Evaluation of Amino Acid Changes and Crumb Hardness of Enriched Bread with Tench (*Tinca tinca* L., 1758) Flesh in Turkey

Seda OĞUR*

Bitlis Eren University, Engineering-Architecture Faculty, Department of Food Engineering, Bitlis, Turkey

*Corresponding author: sdogur@beu.edu.tr

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Abstract The aim of the present study was to evaluate amino acid changes and crumb hardness of enriched bread with tench (*Tinca tinca* L., 1758) flesh. Bread was formulated with washed fish mince at the ratio of 5%, 10%, 15% and 20% and the amino acid changes and crumb hardness of breads were evaluated. Adding washed fish mince into bread resulted in a significant increase in the protein content. The amount of amino acids such as aspartic acid, glutamic acid, serine, glycine, threonine, arginine, tyrosine, cystine, phenylalanine, isoleucine, lysine, hydroxyproline and proline ($p < 0.05$) also increased. Alanine and leucine amino acids were detectable in none of the breads. The crumb hardness value of breads also increased with the addition rate of fish flesh and over time ($p < 0.05$). This study resulted in an alternative product (bread is rich in protein) that can be eaten without changing customs of bread consumption. This enriched bread can be helpful in solving health problems due to protein and essential amino acid deficiency.

Keywords: amino acid, bread, crumb hardness, enrichment, tench

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1. Introduction

Inadequate and unbalanced nutrition influences all human life span groups. But greater negativities especially occur in infants and children, since they are in the growth and development period. Most infant and child mortality are due to preventable diseases, such as growth and development disorders. These diseases are caused by protein, energy, vitamin and mineral deficiencies, based on malnutrition [1].

It is important to fulfill the metabolic role taken by quality protein rather than a high of amount protein, forming the most important group of nutrients, which are needed by an organism. The protein source is attributed as quality protein if it has a high amount of essential amino acids. Also, the biological value of quality protein is high. Essential amino acids are to be found sufficient and balanced quantities in foods of animal origin [2]. Threonine, leucin, isoleucin, valine, lysine, methionine, phenylalanine, tryptophan are basic essential amino acids. Histidine, cystine/cysteine, arginine and glutamine are essential amino acids, depending on the conditions [3].

Biological value is the changeover rate of the protein that is absorbed from the digestive canal into the body. The biological value of egg white protein is considered to be 100 and the biological values of other proteins are determined based on this ratio. So, fish protein has a 75-

80% biological value [4]. The biological value or essential amino acid content of proteins in bread, the basic foodstuff of Turkish people [5], is inadequate according to animal origin foods [6].

Consuming foods that have animal protein with high biological value has become a measure of the development of a nation. While 59.2% of daily protein consumption is provided from animal derived foods in developed countries, this ratio is 12.8% in developing countries. Animal protein consumption in Turkey is 1/3 of the level in developed countries. However, at least one-third of protein needs must be provided from animal products. This means the daily consumption of animal protein is an average of 35 grammes.

Fish consumption is 7-8 kg per year for each person in Turkey, while it is on average 27 kg in developed countries [4]. Fish has a lower fat and a higher more protein content according to meat values and it provides high quality protein, unlike other animal protein sources [7].

In recent years, it has been accepted globally that nutrition is important in the protection of health and in healthy living. Therefore, there has been a focus on functional foods as they became even more important in this respect [8]. Functional foods are associated with health. They are effective in reducing cardiovascular diseases and the risk of cancer, and in prevention of obesity and diabetes. They have a role in the health, especially of muscle, bone, teeth, intestine and eyes, and

in treating these diseases [9]. Low-fat milk and milk products, meat and fish are at the base of functional foods [8]. Enriched foods are also included in the class of functional foods.

Enriched products are cereal and cereal based products, milk and milk products, solid and liquid fats, some special products (salt, monosodium glutamate, sugar, sauces, etc.), tea and other drinks, and baby food [10]. Some firms tried to enrich food in Turkey. But the government or the society gives no importance to this issue. However, one of the short-term measures related to nutrient is to begin food enrichment applications [11].

