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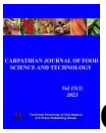

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OPTIMIZATION OF POTATO FLOUR BASED COMPLEMENTARY FOOD USING D-OPTIMAL DESIGN

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Abstract

The huge demand of nutritious, calorie dense, non allergenic and cost effective complementary food is continuously increasing so as to curb the prevalence of malnutrition among children especially in developing countries having limited resources. Therefore, it becomes essential that the synergistic effects of the primary constituents should be exploited to yield maximum optimal functional properties. This study was carried out to develop a weaning mix by using Rice flour (A), Potato Flour (B) and Chickpea Protein Isolates (C) to obtain a formulation having optimal physicochemical properties (Water Absorption capacity, Water Solubility Index, Texture) and sensorial properties by Optimal Mixture Model Design of response Surface Methodology. The lower limit (Rice flour- 50; Potato flour- 20 and Protein isolate-5) and upper limit (Rice flour- 70; Potato flour- 30 and Chickpea Protein isolate-10) for each mixture component was used. Experimental designs had 16 experimental runs with physicochemical and sensorial properties as their responses. The constraint fixed for optimization of the weaning mix was to maximize the overall acceptability and keeping the physicochemical properties WAC, WSI and texture within range. Within these constraints, d optimal design selected the variation 65A:28.1B:6.9C as the most desirable one. The predicted and observed values of the analyzed responses of the optimized formula were compared ($p<0.05$) and the results were found to be in good agreement with the predicted values.

1.Introduction

Complementary feeding period is the period when the diet of older infants (age when toddlers are 18 to 24 months old) and young children (age between 24 months and 5 years old) is transitioned from exclusive breast feeding towards eating the family diet. Infants are able to maintain adequate growth until the age of six months thereafter when additional nutrients are required to complement breastfeeding (Tiwari et al, 2016). WHO has postulated that complementary feeding in the first 6 months along with breastfeeding as one of the potential action to address the issue of malnutrition in lower and middle income countries (WHO, 2017). The most essential characteristics of weaning food or infant formula is to be rich in calories, easily digestible good quality protein content, along with sufficient vitamins and minerals. It should form semisolid soft slurry when mixed with warm or cold water (IAP, 2021).

Traditional Indian processing technologies have pioneered the preparation of weaning food by using different combinations of diverse food components, especially cereals and starches. Cereals like wheat, maize, rice, oats etc. along with legume flours have been used in preparation of weaning mixes that are similar in appearance to conventional food and also have an added advantage of aiding physiological functions along with providing adequate nutrition (Das et al, 2012). All the above mentioned formula basically contain a single

dominant cereal, pulse or fruit as a key ingredient due to which various kinds of mixes have to be administered regularly so as to provide complete balanced nutrients to the child. However, due to large numbers of working mothers and nuclear family settings, there is a huge demand for safe, organic and ready-to-serve weaning food and has ever-growing market (Misra & Dwivedi, 2015).

Qualifying the above-mentioned criteria, ingredients like potato flour, rice flour and chickpea protein isolates have been exploited for their potential of being developed as a cost effective and wholesome weaning mix. Potato flour is an inexpensive, nutritious and an excellent source of carbohydrates. Research involving the development of integrated process for the production of shelf stable potato flour and further baked products mixed with wheat flour should be initiated in order to diversify the use of potato as well as to enhance nutritional quality of products since the quality of protein in potato is the best among other plants protein (Mirajkar et al, 2013). Among several processed products, potato flour is the oldest commercial potato product, which can be stored safely and incorporated into various recipes.

Chickpea possesses good balance of amino acids, non-allergenic highly bioavailable protein and relatively low level of antinutritional factors than soybean. It is a good source of high-quality protein, carbohydrates, vitamins (thiamine and niacin), minerals (calcium, phosphorous, iron, magnesium, and potassium) and its oil is rich with the essential fatty acid. Therefore, it is considered as a suitable source of dietary proteins (Wang et al, 2010). Chickpea protein isolates have been used to increase the protein content of the mix as the protein efficiency ratio (PER), net protein retention (NPR) and net protein utilization (NPU) of chickpea based infant formula were not different to soy or milk based formula. The mean percentage of absorption, retention and biological value of the chickpea formula were 72.4, 26.4 and 35.1 compared to that of soy 69.6, 24.3 and 34.0, respectively (Malunga et al, 2014) which makes it a better choice.

The general acceptability of weaning foods by infants is greatly influenced by the functionality of the ingredients used for their production. Indeed, the functionality of a food is an interactive outcome of the food ingredients in addition to its nutritional value, which has a great impact on its utilization (Kinsella & Melachouris, 1976). Functional properties such as gelation, water holding capacity, viscosity and pasting properties are very important for ensuring the appropriateness of the diet to the growing child. The consistency and energy density (energy per unit volume) of the complementary diet coupled with frequency of feeding are also important factors in determining the extent to which an infant can meet his energy and nutrient requirements (Kikafunda et al, 1997). In the processing of most complementary foods, emphasis is usually on the nutritional quality and quantity of the ingredients rather than their functional properties (Bookwalter et al, 1987).

For example: In a 3 component system:

Where in = 1,2,3 $0 \leq x_i \geq 1$

$$x_1+x_2+x_3=1 \quad (1)$$

The mixture design statistical method is the most suitable method used in optimizing the production process of a complementary food. The mixture design method is usually used in mixture formulation whose sum of all components must be equal to 100 (Brereton, 2003).

There are many types of mixture model design but D-optimal design is constructed to minimize the overall variance of the predicted regression coefficient by maximizing the value of determinant of the information matrix (Esbensen et al, 2014). The experimental region is not simplex but it is irregular (Valko, 2000). As compared with other traditional designs, the benefit of D-optimal configuration is that, it works on straight optimization focussing a chosen optimality criterion along with the suitable model. It has a smaller number of runs

and thus carries low cost of experimentation. Furthermore, combined mixture and process variables can be used in the same experimental design (Valko, 2014).

2. Materials and methods

2.1.Procurement of Raw Material

The processing cultivar of potato "Kufri Chipsona1" was procured from Central Potato Research Institute Campus, Modipuram, Meerut. Kufri Chipsona 1 was used for the preparation of flour due to its low reducing sugar and high starch content. Other ingredients were purchased from local market. All the chemicals used in the study were of analytical grade obtained from Merck-Sigma.

2.2.Experimental designs for the development of Weaning mix formulation:

An optimal mixture model design was used with arbitrary lower and upper bounds as explained by (Esbensen et al, 2014). The three independent variables were Rice flour (A), potato flour (B) and Protein isolates (C). The lower limit (Rice flour- 50; Potato flour- 20 and Protein isolate-5) and upper limit (Rice flour-70; Potato flour- 30 and Protein isolate-10) for each mixture component was worked out for selecting runs of these variables. The design produced 16 experimental formulations (Table 1).

Table 1. Actual and Predicted value of WSI, WAC, Texture and Overall acceptability of the Optimal Mixture Design

RUNS	Rice Flour (A)	Potato Flour (B)	Protein isolate (C)	Actual WAC	Predicted WAC	RSE %	Actual WSI	Predicted WSI	RSE %	Actual Texture	Predicted Texture	RSE %	Actual Overall acceptability	Predicted Overall acceptability	RSE %
F1	62.5	30	7.5	8.90	9.25	3.7429	31.00	32.47	4.5274	8.10	8.21	1.3793	8.30	8.43	1.5094
F2	68.125	23.125	8.75	8.80	8.11	7.8557	27.00	24.72	8.444	8.60	8.26	3.9534	8.50	8.48	0.2639
F3	65	25	10	8.00	8.27	3.2778	29.00	26.19	10.7264	8.70	8.63	0.8471	8.50	8.64	1.6091
F4	65	25	10	8.10	8.27	2.0688	25.00	26.19	4.5462	8.50	8.63	1.4712	8.70	8.64	0.7059
F5	65	30	5	9.10	9.06	0.3866	36.00	34.79	3.4884	8.70	8.67	0.3079	9.00	8.93	0.8385
F6	70	25	5	7.70	7.74	0.4904	24.00	25.66	6.4748	8.20	8.29	1.0854	8.20	8.21	0.1773
F7	63.125	28.125	8.75	8.90	8.91	0.1134	24.00	29.42	18.4172	7.90	8.39	5.8779	8.60	8.47	1.5865
F8	70	20	10	7.00	7.13	1.7942	21.00	21.93	4.2613	7.70	7.77	0.8568	8.10	8.10	0.0242
F9	70	25	5	7.70	7.74	0.4904	24.00	25.66	6.4748	8.20	8.29	1.0854	8.20	8.21	0.1773
F10	70	20	10	7.20	7.13	1.0117	20.00	21.93	8.8203	7.70	7.77	0.8568	8.10	8.10	0.0242
F11	60	30	10	8.70	8.38	3.8158	30.00	28.37	5.7520	8.00	7.88	1.5587	7.60	7.59	0.0989
F12	65	30	5	9.20	9.06	1.4897	37.00	34.79	6.3631	8.80	8.67	1.4608	8.90	8.93	0.2819
F13	65.625	28.125	6.25	9.00	9.02	0.2672	30.00	31.32	4.2282	8.60	8.58	0.2453	8.80	8.80	0.0292
F14	60	30	10	8.30	8.38	0.9573	29.00	28.37	2.2270	8.00	7.88	1.5587	7.60	7.59	0.0989
F15	68.125	24.375	7.5	8.60	8.37	2.8027	27.00	25.97	3.9812	8.60	8.31	3.5375	8.50	8.50	0.0534
F16	70	22.5	7.5	7.40	7.78	5.1351	27.00	23.21	14.0370	7.80	7.88	1.0691	8.10	8.08	0.1999

2.3.Preparation of Potato Powder

The selected potatoes were washed, peeled, shredded and blanched in boiling water at 100±°C for 5 min. After blanching, the shredded potatoes were dried in cabinet tray drier (VAM 934, VAM Instrument Pvt. Ltd., India) for 6 to 8 h at 70°C. The dried potato shreds were ground in a high speed mixer (Inalsa) and sieved through 70 mesh sieve to separate potato powder and potato grits. The potato powder was then packed in laminated LDPE bags, sealed and stored at ambient temperature, until further use.

2.4.Preparation of Chickpea Protein Isolate

Chickpea isolates were prepared from the *desi* cultivar (K-850) by methods given by Mao and Hua (2012) with slight modifications. Chickpea flour was defatted with water in the ratio of (1:20) with the pH adjusted to 10 using 1M NaOH. The slurry was heated at 40º C in water bath for an hour with manual stirring at

every 5 minutes. It was centrifuged and the supernatant was collected with a continuous pH adjustment at 4.5 with 1M HCl. Precipitation of protein isolates was done for about an hour, followed by washing and neutralization of isolates which were then vacuum dried at 40º C for 8 hours at 550 mmHg and finally collected and stored in an air tight container till future use.

2.5.Water absorption capacity and Water Solubility Index of Weaning Mix

The water absorption capacity (WAC) measures the volume occupied by the granule or starch polymer after swelling in excess of water and water solubility index (WSI) determines the amount of free polysaccharide or polysaccharide release from the granule on addition of excess water. WAI and WSI were determined according to the method developed for cereals (Yagci & Goguş, 2008; Chandra et al, 2015). The potato powder and optimized product were suspended in water at room temperature for 30 min, gently stirred during this period, and then centrifuged at 3,000× g for 15 min. The supernatants were decanted into an evaporating dish of known weight. The WAC was the weight of gel obtained after removal of the supernatant per unit weight of original dry solids. The WSI was the weight of dry solids in the supernatant expressed as a percentage of the original weight of sample.

2.6.Texture Analysis and sensory analysis

Consistency of the snacks was examined by TA-XT 2i Texture Analyzer (Stable Microsystems, Surrey, U.K.). The sensory evaluation for color, flavor, taste, texture, crunchiness and overall acceptability was also done on the basis of nine point hedonic scale by a group of thirty semi trained panelists with five replications.

2.7. Statistical Analysis:

The utility of using mixture design is to ascertain the response changes as a function of the relative proportion of components being used in the product optimization. Optimal design is used when the component ranges are used in different proportions having multi-component constraints where the best estimates were provided algorithmically by the chosen model. D Optimal algorithm chooses the best run that has minimum variance –co-variance matrix to the process by creating fractional general factorial experiments. The statistical parameters including the adjusted multiple co-relation co-efficient (R^2), co-efficient of variation (CV%), lack of fit, regression (p value) and regression (F value) were used to evaluate the best fitting mathematical model. The design was expressed by polynomial regression equation to generate the model as follows:

Where

$$y_i = \beta_0 + \beta_1 x_1 + \beta_2 x2 + \beta_3 x3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad (2)$$

Where Y_i, is the predicted response and β_0, β_1 and β Where Yi, is the predicted response and 0, 1 and 2 are linear co-efficient, quadratic co-efficient and interaction co-efficient respectively. The suitable polynomial equation for the design such as linear, quadratic or cubic was chosen according to the fittest model suggested algorithmically. To facilitate better understanding of the response variables and perceive their interaction between the response and causal factor variables, the mixture design space and three dimensional (3D) contour plots of the fittest polynomial regression were generated.

The three independent factors studied were Rice flour (A), Potato flour (B) and Protein Isolates (C). The responses studied were: Y_1 is Water absorption capacity (WAC), Y_2 is Water Solubility Index (WSI), Y_3 is Texture and Y_4 is Overall acceptability (OA).

The Data was subjected to statistical analysis using response surface methodology (Design Expert version 10.0 by Statease Inc.) Selection of a predictive model to accurately describe each response was based on the quality of fit evaluated by analysis of variance

(ANOVA) statistical package on a predefined level of significance of 0.05.

2.8.Optimum ingredients formulation for the weaning mix

The optimization was done by the numerical and graphical methodology. The constraints for optimization were maximizing the overall acceptability and potato flour content; at the same time keeping the Protein Isolates, Water Absorption Capacity (WAC), Water Solubility Index (WSI) and texture in range. The goal of the constraints was to finalize a low cost infant formula having optimal physicochemical properties. The predictive regression models developed for each of the criteria were used to develop ternary contour plots to display the effects of the ingredients on the properties. By superimposing the contour plots of all the selected criteria for an optimal weaning mix the optimum region was determined by generating the overlay contour plot for the optimization criteria. From the selected optimum region obtained, the optimal infant formula variation was selected. Thus, the chosen blend was reformulated and varied in treatment. During the physicochemical analysis; the samples were stored at a controlled temperature of 25±2º C in High density Polyethylene (HDPE) sealed packets. Sensory evaluation of the mix was carried out using a nine point hedonic rating scale. The sensory was done by a panel of thirty semi trained evaluators.

3. Results and discussions

Table No. 1 shows that there were 16 formulations in the physical tests analysis. The actual and predicted data sets of the physical tests are tabulated in Table 2.

Table 2. Predicted and observed values for optimized formulation (65PF:28.1RF:6.9PI)

Responses	Predicted values	Observed values	RSE %
Overall Acceptability	8.74814	8.667	0.2172
Water absorption capacity (WAC)	8.98187	8.8667	1.2816
Water Solubility Index (WSI)	35.3619	34.268	3.0386
Texture	8.41681	8.33	1.0313

Table 3. ANOVA table for WSI, WAC, Texture and Overall acceptability

	WAC Y_1	WSI Y_2	Texture Y_3	Overall acceptability Y_4
Model	Significant	Significant	Significant	Significant
R^2	0.9850	0.9644	0.9813	0.9748
Adjusted R^2	0.9626	0.9122	0.9783	0.9617
Predicted R^2	0.9681	0.7122	0.9119	0.9363
p value	0.0001	0.0011	0.001	0.0001
F value	43.88	18.32	76.17	76.34
CV %	1.69	3.76	0.701	0.9636
Lack of fit	Not Significant	Not Significant	Not Significant	Not Significant
Standard deviation	0.1403	1.19	0.059	0.0865
PRESS	15.53	69.82	0.2041	0.1618

3.1.Effect of variables on Water Absorption Capacity (WAC) Y_1: The software suggested the cubic model as the model to describe the effect of components on the WAC having significant model where lack of fit is insignificant (Table 3). The water absorption capacity of the formulations ranged from 7.2 to 9.2. ANOVA revealed the model's F value as 43.88; implying that the model is significant at p <0.0001 level. The predictive model could explain 98.5% of the influence of variations

composition on the WAC of the blends (Table 3) as elaborated in equation No. 2.

3.2.Water Absorption Capacity (WAC)

$$Y_1=8.477144A+7.135004B+8.84316C+1.636376AB+1.940165AC-1.11189BC \quad (3)$$

Water absorption capacity of flours plays an important role in the food preparation process because it influences other functional and sensory properties. Furthermore, the range of application of flours as food ingredients is dependent, to a large extent, on their interaction with water especially when it comes to development of a complementary food. Potato flour (especially of Kufri Chipsona varieties) has been found to have a very high WAC as compared to the flours of other varieties (Vaishali et al, 2020). Water absorption is a characteristic of the physical and chemical properties of the starch granules, fiber and also protein. The hydrophilic groups bind the water molecules thereby enhancing the gel formation capacity (Hanim et al, 2014). Thus, the enhancement of amylase leaching and solubility along with the loss of crystalline structure increased the WAC.

The protein isolates of *desi* cultivar have been found to have better WAC as compared to *kabuli* cultivar (Kaur & Singh, 2007) which was the primary concern of it as a constituent. It had a positive impact on the WAC of the weaning mix which may be due to the reason that isolates have great ability to swell, dissociate and unfold, exposing additional binding sites which is disrupted by carbohydrate and other flour components present (Kinsella, 1979).Another factor may be that the protein gets dissociated during the production of isolates and thereby increases the water absorbability because of the increase in polar and non polar amino acids occur. The formation of matrices is stabilized by hydrophobic interactions which retains water in their micro structure (Avanza et al, 2012). Kuntz and Brassfield (1971) reported that lower WAC in some flours may be due to less availability of polar amino acids in flours and thus, high WAC warrants better bioavailability of polar amino acids. However, the WAC of proteins follow a different relation when participating in a hydrophilic component system because the selective pH, temperature dependent solubility along with surface hydrophobicity make them to aggregate and form precipitate (Klupšaitė & Juodeikienė, 2015). Thus, in this condition when some component favors the positive effect and the other does not, the cubic equation sums this model to be the best, denoted by cubic equation no.2. In scheffe's model, there are no absolute interactions which mean that apart from the interactions affecting the responses in the model, the individual component is also impacting the response separately which is evident from the nonlinear regression bending (Figure1). Thus, in this model WAC the properties of three components the rice flour, potato flour and protein isolates are affecting the WAC both individually and mutually as exemplified in the equation 2.

3.3.Effect of variables on Water Solubility Index (WSI)

Y_2 of the formulated variations of the weaning mix was significantly affected by the interaction between the varying concentration of rice flour followed by potato flour and protein isolates ($p<0.001$). F value of 18.32 denotes the model terms are significant. The effect of the variables on the WSI could be explained as per the cubic model equation No. 3 which could explain 96.44 % of the interaction observed by the variables on the WSI (Table 3).

