and helped in drafting the manuscript. DU participated in conducting the experiments and analysis of data. CST contributed in drafting and revision of the manuscript. GN supervised the experiment and revised the manuscript.

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