To enrich of cereal products vitamins (A, B1, B2, B6, B12, niacin, folic acid) were used firstly, antioxidants, which converted to vitamin (tocopherol acetate, L-ascorbic acid, beta-carotene, alpha-tocopheryl acetate, palmitate) and minerals (iron, iodine, calcium, magnesium, zinc) [1,11-18]. In later years cereal products were enriched with concentrate and hydrolysate of protein sources, such as lysine, soybean, tomato seed, milk, bean, sunflower, chickpea, pea, gluten, corn germ [19-28]. Fish products were also used in enriching of cereal products: fish protein concentrate, fish protein hydrolysate, omega-3 fatty acid and surimi [29-36]. However, limited information is available related to the enrichment of cereal products with raw fish. Fish mince is a protein source that has an excellent amino acid composition, high nutritional value and is easily digestible yet [37].

In our previous study [38], breads containing tench (*Tinca tinca* L., 1758) mince at the ratio of 5% and 10% were found more acceptable according to panel tests. The aim of this study was to note the amino acid changes of enriched breads with tench (*Tinca tinca* L., 1758) flesh. Also, crumb hardness value of these breads was measured with the instrumental texture test. So, it was decided to discover a suitable bread formula.

2. Material and Methods

2.1. Fish Mince Preparation

In the study tench (*Tinca tinca* L., 1758) provided from fishers found in Isikli Lake (Civril/Denizli) in the spring season were used as material. The fish samples were delivered in ice to the laboratory and the head, internal organs, skin, fins and bones were removed. The fish flesh was washed 3-4 times in water (3-4 times of their weight), then minced to become protein more concentrated by the removal of the water-soluble proteins, an amount of fats and minerals. Also, 0.01-0.3% of salt was added into the final washing water. Ice was added into the water, if needed, because the temperature of water should not exceed 10°C. After washing, the fish mince was squeezed by a press to remove 80% of the water. It was immediately placed in the deep freeze and stored until used for analyses.

2.2. Bread Baking Process

The flour sample (Type: 550 bread wheat flour (ash, max 0.55%)), was transported to the laboratory in suitable conditions and was stored in a sealed state. The bread baking process was by the direct dough method [39]. The fish mince used to produce breads was estimated based on 1000 g flour and was added at the ratio of 5% (950 g flour,

50 g fish mince), 10% (900 g flour, 100 g fish mince), 15% (850 g flour, 150 g fish mince), and 20% (800 g flour, 200 g fish mince) into the dough. The 2% missing water, according to the dough water absorption ratio found in pharinograph experiments [38], 2% yeast and 1.5% salt were added for each addition rate (Table 1). All doughs were kneaded for 7 minutes.

Table 1. The Bread Formulas

Addition Rate	Flour (g)	Yeast (g)	Salt (g)	Water (ml)	Washed Fish Mince (g)
0% (Control)	1000	20	15	557	0
5%	950	20	15	512	50
10%	900	20	15	457	100
15%	850	20	15	408	150
20%	800	20	15	357	200

The bread baking procedure was repeated for the triplicate experiment.

2.3. Chemical Analyses

The protein content was determined in triplicate according to the AOAC 920.87 official standard method [40], the moisture content was determined in triplicate according to the drying method, the ash content was determined in triplicate according to the burning method and the fat content was determined in triplicate according to soxhlet-extraction method in flour and in breads as described by [39]. The dry substance analysis, the moisture analysis, the ash analysis and the fat analysis were made in triplicate according to [41], and the protein analysis was made in triplicate according to AOAC 928.08 method [40] for fish minces.

AOAC 994.12 Alternative I. Performic Acid Oxidation with Acid Hydrolysis-Sodium Metabisulfite Standard Method [40] was used as the reference and Agilent 1100 HPLC equipment was used in amino acid analysis.

The amino acid analyses were repeated for the triplicate experiment in both fish minces and breads.