Water Solubility Index (WSI)

$$Y_2=-29.48898A+20.53575B-63.3744C+8.009893AB+213.9651AC+181.6413BC-336.396ABC-36.7067AB\ (A-B)-163.913AC(A-C)-71.7725BC(B-C) \quad (4)$$

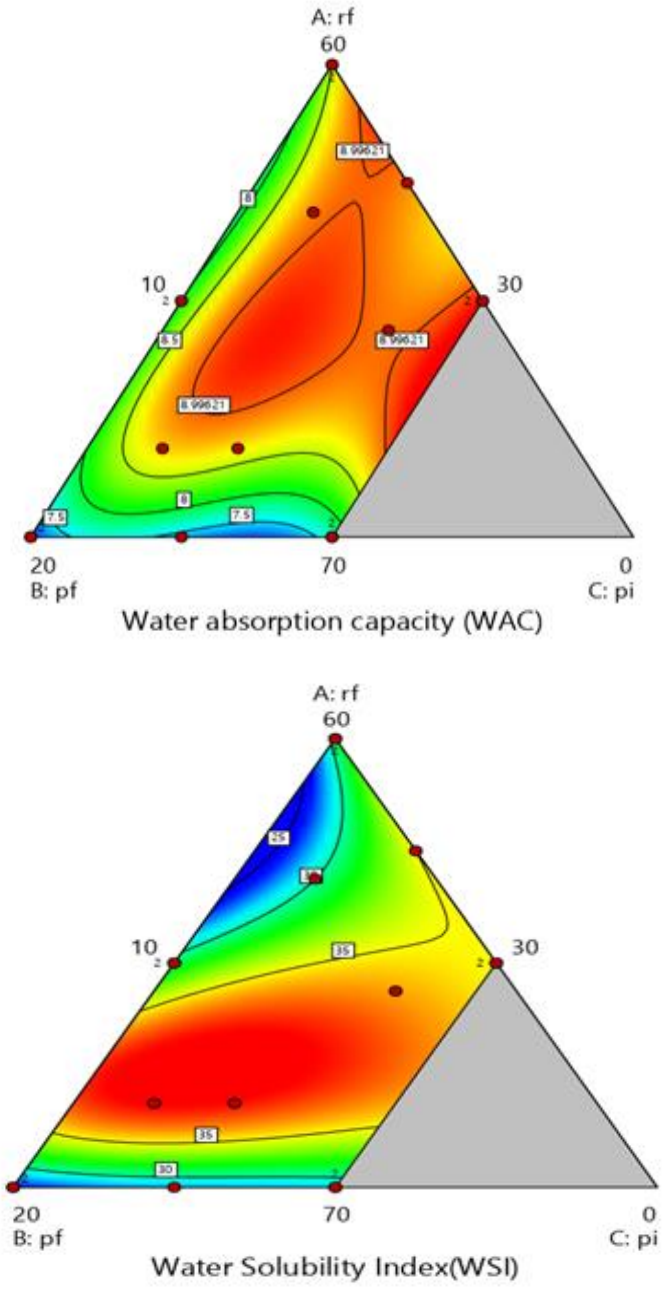

Figure 1. Contour plot illustrating the relationship between component rice flour, potato flour and protein isolates towards the response, (a) Water absorption capacity (WAC) Y_1 (b) Water Solubility Index (WSI) Y_2

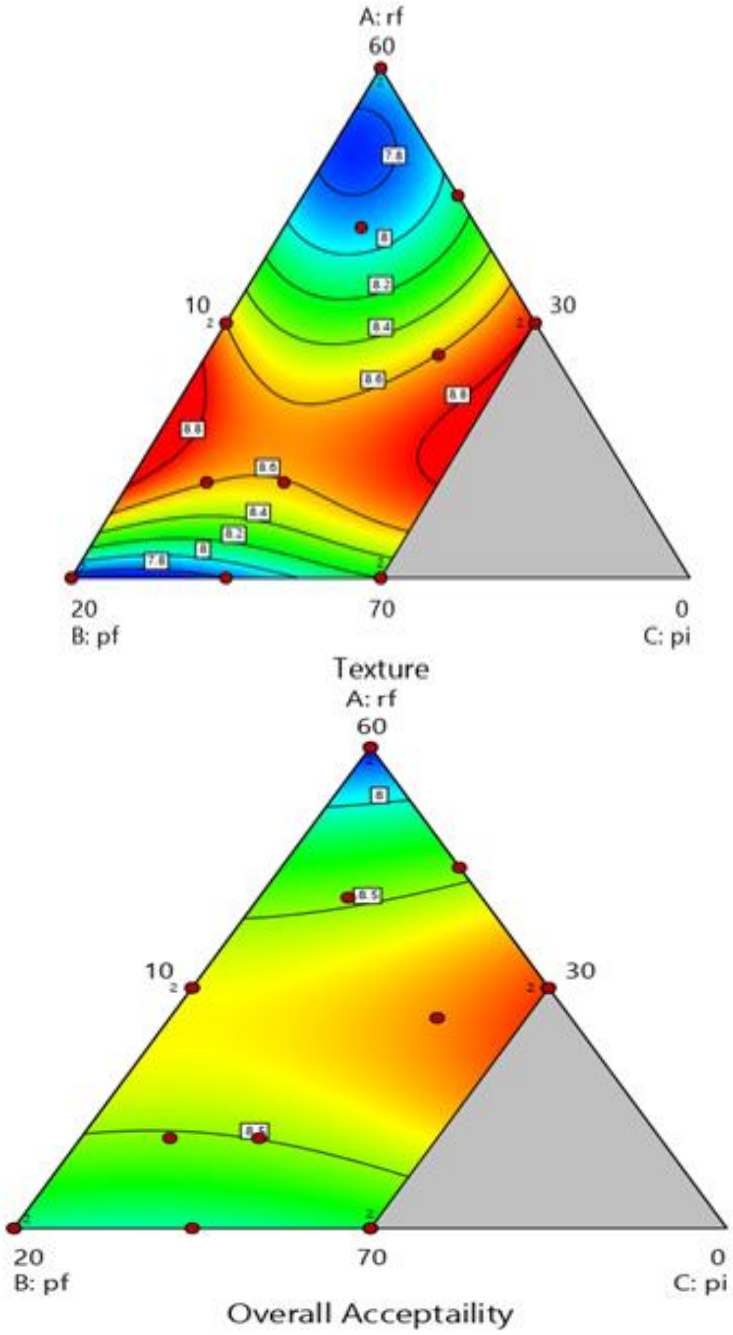

Figure 2. Contour plot illustrating the relationship between component rice flour, potato flour and protein isolates towards the response, (a) Texture Y_3 (b)Overall acceptability Y_4

The effect of A, B and C could be perceived vividly in the contour diagram showing the higher polynomial relation between the components on the response Y_2. The water solubility index increased initially with the increase in moisture content, which may be due to proper gelatinization and lateral expansion of the starch present in potato and rice flour, as potato flour has been found predominant in improving the swelling power and in the rice flour blends (Sun & Yoo, 2011). It justifies better pasting properties of potato starch as compared to sweet potato having better gelatinizing property, paste clarity at lower temperature (Nwokoche et al, 2014) making it suitable for supplementing as an infant food.

The higher WAC and WSI values of potato starch may be attributed to a higher content of phosphate groups on amylopectin, which causes repulsion between phosphate groups on adjacent chains and consequently hydration is increased by the weakening of bonds within the crystalline structure (Hoover, 2001). On the contrary, presence of chickpea protein isolate in the component system even in comparative low proportion could definitely alter the resultant effect of the interactions (Figure 1). The solubility of protein isolate is basically affected by the amount of denatured protein and fat present, as both these reduces the solubility and emulsion forming capacity and thus due to this property higher amount of PI in the weaning mix would make it reconstitution difficult.

3.4.Effect of variables on Texture Y_3:

The effect of the flour and protein isolate blend on the texture is exemplified in the equation no. 4. The F value of 58.48 ($p<0.001$) depicts that the variables had 98.18% effect on the texture of the formulations of the weaning mix (Table 3). The positive cubic equation denotes that the texture of the mix followed high polynomial relation due to the combined effects of factors A, B and C.

Texture Y_3= -4.34382 A+40.79805 B+86.7133 C-0.694434 AB-1.32265 AC-1.57511 BC+0.0659 ABC+0.00649 AB (AB)+0.007064 AC (AC)+0.004248 BC (BC). (5)

It is an important aspect of a proprietary food especially when it has to be accepted by children so as to provide a sumptuous eating experience along with delectable taste and enhanced nutritional properties. Textural properties are affected by the individual functional properties of the flour used and the interaction between the ingredients while formulating a product.

The 3D contour plot (Figure 2) shows that the texture of the complementary mix was improved as the potato flour was increased till it attained a maximum level of 28; this change could be attributed to the soft and pasting property of potato flour. Optimum WAC and WSI are shown to have a conditioning effect on the texture of the mix. Tuberin is a major protein of potatoes, a non-gluten protein, while wheat flour has gluten in which gliadin and glutenin are entangled to form a three-dimensional network. The crude ash contents of medium flour are 0.5-1.3%, while potato flour contains as high as 1.96~2.48% of ash depending on the variety. Thus, the characteristics of tuberin protein and high ash content not only makes it make it micronutrient adequate but also has a beneficial effect on the visco-elastic and pasting properties of the food mix. Rice flour has become a strikingly important ingredient due to its unique attributes such as bland taste, white color and hypoallergenicity (Bazaz et al, 2016), which makes it appropriate to be utilized as a constituent of complementary food. Rice flour has its advantages in designing food products in which textural prerequisites needs to be manipulated in order to get the optimum responses within limited constraints. Hence, the determination of optimal proportion of ingredients becomes crucial in multi-component system which has been appropriately solved by the mixture model design. The capacity of having high water absorption index and low solubility makes protein isolates possess suitable gelation and pasting properties which is a customary property of a complementary infant food.

3.5.Effect of variables on Overall Acceptability Y_4: The contour presented in Fig. 1 describes that the overall acceptability of the complementary food was able to describe 97.48% (Table 1) of effect of variation in the formulation of the complementary mix which is expressed in the quadratic mixture equation no. 4. From ANOVA, the model F 76.34 value of ($p=0.0001$) implies that there was a significant effect of the rice flour, potato flour and protein isolate on the acceptability of the product (fig 2).

Overall Acceptability

$$Y_4 = -29.7081A - 123.229B - 74.5029C + 317.501AB + 269.4074AC + 115.942BC \quad (6)$$

It is observed that individual component A, B and C did not have a strong impact on the overall acceptability of the mix, however, the interactive terms increased the acceptability of the mix a considerably. It may be due to the fact that in the mixture design the interaction between the individual physicochemical properties of the potato flour, rice flour and chickpea protein isolates compliments each other, which may not be possible in using a single food matrix Enhancement of Overall acceptability of the complementary food is due to improvement in the consistency, mouth feel and taste which has been significantly improved by the increment of potato flour and protein isolate. Potato flour has been found to increase the acceptability and textural properties by other studies also (Olatunde et al, 2020).

3.6.Optimization of Complementary food

The optimized formulation was selected with the goal of maximizing the overall acceptability (Y_4) of the complementary food mix and keeping the physicochemical properties WAC (Y_1), WSI (Y_2) and texture (Y_3) within range. Within these constraints, d optimal design selected the variation 65A:28.1B:6.9C as the most desirable one, Where A=Rice flour; B=Potato flour and C= Protein isolates

Verification of the Model: Verification of data is done by the Design Expert software, by calculating the Relative Standard Error (RSE) %.

$$RSE\ \% = \frac{\text{Actual value-Predicted value}}{\text{Predicted value}} \times 100 \quad (7)$$

The predicted and observed values of the analyzed responses (Table 2) were compared in order to check the adequacy of the surface response equation. The observed responses were analyzed against the predicted ones where no significant differences ($p<0.05$) among them showed that the results were found to be in good agreement with the predicted values. This shows that the basic composition of the complementary food mix could be manipulated using the three dimensional special model where variation in more than one factors affects the optimization process and has to find the best fit within a given limit of constraints. Food mix thus developed has optimal physicochemical properties well suited within the limits of multiple factors.

4.Conclusions

In the optimized complementary food mix formula out of the three components, Potato flour had better positive effect on the Water Absorption Capacity (WAC), Water Solubility Index (WSI) and texture; followed by the Chickpea protein isolates and Rice flour. Since, the physicochemical properties of all the three components are diverse the optimization was possible only on the basis of the combined impact between the interactive (intercept) terms. Thus, the lowest content of Rice flour (65g) and maximizing the potato flour (28.1g) with chickpea protein isolate (6.9g) yielded the best possible combination to get the optimal complementary mix. This study exploits the use of starches and chickpea protein isolates to develop a weaning food which is not only utilizes easily available food items with a year-round availability and highly economical but also has optimal physicochemical properties, energy dense and hypoallergenic containing highly bioavailable protein source which could be utilized for mass supplementation programs.

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STUDY ON DETERMINING THE FREEZING MODE OF FROZEN FILLET BIGEYE TUNA (*THUNNUS OBESUS*)

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ABSTRACT

Bigeye tuna (*Thunnus obesus*), a kind of delicious seafood, can be processed to be several valuable products. To maintain the product quality, harvested tunas had been strictly persevered, transported and frozen at low temperature. This study was carried out to determine the technological mode of the freezing process of the fillet tuna to find the optimum temperature and freezing time to reduce mass loss and keep its quality. The combining two-level orthogonal arrays was used to build the relationship between objective functions and income variables. The results found the optimized freezing mode of the fillet tuna: the freezing environment temperature was -42.5°C and the freezing time was 2.12h. Carrying out the experiment with optimized freezing mode, showed that the temperature at the end of the fillet tuna freezing process was reached at –22.5°C and the yield of weight loss was ~~is~~ 3.1%. That meaned all internal water of the product was completely crystallized and the loss of quality was negligible. The freezing mode can be applied in industrial scale for the frozen fillet tuna manufacturing process.

1. Introduction

Bigeye tuna (Thunnus obesus), commonly known as Bò Gù (Vietnamese local name), is a large sea fish belonging to the Scombridae (mackerel) family (Graham J. B. and Dickson K. A., 2004), living in the warm sea, 185 km far from the coastline. In Vietnam, the tuna lives mainly in Binh Dinh, Phu Yen and Khanh Hoa provinces (Lewis A. D.., 2005). The tuna, a kind of delicious seafood, having big and nutritious eyes, can be processed into various delicious dishes and made to be several valuable products for domestic consumption or for exporting to oversea markets. The harvested tunas had been strictly persevered and transported to factories (Lewis A. D.., 2005), in which the tunas were filleted to be plate pieces in size of 300×150×20 mm (length×width×thickness), the tuna chemical ingredients were shown in Table 1 and Table 2.

Table 1. Chemical ingredients and energy in 100g fillet bigeye tuna

Water (wet) (g)	*Protein (g)*	*Lipid (g)*	*Ash~~es~~ (g)*	*Energy (kcal)*
72.6	23.4	1.8	2.2	105

Table 1 and Table 2 showed that the bigeye tunas are rich of nutrients, and are the suitable environment for the growing and developing of bacteria. Thus, during post-harvesting, if it is not having suitable methods for preservation, the quality of the tuna will be rapidly reduced by the effect of internal enzymes, domestic or contaminated bacteria. These effects lead to the debasement of using and economical values of

tunas. To preserve fillet bigeye tunas for domestic consumption and exporting purpose, the freezing method is commonly used to preserve the tunas in seafood processing factories, because only this method can lower the rate of quality decreasing of the products (Dzung N.T, Dzung T.V and Ba T.D, 2012; Dzung N.T., 2015).

Table 2. Chemical ingredients in 100g fillet bigeyes tuna

Mineral				
Calcium (mg)	*Phosphor (mg)*	*Ferrous (mg)*	*Sodium (mg)*	*Potassium (mg)*
68	482	2.4	7.4	6.8
Vitamin				
A (μg)	*B1 (mg)*	*B2 (mg)*	*PP (mg)*	*C (mg)*
162	0.32	0.53	20.4	0.12

However, when using the freezing method for tuna preservation, some of the following problems should be noted:

Firstly, the products at the end of the freezing process have to reach an optimum temperature (Dzung N.T, Dzung T.V and Ba T.D, 2012; Dzung N.T., 2015). At that time, all liquid water inside the products has been completely crystallized then the shelf-life has been extended. At this temperature, the operating freezing systems can be stopped to save energy and product costs.

Secondly, if the freezing rate is low, the size of crystalized water inside the tissue of products will be large lead to destroying the texture of cells, when the frozen products are defrosted, the fluid in cells will be leaked, and it causes the large mass loss of products. Then, their quality will be decreased because nutrition in the cell fluid will be loosed. If the freezing rate is quick or super quick, the water inside the products is crystallized at its position, and the size of the crystallized water is small in micro or nano size, it does not destroy the texture of cells. When the products are defrosted, their quality are kept as that of initial. But quick frozen or super quick frozen have to be carried out in the deep low temperature, lower than –40°C, since the freezers will spend a large amount of energy. These are the unexpected problems occurring in manufacturing (Dzung N.T, Dzung T.V and Ba T.D, 2012; Dzung N.T., 2015).

Thus, the problem that needs to be solved is how to build the optimum freezing temperature and how to minimize The freezing time making products – the frozen fillet bigeye tunas in size of 300 mm × 150 mm × 20 mm – reach the optimum temperature, making all water inside the products is crystallized, and making the mass loss of post-defrosted frozen products is lowest.

According to some authors (Cleland A. C. and Earle R. L.., 1976; Holman J.., 2009; Dzung N.T., 2015), crystallizing temperature of fillet tuna internal water is –1.24°C, and the average temperature of crystallized water inside the final frozen fillet tunas are –22.5°C – the Eutectic point of internal water of products. There are the fundamentals to build the optimized mathematical problem to find the freezing technology of the frozen fillet bigeye tuna.

2. Materials and methods

2.1. Materials

Whole harvested bigeye tunas caught from Binh Dinh to Khanh Hoa, were stored at –45°C to –40°C, then they were transported to factories. At the factories, they were filleted and cut to be plat pieces in size of 300mm length, 150mm width, and 20mm thick (see 01), each piece was put into a PE bag to avoid loss of water and cold burning on the surface, put into trays and frozen (Charm S. E. and Slavin J.., 1962; Heldman D. R.., 1982; Dzung N.T., 2014).

Figure 1. Frozen fillet bigeye tuna pieces

2.2. Apparatus

Scale Sartorius, Basic Type BA310S ((Sartorius, Germany): accuracy class: F1 (Guided by International Organization Of Legal Metrology), Dual Digital Thermometer – range (−50 ÷ 70)^{o}C, readability ± 0.05^{o}C – (Omron, Japan), the freezer DL-3 – build by the Faculty of Chemical and Food Technology, Ho Chi Minh city University of Technology and Education, Vietnam (02) – controllable freezing temperature: lowest –50^{o}C, the cooling system controlled by computer (Dzung N.T., 2015).

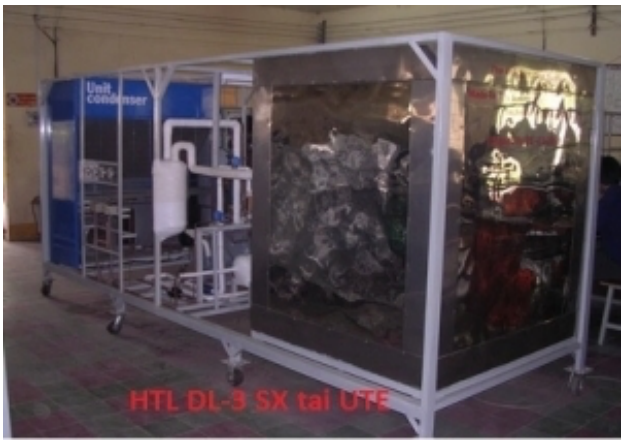


Figure 2. Freezing system DL-3

2.3. Methods

In this study, some methods had been used:

2.3.1. Temperature measuring

The temperature of the freezing environment was measured by temperature sensors, and calculated automatically by a computer (Charm S. E. and Slavin J.., 1962; Dzung N.T., 2014). The temperature of the surface and center of the frozen fillet tuna fish were measured by the Dual Digital Thermometer. To measure the center temperature of the fillet tuna products at the end of freezing, the lenght of the PT100 sensor was placed paralelled to the length of the frozen piece so that the sensor was inside of the center position of the piece

Average final temperature of the frozen fillet tuna pieces was determined by the following formula (Dzung N.T, Dzung T.V and Ba T.D, 2012; Dzung N.T., 2015):

$$Y_1 = T_a = \frac{T_s + T_c}{2} \quad (1)$$

Where: T_a, T_s, T_c (^{o}C) were average, surface and center temperatures of the fillet tuna products at the end of freezing, respectively.

2.3.2. Defrosting of frozen fillet tuna

Frozen fillet tuna pieces were defrosted by 3.5 MHz ultrasonic at 28^{o}C cooling chamber for 45 minutes. The ultrasonic was regularly turned on and off every minute.

2.3.3. Mass loss determining

Mass of the pre-frozen fillet tuna products, and that of post-defrosted frozen fillet tunas were determined by the Scale (Sartorius), and the yield of mass loss of post-defrosted frozen fillet tuna products was calculated by formula (Dzung N.T, Dzung T.V and Ba T.D, 2012; Dzung N.T., 2015):

$$Y_2 = \frac{m_1 - m_2}{m_1}.100\% = \frac{\Delta m}{m_1}.100\% \quad (2)$$

Where: m_1, m_2 (g) were mass of the pre-frozen and post-defrosted frozen fillet tuna products, respectively.

Y_2 (%) Yield of mass loss of post-defrosted frozen fillet tuna products.

2.3.4. Building the freezing mode

Using the combining two-level orthogonal arrays to build the relationship between objective functions (Y_1, Y_2) including average final temperature of frozen fillet tuna products (Y_1, ^{o}C), and the yield of mass loss of post-defrosted frozen fillet tuna products (Y_2, %); and technological factors (X_1, X_2) directly affecting to the process such as: temperature of freezing environment (X_1, ^{o}C), time of freezing process (X_2, h), and the thickness of the fillet tuna plat (X_3, mm). Because the thickness of the bigeye tuna is standardized as X_3 = 20mm, X_3 had been omitted to the variable sheet. Thus, the relationship between objective functions (Y_1, Y_2) and technological factors (X_1, X_2) is described by the following formula (Dzung N.T, et al., 2012):

$$Y = b_0 + \sum_{j=1}^{k} b_j x_j + \sum_{j\neq i;j=1}^{k} b_{ji} x_j x_i + \sum_{j=1}^{k} b_{jj}\left(x_j^2 - \lambda\right) \quad (3)$$

Where x_1, x_2 are variables coded from the real variables X_1, X_2 as following formula:

$$x_i = \frac{X_i - X_i^0}{\Delta X_i};$$

$$X_i = x_i.\Delta X_i + X_i^0 \quad (4)$$

Where:

$X_i^0 = (X_i^{max} + X_i^{min})/2;$

$\Delta X_i = (X_i^{max} - X_i^{min})/2;$

$X_i^{min} \le X_i \le X_i^{max}; i = 1 \div 2$

Number of experiment was identified by (Dzung N.T., 2012):

$$N = n_k + n_* + n_0 = 2^k + 2k + n_0 = 9 \quad (5)$$

Where:

$k = 2;$

$n_k = 2^k = 2^2 = 4;$

$n_* = 2k = 2\times2 = 4; n_0 = 1$

Swing arm of orthogonal matrix was identified (Dzung N.T., 2012):

$$\alpha = \sqrt{\sqrt{N.2^{(k-2)}} - 2^{(k-1)}} = \sqrt{\sqrt{9.2^{(2-2)}} - 2^{(2-1)}} = 1 \quad (6)$$

Conditions of empirical orthogonal matrix (Dzung N.T., 2012):

$$\lambda = \frac{1}{N}\left(2^k + 2\alpha^2\right) = \frac{1}{9}\left(2^2 + 2.1^2\right) = 2/3 \quad (7)$$

2.3.5. Other measurements

In addition, mathematical and information technical tools had been used to solve the optimized problems describing the freezing process, and to identify the technical models of the freezing process of fillet tuna pieces.

3. Results and discussions

3.1. Building the mathematical models of the freezing process of fillet tuna

The average final temperature of fillet tuna products at the end of the freezing process (Y_1, ^oC), and the yield of mass loss of post-defrosted frozen fillet tuna products are dependent on the freezing process, and directly affected by technological factors such as: the temperature of freezing environment (X_1, ^oC), time of the freezing process (X_2, h).

Technological factors X_1 and X_2 were individually surveyed to find the extreme domain of Y_1, Y_2. The vital surveying domain was found and shown in Table 3, (Dzung N.T., 2012).

The experiments, with levels of technological factors X_1, X_2 in Table 3, were carried out with the combining two-level orthogonal arrays shown in Table 4 to build the mathematical model $Y_1 = f_1(X_1, X_2) = f_1(x_1, x_2)$ và $Y_2 = f_2(X_1, X_2) = f_2(x_1, x_2)$ describing the freezing process. Results identified Y_1, Y_2 depended on x_1, x_2 shown in the column "Objective function values" of Table 4, (Dzung N.T., 2012).