2.4. Texture Analysis

Measuring crumb hardness was made with a TA.XTPlus Texture Analyzer (Stable Microsystems Ltd.), according to the AACC 74-09 Standard Method [42]. Two slices (about 0.8 mm) were superposed and adjusted pre-test speed: 2 mm/s, test speed: 1 mm/s, post-test speed: 1 mm/s, deformation rate: 40% and trigger force: 10 g [43,44,45,46,47]. This procedure was repeated for the triplicate analysis.

2.5. Statistical Analysis

The analysis results, obtained in the experiments, were converted into suitable tables and were subjected to a one-way variance analysis by using the Minitab 15® program [48]. The average for each analysis was evaluated according to the Tukey test and the significant difference was determined between values. $p < 0.05$ variation was accepted as the significant discrepancy between the groups.

3. Result and Discussion

The chemical composition of tench mince before and after washing is given in Table 2. The dry substance, the ash content and the fat content decreased, at a ratio of 7.22%, 70.75% and 50.36% respectively, but the protein ratio increased 3.96%, depending on the applied washing process during preparing fish mince.

Table 2. The Chemical Composition of Tench Mince (%)

	Dry Substance	Water	Protein (Nx6.25)	Ash	Fat
Unwashed Fish Mince	20.07	79.93	16.15	1.06	2.74
Washed Fish Mince	18.62	81.38	16.79	0.31	1.36

Reference [49] reported that tench mince, used for making fish balls, contained 83.41% moisture, 12.68% protein, 1.10% fat and 1.66% ash. Reference [50] implied that tench mince had 18.78% dry substance, 16.00% protein, 1.79% fat and 0.73% ash content. The composition of tench mince used in our study showed no likeness with the composition of these fish minces. The cause of this difference may be in the region that these fish were caught, nutritional status, environmental temperatures, sizes, ages, gender and many other factors that have shown dissimilarity as suggested by [51]. After the fish mince was washed, dry substance, ash and fat content decreased with the removal of some water soluble substances, and the protein concentration increased, as reported in a study made by [52].

The chemical composition of Type: 550 bread wheat flour is given in Table 3. Protein, ash and fat values were calculated as a percentage of dry substance. The protein in washed fish mince was 90.17% of the dry substance in fish mince, but the protein in flour was 12.81% of the dry substance in flour.

Table 3. The Chemical Composition of Type: 550 Bread Wheat Flour (%)

Dry Substance	Water	Protein (Nx5.70)	Fat	Ash
86.04	13.96	11.03	0.94	0.55

The amino acid composition of tench mince is given in Table 4. Breads contained fish mince and control bread was prepared based on the result of baked experiments. Their chemical compositions are given in Table 5. Protein, ash and fat contents were calculated as a percentage of dry substance.

Table 4. The Amino Acid Composition of Tench (*Tinca tinca* L., 1758) Mince

Amino Acid	Amount (mg/100 g)	Amino Acid	Amount (mg/100 g)
Leucine*	217	Tyrosine	538
Lysine*	1510	Cystine	433
Isoleucine*	3362	Aspartic acid	2578
Valine*	379	Glutamic acid	3773
Methionine*	2951	Asparagin	142
Phenylalanine *	645	Serine	1305
Threonine*	326	Glycine	229
Tryptophan*	784	Alanine	162
Histidine*	136	Hydroxyproline	552
Arginine*	341	Proline	1134

*Essential amino acid

The amount of dry substance was found to be 61.76% in the control group bread, and decreased gradually as additional fish mince was added (Table 5). There was no significant difference ($p>0.05$) in the proportion of dry substance between the control bread and the 5% fish mince-added bread. However, the 10-15-20% fish mince-added bread showed a significant difference ($p<0.05$). When the fish mince added breads were evaluated between themselves, the change in their dry substances was insignificant ($p>0.05$).

Table 5. The Chemical Composition of Breads (Protein Conversion Factor was Used as Nx5.70 in the Control Group, as Nx6.25 in Other Groups.)

Addition Rate	Dry Substance (DS) (%) *	Protein (DS) (%) *	Ash (DS) (%) *	Fat (DS) (%) *
0% (Control)	61.76 ± 0.516 ^a	13.89 ± 0.523 ^d	1.76 ± 0.156 ^b	1.73 ± 0.353 ^a
5%	61.49 ± 0.530 ^{ab}	16.06 ± 0.205 ^c	1.90 ± 0.106 ^{ab}	2.08 ± 0.120 ^a
10%	60.32 ± 0.057 ^b	19.71 ± 0.078 ^b	1.96 ± 0.156 ^{ab}	2.20 ± 0.106 ^a
15%	60.16 ± 0.247 ^b	20.81 ± 0.141 ^b	1.90 ± 0.106 ^{ab}	2.28 ± 0.092 ^a
20%	60.16 ± 0.481 ^b	22.85 ± 0.587 ^a	2.24 ± 0.056 ^a	2.37 ± 0.113 ^a

*: The difference is significant between averages indicated different letters in the same column ($p<0.05$).

±: Standard deviation.

The protein amounts in the breads increased with the increased addition of fish mince into dough. The amount of protein contained in fish mince samples at the ratio of 10% and 15% were similar ($p>0.05$), however, differences ($p<0.05$) were found between other application groups (Table 5).

It was established that the amount of protein in fish mince added-breads was higher than the amount of protein in the control bread and in breads that had had different types of fish protein concentrate added, as seen in the previous study [30]. When their nutritional values were considered, the acceptability of fish mince added breads were higher as the protein amounts in tench mince added breads were higher than the control bread (Table 5).

The amino acid composition of breads is given in Table 6. Although leucine (essential amino acid) and alanine amino

acids were determined in fish mince (Table 4), these were not detectable in the control bread and in any of the fish mince added breads (Table 6).

When the ratio of fish mince to dough increased the amount of lysine, isoleucine, phenylalanine, threonine, arginine, tyrosine, cystine, aspartic acid, glutamic acid, serine, glycine, hydroxyproline, proline also increased ($p<0.05$) (Table 6).

As expected, the amount of valine was high in all fish mince added breads because valine was found in higher levels in the fish mince (Table 4). Results showed a decrease in fish mince added breads at the ratio of 5% and 10% and these levels were below the valine level of the control bread (Table 6). The amount of valine increased to the ratio of 15%. Whilst there was little difference ($p>0.05$) between the valine amount in the control bread and fish

mince added breads with a ratio of 5%. Significant differences ($p < 0.05$) were found between other application groups according to their valine amounts (Table 6).

The amount of methionine and histidine was expected to increase in fish mince added breads, because the amount of methionine and histidine in fish mince was higher (Table 4) than the amount of methionine of the

control bread. However, the amount of methionine and histidine showed an inversely proportional change to the addition rate of fish mince and decreased gradually, if the addition rate of fish mince increased (Table 6). Significant differences ($p < 0.05$) emerged between control bread and fish mince added breads at different rates, according to the amount of methionine and histidine.

Table 6. The Amino Acid Composition of Breads (mg/100 g)