MS Excel 2019 (Microsoft, US) was used to calculate the experimental data in Table 4, to calculate coefficients, b_j and b_{ji}. The significant coefficients of regression equations (3) were tested by Student's test, and the compatibility of the regression equation was tested by Fisher test (Fisher R.., 1929; Dzung N.T., 2012). Results built mathematical modeling of experiments describing the freezing process of the fillet tuna pieces.

Table 3. Levels of technological factors affect to freezing process of fillet tuna pieces

Factors		X_1 (^oC)	X_2 (h)
Levels of experiment	$-\alpha$ *(-1)*	-45	0.4
	Lower level, (-1)	-45	0.4
	Medium level, (0)	-40	2.2
	Upper level, (+1)	-35	4.0
	$+\alpha$ *(+1)*	-35	4.0
Variance range ΔZ_i		5	1.8

Table 4. The combining two-level orthogonal arrays and the experiment results finding objective function (Y_1, Y_2) of the freezing process of fillet tuna pieces

Number of experiments		Real variable		Coded variable		Objective function values	
		X_1, ^oC	X_2, h	x_1	x_2	Y_1	Y_2
2^k	1	–35	4.5	+1	+1	–27.77	5.15
	2	–45	4.5	–1	+1	–36.38	4.74
	3	–35	0.5	+1	–1	–2.19	1.52
	4	–45	0.5	–1	–1	–2.83	0.99
2k	5	–35	2.5	+1	0	–20.45	3.61
	6	–45	2.5	–1	0	–24.34	3.15
	7	–40	4.5	0	+1	–31.24	4.65
	8	–40	0.5	0	–1	–2.77	1.03
n_0	9	–40	2.5	0	0	–21.93	3.19

After testing the compatibility by Fisher test (Dzung N.T., 2012), the results confirmed that the mathematical models (8), (9) are totally compatible with the experimental data shown in Table 4. Thus, they can describe the freezing process of the fillet tuna.

3.1.1. Temperature of fillet tuna products at the end of freezing process

$$Y_1 = f_1(x_1, x_2) = -18.88 + 2.2x_1 - 14.59x_2 + 2.01x_1x_2 - 0.34\left(x_1^2 - \frac{2}{3}\right) + 5.04\left(x_2^2 - \frac{2}{3}\right) \quad (8)$$

3.1.2. Yield of mass loss of post-defrosted frozen fillet tuna products

$$Y_2 = f_2(x_1, x_2) = 3.11 + 0.231x_1 - 1.829x_2 - 0.03x_1x_2 + 0.24\left(x_1^2 - \frac{2}{3}\right) - 0.31\left(x_2^2 - \frac{2}{3}\right) \quad (9)$$

Simulating the mathematical models (8) and (9), the results received responsive or curved surfaces $Y_1 = f_1(x_1, x_2)$ and $Y_2 = f_2(x_1, x_2)$ shown in 03 and 04.

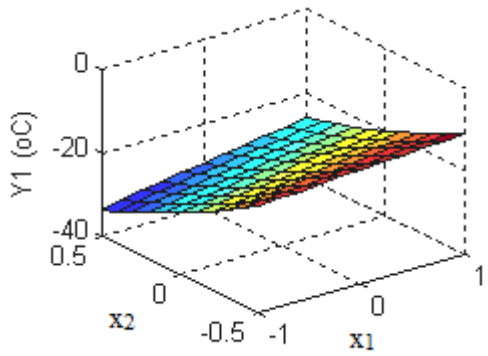


Figure 3. Relationship between frozen fillet tuna pieces and technological factors: temperature of freezing environment, and freezing time

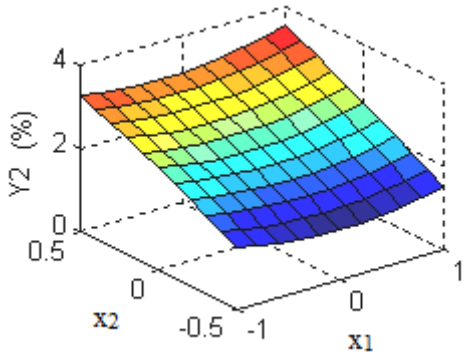


Figure 4. Relationship between yield of mass loss of post-defrosted of the frozen fillet tuna products and technological factors: temperature of freezing environment, and freezing time

The results showed that the relationship between objective functions (Y_1 (°C) and Y_2 (%)) and the technological factors (x_1 and x_2) directly affecting the freezing process, is the quadratic nonlinear. It is more complicated than that of first-order linear. Normally, the quadratic nonlinear function consists of two value ranges on two branches, one branch is covariant and the other is inverse depending on a quadratic coefficient. If the coefficient is negative, the parabola curve is convex. In contrast, the parabola curve is concave. The boundary point between these two branches is the extreme pitch of the parabola.

The formula (8) showed the coefficient 5.04 linking to x_2^2, thus it is misunderstood that if freezing time (x_2) is prolonging - time increase, the temperature of fillet tuna products at the end of freezing process will be increasing; It is the wrong situation. In fact, the freezing process, assessed in the negative temperatures (x_1), is belonging to the inverse brand. It means that when the freezing time is increasing, the product temperature during the freezing process will be decreasing, see 03. Similarly, the model (9) showed the quadratic coefficient –0.31 linking to x_2^2, thus the freezing process is assessed in the covariant brand of parabola, which means that when the time is increasing, the Yield of mass loss of post-defrosted frozen fillet tuna products will be increasing, see 04 (Dzung N.T., 2012).

3.2. Building and solving the optimized problems describing freezing process of fillet bigeye tuna

3.2.1. Building the optimized problems describing freezing process of fillet bigeye tuna

Based on technical conditions answering the requirements of economy and engineering, the freezing process of the fillet tuna (in size of 300mm×150mm×20mm) had been carried out as quick as possible, and not only whether all internal water of the tuna is completely crystallized or temperature of the product reaches the optimum temperature T_{Fopt} = –22.5°C but also the quality of post-frozen products have to be paid attention (Banin A. and Duwayne A. M.., 1974; Dzung N.T, et al., 2012). To solve this problem, the optimized problem had been established as follow: Let's determine the optimized problem $x^{opt} = (x_1^{opt}, x_2^{opt}) \in \Omega_x = \{-1 \le x_1, x_2 \le 1\}$ to:

$$\begin{cases} Y_1 = f_1(x_1^{opt}, x_2^{opt}) = T_{Fopt} = -22.5 \\ Y_{2\min} = f_2(x_1^{opt}, x_2^{opt}) = \text{Min}\{f_2(x_1, x_2)\} \\ \forall x = (x_1, x_2) \in \Omega_x = \{-1 \le x_1, x_2 \le 1\} \end{cases} \quad (10)$$

3.2.2. Solving the optimized problems describing freezing process of fillet bigeye tuna

Solving the optimized problem (10) by Lagrange method as follow:

$$L(x_1, x_2, h) = f_2(x_1, x_2) + h.(f_1(x_1, x_2) + 22.5) \quad (11)$$

Find extremes by Lagrange method (11) as follow:

$$\begin{cases} \dfrac{\partial L(x_1, x_2, h)}{\partial x_1} = 0 \\ \dfrac{\partial L(x_1, x_2, h)}{\partial x_2} = 0 \\ \dfrac{\partial L(x_1, x_2, h)}{\partial h} = 0 \\ -1 \le x_1, x_2 \le 1 \end{cases} \quad (12)$$

Equation (12) can be written as follow

$$\begin{cases} 0.231 - 0.03x_2 + 0.048x_1 \\ \qquad + h \times (2.2 + 2.01x_2 - 0.68x_1) = 0 \\ -1.829 - 0.03x_1 - 0.62x_2 \\ \qquad + h \times (-14.59 + 2.01x_1 + 10.08x_2) = 0 \\ -18.88 + 2.2x_1 - 14.59x_2 + 2.01x_1x_2 - \\ \qquad 0.34 \times \left(x_1^2 - \dfrac{2}{3}\right) + 5.04 \times \left(x_2^2 - \dfrac{2}{3}\right) = 0 \\ -1 \le x_1, x_2 \le 1 \end{cases} \quad (13)$$

With meshing algorithm under the supporting of Visual Basic 6.0 to solve equations (13), the results found that roots of the equations (10) together with Lagrange multiplier coefficient, h = 0.109, are:

$$x_1^{opt} = -0.502; \quad x_2^{opt} = -0.044; \quad (14)$$

Converting to real variable by equation (4) received:

$$X_1 = -42.5^oC; \quad X_2 = 2.12h \quad (15)$$

Replacing the optimal roots x_1^{opt} and x_2^{opt} into equations (8) and (9), found:

$$Y_1 = -22.5^oC; \qquad Y_2^{min} = 3.1\% \quad (16)$$

3.2.3. Verification of the found optimized problems described the freezing process of fillet tuna

Carrying out the Freezing of the fillet tuna (in size of 300mm×150mm×20mm) on the freezing system DL-3 at the optimized freezing modes: the environment temperature is –42.5°C, and kept constantly during freezing process, and the freezing time is 2.12h. Temperature of products and yield of mass loss were measured as described in ***Error! Reference source not found.***. At the end of the freezing process, the results are followed: the average temperature of the center products is –22.57°C, Yield of mass loss of post-defrosted frozen fillet tuna products is 3.19%.

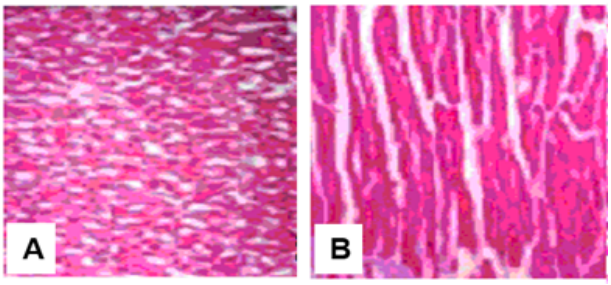


Figure 5. The SEM image of ice crystal structure in frozen fillet tuna in size of 300mm×150mm×20mm. A. The freezing mode: temperature of freezing environment, -42.5°C, and freezing time, 2.12h; B. The freezing mode: temperature of freezing environment, -38.55°C, and freezing time, 3.95h. The images A and B were scanned and captured at the same magnification (1000×)

It can be seen that the optimized freezing process model of fillet tuna is completely consistent with reality. This technical condition, thus, can be applied to real manufacturing to preserve the fillet tuna pieces to serve for domestic consumption and export. Besides, in this study, the fillet tuna had been frozen in another mode that differs from the optimized mode: the temperature of freezing environment –38.5°C to make the temperature of the products at the end of the freezing process reach –22.5°C then all internal water of products is crystallized, and the freezing time is 3.95h. At that time, yield of mass loss of post-defrosted frozen fillet tuna products is 6.79%.

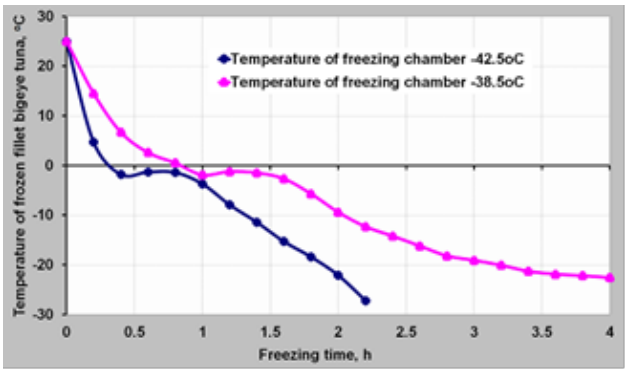
What is the reason why when the freezing environment temperature is –38.5°C, and the freezing time is 3.95h, the yield of mass loss of post-defrosted frozen fillet tuna products increases to 6.79%. The reason is that in the low rate freezing process, ice crystals in the products are formed in a large size (see Figure 5B), then they do destroy the texture of cell membranes and tore the tissue membranes (Banin A. and Duwayne A. M.., 1974; Haugvalstad G. H., Skipnes D. and Sivertsvik M.., 2005). When the products are defrosted to consume, the melting process of ices inside the products will attract cell fluid, lead to mass and nutrition loss will increase. When freezing at the optimized mode, the mass loss is very low and considered negligible. The reason is that ices inside the products are formed with a small size (see Figure 5A), they cause tiny injury to cell membranes. Then, when the products are defrosted, nutrients are maintained as initial, and the frozen products are high quality.

Table 5. Experimental data of the process of freezing of fillet tuna pieces: $T_1 = f_1(t)$ when temperature of freezing chamber -42.5°C; $T_2 = f_2(t)$ when temperature of freezing chamber -38.5°C

Time of freezing process, t (h)	Temperature of fillet tuna pieces $T_1 = f_1(t)$ when temperature of freezing chamber -42.5°C	Temperature of fillet tuna pieces $T_2 = f_2(t)$ when temperature of freezing chamber -38.5°C
0.0	25.0	25.0
0.2	4.7	14.5
0.4	-1.8	6.7
0.6	-1.3	2.6
0.8	-1.4	0.5
1.0	-3.7	-1.9
1.2	-7.9	-1.24
1.4	-11.4	-1.48
1.6	-15.3	-2.68
1.8	-18.4	-5.7
2.0	-22.1	-9.4
2.2	-27.2	-12.3

2.4		-14.2
2.6		-16.2
2.8		-18.2
3.0		-19.1
3.2		-20.1
3.4		-21.3
3.6		-21.9
3.8		-22.2
4.0		-22.6

Table 5 and Figure 6. showed that the crystallizing temperature of water in the fillet tuna products is –1.24°C, not 0°C, the reason is that product internal water is not pure water, it is solution form.



06. Dynamics graph of the freezing process fillet tuna pieces built by experiment

The crystallizing temperature of the pure solvent is not been changing during the freezing process, but that of solution is being decreased continuously during the process (Banin A. and Duwayne A. M.., 1974; Haugvalstad G. H., Skipnes D.and Sivertsvik M.., 2005) to reach the Eutectic point where all amount of product internal water is crystallized. When the temperature of the product reduces to –1.24°C, some amount of water is crystallized, and the crystallized water will separate from the solution. It increases the solution concentration, and leads to a decrease in the crystallized temperature. Thus, when the temperature of products decreases to –22.5°C, all internal water in the products is crystallized (This point is called optimal freezing temperature or the Eutectic point). However, crystallized water is only free and physio-chemically bonded water, chemically bonded water is nearly not crystallized.

4. Conclusions

This research found the optimized freezing mode of the fillet bigeye tuna (in size of 300mm length, 150mm width, and 20mm thick): environment temperature was –42.5°C and the freezing time was 2.12h. When this mode was applied for freezing, the temperature of fillet tuna at the end of the freezing process reached –22.5°C and the yield of weight loss was 3.1%. It meaned that all internal water of the product was crystallized and the quality loss is negligible. This technology regime, thus, can be can be applied in industrial scale for the frozen fillet tuna manufacturing process and served for domestic trade and exporting purposes.

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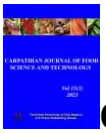

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DEVELOPMENT AND EVALUATION OF FREE SUGAR JELLY MADE WITH LEAFY VEGETABLES AS A FUNCTIONAL FOOD

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ABSTRACT

Commercial jellies are infamous for their excessive sugar content and for offering no nutritional or functional benefits. Hence, this research is intended to produce sugar-free, healthy jellies utilizing leafy vegetables, including leek, Swiss chard, and celery juice. The findings demonstrated that these products were softer, less gummy, easier to chew, and had nearly the same range of springiness and cohesiveness compared with commercial ones. Also, they have an appealing green hue. Besides, these products are rich sources for manganese, magnesium, iron, zinc, potassium, and calcium, providing an average of 110, 72, 52, 30, 25, and 22.9% of the daily values (DV), respectively. They are also considered a moderate source of vitamins A, C, and B6, giving an average of 16.7, 10.36, and 8.8% DV. Moreover, they could cover up to 55% of the daily value required from dietary fibers. Conversely, they have a lower percentage of carbohydrates, energy density, and glycemic index than the commercial equivalent. Additionally, they showed a high amount of flavonoids (100–348 mg QE/100 g), total phenolic components (318–824 mg GAE/100 g), and significant antioxidant activity (112.91–255.43 mg TE/100 g). Therefore, these novel varieties of jellies provide a nutritious and multifunctional source to satisfy consumers' desire for value-added products.

1. Introduction

Malnutrition and weight growth are significant indicators of health issues in low- and middle-income countries, as highlighted by the World Health Organization (WHO, 2020). The influence of television commercials promoting unhealthy diets has made children more prone to making poor dietary choices. This, in turn, has led to an increase in obesity among children, which is associated with abnormal lipid profiles, poor glucose metabolism, and a higher risk of diabetes (Ibrahim et al., 2020). It is estimated that 300 million people will develop diabetes mellitus by 2025 (Animaw and Seyoum, 2017).

Jams and jellies are products containing sugar, sucrose syrup, or glucose with gelling agents, acids, aromas, and food colorants (Cano-Lamadrid et al., 2020). A high sugar content is a major health concern. Researchers are working on finding natural sugar substitutes that deliver sweetness without harmful effects. Stevia extract, a natural low calorie alternative from the *Stevia rebaudiana* plant, has been studied for its potential to help individuals with diabetes and obesity by positively regulating blood glucose levels (Gao et al., 2016). Using stevia as a substitute for sucrose in food products not only reduces the calorie content but also provides sweetness without the negative health effects of sugar, making it a suitable option for individuals looking to reduce their sugar intake. Moreover, the stable nature of stevia allows for a longer shelf life of the final product (Schiatti-Sisó et al., 2023).

Recent attempts have been made to make reduced or free sugar jelly, but in most cases, the texture of jelly made with gelatin or pectin was negatively affected (Riedel et al., 2015; Ben Rejeb et al., 2020). Agar agar, extracted from red algae, is a high-performance gelling agent. Its ability to produce clear, colorless, odorless, and natural gels without the support of other colloids has been exploited by the food industry as a stabilizer and gelling agent, as well as in the manufacturing of confectionery (Stephen and Phillips, 2006).

The growing public interest in healthy foods has led to the promotion of leafy vegetables, which offer essential nutrients like minerals, vitamins, dietary fibers, and phytochemicals. These vegetables can restore balance in diets, address malnutrition and chronic issues, and contribute to overall health (Ashok et al., 2020).

Among known leafy vegetables is the Chenopodiaceae plant family member Swiss chard (*Beta vulgaris* subsp. Cycla). Which is considered a rich source of vitamins like A and C as well as minerals like phosphorus (Gamba et al., 2020). Additionally, it is used as an anti-inflammatory, anti-cancer, hypoglycemic, and hematopoietic system stimulant (Geziginci-Oktayoglu et al., 2014; Zein et al., 2015).

Another valuable plant is celery (*Apium graveolens*), a biennial plant commonly referred to as karafs. It belongs to the Apiaceae family. Celery possesses antifungal, antihypertensive, hypolipidemic, hepatoprotective, and anticancer effects (Fazal and Singla, 2012). On the other hand, the leek (Allium porrum L.) displays a pronounced antioxidant and free radical scavenging activity owing to the presence of phenolic acids and flavonoids, especially rosmarinic acid and quercetin. The ethanolic extracts of *Allium porrum* L. showed favorable antimicrobial and anticancer activity when applied in vitro (Radovanovi et al., 2015).

Leafy vegetables are often sold in fresh form, and their products are rarely found in the market. This scarcity may be due to various reasons, such as their perishable nature, limited availability, and shelf life. Hence, this research investigates the possibility of utilizing leafy vegetables to create a sugar-free jelly product that will not only address the scarcity of such products in the market but also cater to the increasing demand for a healthier and more sustainable alternative to traditional jellies. The use of Agar-agar as a gelling agent and stevia for sweetness would make this jelly suitable for those with dietary restrictions or concerns about sugar intake. By studying the sensory, physicochemical, nutritional, and functional properties of these products, valuable insights can be gained to ensure their quality and appeal to consumers.

2. Materials and methods

2.1. Materials

The ingredients utilized were agar-agar powder (agar-agar food-grade powder, Foodchem, China) and Stevia powder (Mozn, Germany). Celery (*Apium graveolens*), leeks (*Allium porrum L.*), Swiss chard (*Beta vulgaris subsp. Cycla*) and peppermint (*Mentha piperita L.)* were purchased from the Egyptian market. All used vegetables were authenticated through the "Vegetable Crop Department, Faculty of Agriculture, Cairo University, Egypt".

2.2. Preliminary formulation

Preliminary palatability tests were carried out to assess the potential of conjugation to improve the ratio of agar and the optimal flavor ratio. This combination comprises stevia (30, 60, and 90 mg/100 mL), lemon juice (1, 3, 5 mL/100 mL), and mint water extract (1, 2, 3 mL/100 mL) (results not disclosed). To accomplish the sensory attribute, the study used an acceptable blend of these additives combined with a variety of vegetable juices (10, 20, and 30%).

2.3. Development of vegetable jellies

For the production of vegetable juice, 500 g of each variety of vegetable plant (leaves and stem) was washed, sliced, and blended for 10 minutes in a typical home blender with 200 mL of distilled water. A muslin cloth filter was used to remove the final juice. At the same time, mint

leaves were extracted at 10% in boiling water for 20 minutes and filtered.

Agar with a percent of 2% was prepared to be double concentrated as follows: the needed weight of the agar is dissolved in half of the required distilled water on a magnetic stirrer (60 ºC) till completely dissolved. The vials of jelly were prepared to contain the same volumes (50 mL) of agar solutions. Then, different concentrations of vegetable juice (0 as a control, 10, 20, and 30% mL/mL) with a 0.06 % stevia addition were achieved. At this point, mint extract was added at 2% mL/mL.

The volume of each type was diluted to 97 mL with water. After being pasteurized at 100°C for 1 minute, the jelly was quickly cooled to 40°C. 3% v/v of freshly prepared lemon juice was added at this point. Finally, the silicone molds were filled with jelly.

Table 1. Formulation of vegetable jelly

Percent of formulation (%)	Control	T1	T2	T3
Agar powder	2	2	2	2
Vegetable leaves juice	0	10	20	30
Stevia powder	0.06	0.06	0.06	0.06
Lemon juice	3	3	3	3
Mint extract	2	2	2	2
Water	92.94	82.94	72.94	62.94

After being completely solidified and refrigerated at 4°C for 30 minutes, the jelly beans were inverted and packaged in a plastic container. These jelly beans were examined for sensory and physical properties (Table 1, Figure 1). Other vials were created in jars that had been sanitized with hot water, cooled, and then had metal covers placed over them. For shelf life testing, these vials were kept in the refrigerator at 4 °C.