Amino Acid	Addition Rate				
	0% (Control)	5%	10%	15%	20%
Leucine ^e	-	-	-	-	-
Lysine ^e	373.01 ± 1.48 ^c	397.63 ± 2.14 ^d	417.70 ± 0.85 ^c	443.23 ± 1.58 ^b	527.14 ± 1.48 ^a
Isoleucine ^e	1728.24 ± 1.26 ^c	1827.65 ± 0.93 ^d	1923.41 ± 2.28 ^c	2010.96 ± 1.07 ^b	2396.74 ± 2.06 ^a
Valine ^e	187.87 ± 0.48 ^d	185.14 ± 2.80 ^{cd}	180.58 ± 1.54 ^c	193.34 ± 0.91 ^b	214.32 ± 0.69 ^a
Methionine ^e	1524.86 ± 0.66 ^a	1503.89 ± 1.65 ^b	1357.06 ± 0.95 ^c	1104.43 ± 1.03 ^d	1068.86 ± 0.24 ^e
Phenyl-alanine ^e	93.94 ± 1.47 ^e	142.27 ± 0.47 ^d	154.13 ± 0.03 ^c	166.90 ± 0.04 ^b	237.12 ± 1.05 ^a
Threonine ^e	67.49 ± 1.43 ^e	89.38 ± 2.87 ^d	138.62 ± 0.81 ^c	162.34 ± 0.85 ^b	178.75 ± 2.22 ^a
Tryptophan ^e	101.23 ± 2.88 ^c	99.41 ± 2.19 ^c	144.10 ± 1.15 ^b	152.30 ± 1.37 ^b	309.17 ± 1.33 ^a
Histidine ^e	57.46 ± 1.15 ^a	53.81 ± 0.99 ^b	49.25 ± 0.82 ^c	20.06 ± 0.42 ^d	15.50 ± 0.65 ^e
Arginine ^e	189.70 ± 2.18 ^c	204.29 ± 0.95 ^d	219.79 ± 1.16 ^c	258.10 ± 0.59 ^b	274.51 ± 1.17 ^a
Tyrosine ^e	83.90 ± 2.31 ^e	116.74 ± 0.68 ^d	130.42 ± 1.78 ^c	150.48 ± 0.20 ^b	195.17 ± 0.64 ^a
Cystine ^e	207.94 ± 1.20 ^e	220.70 ± 1.44 ^d	233.47 ± 1.48 ^c	249.89 ± 0.91 ^b	269.95 ± 2.43 ^a
Aspartic acid ^e	1836.50 ± 1.43 ^c	2074.8 ± 2.6 ^d	2228.02 ± 2.64 ^c	2290.94 ± 0.01 ^b	2484.29 ± 0.14 ^a
Glutamic acid ^e	1646.16 ± 1.92 ^d	1751.95 ± 1.68 ^c	1820.35 ± 0.69 ^b	1824.91 ± 0.83 ^b	1930.70 ± 0.57 ^a
Asparagin ^e	98.50 ± 0.47 ^e	165.98 ± 1.91 ^d	195.17 ± 0.06 ^b	248.98 ± 1.48 ^a	183.31 ± 0.96 ^c
Serine ^e	106.70 ± 1.09 ^c	117.65 ± 0.83 ^d	130.42 ± 2.87 ^c	144.10 ± 1.58 ^b	190.61 ± 2.15 ^a
Glycine ^e	370.27 ± 1.88 ^c	428.64 ± 0.34 ^d	439.58 ± 0.04 ^c	452.35 ± 1.39 ^b	462.38 ± 0.85 ^a
Alanine ^e	-	-	-	-	-
Hydroxy-proline ^e	223.44 ± 1.22 ^c	263.57 ± 0.25 ^d	274.51 ± 0.06 ^c	343.82 ± 0.08 ^b	414.96 ± 1.17 ^a
Proline ^e	3095.33 ± 2.80 ^c	3162.82 ± 2.87 ^d	3269.52 ± 1.80 ^c	3319.68 ± 2.15 ^b	3467.42 ± 1.30 ^a

e: Essential amino acid

*: The difference is significant between averages indicated different letters in the same row ($p < 0.05$).

±: Standard deviation

The amount of tryptophan found in fish mince added bread (at the ratio of 5%) showed a slight decrease compared with its level in control bread (Table 6), but this decline was insignificant ($p > 0.05$). The amount of tryptophan increased gradually with the added fish mince ratio of 10% and at the following rates ($p < 0.05$). But it was observed that the amount of tryptophan in fish mince added breads at the ratio of 10% and 15% were similar ($p > 0.05$) (Table 6).

The amount of asparagin in fish mince added breads increased to over 142 mg/100 g, the amount of asparagin in fish mince (Table 4). Because the highest amount of asparagin (248.98 mg/100g) was reached at the ratio of 15%, increasing the addition rate of fish mince after the ratio of 15% would not be beneficial.

Although the essential leucine amino acid was found at a level of 217 mg/100 g and the alanine amino acid was found at a level of 163 mg/100 g (Table 4) in the fish mince, it was not detectable in the control bread or in any of the fish flesh added breads (Table 6). The cause of this result may be the leucine and alanine amino acids, that are found in fish mince degraded their chemical structure at high baking temperatures (200 °C) or changed into different forms via chemical reactions with other components or formed aromatic compounds catabolizing during the fermentation by yeast.