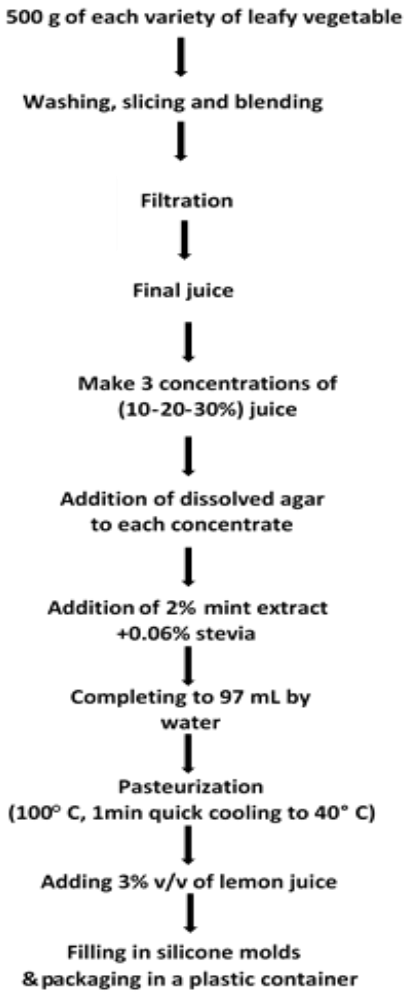

Figure 1. A Flowchart for the production of vegetable Jelly

2.4. Organoleptic evaluation of vegetable jelly

An organoleptic evaluation of nine varieties of vegetable jelly along with a control (free of vegetable juice) and a commercial jelly item was conducted. Each of the three treatment types on the panel had three different concentrations of vegetable juice. A panel of semi-trained judges performed the evaluation (including members of the Food Research Technology Institute, Egypt; 30 females and 20 males, n = 50; age: 25–60 years). The nine-point hedonic scale was used for character traits (Lawless and Heymann, 2010). All panel members were informed of the product constituents, and none of them reported any food allergies. And the sensory assessment

was carried out following Regulation No. 1924/2006.

2.5. Physicochemical analysis

2.5.1. Color analysis

A homogenous sample was obtained by blending each jelly sample. Each sample received five measurements from the chromameter (CR-400, Konica Minolta) (Pathare et al., 2012). In the color system, the L-value denotes the degree of lightness, with 0 denoting complete darkness and 100 denoting complete lightness. The a and b values, meanwhile, measured the intensity of green versus red and yellow versus blue in the range of 0 to 100 and -100 to 0, respectively. Additionally, samples were examined using their chroma values ($C^* = (a^{*2} + b^{*2})^{1/2}$), which represent the intensity or color saturation, and hue angle, h (0-360°), calculated using $\tan^{-1} b^*/a^*$, and colour difference represent by delta E (ΔE) estimated using the formula:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2} \quad (1)$$

2.5.2 Analyses of Texture Profiles (TPA).

A TPA analysis of the jelly's consistency was performed after 24 hours of its production. The test was done at room temperature using a half-inch diameter acrylic cylinder probe and a TA.XT2 texture analyzer (Stable Micro System Ltd., Scarsdale, NY). Parameters were established as follows: a data acquisition rate of 200 pp, a trigger force of 5.0 g, a distance of 4.0 mm, a test speed of 1.0 mm/s, and a post-test speed of 1.0 mm/s. Using Texture Expert Version 1.22 Software (Stable Micro System Ltd., Scarsdale, NY), the data were examined to determine the jelly's firmness, cohesion, and springiness following the method of Bourne (1978).

The following formulas were used to determine other parameters:

A product's springiness refers to how much it physically recovers after being distorted during the initial pressure and may wait between the two strokes.

Springiness = Distance 2 traveled by the probe during the second compression cycle / Distance 2 traveled by the probe through the first compression cycle (Distance 1).

Gumminess is equal to the sum of hardness and cohesiveness. Whereas chewiness is the result of the sum of gumminess and springiness. Resilience is equal to the product of the upstroke energy (Area 4) and the downstroke energy of the initial compression (Area 3). For each item, this test was carried out in triplicate.

2.5.3. Soluble Solids Content (TSS)

The concentration of TSS in the jelly was determined using a tabletop Reichert-Jung ABBE MARK II refractometer (Cambridge Instruments, Buffalo, NY). About 1-2 droplets of chilled jelly were put into the instrument prism (Pilgrim et al. 1991). The shaded area was changed to represent the proportion of soluble solid material before the digital reading was obtained.

2.5.4. Water activity (WA)

WA readings were taken using an Aqua Laboratory (AQUALAB 4TE, Pullman, USA). Before being placed in the measurement chamber, jelly was partially poured into the sample plastic cups. At room temperature, WA values were immediately recorded from the digital display.

2.5.5. Syneresis

At room temperature, the jelly samples' syneresis was assessed after the end of their storage time (60 days). By turning the jelly jars upside down and letting the separated liquid drop into a 10-mL graduated cylinder, the amount of the separated liquid from the jelly was calculated (Khouryieh et al., 2005). The following equation was used to determine the syneresis percentage:

$$\% \text{ Syneresis} = \text{Total weight of separated liquid (g)} / \text{Total weight of the jelly sample (g)} \times 100 \quad (2)$$

2.5.6. pH-measurements

For evaluating acidity, a pH meter (Fisher Scientific in Pittsburgh, PA) was used. After blending 1 g of each product in 10 ml of

distilled water, the pH level was monitored at ambient temperature.

2.6. The nutritional composition of vegetable jelly

2.6.1. The proximate composition analysis, energy content, and glycemic indicator.

Dry ashing, gravimetric, oven drying, soxhlet procedures, and The Kjeldahls (AOAC, 2000) were used to assess the composition of jelly products, including their, ash content, crude fibers, moisture content, fat content, and protein content after multiplying with 6.38. The difference calculation method was used to calculate the carbohydrates.

Each type of gummy jelly's energy output (kilocalories) was estimated by combining the contents of protein, carbohydrate, and fat after multiplying them by the relevant Atwater factors of 4, 4, and 9. The energy value was also multiplied by 4.184 to get the result in kilojoules. Additionally, the calculation methods recommended by Dodd et al. (2011) were used to construct the glycemic index of the meal.

$$GI\ (product) = \{(GI_{component\ a} \times CHO_{Component\ a}) + (GI_{component\ b} \times CHO_{component\ b}) + \ldots..\}/CHO_{Product} \quad (3)$$

Whereas $GI_{component\ a}$ is the glycemic index of each component in the product, $CHO_{component\ a}$ is the available gram carbohydrate content contributed by the component a in the product, and $CHO_{product}$ is the total carbohydrates in grams of the product.

2.6.2. Estimation of minerals

The mineral quantification in jelly products was assessed using the atomic absorption spectroscopy method (AAS, Varian model) for both trace minerals (manganese (Mn), Zinc (Zn), iron (Fe), and copper (Cu)) and macro-minerals (calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na)).

2.6.3. Chromatographic determination of vitamins

Standard vitamins, including water-soluble ones (ascorbic acid-C, pyridoxine-B6, pantothenic acid-B5, nicotinamide-B3) and fat-soluble ones (vitamin A), were acquired from Sigma-Aldrich. The vitamins B3, B5, and B6 were extracted from samples according to Antakli et al. (2015). The HPLC 1200 series (Agilent Technologies, Germany) equipped with an autosampler, quaternary pump degasser, and column compartment C18 BDS (100 x 4.6 mm; 3 μm), a flow rate of 1.6 mL/min, a column temperature of 40°C, and the appropriate detection wavelength recorded at 270 nm at room temperature were used. The mobile phase's components were solvents (A): 5.84 mM of hexane-1-sulfonic acid sodium: acetonitrile (95:5,v/v) with 0.1% triethylamine at pH 2.5, and solvent (B): 5.84 mM of hexane-1-sulfonic acid sodium: acetonitrile (50:50, v/v) with 0.1% triethylamine at pH 2.5.

Whereas, a mobile phase (A/B 33/67; solvent A: 0.1 M potassium acetate, pH = 4.9; solvent B: acetonitrile: water [50: 50]) and a flow rate of 1 mL/min with a UV absorption of 254 nm at an ambient temperature were applied to measure vitamin C (Romeu-Nadal et al., 2006).

The saponification technique outlined by Dennison and Kirk (1977) was applied to calculate the vitamin A levels. A cooled autosampler at 4ºC and a heated column compartment at 30 ºC are used in the delivery system, and a 330 nm variable wavelength detector was used. Water and methanol (5:95) flowing at a rate of 1 mL/min were used in the mobile phase. All vitamins were identified by comparing retention times to real standards.

2.7. Functional properties

2.7.1. Extraction of active compounds

Following the methodology of Cano-Lamadrid et al. (2020), phytochemical components were extracted from the jelly products. This method was used to prepare methanol extract: 1 gram of each jelly product was combined with 10 mL of an 80:20 mixture of MeOH and water, homogenized with 1% HCl, and then kept at 4°C for 24 hours (TSD-J 0.7 L, 50 W, 40 kHz) before being centrifuged at 15,000 g for 10 minutes (Sigma 3–18 K, Osterode and Harz, Germany). The recoverable

supernatants were collected and stored at -20 °C in the dark pending analysis.

2.6.2. Total polyphenols content (TPC)

Using the technique developed by Singleton et al. (1999), the TPC in the previously collected supernatant was estimated. In a nutshell, 1.5 mL of distilled water, 0.1 mL of the Folin-Ciocalteu phenol reagent (1:1), and 0.1 mL of the sample or the standard dilutions (100–500 μg/mL) were combined. This was vortexed with 0.3 mL of a 20% Na2CO3 solution after homogenization for 5 minutes. After that, it was maintained at ambient temperature for 60 minutes without exposure to light. Finally, all solutions were measured at 740 nm in comparison to an acidified methanol blank using a Spectro 23 digital spectrophotometer (Labomed, Inc., Los Angeles, California, USA). The experiment was conducted three times. The findings were represented as mg gallic acid equivalents (GAE) per 100 g of dry weight, employing a standard curve of 25–2500 mg/L.

2.6.3. Total flavonoids content (TFC)

TFC was calculated using the colorimetric technique developed by Zhishen et al. (1999). 4 mL of dist. water was mixed with 1 mL of extract solution and dilutions of a standard solution (10–100 g/mL). The latter mixture was treated with 0.3 mL of 5% NaNO2. 0.3 mL of 10% $AlCl_3$ was added after 5 minutes. 2 mL of 1 M NaOH were further added after 6 minutes. Finally, dist. H_2O was then added to bring the total amount to 10 mL, and it was thoroughly mixed. At 510 nm, the absorbance of solutions was measured in comparison to a freshly prepared blank. Milligrams of quercetin equivalent per gram of dry matter (mg QE/g) were used to represent the TFC. Results were shown as the average of three replicates.

2.6.4. Antioxidant activity (AOX):

Vegetable jelly's antiradical activity was assessed using the technique outlined by Sánchez-Moreno et al. (1998). Thus, 3.9 mL of DPPH (1,1-diphenyl-2-picryl hydrazyl, 0.025/L methanol) was mixed with 0.1 mL of each extracted sample. Using a Vis spectrophotometer, absorbance was measured at 515 nm against a blank after stirring and storing in a dark place for 15 minutes. The percentage of inhibition for each sample served as a measure of the scavenging activity. Trolox, with a concentration range of 0.50 to 5.0 mM, was used as the standard. Calibration curves were used for the quantification of the antiradical activity, showing good linearity ($R2 \geq 0.998$). The findings were presented in milligrams of Trolox equivalents (TE) per 100 g of gummy jelly (mg TE/100 g). The test was performed in triplicate.

2.6.4. Chromatographic analysis of the generated jelly's phenolic components

All standards have been acquired from Sigma-Aldrich (USA). The extract of the samples was first prepared for determining the phenolic compound profile of jelly via chromatography (Devi Ramaiya et al., 2013). 20 mL of 70% (v/v) HPLC-grade methanol was used to homogenize 2.5 g of the sample before it was maintained at room temperature in an ultrasonic bath for an hour to facilitate extraction. Each extract was centrifuged at 8.832 × g for 15 minutes at 4 °C and filtered through filter paper. Gradient elution with a mobile phase of a solvent A (2% acetic acid) and solvent B (0.5% acetic acid: acetonitrile (1:1)) was used to estimate phenolic chemicals. For flavonoids, a mobile phase of solvent A (2% acetic acid) and solvent B (methanol) was utilized to carry out gradient elution. The injection volume was 20 L, and the flow rate was 1 mL/min. At nm 220-365, UV absorption was measured. Phenolic compounds were identified and quantified by comparing them to a standard sample in terms of retention duration and peak area. The results were expressed as mg/100 g of vegetable jelly.

2.7. Microbiological analysis

The quality of the jelly products was assessed microbiologically along with free mint extract control on days 1, 15, 30, and 60. In 90 mL of peptone water, 10 grams of each jelly were diluted and homogenized using Stomacher® bags (Fisher Scientific, Pittsburgh, PA). For each sample, six further dilutions were made. Testing for total aerobic mesophilic bacteria (TBC) on aerobic count plates (3M

Petrifilm TM, 3M Microbiology Products, St. Paul, MN) was done after incubation at 35 °C for 48 hours. On days 3 and 5, the yeast and mold (Y&M) were counted using Y&M count plates (3M Petrifilm TM, 3M Microbiology Products, St. Paul, MN) after being kept at room temperature. Log CFU/g was used to express the counts (Salfinger and Tortorello, 2015).

2.8. Statistical analyses

The associated results were statistically assessed using SAS, the Statistical Analysis System (2017). Duncan's multiple range analysis was used to establish the significance of the discrepancy at $p < 0.05$. Different series of superscripts show the difference in significance (e.g., a, ab, b, etc.). Whereas the Pearson correlation coefficient was used to find the correlation between some variables.

3. Results and discussions

3.1. Nutritional composition of raw materials

Many individuals are interested in green, leafy vegetables because of their various health advantages. Nonetheless, children rarely eat vegetables, especially leafy ones. In the current study, the nutritional content of these vegetables was first investigated (Table 2).

Table 2. Proximate analysis of vegetable plants used through study.

Parameters (%)	Chard juice	Celery juice	Leek juice	Mint extract
Moisture	91.45±0.48^{b}	93.51±0.59^{a}	89.58±1.52^{b}	93.53±0.57^{a}
Fat	0.08±0.044^{c}	0.18±0.00d^{b}	0.24±0.00^{b}	0.55±0.23^{a}
Protein	1.74±0.07^{a}	0.69±0.01^{c}	1.08+±0.06^{b}	2.01±0.31^{a}
Carbohydrate	4.51±0.47^{b}	4.83±0.43^{b}	8.05±0.23^{a}	8.03±0.54^{a}
Ash	2.22±0.07^{a}	0.79±0.03^{c}	1.25±0.12^{b}	1.91±0.17^{a}
Fibre	2.7±0.05^{a}	1.62±0.16^{c}	1.8±0.03^{b}	1.55±0.07^{c}

Each value in the table was obtained by calculating the average of three experiments, and the data are presented as mean ± Standard error of the mean within the same row. Statistical significance was accepted at the $p < 0.05$ level. The superscript letters a, b, etc. denote the significance of one parameter between different vegetable types in the same raw.

Based on the proximate composition, chard substantially ($p<0.05$) had the highest protein, ash, and fiber levels and the lowest carbohydrate levels when compared to celery and leeks, which are within the range described by other authors (Gamba et al., 2020). The moisture content was the highest in celery juice (93.51%). In contrast, leek juice has the most carbohydrates (8.05 ± 0.04%), which is within the range reported by the USDA (2010) and higher than the 4.5% designated by Pak et al. (2014) (Table 2). These vegetables are recognized to be high in minerals and vitamins, as well as having a high antioxidant capacity (Fazal and Singla, 2012; Radovanovi et al., 2015; Gamba et al., 2020).

3.2. Sensory evaluation of vegetable jellies

The sensory investigation aimed to select suitable vegetable concentrations for jelly products with a suitable flavor. According to the primary investigation, the best flavors were stevia (0.06 g/100 mL), mint extract (2%), and lemon juice (3%). Stevia powder was carefully selected to prevent a bitter taste by adjusting the amount of stevia powder and lemon juice. Consequently, it was used in conjunction with all treatments. Afterwards, three concentrations of vegetable juice were made into jelly to accomplish the sensory attributes. The samples were compared to both an agar jelly sample without vegetable juice (the control) and a commercial sugar-added product.

Table 3. Sensory characteristics of three preparations of sugar-free vegetable jelly

Sample	Conc. (%)	Color	Appearance	Odour	Texture	Taste	Overall acceptability
Commercial jelly	-	8.81 ± 0.13^{A}	8.83 ± 0.12^{A}	8.01 ± 0.18^{C}	8.9 ± 0.36^{A}	8.5 ± 0.34^{A}	8.18 ± 0.35^{B}
Control	0	6.72 ± 0.18^{D}	6.18 ± 0.35^{F}	8.31 ± 0.36^{A}	8.71 ± 0.36^{A}	6.09 ± 0.34^{D}	6.18 ± 0.35^{D}
Chard jelly	10	8.5 ± 0.30^{Bc}	8.27 ± 0.35^{Db}	8.09 ± 0.34^{Cc}	8.61 ± 0.31^{Ba}	7.73 ± 0.45^{Bc}	8.12 ± 0.36^{Bb}
	20	8.73 ± 0.28^{Aa}	8.50 ± 0.29^{Ca}	8.36 ± 0.34^{Aa}	8.55 ± 0.32^{Ba}	8.23 ± 0.36^{Aa}	8.45 ± 0.34^{Aa}
	30	8.67 ± 0.33^{Bb}	8.55 ± 0.28^{Ca}	8.18 ± 0.44^{Bb}	8.27 ± 0.46^{Cb}	8.00 ± 0.42^{Ba}	7.91 ± 0.39^{Bc}
Celery jelly	10	7.2 ± 0.28^{Db}	8.64 ± 0.24^{Ba}	8.18 ± 0.31^{Bb}	$8.45\pm.312^{Ba}$	8.32 ± 0.29^{Aa}	8.09 ± 0.31^{Bb}
	20	8.22 ± 0.24^{Ca}	$8.73\pm.24^{Aa}$	8.40 ± 0.34^{Aa}	8.45 ± 0.25^{Ba}	8.45 ± 0.34^{Aa}	8.27 ± 0.33^{Aa}
	30	$8.32\pm.371^{Ca}$	8.14 ± 0.38^{Db}	8.44 ± 0.35^{Aa}	8.09 ± 0.39^{Db}	7.86 ± 0.38^{Ba}	7.95 ± 0.47^{Bb}
Leek jelly	10	8.19 ± 0.32^{Cc}	7.77 ± 0.29^{Eb}	7.59 ± 0.33^{Db}	8.49 ± 0.32^{Ba}	7.45 ± 0.43^{Cb}	7.73 ± 0.31^{Ca}
	20	$8.36\pm.39^{Cb}$	8.50 ± 0.31^{Ca}	7.95 ± 0.30^{Ca}	8.36 ± 0.39^{Ca}	8.00 ± 0.33^{Ba}	8.09 ± 0.37^{Ba}
	30	8.41 ± 0.31^{Ca}	7.55 ± 0.343^{Ea}	7.18 ± 0.40^{Dc}	8.21 ± 0.39^{Cb}	7.41 ± 0.43^{Cb}	7.55 ± 0.34^{Ca}

Control: jelly free of vegetable juice. Values are expressed as means ± standard deviations (A, B, C, etc.) donated the significant difference ($p<0.05$) between all treatments in the same column for one parameter, (a,b,c, etc.) donated the significant difference ($p < 0.05$) between different concentrations in the same treatment under the same parameter. Means sharing similar letters within a column are statistically nonsignificant ($p > 0.05$). Hedonic scale: 1, dislike extremely; 5, neither like nor dislike; 9, like extremely.

The sensory acceptability test revealed that 90% of panelists found green vegetable jellies suitable, moderately sweet, mildly sour, and fresh mint-flavored. They reported that the combination of lemon and mint gives the jelly a refreshing and zesty twist, balancing out the flavors of the vegetables. The findings showed that celery had the highest flavor rating, while chard jelly had the best color among other treatments (Table 3). The texture was acceptable for the three products as compared to the commercial product, but as the percentage of vegetable juice increased, it became less hard. The bright green color was appreciated as a healthy indication.

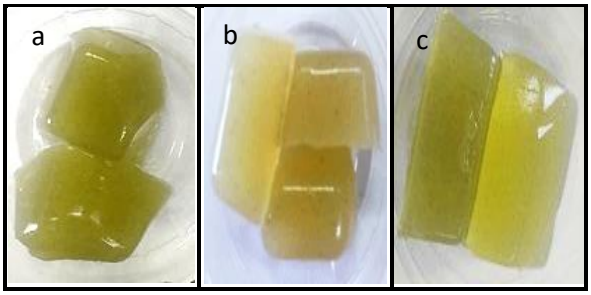

Figure 2. Vegetable jelly made from 20% of chard (a), celery (b), and leek (c) juice.

The optimal treatment was the 20% vegetable content (Figure 2), which scored higher in color, appearance, texture, flavor, taste, and overall acceptability.

3.3.Physicochemical properties of vegetable jelly

3.3.1. Texture profiles of vegetable jellies

Texture is a crucial factor in jelly production, and all produced jellies have acceptable firmness, hardness, and flexibility (Table 4). Nevertheless, commercial gelatine jelly registered the highest hardness, gumminess, and chewiness values with the lowest resilience. Increasing vegetable juice results in a less hard product, possibly due to limited free water availability (Kronberga et al., 2011). On the other hand, leafy vegetables also have soluble fibers that form a jell, boosting water retention and resulting in a stable, hard product (Chawla & Patil, 2010). Jelly with 20% vegetable juice seems to have a balanced state of stiffness. The present hardness measurements for vegetable jelly were closely in the range stated for orange jelly (4.77 N) (Teixeira-Lemos et al., 2021). Still, increasing vegetable juice lowers

adhesiveness, gumminess, and chewiness while increasing resilience and cohesion values.