There are a limited number of studies mentioned in the literature about the cause of the decrease of some amino acid rates. But there is no study in the literature concerning the amino acid changes of fish mince added bread.

Free amino acids, released because of peptide hydrolysis or proteolysis during the sourdough fermentation, serve as substrates for yeast. They influence the taste of bread and are important precursors for volatile flavor compounds. Amino acid turnover during sourdough fermentation. Yeast consumes amino acids during growth, and the amino acid levels increase only after the yeast growth has ended. The hydrolysis of peptides (secondary proteolysis) by sourdough lactobacilli accumulates amino acids in the dough in a strain dependent manner, whereas yeast decreases amino acid levels in dough [53, 54].

Reference [55] states that the most important factors governing the levels of amino acids in wheat dough are dough pH, fermentation time and the consuming of amino acids by the fermentative microbiota.

Reference [56] reported that glutamic acid, isoleucine and valine were essential for the growth of *L. brevis* subsp. *lindneri* CB1 and *L. plantarum* DC400. Arginine, methionine and lysine were stimulatory and alanine, glycine, aspartic acid, lysine, histidine, cysteine, tyrosine, serine, threonine and proline were without influence. Each individual amino acid, except lysine, cysteine and histidine, were suitable nitrogen sources for the growth of *S. cerevisiae* 141 and *S. exiguus* M14.

Proteolytic strains of sourdough lactic acid bacteria (LAB) may influence the amino acid level in doughs, but there is considerable evidence showing that activating of cereal proteases are a major driving force for protein degradation in sourdoughs [57].

Reference [58] studied the evolution of free D- and L-amino acids in sourdoughs started with various LAB and

yeasts. *Lactobacillus brevis* subsp. *lindneri* CB1 and *Lactobacillus plantarum* DC400 had high proteolytic activity. During sourdough fermentation, *Saccharomyces cerevisiae* 141 and *Saccharomyces exiguus* M14 sequentially utilized free amino acids produced by bacterial activity. Because of increased cell yeast autolysis, more *S. exiguus* M14 inocula caused more free amino acids, which were partially utilized by LAB without causing hydrolysis of wheat flour protein. D-alanine, D-glutamic acid and traces of other D-isomers were observed in sourdoughs fermented with *L. brevis* subsp. *lindneri* CB1 and *S. cerevisiae* 141. Free total D- and L-amino acid content decreased by more than 44% after baking the sourdoughs.

The most important consideration must with lysine amino acid as lysine is at a limited level in bread and is essential for the body [6]. Amino acids are lost during the cooking of bread. During cooking lysine 73%, threonine 66%>, tryptophan 86%, methionine 57% is destroyed in the crust, while lysine 23%, threonine 13%, tryptophan 26%, methionine 14% is destroyed in the crumb. Since amino acids, especially lysine, take part in the Maillard reaction in the crust during cooking, their biological value has suffered loss [59].

When reference [60] baked pizza crusts at 316 °C for 4.5 min, this process did not change protein contents of pizza crusts. But high-temperature and short-time baking did reduce the lysine and to a lesser extent, tyrosine, cystine, and threonine in pizza crusts.

The role of the elimination of lysine deficiency has been conducted by different enrichment studies. It was reported that breads reached the desired level of lysine by adding different protein sources (chickpea flour+soybean flour, pea protein, fish protein concentrate), and lysine amino acid [29]. Therefore, adding the fish mince into bread would perhaps overcome the deficiency of lysine.

Despite the high amount of methionine and histidine in fish mince, the reason for the decreasing in the amount of methionine and histidine in fish mince added breads may be due to the reaction of methionine with some compounds in breads or by turning into another form catabolizing by yeast. Reference [25] reported that by adding bean protein to bread also decreased the amount of methionine. The histidine amino acid is essential for infants. But it could be determined in any bread at the amount of the daily requirement of infants [2]. Therefore, it can be said that by adding the fish mince to bread does not improved at the desired level this essential amino acid. On the contrary, this application caused a loss of histidine. It was implied that 30% of the methionine requirements can be met with cystine amino acid, because of they have the same chemical structure [2]. It was possible to say that 30% of the methionine deficiency will could be supplied thanks to the amount of cystine increased in direct proportion to the addition rate of fish mince.