Table 4. Texture profile analysis of vegetable jelly

Types of jelly	Conc.	Hardness (N)	Adhesiveness (mj)	Springiness (mm)	Gumminess (N)	Chewiness (mj)	Resilience%	Cohesiveness
Commercial jelly		12.01±0.28^{a}	0.21±0.05^{e}	0.93±0.01^{Ab}	11.5±0.51^{Aa}	8.5±0.25^{Aa}	0.09±0.04^{Ae}	0.87±0.02^{Aa}
Control	0	7.74±0.23^{b}	0.33±0.03^{d}	0.92±0.04^{Aa}	6.88±0.11^{Ab}	5.26±0.14^{Ab}	0.11±0.01^{Ae}	0.89±0.01^{Aa}
Chard Jelly	10%	6.01±0.08^{Ac}	0.6±0.02^{Aa}	0.95±0.02^{Aa}	4.57±0.01^{Ac}	4.26±0.01^{Ac}	0.32±0.28^{Cc}	0.75±0.06^{Bc}
	20%	5.28±0.06^{Bd}	0.5±0.03^{Bb}	0.93±0.01^{Bb}	4.14±0.05^{Ac}	3.80±0.28^{Bd}	0.37±0.28^{Bc}	0.78±0.03^{Bc}
	30%	3.79±0.02^{Cf}	0.1±0.0^{Ce}	0.92±0.00^{Cb}	3.24±0.04^{Cd}	2.99±0.04^{Ce}	0.51±0.02^{Aa}	0.86±0.02^{Ab}
Celery Jelly	10%	4.49±0.03^{Ae}	0.4±0.02^{Ac}	0.93±0.03^{Ab}	3.99±0.04^{Ad}	3.78±0.08^{A}	0.44±0.01^{Cb}	0.88±0.04^{Aa}
	20%	3.85±0.05^{Bf}	0.2±0.05^{B}	0.90±0.02^{Bc}	3.38±0.03^{Bd}	3.04±0.05^{Be}	0.49±0.08^{Bb}	0.89±0.01^{Aa}
	30%	2.58±0.03^{Cg}	0.1±0.00^{Cc}	0.89±0.02^{Bc}	2.27±0.05^{Ce}	2.036±0.08^{Cf}	0.54±0.018^{Aa}	0.89±0.02^{Aa}
Leek jelly	10%	5.41±0.08^{Ad}	0.6±0.03^{Aa}	0.95±0.01^{Aa}	4.28±0.08^{Ac}	3.97±0.03^{Ac}	0.39±0.02^{Bb}	0.78±0.02^{Bc}
	20%	4.79±0.03^{Be}	0.5±0.01^{Ab}	0.92±0.01^{Bb}	4.07±0.06^{Bc}	3.74±0.03^{Bd}	0.41±0.02^{Bb}	0.85±0.03^{Ab}
	30%	4.14±0.02^{B}f	0.1±0.02^{Be}	0.92±0.02^{Bb}	3.52±0.08^{Bd}	3.23±0.02^{Ce}	0.45±0.02^{Ab}	0.85±0.02^{Ab}

Control: jelly free of vegetable juice; Values are expressed as means± standard deviations, (a,b,c etc.) for the significance difference (P 0.05) between all treatments in the same column for one parameter. (A, B, C, etc.) refers to the significant difference ($P < 0.05$) between different concentrations in the same treatment under the same parameter. Means sharing similar letters within a column are statistically nonsignificant ($p > 0.05$).

Consumer acceptance is influenced by cohesiveness, whose low value indicates ease of chewing and swallowing (Kawano et al., 2017). Although jelly candies have slightly different hardness values compared to commercial products, they still have nearly similar cohesiveness values and are still in the range of 0.54 to 0.82 described for jelly candies (Mutlu et al., 2018). Elasticity measures the speed at which a deformed sample returns to its original shape (Hamedi et al., 2018). As the product becomes harder, its elasticity decreases, resulting in lower spring values. Because the agar percentage is the same, the elasticity also remains consistent across products and was within the 0.90–1.50 range described for jelly candies (Khouryieh et al., 2005). Another elasticity parameter is resilience, which is the ability of a food to spring back into shape (Cruz et al., 2015). The results also showed that when hardness decreases, resilience also increases slightly.

Gumminess value represents the energy needed to reshape food into a swallowable form. Harder products have higher gumminess and chewiness (Mutlu et al., 2018). But Chewiness is more accurately attributed to gummy jelly (Delgado and Bañón 2015). According to the current findings, vegetable jellies have lower gumminess and chewiness values than commercial product. In conclusion, vegetable jellies with 20% vegetable juice were softer, less gummy, easier to chew, and had nearly the same range of springiness and cohesiveness compared with the commercial product

3.3.2. Color analysis

Vegetable jelly's color characteristics were expressed on a L*, a*, and b* scale (Table 5). Vegetable jelly had a negative (a*) value, signifying that it was greenish. It was noticed that T1 was the greenest in color, followed by T3 and T2 jelly.

Table 5. Colour parameters of vegetable jellies expressed in terms of the CIELab scale parameters:

Treatments		Color parameters					
		C*	L*	a*	b*	h	ΔE
Control	0	40.49±1.21^{d}	84.2±1.14a	0.5±0.04^{d}	16±1.55^{e}	40.05	0
Chard Jelly	10%	39.04±1.04^{d}	56.6±2.03^{c}	-9.02±1.13^{c}	38.2±1.03^{d}	46.01±1.25^{b}	36.67±0.03^{d}
	20%	65.34±1.03^{b}	45.15±1.53^{e}	-12.45±1.03^{b}	44.5b±1.61^{b}	48.4±1.33^{b}	50.083±0.04^{b}
	30%	89.99±1.21^{a}	41.48±2.03^{e}	-15.2±1.11^{a}	46.89±1.33^{a}	52.65±1.23^{a}	55.03±1.03^{a}
Celery Jelly	10%	30.04±2.03^{e}	67±1.06^{b}	-4.66±1.08^{e}	25±1.73^{e} f	43.84±1.63^{e}	20.08±0.03^{g}
	20%	38.81±2.03^{d}	64.7±1.23^{b}	-5.65±1.05^{e}	29.12±1.57^{e}	45.5±1.25^{c}	24.34±0.03^{f}
	30%	39.2±1.06^{d}	60.3±1.24^{c}	-6.65±1.03^{e}	35.2±1.23^{d}	46.86±1.21^{c}	31.47±0.03^{e}
Leek jelly	10%	33.99±1.03^{e}	62.2±1.21^{b}	-7.4±1.25^{e}	37±1.13^{d}	45.14±1.24^{c}	31.42±0.03^{e}
	20%	40.96±1.03^{d}	60.56±1.11^{c}	-9.1±1.23^{c}	42.3±0.03^{c}	47.15±1.54^{c}	36.64±0.03^{d}
	30%	44.36±1.33^{c}	58.8±1.23^{c}	-12.5±1.06^{b}	44.2±1.53^{b}	49.69±1.03^{b}	40.11±0.03^{c}

Control: jelly free of vegetable juice Values are expressed as means ± standard deviations (a,b,c, etc.) for the significant difference between all treatments in the same column for one parameter. Means sharing similar letters within a column are statistically nonsignificant ($p > 0.05$).

The green color in jelly was preferred in our study because it denoted a link to healthy products (Schuldt, 2013). This green hue is related to the presence of chlorophyll pigment (Khoo et al., 2011). The sensitivity of chlorophyll depends on the duration of the cooking process and the type of plant. Chard pigment exhibits the least amount of decrease (19–36%) after boiling among the numerous vegetables studied (Mitić et al., 2013). This observation was consistent with the current chard jelly's measured chlorophyll concentration, since T1, followed by T3, had the highest chlorophyll content (Table 7). The green color was marginally affected, because of the addition of vegetable juice after solubilizing the agar. However, a positive b* value indicates a yellowish degree of chlorophyll degradation, possibly caused by heat or lemon juice addition (Al-Dabbas et al., 2017). Increasing vegetable juice concentration reduces lightness (L*) and increases color saturation (C*). Chard jelly has the greatest color difference (E) due to its stable pigment after boiling. Finally it was concluded from the results that treatments with the most shine and saturated color were those with 20% juice.

3.3.3. pH, syneresis, TSS, and water activity analysis

The study investigated vegetable jelly types with 20% vegetable juice based on their organoleptic, textural, and visual characteristics. The results showed a reduction in acidity and a minor increase in total solids compared to the control (Table 6). The low TSS values (1.18 to 2.27 °Brix) were due to the absence of sucrose. Herein, stevia powder was used in place of sugar, resulting in a minimal increase in soluble solids. These results align with previous studies on porang jelly (Herawati and Kamsiati, 2022) and green tea jelly made with stevia (Akesowan and Choonhahirun, 2021).

The pH measurements indicate an acidity range of 4.36 to 4.51 due to the use of lemon juice. This range is quite comparable to Mutlu et al. (2018)'s stated range of 4.26–5.03. Since most bacteria can grow well at a pH of 7, acidity acts as a barrier to their growth (Padan et al., 2005). Additionally, it is well known that jelly desserts' microbiological viability and freshness are impacted by water activity. Vegetable jelly has a water activity index that is marginally higher than 0.7, but it is less than the 0.9 informed by Belova et al. (2021). It is stated that

the use of sweeteners causes a decrease in water activity compared to sucrose, which is related to their higher osmotic pressure. This decrease in water activity can contribute to the preservation and stability of jelly desserts

Table 6. pH, TSS, water activity and syneresis values of vegetable jelly

Parameters	Commercial product	Control	T1	T2	T3
Water activity	0.71±0.01^{c}	0.782±0.01^{a}	0.751±0.02^{b}	0.792±0.1^{a}	0.731±0.04^{b}
pH	3.2±0.01$^{b\,a}$	4.1±0.05^{b}	4.37±0.05^{b}	4.51±0.03^{c}	4.36±0.03^{b}
TSS (0Brix)	76.2±1.9^{a}	0.92±0.03^{d}	1.18±0.03^{c}	2.16±0.04^{b}	2.27±0.03^{a}
Syneresis value (%)	0.0	0.0	0.0	0.2^{a}	0.1^{b}

Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice. Values are expressed as means ± standard deviations, (a,b,c,..) donated the significant difference ($P<0.05$)between all treatments in the same raw for one parameter. Means sharing similar letter within a raw are statistically non significant ($p>0.05$)

The syneresis percentage, which refers to the release of liquid from the jelly, was found to be low in vegetable jelly. It was noticed that after 60 days of storage at 4ºC, the syneresis value increased slightly in celery jelly than the other treatments. This observation aligns with a study by Figueroa and Genovese (2019), who found that fibers could decrease jelly syneresis due to their water-holding capacity.

3.4.Nutritional composition of vegetable jellies

3.4.1.Proximate analysis and energy content

Table (7) summarizes the nutritional profiles of three vegetable jellies. The moisture content of the three products was similar, with celery jelly having the highest moisture content. The moisture percentage ranged from 93.70 to 96.58%, falling within the range described by Khouryieh et al. (2005) and Belova et al. (2021). That result was expected because of the reduced total solids compared to commercial jelly. There was no significant difference in fat content between treatments.

All manufactured jelly types contained a significantly lower carbohydrate and protein content compared to commercial jelly. This is because agar, the hydrocolloid used in vegetable jelly, lacks protein, and vegetables themselves have low protein content. The protein content of vegetable jelly is even lower than that of fruit jelly (Teixeira-Lemos et al., 2021). Additionally, the lower carbohydrate content in vegetable jelly is due to the absence of sugar, nonetheless its concentration is still lower than that of free sugar-berry jelly (Teixeira-Lemos et al., 2021).

Vegetable jellies are rich in dietary fiber (DF), ranging from 2.09±0.16 to 4.2±0.04 g/100g (Table 7). These fibers contribute to the soluble fraction of agar and vegetable juice, ensuring a firm texture without sugar addition (Riedel et al., 2015). It was reported that soluble agar fibers delay gastric emptying and do not affect glycemic response (Clegg and Shafat, 2014). Additionally, DF has physiological benefits, such as reducing intestinal transit time, blood cholesterol levels, and postprandial blood glucose or insulin levels (Gill et al., 2021). Consuming 7 grams of plant-based dietary fiber daily is believed to significantly reduce the risk of cardiovascular and coronary heart diseases (McRae, 2017). Hence, it is good to figure out that consuming 100 g of vegetable jelly would cover roughly a range of 30 to 55% of this daily required dosage.

Table 7. Proximate analysis, energy content, glycemic indicator and chlorophyll of vegetable jelly.

Parameters	Commercial product	Control	T1	T2	T3
Fat (%)	0.01±0.00^{b}	0.02±0.22^{b}	0.25±0.01^{a}	0.10±0.01^{a}	0.15±0.01^{a}
Protein (%)	7.9±0.12^{a}	1.3±0.002^{c}	3.7±0.54^{b}	2.2±1.02^{b}	3.4±1.03^{b}
Carbohydrate (%)	65.5±1.14^{a}	0.15±0.00^{c}	1.6±0.35^{b}	1.12±1.3^{b}	2.75±0.25^{b}
Ash (%)	-	0.085±0.00^{c}	0.47±0.004^{a}	0.24±0.01^{b}	0.29±0.00^{b}
Dietary Fibre (%)	-	1.76±0.06^{c}	4.2±0.04^{a}	2.09±0.16^{b}	3.87±0.07^{a}
Moisture content (%)	26.59±0.04^{d}	97.53±0.44^{a}	94.45±0.14^{c}	96.58±0.25^{b}	93.7±0.34^{c}
Total energy (kcal/100 g)	294.5±2.24^{a}	9.49±0.01^{c}	31.8±0.70^{d}	18.3±0.34^{c}	33.49±1.7^{b}
Total energy (kj/100 g)	1251.5±9.3^{a}	39.47±6.3^{e}	132.9±4.2^{c}	76.86±5.3^{d}	140.21±2.3^{b}
Glycemic index	78±0.22^{a}	2.26±0.11^{b}	7.45±0.13^{a}	16.4±0.12^{c}	18.78±0.14^{a}
Chlorophyll (mg/100g)	-	0.003±0.0^{d}	0.79±0.02^{a}	0.045±0.00^{c}	0.68±0.00^{b}

Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice. Values are expressed as means ± standard deviations, (a,b,c,..) donated the significant difference ($p<0.05$)between all treatments in the same raw for one parameter. Means sharing similar letter within a raw are statistically non significant ($p>0.05$).

Consumers are commonly concerned about the calorie content of products, particularly due to obesity and related diseases. Commercial jellies are typically a high-energy products, due to their high sugar content. A zero-calorie plant extract, stevia, has been used in this study, resulting in a vegetable jelly with a low energetic density (18.3 to 33.49 kcal/100 g) (Table 7), making it designed as sugar-free according to European regulations 1924/2006 (European Commission, 2006) and 1047/2012 (European Commission, 2012). Vegetable jelly had a significantly lower glycemic indicator value ($p<0.5$) than the index of the commercial product by an average of approximately 77.97%. This decline is larger than the 40% reported by Hadjikinova et al. (2019) for reduced sugar-fruit jelly. Which is an encouraging finding for the use and marketing of that type of jelly.

Table 8. Minerals and vitamin content in the vegetable jelly

Parameters	Control	T1	T2	T3
Minerals (mg/100g)				
Cu	0.14±0.01^{b}	0.43±0.00^{a}	0.45±0.00^{a}	0.43±0.00^{a}
Fe	5.16±0.49^{c}	10.05±0.17^{a}	8.25±0.17^{b}	9.79±0.10^{a}
Mn	1.32±0.00^{c}	2.69±0.012^{a}	2.39±0.00^{b}	2.53±0.00^{a}
Zn	1.04±0.00^{b}	3.73±0.06^{c}	2.68±0.00^{b}	3.46±0.08^{a}
Ca	105.8±4.5^{d}	310.7±4.33^{a}	297.5±3.93^{c}	286.9±2.18^{b}
Mg	144.9±0.85^{d}	346.4±0.93^{a}	332.5±0.58^{c}	339.1±1.20^{b}
Na	10.9±2.6^{d}	90.3±1.6^{a}	53±2.02^{b}	35.3±1.45^{c}
K	780.3±8.9^{d}	1218±2.5^{b}	1229±3.8^{a}	1191±4.3^{c}
Vitamins				
A (IU/100g)	286.3±5.9^{d}	585±8.82^{b}	414±5.72^{c}	510±0.21^{a}
C (mg/100g)	5.24±0.23^{d}	11.13±0.18^{a}	7.41±0.18^{c}	9.45±0.36^{b}
B6 (mg/100g)	0.002±0.00^{c}	0.05±0.00^{b}	0.021±0.00^{b}	0.38±0.00^{a}
B5(mg/100g)	0.007±4.5^{c}	0.041±0.00^{b}	0.057±5.1^{a}	0.037±0.00^{c}
B3 (mg/100g)	0.035±0.08^{b}	0.08±0.00^{a}	0.10 ±0.00^{a}	0.11±0.00^{a}

Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice. Values are expressed as means ± standard deviations (a,b,c, etc.) donated the significant difference ($p<0.05$) between all treatments in the same raw for one parameter. Means sharing similar letters within a raw are statistically nonsignificant ($p > 0.05$).

3.4.2. Minerals and vitamins

In terms of mineral content, all vegetable jellies had a significant amount of minerals, and the highest percentages were for K, Mg, and Ca (Table 8).

According to the US Food and Drug Administration (FDA, 2016), the produced vegetable jellies could cover a good percent of the DV recommended for adults and kids aged 4 and older and are considered a rich source of minerals (Figure 3). It was noticed that vegetable jelly could provide 110, 72, 52, 30, 25, and 22.9% of the daily values (DV) for Mn, Mg, Fe, Zn, K and Ca (DV: 1300 mg), respectively.

These minerals are associated with many health aspects. Magnesium, for example, is essential for energy generation, heart rhythm, bone formation, and nerve signal conduction (Ross et al., 2012). Besides being identified as a "nutrient of public health concern" (U.S. Department of Agriculture, 2015), potassium levels are linked to higher rates of type 2 diabetes, insulin resistance, and fasting blood sugar. Calcium also is essential for bone strength and osteoporosis (Song, 2017). Iron is essential for child development and female anemia prevention (Wang et al., 2019). Zinc also plays a role in cellular metabolism, cell-mediated immunity, bone formation, and acute diarrhea treatment in children (Bagherani and Smoller, 2016).

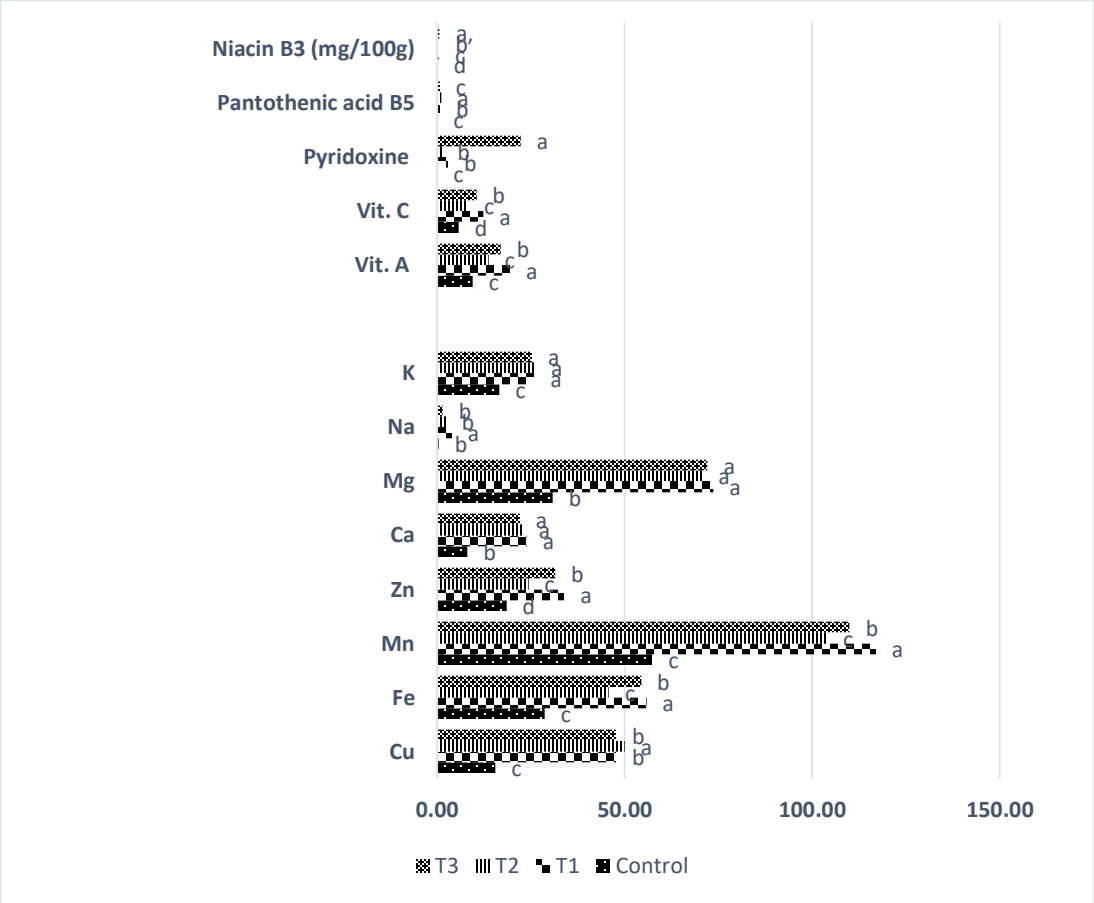

Figure 3. Vitamins and minerals expressed as a percent of the Daily Value (% DV) covered by each vegetable jelly Control: jelly free of vegetable juice T1, T2, and T3: Vegetable jellies made with 20% chard, celery, and leek juice. Results are expressed as the mean of three replicates. Significant differences ($p < 0.5$) are shown by (a, b, c, etc.) between jelly types in the same parameter.