Reference [61] examined free amino acid changes related to the nutritional requirements and proteolytic activities developed by three strains of LAB species (*Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40)). Nutritional requirements of lactobacilli and *Enterococcus* for glycine were evidenced at the beginning of fermentation (0-4 hours), whereas asparagin was consumed later (4-24 hours). B33 metabolized glutamine and serine first, and aspartic

acid, glutamic acid, gamma-aminobutyric acid, tryptophan, and proline later; alanine was assimilated during the entire fermentation (0-24 hours). B39 showed preferential uptake of aspartic and glutamic acids, glutamine, serine, alanine, ornithine, and threonine. During fermentation (0-24 hours), valine, leucine, and lysine gradually increased; however, in all doughs proline content increased only during the first 4 hours. Aspartic acid and histidine accumulated only in doughs fermented with B40. Methionine and tryptophan accumulated in doughs fermented with B39 and B40.

It was observed, that the amount of phenylalanine in the control bread was well below the amount that infants, children and adults need to maintain their metabolism, according to the table of “the daily requirement of essential amino acid of the people to keep normal protein metabolism” mentioned by [2]. However, the addition rate of fish mince increased, so the amounts of phenylalanine of breads increased and became to the needed level. It was determined that the amount of threonine of the control bread was well below people’s daily requirement [2], however the level of threonine improved if fish mince was added to bread. It can be said that bread containing essential amino acid tryptophan at this level could meet the daily needs of adults [2].

Reference [62] suggested that the variation in the amino acid content after fermentation could be explained by yeast metabolism and their release by proteases. For example, there was an increase mainly in alanine and serine during wheat bread-making that could partially be because of the fermentation process, a metabolic state that consumes, as well as releases, different amino acids. Rye crisp bread fermentation and baking showed that asparagin, and aspartic acid decreased during bread-making as well. But glycine, serine, proline and lysine showed a smaller increase, as occurring in our study.

The amount of amino acids in bread varies according to applied temperature and the time during fermentation and cooking stages. Therefore, selecting a more favorable bread group should be according to sensory evaluations [38]. The fermentation and cooking stages should be studied to apply different temperatures and durations, and to fully understand the amount of change various amino acids at various stages.

Crumb hardness values, measured by Texture Analyzer equipment are presented in Table 7. The crumb hardness value of breads, determined 24, 48 and 72 hours after, also increased because of the increased addition rate of fish flesh and the extended time ($p<0.05$). When the fish flesh was added to the bread, volume and water absorption [38] decreased. Decreasing of gluten ratio causes this influence. Also, a harder structure formed in the bread.

The resistance of the crumb and crust to deformation is the textural character referred to as “hardness” and is an important factor in bakery products, as it strongly correlates with consumers’ perception of bread freshness [63].

According to instrumental texture measurement, a harder structure formed in the bread with the fish flesh because of the addition of fish flesh into the bread formula reduced the amount of gluten and thus, the volume of bread. The amount of gluten and water absorbed by the dough also decreased, by adding the fish flesh, rich in animal protein, into the dough. The crumb became harder

by reducing of the volume of bread. Reference [64] has reported previously the converse relationship between the hardness and the moisture content.

Table 7. Instrumental Hardness Value of Crumb (gforce)

Addition Rate	After 24 h [*]	After 48 h [*]	After 72 h [*]
0% (Control)	501.2 ± 56.6 ^c	778.0 ± 81.5 ^c	1084.0 ± 43.8 ^c
5%	920.7 ± 97.6 ^b	1177.7 ± 82.5 ^b	1297.5 ± 141.8 ^c
10%	1052.8 ± 2.6 ^b	1344.3 ± 1.1 ^b	1603.2 ± 13.2 ^b
15%	1282.9 ± 110.7 ^{ab}	1649.7 ± 52.6 ^a	1913.5 ± 55.7 ^a
20%	1479.5 ± 104.5 ^a	1750.9 ± 33.9 ^a	2010.4 ± 48.3 ^a

*: The difference is significant between averages indicated different letters in the same column (p<0.05).