Based on the information provided in table (8) and figure (3), T1 and T3 jelly can provide the best coverage of the recommended daily amounts for vitamins A, C, and B6. And can be considered moderately vitamin-rich foods (FDA, 2016). Since they could provide an average of 16.7, 10.36, and 8.8% of the DV% prescribed for vitamins A, C, and B6 (FDA, 2016). These vitamins are important for cellular interaction, inhibition of some cancers, IL-2 synthesis, hemoglobin production, and overall immune system function (Akram et al., 2020).

Micronutrients aid the immune system in combating viral infections, and doctors worldwide are interested in supplementing them for COVID-19 prevention or treatment (El Sabbagh et al., 2022). According to the 2020–2025 Dietary Guidelines for Americans, nutritional needs should be supplied through meals, and vegetable jellies can be a convenient and effective way to fulfill the nutritional requirements of these patients.

3.5. Functional properties of vegetable jelly

The popularity of polyphenols derived from plants, particularly vegetables, is steadily rising.

Table 9. Phytochemicals content and antioxidant activity of vegetable jelly

Parameters	Control	T1	T2	T3
TPC (mg GAE/ 100g dw)	190±0.1^{d}	790±0.17^{c}	318±1.9^{a}	824±0.3^{b}
TFC (mg QE/ 100g dw)	66±0.6^{d}	236±0.27^{b}	108±0.01^{c}	348±0.21^{a}
Antioxidant AOX (%)	9.79±0.1^{c}	56.47±0.06^{b}	32.67±0.35^{a}	73.54±0.43^{a}
AOX (mg TE/100gm)	36.4±0.1^{d}	195.7±0.16^{b}	112.9±0.25^{c}	255.4±0.65^{a}

Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice. Values are expressed as means ± standard deviations (a,b,c, etc.) donated by the significant difference (P < 0.5) between all treatments in the same raw for one parameter. Means sharing similar letters within a raw are statistically nonsignificant (p > 0.05).

The findings for total phenolics and flavonoids differed significantly (p<0.5) among jelly samples and ranged from 8.24 to 3.18 mg GAE/g and 3.48 to 1.08 mg QE/g, respectively (Table 9).

T3 displayed the highest value in both categories. The reported range of flavonoids and phenolic compounds was higher than that established by Ben Rejeb et al. (2020) for citrus jellies (192.76 mg GAE/100 g for total phenolic and 9.06 mg QE/100 g for flavonoids, respectively). Previous studies have shown that a short heat cycle can greatly increase the levels of total phenolics and flavonoids in vegetables, but prolonged heating can have the opposite effect (Kim et al., 2008). This research also analyzed vegetable jelly's phenolic components by HPLC, revealing a rich and diverse range of compounds (Table 10). The most elevated phenolic compounds were E-vanillic and salicylic acids in T1, E-vanillic and pyrogallol in T2, and caffeine and chlorogenic acid in T3. While the predominant flavonoids in T1, T2, and T3 were luteolin, rosmarinic acid, and kaempferol, respectively. These findings align with previous studies on the phenolic profiles of Swiss chard, Egyptian leeks, and celery leaves (Zein et al., 2015; El-Rehem et al., 2013; Yao et al., 2010).

In the same manner, cooking has been found to boost antioxidant activity by softening tissues and releasing phytochemicals (Sharma et al., 2015). T3 showed the highest antioxidant activity (81.47%), followed by T1 (69.5%) and T2 (53.06%), respectively. Giving a range of 255.43 to 112.91 mg TE/100 g, which is larger than what was observed by Teixeira-Lemos et al. (2021) for the berries (83.7 mg TE/L) and the orange with honey jelly (50.4 mg TE/L).

Furthermore, the antioxidant activity of leafy vegetables has been found to be positively correlated with their flavonoid (r = 0.97, p < 0.05) and phenolic content (r = 0.96, p < 0.05). These compounds have been increasingly studied for their various health benefits, such as their potential to provide protection against allergies, inflammation, pain, heart diseases, liver damage, viral infections, and even certain types of cancer (Ghasemzadeh, 2011). Leafy vegetables' antioxidant activity is also

associated with pigment content, including chlorophyll, betalains, and carotenoid content (Ivanović et al., 2019).

Table 10. HPLC analysis for phenolic profile of vegetable jelly

Compounds	T1	T2	T3
Phenolic compounds (mg/100g)			
Gallic	11.7±015^{a}	3.95±0.59^{c}	9.8±0.25^{b}
Pyrogallol	15.68±0.54^{b}	107.9±0.55^{a}	10.46±0.54^{c}
Caffeic Acid	11.87±0.41^{a}	7.96±0.21^{b}	6.8±0.57^{c}
Protocatchoic	45.6±1.59^{a}	9.89±0.27^{b}	5.29±0.14^{c}
Chlorogenic	5.6±0.11^{c}	44.68±0.12^{b}	60.66±1.5^{a}
Epi-Catechin	17.9±0.88^{a}	9.41±0.12^{b}	3.56±0.54^{c}
Catechin	9.5±0.33^{b}	9.85±0.45^{b}	21.13±0.51^{a}
Caffeine	9.9±0.14^{c}	22.56±0.22^{b}	50.2±0.21^{a}
P-OH.Benzoic	13.7±0.16^{c}	25.37±1.5^{a}	23.9±0.21^{b}
Vanillic	21.26±0.14^{a}	5.78±0.11^{b}	4.53±0.14^{b}
Ferulic	26.52±0.17^{b}	33.25±0.57^{a}	5.07±054^{c}
Catechol	27.7±0.21^{a}	3.44±0.14^{b}	4.73±0.41^{b}
E- Vanillic	141.8±0.39^{a}	123.3±0.71^{b}	5.4±0.77^{c}
Ellagic Acid	22.7±0.14^{b}	78.9±0.55^{a}	7.53±0.41^{c}
Benzoic	30.7±0.51^{b}	53.89±0.54^{a}	10.47±0.45^{c}
Salycillic	130±0.11^{a}	----	3.73±0.24^{b}
3,4,5 Methoxy Cinnamic	4.7±0.53^{b}	5.1±0.25^{b}	19.8±0.32^{a}
Coumarin	3.3±0.41^{c}	10.52±0.54^{a}	8.4±0.19^{b}
P- Coumaric	25.38±0.52^{a}	3.54±0.14^{c}	26.7±0.12^{b}
Cinnamic	4.72±0.55^{b}	0.77±0.04^{c}	84.6±0.07^{a}
Syringic	33.5±0.14^{a}	1.6±0.04^{b}	2.5 ±0.01^{b}
Flavonoid (mg/100g)			
Luteolin	151.39±0.51^{a}	27.21±0.11^{c}	67.98±0.45^{b}
Naringin	31.3±0.42^{a}	---	4.4±0.14^{b}
Rutin	25.6±0.22^{b}	3.34±0.02^{c}	50.02±0.52^{a}
Hesperidin	77.9±0.82^{a}	21.32±0.54^{b}	3.96±0.05^{c}
Rosmarinic	5.6±0.07^{c}	199.02±0.12^{a}	22.6±0.52^{b}
Quercetrin	10.64±0.72^{c}	32.21±0.71^{a}	23.58±0.12^{b}
Quercetin	1.2±0.02^{b}	0.35±0.02^{c}	122.32±1.7^{a}
Kaempferol	7.5±0.09^{c}	10.96±0.08^{b}	195.36±0.25^{a}
Hespertin	18.34±0.78^{a}	2.48±0.12^{c}	3.25±0.45^{b}
Apegenin	0.16±0.00^{b}	0.18±0.01^{b}	10.69±0.52^{a}

Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice. Values are expressed as means ± standard deviations (a,b,c, etc.) donated by the significant difference ($P < 0.5$) between all treatments in the same raw for one parameter. Means sharing similar letters within a raw are statistically nonsignificant ($p > 0.05$).

3.6. Microbial analysis

Since the existing jellies didn't include any additional sugar, they should be susceptible to spoiling. But satisfactory, the current vegetable jellies had total bacterial counts ranging from 0.22 to $2.3*10^{2}$ CFU/g (Figure 4), which was below the 10^{3} CFU/g limits specified by EU law (2005).

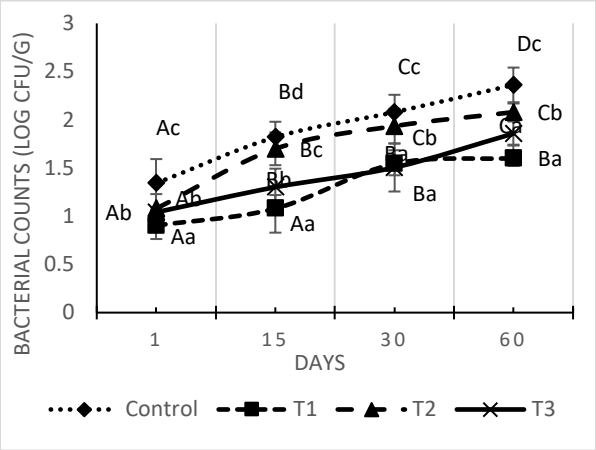

Figure 4. Total mesophilic counts during storage for two months. Control: jelly free of vegetable juice. T1, T2 and T3: Vegetable jellies made with 20% chard, celery and leek juice Results expressed as a mean of three replicates ± standard deviation. A significant difference ($p < 0.5$) is shown by different letters (A, B, C, etc.) in the same treatment for different times of storage and by (a, b, c, etc.) in the same storage day for different treatments.

Among the different vegetable jellies tested, chard jelly had the smallest bacterial population, followed by leek jelly. The bacterial counts slightly increased during storage, with celery jelly having the highest count after two months. Moreover, there was no discernible yeast or mold.

The obtained preservation period is comparable to that of sugar-free jelly preserved with chemical preservatives and a low pH (Khouryieh et al., 2005), as well as fruit jellies with or without sugar (Ben Rejeb et al., 2020). Herein, mint leaf extract is used as a flavoring and preservation element to increase safety due to its antimicrobial activity (Sugandhi and Meera, 2011). Additionally, the low pH of lemon juice creates an unfavorable environment for bacterial survival and reproduction. Other factors like low water activity, antioxidants, phenols, and flavonoids also contribute to the safety, preservation, and quality of vegetable jellies.

4. Conclusions

To the best of our knowledge, this research formulated an agar-based sugar-free jelly by incorporating leafy vegetables, including Swiss chard, celery, and leek, which is flavored with lemon and mint and sweetened with stevia. The results of this investigation showed that the incorporation of leafy vegetables not only adds nutritional value to the jelly but also enhances its physical and functional properties. Since it was found that vegetable jelly has greater amounts of dietary fiber, vitamins, minerals, and phytochemicals while still being considered a low-calorie product with a low glycemic load compared to commercial jelly. Furthermore, the jelly's texture can provide a satisfying mouthfeel, making it an appealing option for individuals with difficulty swallowing or chewing. This innovative approach could benefit individuals with diabetes, obesity, malnutrition, and cardiovascular diseases and also be a suitable option for people following vegetarian or vegan diets. Additionally, they are low-cost, multifunctional, and open up opportunities for diversifying the market for healthy, sustainable, and value-added products.

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MOLECULAR CHARACTERIZATION, PRODUCTION AND OPTIMIZATION OF PECTINASE PRODUCER AND ITS INDUSTRIAL APPLICATIONS

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ABSTRACT

A naturally occurring component called pectin is present in many fruits, including berries and apples. Almost all plants contain it, where it adds to the structure of the cell. Pectinase is an enzyme product that is used professionally to break down pectin. Pectinases are widely used in the wine and citrus juice industries. It is used in the fruit juice business for clarification because it lowers viscosity, which results in the creation of clear juice. In the current research, soil samples were taken from six different regions in the Ahmedabad district in an effort to isolate bacteria that produce pectinase from those samples. Primary screening produced a total of 41 strains, of which 14 showed pectinase activity. Screening was done by using Vincent's agar medium containing pectin. Best pectinolytic activity was determined by clear zone of hydrolysis on selective media. Among 14 isolates, Isolate 1 was showing highest zone of utilization which was selected for further study. Pectinase was produced by submerge fermentation technique and physicochemical parameters were optimized in which isolate 1 showed highest activity at pH 7.4, temperature 37°C, incubation period 48hrs., inoculation size, substrate, carbon source, nitrogen source. Isolate 1 was characterized by its cultural, morphological, biochemical and molecular basis by 16S rRNA sequencing and designated as *Bacillus subtilis.* This isolate was applied for fruit juice clarification and demucilization.

1. Introduction

Since bacteria are responsible for creating vital goods like enzymes, antibiotics, vaccines, cheese, bread, and many other things, they are very advantageous to society (Antranikian, 1992). From soil bacteria, different enzymes are extracted that are used in a variety of commercial fields. In both technical and commercial processes, microbial enzymes take the place of some harsh chemicals, reducing pollution. Pectinase is one such enzyme that can be produced from various microorganisms, including bacteria and fungi, and is used in a variety of sectors (Kaur *et al.* 2004). Pectic substances are chemicals that are catalysed by pectinolytic enzymes. These are negatively charged and corrosive (Danielle *et al.* 2009). Fruits are an abundant source of pectin, and the mechanical crushing of pectin-containing fruits results in the high viscosity of fruit juices. Juice extraction by mechanical means is challenging (Semenova *et al.* 2006). Pectinase and a few other enzymes take the role of mechanical extraction to clarify fruit juice.

2. Materials and methods

2.1. Sample Collection and Evaluation for Pectinase Producers

Bacteria for the production of pectinase were isolated from soil sample collected from 6 different region of Ahmedabad district including garden area, fruit market and vegetable market. Soil sample was enriched by placing 100 g of soil sample and 1g of pectin powder in a sterile beaker for few days at room temperature. 1g of enriched soil sample was inoculated into enrichment broth containing (g/100ml): Pectin-1, Yeast extract-0.1, Peptone-0.5, $CaCO_3$-0.2 and NaCl –0.2 which was then shook for 10 days at 200 rpm on a rotatary shaker. The enriched soil sample was diluted and inoculated on Vincent's agar medium containing (g/1000ml): sucrose, KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, K_2HPO_4, KCl, yeast extract, pectin, and agar. Following 24 h incubation at 37°C, the clearing areas of the medium after the addition of Lugol's iodine solution were used to classify pectinase secretion. Colony with maximum zone diameter was preceded for further studies.

2.2. Identification of the Bacterial Strain

Different staining methods, as well as biochemical and molecular techniques, were used to classify the bacteria that had been isolated.

2.2.1. Sequencing of the Expanded 16S rrna Gene to Identify the Isolated Bacteria

Amplified PCR product was purified using a Helini DNA purification package, and the PCR product was then commercially sequenced. The collected sequence was blasted in the NCBI database, and a Bacillus phylogenetic analysis was performed. FASTA sequence was built using the neighbour-joining (N-J) process.

2.2.2. Genomic DNA Isolation of the bacterial Isolate

The above sequence was blasted against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree. Based on nucleotide homology and phylogenetic analysis, the sample displayed a high degree of resemblance to *Bacillis subtilis*. CLUSTAL W. Performed a molecular phylogenetic study using the Maximum Likelihood approach for pectinase producers. The final dataset included 1434 locations. MEGA7 was used to run evolutionary studies.

2.2.3. Sequences for Scanning Electron Microscopy of bacterial strain

Bacterial isolate were fixed in 6% buffered glutaraldehyde for 24 hours. Scanning Electron Microscope photographs were obtained from mediwave labs, Mumbai**.** The absolute ethyl alcohol was acquired from Hayman Ltd., England. The microbial identification by 16srrna was conducted by Eurofins Genomics India Pvt Ltd. Bangalore 560048, Karnataka, India.

2.3. Optimization physico-chemical parameters for pectinase biosynthesis by *Bacillus subtilis*

Any bacterium species' ability to produce pectinase can vary and be influenced by a variety of physico-chemical factors. Different amounts of these variables may have an impact on the enzyme's secretion. Therefore, in the current study, pectinase enzyme production was optimised at various temperature ranges (28 to 60°C), pH ranges (6 to 12), substrates (sugarcane, paper pulp, tea waste, cassava waste, orange peel, molasses, wheat bran, and agricultural waste), carbon sources (lactose, maltose, mannitol, sucrose, glucose, fructose, cellulose, and starch), and nitrogen source (CTAB, EDTA, triton X-100, SDS, glycerol, and tween 20). The experiment was carried out in 250 ml Erlenmeyer flasks holding the production medium (yeast extract pectin) for the pectinase enzyme (YEP). Following sterilization, flasks were cooled and infused with pure *Bacillus subtilis* cultures that had been separated using selective media.

2.4. Production of Pectinase by Submerged Fermentation (smf)

The best initial ph, temperature, time, nitrogen supply, inoculum age, inoculum size,

incubation period, agitation rate and substrate concentration for pectinase development were determined. Pectinase fermentation medium comprising of Peptone1.0 gl^{-1}, Yeast extract1.0 gl^{-1}, $NaNO_3$ 2.0 gl^{-1}, KH_2PO_4 1.0 gl^{-1}, $MgSO_4.7H_2O$ 0.5 gl^{-1} and substrate 5.0 gl^{-1} was used for submerged fermentation process. After being sterilized, containers were cooled, infused with pure *Bacillus subtilis* cultures, and then incubated for three days at 37 °C with agitation. After centrifuging the production medium, the supernatant was used as a supply of crude enzymes in subsequent research.

2.4.1. Pectinase assay by DNS method (3,5-dinitrosalicyiic acid)(Miller 1959)

Pectin was used as the substrate for the pectinase test (Janani et al. 2011). In a 0.1M acetate buffer with a pH of 6.0, 0.5 ml of the crude enzyme and 0.5 milliliter of pectin solution are combined to create the reaction mixture. It was then warmed for 10 minutes at 40 °C. Following the addition of 1 ml of DNS reagent, the combination was boiled for 5 minutes at 90 °C. Rochelle's salt, one millilitre, was applied to halt the reaction. At 595 nm, the absorption was measured. The creation of a standard graph was done using a normal glucose solution. The amount of enzyme that releases 1 mmol of glucose per minute was used to determine one unit of cellulase activity.

2.4.2. Partial purification of pectinase enzyme Ammonium sulfate precipitation

After adding solid ammonium sulphate to about 20 ml of the crude enzyme solution, the combination was allowed to sit at 40 °C overnight to precipitate. Centrifugation was used to separate the precipitates, which were then dissolved in 10 ml of 50 mm sodium acetate solution with a pH of 5.5.

2.4.3. Dialysis

Enzyme collected after ammonium sulphate precipitation was dialyzed against 30 mm sodium acetate buffer (pH 5.5) at 4°C with three buffer adjustments for partial purification. Enzyme activity and protein content were assessed in the sample that had only partly been purified.

2.5. Statistical evaluation

The data gathered were statistically analyzed using SPSS 16.0. The information is presented as a mean and standard variation (SD). In the one-way ANOVA, p0.05 was found.

2.6. Application of pectinase in fruit juice clarification

Enzymatic treatment is effective way to reduce cloudiness in the fruit juices (Singh and Singh, 2015). In this study enzyme treatment was applied on two fruit juices grapes and oranges. For sample preparation both fruites are washed with purified water and then fine pulp was obtained by use of blender. After that pulp was pasteurized at 85oc for 3 minutes. After cooling different concentration of pectinase (crude enzyme) treatment like 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/20g of pulp was given to both fruit pulp separately in a glass tube. Tubes are incubated at 50^OC at different time interval like 30, 60, 90, 120 and 160 minutes. After that pectinase activity is inactivated by exposed tubes at 95^OC for 5 minute in water bath. After cooling centrifuge the content of both tubes at 2000 rpm for 10 minutes. After that filter all content through muslin cloth. Extracted juice was analysed for its clarity (%transmission) and yield (%w/w). Juice yield was determined by following formula:

Juice yield % = Weight of clear juice × 100/Weight of sample

(1)

2.7. Removal of Mucilage from Coffee Beans Using pectinase

Manually pulped fresh coffee grounds were used. The mucilage was removed from the pulped beans by soaking them in the enzyme mixture under still circumstances. As per custom, the demucilized coffee beans were ultimately washed and sun dried after being thoroughly felt by hand. The pulped coffee beans were soaked in water without any additional enzymes in order to compare

enzymatic demucilization with spontaneous fermentation.

3. Results and discussions

3.1. Results of pectinase screening, production and optimization

After primary and secondary screening, we identified total 14 isolates from 6 different region of Ahmedabad district including garden area, fruit market and vegetable market. On the premise of having the highest zone of pectin degradation among the 14, one isolate was chosen for additional research. It was named isolate 1 for ease of study. Jaysankar and Graham (1970) stated that pectinolytic organisms are detected by clearing zones on agar plate. Isolate was gram positive rod on the basis of its gram rection. Biochemical characterization was also done in which it gives positive result in starch utilization test, citrate utilization test, Gelatine hydrolysis test and carbohydrate fermentation test (Glucose, Lactose, Maltose, Mannitol, Mannose and Fructose). Identity and phylogeny of the isolate was analysed using 16S rdna analysis. The amplified PCR product was then commercially sequenced after being purified using a Helini DNA purification kit. The collected sequence was blasted in the NCBI database, and a Bacillus phylogenetic analysis was performed. The 16S rdna nucleotide sequences were identified, and a phylogenetic tree based on the 16S rdna FASTA sequence was constructed using the neighbour-joining (N-J) method to categorise the strain. The gene sequence was sent to NCBI, and the genbank library assigned it the accession number MK034151. The sequence was blasted against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree. The bacterial isolate was identified as *Bacillus subtilis* based on the 16s rrna sequence (Figure 1). The majority of bacterial pectinase producers were classified using morphological and biochemical tests, with *Bacillus sp.* Being the most common. Among the many microorganisms, *Bacillus subtilis* is recognized for producing a broad range of extracellular enzymes and has a number of industrial uses.