±: Standard deviation

A negative correlation was found between loaf volume and crumb hardness. This signifies that smaller loaves (as in the case of the control) were denser and had a tightly packed crumb structure, resulting in higher crumb hardness readings. In one study, breads baked from lower (10.4%) protein flours staled at a faster rate than those from higher (13.1%) protein flours, and it was concluded that the flour component primarily responsible for the shelf life of bakery products was, in fact, gluten. It has also been stated that gluten present in wheat bread slows down the movement of water by forming an extensible protein network, thus keeping the crumb structure together. Therefore, the absence of gluten should increase the movement of water from the crumb to crust, resulting in a firmer crumb and a softer crust [28].

Although the water holding capacity of fish protein concentrate is higher than egg albumin and soy protein [37], when fish flesh is added to dough, water absorption decreased. The cause of this may be due to reducing the amount of gluten protein, having the water binding capacity, undergoing dilution of the gluten structure by adding fish protein [27] or water contained in the fish flesh. When whey protein concentrate was added into the French bread, there was a decrease in the water absorption of dough [22].

When two recent studies added gluten to bread, they found the hardness value decreased at the end of 24, 48 and 72 hours [23,28]. On the other hand, they reported that adding gluten improves the strength properties of bread by causing a slowing in the increase in hardness over time [23, 28].

The increase in crumb hardness causes bread staling. The cause of the increase in bread hardness and so causing bread staling, is starch retrogradation. Retrogradation occurs because of crystalline structures combining with each other, like a clothes zipper, thanks to hydrogen bonding or physical attraction forces formed between the free hydroxyl groups and amylose molecules, when two or more amylose molecules come near each other in the media [6].

The breadmaking process (including dough recipe) the method of mixing and proofing, the temperature of the dough during baking, and the final packaging, influences the staling of bread loaves [28]. Gluten-free breads exhibit a higher staling rate in comparison to wheat-containing bread. The absence of gluten increases the movement of water from the crumb to the crust, resulting in a firmer crumb and softer crust. This acceleration in the rate of changes associated with the staling phenomenon lends

support to the role of gluten in modulating the textural properties of baked goods during storage [65].

When comparing texture scores (obtained from sensory panel test in our previous studies [38]) and instrumental hardness values after 24 hours, it was observed that the instrumental crumb hardness increased, whilst the sensory texture scores decreased after the increasing additional of fish mince. It can be said, according to hardness values in all three measurement periods, that bread with added fish flesh at the ratio of 5% will be more acceptable to the sensory evaluation of bread texture [38], since the hardness value of this bread is lower than the hardness value of bread with further added fish protein. Also, the bread with on added fish flesh at the ratio of 5%, was preferred compared to the control bread according to chewing scores [38].

Anti-staling agents are used for prevention of the rapid staling in gluten-free bread by increasing hardness values. Reduced and softer crumb structure was obtained compared to control bread in a study using some hydrocolloids as anti-staling agent [66]. The increased hardness in bread produced by us may be removed with the use of similar agents.

On the other hand, it is possible to say that because of the decreasing water content of fish flesh added bread microbiological deterioration may be delayed. The possibility of this influence will be researched in a further study.

4. Conclusions

It is considered that adding fish mince to bread is helpful in the elimination of human health problems, based on animal protein and essential amino acid deficiency, as it enriches protein and amino acid content (for the most part). The application of added fish mince may contribute to bread consumed in developing countries, whilst rich in carbohydrates, to reach the level to meet the protein needs of people by protein enrichment. This study developed an alternative product (bread is rich in protein) that can be eaten whilst not changing customs of bread consumption. This method may be a new process to utilize fish that cannot be consumed fresh, be of low economical value or have been over hunted, cannot be preserved with the proper technological process, or for waste fish products resulting from fish processing. Fish mince may also be used as a more effective means of healthy nutrition for people as well as enriching of other food products with protein.

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