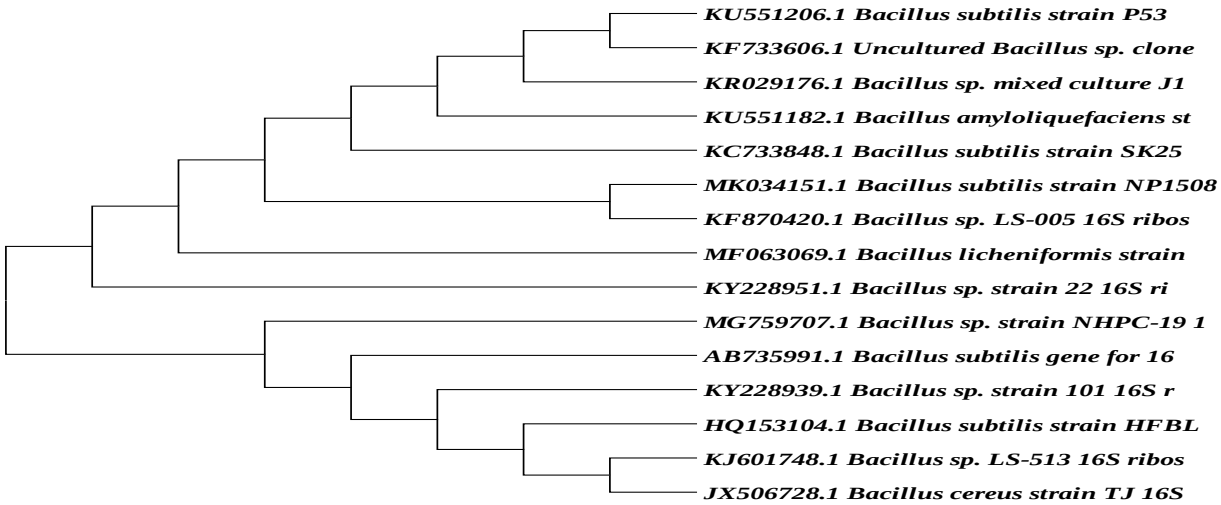

Figure 1. Maximum Likelihood Molecular Phylogenetic study of Isolate 1 reveals *Bacillus subtilis.* The FASTA sequence was BLASTed against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree.

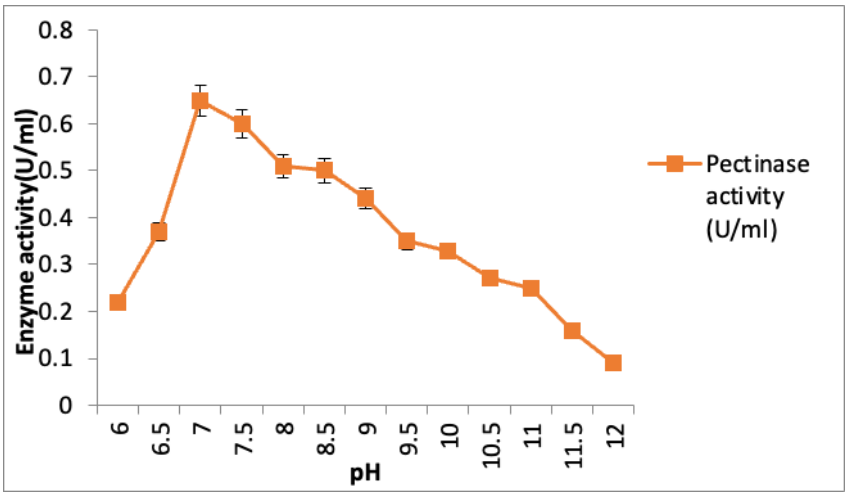

Figure 2. enzyme activity of *Bacillus subtilis* NP1508 With respective to different pH

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

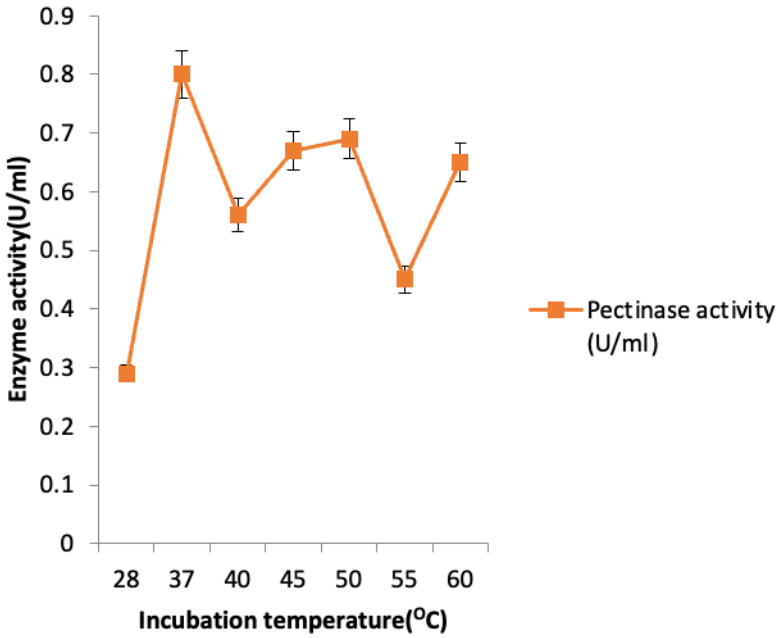

Figure 3. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different incubation temperature

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

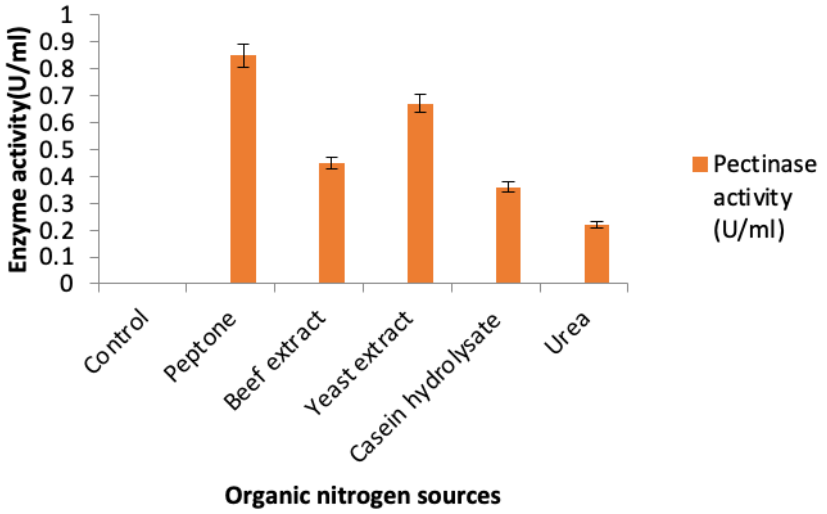

Figure 4. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different organic nitrogen source

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

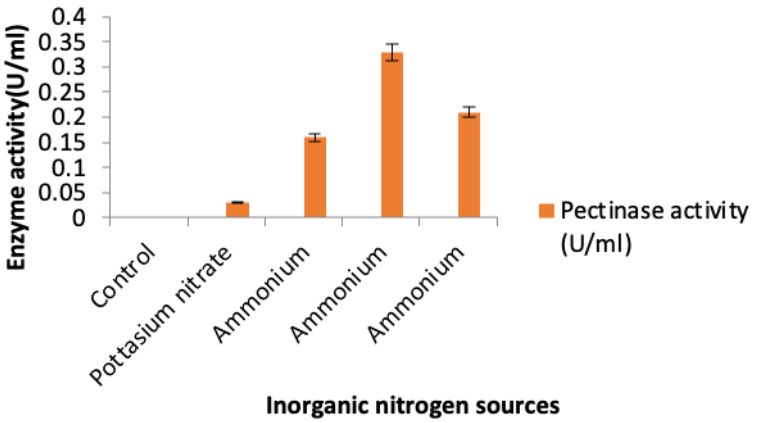

Figure 5. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different inorganic nitrogen source.

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

Bacillus subtilis was studied in detail for the pectinase production with respect to development of low-cost and easy available medium ingredient by submerged fermentation. As per the swain, submerged fermentation is the preferred method for the production of pectinase. Production medium was optimized for different physicochemical parameters. Among raw substrate, cassava wastes showed highest pectinase activity 0.94±0.07 U/ml (Table 1). The use of cost effective agro-residue for pectinase production in submerged fermentation would cause a substantial reduction in the cost of enzyme (Smith and Aidoo, 1988; and Pilar *et al.*, 1999).

Table 1. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different raw substrates

Raw substrates used	Enzyme activity (U/ml)	P value
Sugarcane	0.44+0.06	0.12
Paper pulp	0.67+0.08	0.13
Cassava waste	0.94+0.07	0.05
Tea waste	0.90+0.06	0.12
Orange peel	0.77+0.07	0.19
Molasses	0.84+0.08	0.01
Wheat bran	0.67+0.06	0.17
Agriculture waste	0.31+0.08	0.15

Pectinase production was higher at pH 7(Fig. 2), 37^{O}C temperature(Fig. 3), 2.5% inoculum, 24 hrs old culture, 48 hrs of incubation period, 150 rpm agitation rate. As shown in Table 1, cassava waste, tea waste and molasses shows highest activity. According to Kashyap *et al.* (2003), the pH and temperature conditions at which the soil strain Bacillus sp. DT7 produces the most extracellular pectinase are 7.2 and 37 °C. According to Sunnotel et al. (2002), using Bacillus sp. At 37 ^{O}C and pH 7.2 increases pectinase output. Our study shows similar results of optimum temperature and pH. This study shows that peptone and ammonium sulphate gives maximum pectinase activity as an organic and inorganic nitrogen source respectively (Fig. 4 & 5). Optimization parameters are represented in graphical form (See Fig. 2 to 5). According to Galiotou-Panayatou and Kapantai (1993), ammonium phosphate and ammonium sulphate did have a beneficial impact on pectinase production.

The greatest amount of pectinase could be produced, according to Phutela *et al.* (2005) when yeast extract and ammonium sulphate were present in the growth medium. According to Sarvamangala and Dayanand (2006) pectinase synthesis in submerged fermentation is increased by glucose and sucrose.

3.2. Application of pectinase in fruit juice clarification

Fruit juice clarification method used crude enzyme. According to this study, juice recovery increased along with rising enzyme concentration and incubation time. By combining these juice extraction methods with different pretreatments, such as cold, hot, and enzymatic extraction, the yield of juice can be improved (Chadha *et al.* 2003). Enzymatic treatment, when compared to cold and heated extraction methods, significantly increases juice recovery (Joshi *et al.* 1991). Juice that had been clarified by enzymes experienced a decrease in viscosity and the creation of clusters, which makes centrifugation or filtration easier. Because of this, the juice has greater clarity and more intense taste and colour (Abdullah *et al.* 2007). The sort of enzyme, incubation duration, temperature, enzyme concentration, agitation, ph, and use of various enzyme combinations all play a role in the biomaterial's enzymatic degradation (Baumann 1981). Grape and orange juice yield increased by increasing enzyme concentration and incubation time up to 180 min. (Table 2 and Table 3).

Table 2. Grape juice yield (%w/w) optimization by enzyme concentration and incubation time

Enzyme concentration mg/20g of pulp	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**180 min.**
0.5	63	62.5	63.5	63.5	64.5
1.0	63	63	63	64.5	64.5
1.5	64	64	64	65.5	65.5
2.0	64.5	64.5	65	65.5	66
2.5	64.5	65.5	65.5	66.5	66.5
3.0	65	65.5	66.5	66.5	66
3.5	65.5	66	66.5	66	68

Table 3. Orange juice yield (%w/w) optimization by enzyme concentration and incubation time

Enzyme concentration mg/20g of pulp	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**180 min.**
0.5	52	53.5	54.5	55	56
1.0	52	53	54.5	56	56.5
1.5	52.5	53.5	55	55.5	56
2.0	53	54.5	55	56	57
2.5	53.5	53.5	55	56.5	57.5
3.0	53.5	54	55	56	57.5
3.5	54	54.5	55	56.5	58

According to Yusof and Ibrahim (1994), the yield of juice increased with the volume of enzyme used and the length of the incubation period. Juice clarity increases after enzymatic therapy. As long as the temperature is below the enzyme's denaturation temperature, the temperature speeds up enzymatic processes and, consequently, the rate of clarification. In my study percentage transmission of grape and orange juice increase by increasing enzyme concentration and incubation time up to 180 min. (Table 4 and 5). When the incubation period changed for bananas, the brightness exhibited a similar pattern of behaviour (Lee *et al*. 2006).

Table 4. Effects of enzyme concentration and incubation time on the clarity of grape juice (%Transmission)

Enzyme concentration mg/20g of pulp	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**180 min.**
0.5	2.2	2.2	2.3	2.4	2.5
1.0	2.9	2.8	2.8	2.9	3.0
1.5	3.1	3.3	3.3	3.4	3.3
2.0	3.8	2.6	2.8	2.9	3.0
2.5	4.2	4.3	4.3	4.5	4.4
3.0	4.4	4.5	4.5	4.8	4.9
3.5	4.7	4.7	4.9	5.0	5.3

Table 5. Effects of enzyme concentration and incubation time on the clarity of orange juice (%Transmission)

Enzyme concentration mg/20g of pulp	30 min.	60 min.	90 min.	120 min.	180 min.
0.5	0.7	0.7	0.8	0.8	0.8
1.0	0.9	1.0	1.2	1.2	1.1
1.5	1.4	1.5	1.5	1.6	1.6
2.0	1.5	1.5	1.6	1.7	1.7
2.5	1.8	1.8	1.9	2.0	2.2
3.0	1.9	2.0	2.1	2.1	2.4
3.5	2.1	2.2	2.2	2.5	2.8

3.3. Use of pectinase for the removal of mucilage from coffee beans

Manually pulped fresh coffee grounds were used. The crude pectinase-infused water was used to soak half of the pulped legumes, while the other half underwent spontaneous fermentation. Within 24 hours of incubation, fully demucilized coffee seeds with pectinase treatment were visible. Demucilization, however, was not finished in the instance of natural fermentation within 36 hours of fermentation. These findings are consistent with the research conducted by Duy et al (2016). In the fermentation of coffee, pectinolytic bacteria are used to remove the mucilaginous coat from the coffee beans (Carr,1985; Kashyap et al., 2000 and Jayani et al., 2005).

4. Conclusions

Bacillus subtilis NP1508 produced pectinase in considerable amounts after 48 hours of fermentation medium incubation at 37 °C and 7.0 ph. With glucose and sucrose as the carbon source, peptone and yeast extract as the nitrogen source, and cassava waste, tea waste, and molasses as the substrate, enzyme output was at its highest. This bacterial enzyme was used to demucilate coffee beans and clarify fruit liquid. Increases in enzyme concentration and incubation time resulted in increases in fruit juice output and clarity. On coffee seeds that had received pectinase treatment, complete demucilization was seen within 24 hours of incubation.

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PHYTOCHEMICAL PROFILE OF *AGRIMONIA EUPATORIA* L. FROM BULGARIA AND EFFECTS OF ITS EXTRACTS ON *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA: PYRALIDAE) LARVAE

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ABSTRACT

Plants contain polyphenolic compounds such as phenolic acids, flavonoids, stilbenes, lignans, essential oils, *etc*., which are endowed with antioxidant properties. This study aimed to correlate the total phenolic compound and flavonoid content of *Agrimonia eupatoria* L. with its antioxidant properties and to determine some mineral elements (Na, K, Ca, Mg, P). The antioxidant capacity of the extract was tested by three methods (DPPH, FRAP, and TEAC). According to the results, the antioxidant content of *A. eupatoria* was found to be high (IC_{50}=38.03±0.01µg/mL). Total phenolic and flavonoid contents were found as 13.66±0.38 mg GAE/g and 4.65±0.01mg QE/g, respectively. Besides, the major components found in *A. eupatoria* were α-pinene (62.72%), n-hexadecanoic acid (11.41%), (5E,9E)-farnesyl acetone (6.64%), and (5E,9Z)-farnesyl acetone (3.65%). Heavy metal content in *A. eupatoria* was found within WHO limits. It was also investigated whether *A. eupatoria* has toxic effects. Because a medicinal plant is not supposed to harm metabolism, for this investigation, larvae stage of *Galleria mellonella* had selected as a test organism. In order to investigate the toxic effects, oxidative stress parameters (SOD, CAT, GST, GPx, MDA) and AChE activity were measured. And no harmful effects were observed at the doses administered at 24, 48, and 72 h.

1.Introduction

The vast variety of medicinal plants in Bulgaria, used in different forms, is increasing. Over 80% of the world's population uses various herbal supplements and infusions for treatments. The genus *Agrimonia* are perennial herbaceous flowering plants classified in Division Magnoliophyta, Magnoliopsida class, Rosaceae family, and the *Agrimonia* genus. Two plant species, *Agrimonia eupatoria* L., and *Agrimonia procera* Wallr., are naturally spread in Bulgaria (Petrova *et al*., 1999).

Polysaccharides, tannins, flavonoids, catechins, procyanidins, phytosterols, B vitamins, and vitamin K have been previously identified in *A. eupatoria* (Garcia-Oliveira *et al*., 2020). Polyphenols constituents in the plant species reduce the risk of several diseases or cause a curative effect (Pamukov, 1989). The content of flavonoids and other biologically active components correlates with other secondary metabolites and increases plant species' antioxidant potential and extracts.

Therefore, they form specific structural parts that inactivate free radicals in the body (Pamukov, 1989; Muruzovic *et al*., 2016).

Agrimony is well known for its beneficial effects on various diseases such as liver complaints, gall-bladder stones, diarrhea, edemas, and kidney diseases. Thanks to its diuretic properties, the herb is widely used against atony of the bladder and dysuria. Many other uses of Agrimony in Bulgarian folk medicine are known, such as rheumatism, hemorrhoids, bleeding gums, varicose ulcers; laryngitis; pulmonary and cutaneous tuberculosis. The extracts could be used externally as compressor gargle and internally as an infusion (Pamukov, 1989).

It was reported that more than 50 components have been identified in the essential oil composition of the plant's leaves, flowers, and roots. The major constituents in the wild growing leaf oil were β-caryophyllene, caryophyllene oxide, α-humulene, and E-β-farnesene, and in the cultivated leaf oil were α-pinene, β-caryophyllene, E-α-farnesene, and cumin aldehyde. The main constituents of the essential oil obtained by the wild-growing flowers were β-caryophyllene, E-β-farnesene, and a-humulene, while β-caryophyllene, caryophyllene oxide, and α-copaene were among the main constituents in the cultivated flowers (Muruzovic *et al.*, 2016; Navaei and Mirza, 2009).

The essential oil constituents from the aerial part of *A. asiatica* Juz. were obtained using a steam-distillation method in wild-growing conditions in Kazakhstan. The essential oil extracted from the aerial part of the plant was analyzed by gas chromatography-mass spectroscopy, and its major components amounting to 100% were found to be β-selinene (36.370%), α-panasinsene (21.720%), hexadecanoic acid (7.839%), and 1,2-nonadiene (6.199%) (Kozykeyeva *et al*., 2020).

Galleria mellonella (L.) (Lepidoptera: Pyralidae) is a greater wax moth and found throughout the world. *G. mellonella* has four life stages and eggs are laid in the spring. Investigating the toxicity of medicinal plants is a necessity prior their usage. The vertebrate model organisms used for this objective are often subject to ethical considerations. This has led researchers to prefer invertebrate models. Invertebrate models, including *Galleria mellonella*, are used to assess the toxicity of various products. The larvae of *G. mellonella* are also often used as a model organism in researchs (Arsene *et al.*, 2021).

A detailed phytochemical profile of medicinal plants was necessary due to the local differences in their composition. The studies contributed to the popularization of medicinal plants in Bulgaria and provided an opportunity for their application in phytotherapy and pharmacy. The discovery of new biologically active components in the composition of plant species gives a strong impetus to creating herbal pharmaceutical prototypes in modern medicine.

Therefore, the present study aimed to analyze the phytochemical profile of a traditional Bulgarian phytotherapy species - *Agrimonia eupatoria* L. In addition, since the toxicity tests of medicinal plants are essential for safe use, it was also investigated whether *A. eupatoria* has harmful effects on *Galleria mellonella* larvae.

2. Materials and methods

2.1. Materials

2.1. Plant material

The plants were collected in October 2016 from South Bulgaria (at 553 m elev., 42.85 ° N 26.15 ° E) - these are the coordinates of the village of Bozhevtsi. The samples were collected by hand and dried in ventilated rooms without direct sunlight, and the room air temperature was regulated (18±2°C). After drying, the samples were placed in paper bags for storage. The aerial plant parts were separated into leaves before analysis.

2.2. Methods

2.2.1. Chemical composition of the plants

The moisture of the plants was determined by drying up to the constant weight at 105°C and the results from the chemical analyses were given on a dry weight (DW) basis. The ash

content was determined according to AOAC (2005), by mineralization of the samples at 550°C for 5 h.

2.2.2. Isolation of essential oil

The air-plants (50 g) were cut to a size of 0.5 cm. The essential oil was isolated by hydrodistillation (ratio plant:water = 1:10) for 3 h in a Clevenger-type laboratory glass apparatus (Balinova and Diakov, 1974). The oil obtained was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until analysis.

2.2.3. Chromatography–Mass Spectrometry (GC-MS) analysis

The compounds of the essential oil were detected with gas chromatography (GC) (Agilent 7890A), HP-5 column MS (30 m × 250 mm × 0.25 μm), temperature: 35°C/3 min, 5°C/min to 250°C for 3 min, 49 min in total, helium as carrier gas, 1 mL/min constant speed, 30:1 split ratio. A gas chromatography–mass spectrometric (GC/MS) analysis was carried out on an Agilent 5975C mass spectrometer, helium as a carrier gas, column and temperature the same as in the GC analysis. The identification of the chemical compounds was made by comparison to their relative retention time and library data (NIST 08 database; own libraries) (Adams and Robert, 2007). Components were listed according to their retention (Kovat's) indices, calculated using a standard calibration mixture of C_8 - C_{40} n-alkanes in n-hexane. Compound concentration was computed as percentage of the total ion current (TIC).

2.2.4. Protein content

The total protein content was analyzed according to the method of AOAC (2016) with a UDK 152 Kjeldahl System (Velp Scientiffica, Italy). The samples 1.0 g each, were mineralized in 15 mL concentrated H_2SO_4 and catalysts: anhydrous K_2SO_4 and CuSO4. The process was run at 420°C for 60 min. With this method, 40% NaOH was used to produce an alkaline distillation medium and 4% H_3BO_3 in order to collect the distilled ammonia. The titrations were carried out with a standard HCl (0.2 N) solution.

2.2.5. Cellulose content

The content of cellulose (crude fiber) in leaves was determined by a modification of the method by Brendel *et al.* (2000). Hydrolysis of cellulose and hemicellulose was carried out by boiling 1 g of leaves with 16.5 mL of 80% CH_3COOH and 1.5 mL concentrated HNO_3 for 1.5 h. After filtration of the suspension, the solid residue was dried at 105°C for 24 h and weighed.

2.2.6. Total chlorophylls and carotenoid content

For evaluation of chlorophyll a, chlorophyll b and the total carotenoids content, 0.5 g of fresh leaf sample was homogenized with 10 ml extract (80% alkaline acetone) and stored in the dark at 25°C for 24 h. After that, the homogenate was centrifuged at 1500 g for 10 min. Absorbance was measured at 470 nm, 645 nm and 663 nm; then, the results were calculated by the corresponding formulas (Côrte-*Real et al*., 2017):

Chlorophyll a (mg/L) = 9.784*A663-0.990*A645 (1)

Chlorophyll b (mg/L) = 21.426*A645-4.650*A663 (2)

Total carotenoids content (mg/L) = 4.695*A470-0.268*(chl a + chl b) (3)

Finally, mg/L unit was converted into μg/g.

2.2.7. Nutrient contents

Plant samples (areal part) were dried and 0.5 g was weighed. Then, each sample put into a porcelain crucible. All samples were burned until gray ash (550°C). After burning the 0.5 g weighed samples, the ashes were dissolved in 4 mL 0.1 N HCl and filtered (Whatman No 1), and completed with distilled water (10 mL) (Kaçar and İnal, 2010). Mineral and heavy metal contents was determined by Yozgat Bozok University, Science and Technology Application and Research Center using iCAP-Qc ICP-MS spectrometer (Thermo Scientific).

2.2.8. Total Phenolic Contents

Folin-Ciocalteu Reagent (FCR) method was used to determine the total phenolic content of the extracts (Singleton *et al.,* 1999). The prepared samples were incubated at room temperature (20±1°C) for 2 h and absorbance

measurement was performed at 765 nm. Gallic acid was used for standard phenolic substance control. The values obtained are expressed as gallic acid conjugate. Spectrophotometric measurements to determine the total phenolic content PerkinElmer Lambda 25 UV / VIS made in spectrophotometer device.

2.2.9. Determinations of Total Flavonoid Assay

The total flavonoid compound amounts of the 40 mL methanol extracts were determined by optimizing the aluminum chloride colorimetric method of Biju *et al*., 2014. Then absorbance was measured at 510 nm. As result of quercetin equivalents (QE) g-1 of extract was calculated.

2.2.10. DPPH Free Radical-Scavenging Activity

The plant (leaf) sample (4 g) was mixed by methanol (40 mL) (1/10 w/v). The prepared samples were incubated for 24 h at 40°C in an oven (Electo-mag M 5040 P). Then, it was filtered into balloon flasks (Whatman No 1 filter paper). The methanol in the samples was removed with the help of a rotary evaporator (Heating Bath B-491, BUCHI). The balloon bottles, which were blown up, were kept in the oven for 24 h and completely dried. The extracts were taken into falcon tubes and closed with parafilm and stored at +4°C to be used in the analysis.

The free radical activities of the extracts were determined using DPPH (1,1-diphenyl-2-picrilhydrazyl) free radical, a known and commonly used radical (Gezer *et al*., 2006). Firstly, the amount of extract that defines a certain amount of DPPH radical has been determined, and a comparison has been made between these samples. 16 mg DPPH radical solution was prepared in 100 mL CH_3OH. The DPPH solution to be used in the analysis was prepared as 0.1 µM. By setting 517 nm in the spectrophotometer, DPPH reading was done and dilution was made with methanol until the absorbance value was 1.000. One mg/mL extract solution was prepared as main stock and 6 different concentrations were obtained by dilution. 3 mL samples were taken from each concentration (50, 75, 100, 150, 200, 300) and 1 mL 0.1 µM DPPH was added on top. The reaction mixture was incubated for 30 min in the dark. BHT (butyl hydroxytoluene) and BHA (butyl hydroxyanisol) were used as reference. Radical scavenging activity DPPH was determined as the inhibition percentage and the following formula is used:

Radical scavenging activity DPPH %= [A blank −A sample)/A blank] x100

Spectrophotometric measurements for DPPH radical scavenging activity determination were performed with the aid of PerkinElmer Lambda 25 UV / VIS spectrophotometer device.

2.2.11. Ferric reducing antioxidant power assay (FRAP)

The antioxidant capacity of samples was detected by Benzie and Strain's FRAP assay. Dried samples (4 g) were extracted with distilled water (40 mL) at a temperature from 80 to 105°C for 20 min for fraction I. The residues were extracted with distilled water (60 mL) at a temperature from 100 to 130°C for 30 min for fraction II. After cooling to 25°C, both fractions were filtered. They were combined and dried at 40°C and weighed to detect the yield (Benzie and Strain, 1999). This method measures the ability of antioxidants to reduce ferric iron. This assay evaluates the alteration in absorbance at 620 nm because of the generation of FeII-tripyridyltriazine from oxidised FeIII. The reagent was made ready via mixing acetate buffer (300 mmol/L) with 2,4,6-tripyridyl-s-triazine (10 mmol/L) (TPTZ) in HCl (40 mmol/L) and with ferric chloride (20 mmol/L) at low pH. As the standard, Trolox® was used. Samples were quantified by a spectrophotometer (PerkinElmer Lambda 25 UV / VIS).

2.2.12. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is consisted in the reducing of the absorbance of the ABTS+ (Re *et al*., 1999) at 734 nm. Dried samples (4 g) were extracted with distilled water (40 mL) for 20 min at a temperature of 80 to 105°C to give Fraction I. The residues were extracted with distilled water (60 mL) at a temperature of 100 to 130°C for 30 min to give Fraction II. After cooling to 25°C, both fractions were filtered. These were

combined and dried at 40°C and weighed to determine yield (Benzie and Strain, 1999).

ABTS+ was prepared by reacting ABTS solution with potassium persulfate (2.45 mM). ABTS+ solution was diluted with phosphate buffer for obtaining an absorbance of 0.7±0.02 at 734 nm. Diluted ABTS+ was added to Trolox® standard or biological sample, then this admixture was incubated for 15 min. After this step, at 734 nm, the inhibition in absorbance was evaluated. All evaluations were performed in 6 repetitions. Samples were quantified by a spectrophotometer (PerkinElmer Lambda 25 UV / VIS).

2.2.13. Determination of hydroxyl radical scavenging capacity

Hydroxyl radical scavenging capacity was assessed by detected the ability of leaf sample extracts to reduce the generation of 2-hydroxyterephthalate which is a strongly fluorescent in a reaction between terephthalic acid and hydroxyl radical (Gutteridge and Halliwell, 2010).

2.2.14. Determination of superoxide scavenging capacity

Superoxide scavenging capacity was determined as the superoxide radical inhibition caused decreasing of nitro blue tetrazolium to formazan (McCord and Fridovich, 1999).

Cultivation of Galleria mellonella and application of Agrimonia eupatoria extract to Galleria mellonella larvae

Galleria mellonella were taken from the stock culture of Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Plant Protection. Corn flour, water, bran, milk powder, honey, glycerol, yeast, honey nutrients were used in the cultivation of *G. mellonella*. The last stage larvae were taken from the prepared cultures and used in the experiments. Cultures were placed in an incubator adjusted to 28±2°C, 65±5% relative humidity.

Four groups are formed for this study (control, 5% extract concentration group, 10% extract concentration group and 20% extract concentration group). Different concentrations of *A. eupatoria* extract (5%, 10%, 20%) were injected into the application groups with a microinjector in the amount of 5 µl to the left last leg of each larva. The same amount of distilled water was given to the control group. The duration of the experiment is 24 h, 48 h and 72 h. 20 insects were used for each group. 3 repetitions were made.

Measurement of malondialdehyde (MDA) levels, acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities

Oxidative stress parameters (MDA levels, SOD, CAT, GST, GPx) and AChE activities were measured according to following procedures.

MDA: Ohkawa *et al.* (1979)
GST: Habig *et al.* (1974)
SOD: Marklund and Marklund (1974)
CAT: Aebi (1984)
GPx: Paglia and Valentine (1961)
AChE: Ellman *et al.* (1961)

2.2.15. Statistics

All measurements (antioxidants and mineral matters) were carried out in triplicates. The results were expressed as mean ± SD and analyzed using MS-Excel software. The data obtained in the study (Galleria mellonella analyses) were evaluated using the One-Way Analysis of Variance (ANOVA) and Tukey test in the Windows SPSS 26.0 computer program. A P value of <0.05 was considered statistically significant.

3. Results and discussions

3.1. Chemical composition

A. eupatoria has been used to treat various diseases for centuries in many parts of the world, especially in Europe. It is also widely used as an herbal tea due to the plant's flavor (Tomlinson *et al.*, 2003). The chemical composition of *A. eupatoria* is presented in Table 1.

According to Table 1, the essential oil (EO) rate was found to be 0.02%. According to the studies, the oil yield based on the dry weight of samples was 0.2% per leaf (Muruzović *et al.*, 2016; Kozykeyeva *et al.*, 2020). *A. eupatoria* also contains 1.2-1.6% of flavonoids, which belong to different subgroups. According to the

literature, the amount of polyphenol in the. *A. eupatoria* plant was obtained as 124.5±0.032 GAE/g (Lee *et al*., 2010; Ciobanu *et al.*, 2018).

The antioxidant activity of *A. eupatoria* is shown in Table 2. DPPH radical scavenging activity was determined as 38.03 µg/mL and also in comparison with BHA and BHT samples. BHA and BHT are synthetic antioxidants used as food additive to prevent deterioration (Norhasidah *et al*., 2014). Some extracts contain less essential oil flavonoid antioxidants and their observed antioxidant activity is comparable to BHT such as ginger, rosemary, and sage (Sekretar *et al*., 2004). A study of the antioxidant activity of *A. eupatoria* (Agrimony) extracts was measured and evaluated in the DPPH radical scavenging and ABTS radical decolorization reaction systems. The radical scavenging capacity of *A. eupatoria* extracts varied in a wide range (9.1-97.5% in DPPH reaction and 6.7-79.5% in ABTS reaction) depending on the polarity of the solvent used to obtain the extract (Venskutonis *et al.*, 2007). A study found the most significant antioxidant capacity for *Agrimonia* herba (IC_{50} 45.55 µg/mL) (Ciobanu *et al*., 2018). Our results are lower than that reported by Muruzović *et al.* (2016) who studied the concentration of total phenols, flavonoids, the antioxidant activity of the water, diethyl ether, acetone, and ethanol extracts of *A. eupatoria*. The concentration of total phenols was obtained ranged from 19.61 mg GA/g to 220.31 mg GA/g. The concentration of flavonoids was obtained ranged from 20.58 mg RU/g to 97.06 mg RU/g. Differences obtained for the antioxidant activity of the extracts and their phenolic and flavonoid content could be due to the geographical characteristics of the plants and differences in the methods used for examining the activities.

The *A. eupatoria* essential oil chemical composition is shown in Table 3.

Table 1. The chemical composition of *A. eupatoria*

Parameters	Leaves
Moisture, %	6.69
Yield of essential oil, % (v/w)	0.02
Protein, %	14.13
Cellulose, %	21.01
Ash, %	7.48
Chlorophyll a, µg/g dw	72.22
Chlorophyll b, µg/g dw	1456.00
Total carotenoids, µg/g dw	82.51
Total phenol contents, mg GAE /g	13.66 ± 0.38
Total flavonoid assay, mg QE/g	4.65 ± 0.01

Table 2. Antioxidants activity of *A. eupatoria*

Methods	Leaves
FRAP assay, µmol/L	864.00± 0.01
TEAC assay, µmol/L	57.97± 0.02
IC50 value, µg/mL	38.03± 0.01
BHA	19.66± 0.0
BHT	13.81± 0.0
Hydroxyl radical scavenging capacity, mM EtOH/mL	16.8 ± 5.8
Superoxide scavenging capacity, unit SOD/mL	**14.6 ± 3.7**

Table 3. Chemical composition of *A. eupatoria* essential oil

№	RT, min	RI a	Compounds	Content (% of TIC b)
1	10.05	930	α-Pinene	62.72 ± 0.61
2	11.50	976	β-Pinene	1.23 ± 0.01
3	12.31	999	n-Octanal	0.33 ± 0.0
4	13.22	1024	D-Limonene	0.71 ± 0.0
5	15.57	1099	n-Nonanal	0.85 ± 0.0
6	16.28	1110	6-Campholenol	0.52 ± 0.0
7	25.24	1450	Geranyl acetone	0.98 ± 0.0
8	26.00	1480	methyl-γ-Ionone	1.37 ± 0.01
9	26.78	1512	Tridecanal	0.57 ±0.0
10	27.88	1562	(E)-Nerolidol	0.51 ±0.0
11	28.37	1577	Spathulenol	0.75 ±0.0
12	28.58	1581	Caryophyllene oxide	1.44 ± 0.01
13	29.29	1630	γ-Eudesmol	1.38 ± 0.01
14	34.17	1861	(Z,Z)-Farnesyl acetone	1.72 ± 0.01
15	34.49	1883	(5E,9Z)-Farnesyl acetone	3.65 ± 0.03
16	36.30	1922	(5E,9E)-Farnesyl acetone	6.64 ± 0.016
17	36.69	1957	n-Hexadecanoic acid	11.41 ± 0.11
18	39.73	2130	Linoleic acid	0.46 ± 0.0
19	39.95	2141	Oleic acid	1.05 ± 0.0
20	40.03	2152	Linolenic acid	0.12 ± 0.0
Oxygenated aliphatics,%				15.02
Monoterpene hydrocarbons,%				65.70
Oxygenated monoterpenes,%				2.93
Oxygenated sesquiterpenes,%				16.35

a RI – retention (Kovat's) index; b TIC – total ion curren

A. eupatoria EO obtained from flowers was characterized by 20 constituents representing 98.41% of the total oil content. Ten of the EO constituents were with concentrations above 1%. The main EO constituents (over 3%) were: α-pinene (62.72%), n-hexadecanoic acid (11.41%), (5E,9E)-farnesyl acetone (6.64%), and (5E,9Z)-farnesyl acetone (3.65%). The distribution of significant groups of aroma substances in oils is shown in Table 3. Monoterpene hydrocarbons (65.70%) are the dominant group in the oil, followed by oxygenated sesquiterpenes (16.35%), oxygenated aliphatics (15.02%), and oxygenated monoterpenes (2.93%).

Due to its wide application opportunities, the *A. eupatoria* essential oil was studied by several researchers. Navaei and Mirza (2009) analyzed the essential oils of leaves and flowers in wild and cultivated forms of *A. eupatoria* distributed in Iran. As a result of the study, they reported the main components of wild leaves as β-caryophyllene (59.6%), caryophyllene oxide (10.4%), and the main components of culture leave as α-pinene (28.2%), β-caryophyllene (20%).

Jin *et al.* (2010) obtained the chemical composition of essential oils of *A. pilosa* collected from 3 different regions of China by micro distillation and traditional distillation methods and compared them by making their

analyzes. In total, 49 compounds were identified. They determined hexadecanoic acid with 11.83-41.18% as the main component. Other components were linolenic acid methyl ester (1.93-13.45%), α-curcumene (trace-7.88%), p-propenylanisol (trace-6.55%), and α-bisabolol (0.94% 6.27%) (Wang *et al.*, 2012). The essential oil obtained by water distillation of the leaves and roots of *A. eupatoria* grown in the Zhejiang region of China was analyzed by GK-KS, and 68 compounds from the root and 65 compounds from the leaves were identified. Common main components in both leaves and roots were determined as cedrol (14.37%), α-pinene (8.31%), and linalool (5.72%) (Feng *et al.*, 2013). The essential oil obtained by steam distillation of the aerial part of *A. aitchisonii* (Rosaceae) collected from India was examined by GK/KS and NMR spectroscopy, and it was reported that the oil was rich in methyl mirtenate (62.4%). Other major constituents were defined as limonene (7.2%), linalyl acetate (5.9%), linalool (4.8%), mirtenyl acetate (4.6%), and zingiberene (2.4%) (Melkani *et al.*, 2007).

3.2. Nutrient contents

Today, the analytical determination of heavy metals in medicinal and aromatic plants is among the most important quality parameters in determining these plants' purity, safety, and efficacy.

In our study, macro-and microelements of *A. eupatoria* are presented in Figure 2 and Figure 3. With regard to total dry matter, the order of limiting nutrients was K > Ca >Mg> P >Na for macro-elements and Mn > Fe > Cu > B for micro-elements. Also, the order of heavy metals contents were Sr > Co >Rb > Zn> Ba >Ni>Cr (Fig 4).

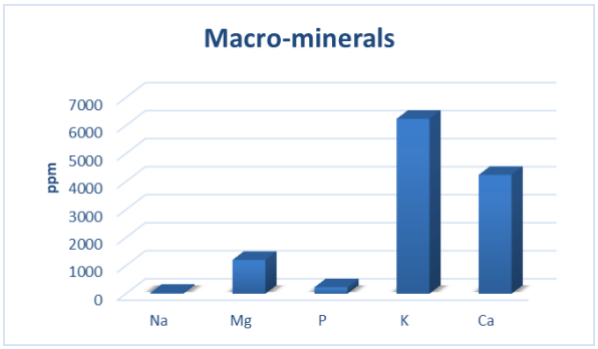

Figure 2. Macro- minerals in *Agrimonia eupatoria* L. (ppm)

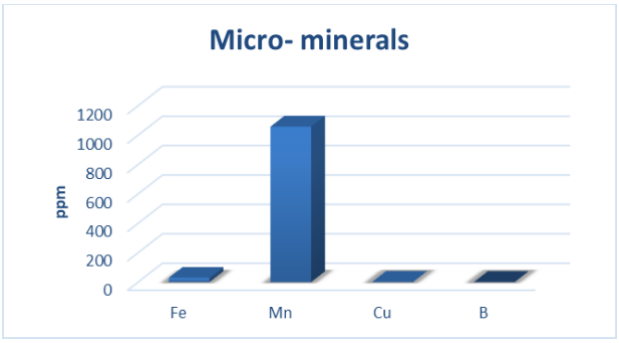

Figure 3. Micro- minerals in *Agrimonia eupatoria* L. (ppm)

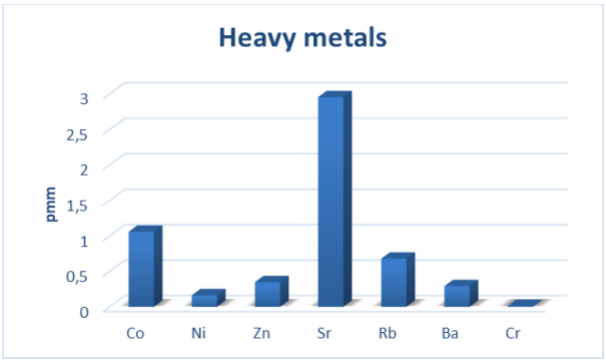

Figure 4. Heavy metals in *Agrimonia eupatoria* L. (ppm)

The limit values for Cr, Ni, and Zn determined by the WHO/FDA are 1.30, 0.02, and 50 ppm. Zinc (Zn) is an essential trace element that plays a vital role in many organisms' physiological and metabolic processes. Nevertheless, higher zinc

concentrations can be toxic to the organism (Doğan, 2020; Şenkal *et al.*, 2019).

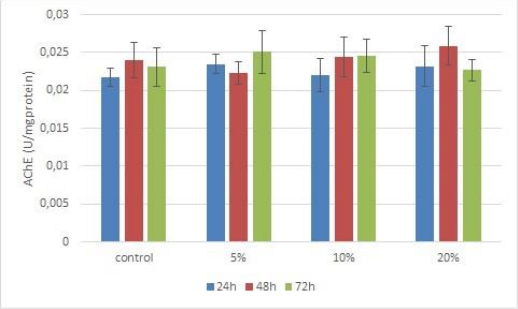

Figure 5. Effects of *A. eupatoria* extract on AChE activities of larvae of *Galleria mellonell*a. (There are no statistical differences between groups.)

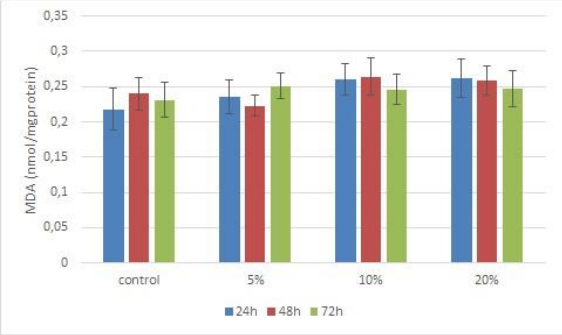

Figure 6. Effects of *A. eupatoria* extract on MDA levels of larvae of *Galleria mellonella*. (There are no statistical differences between groups.)

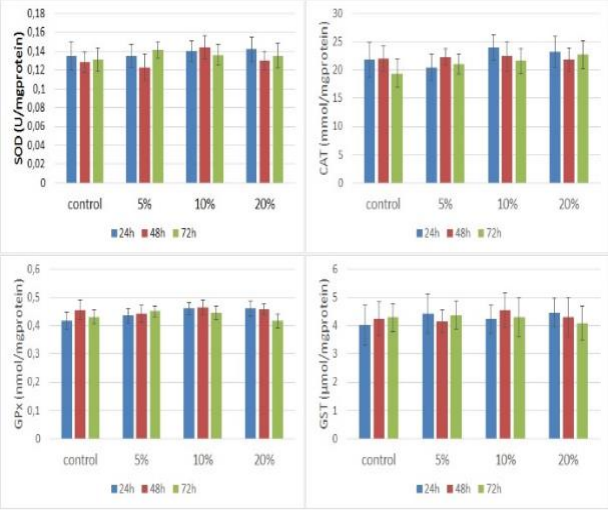

Figure 7. Effects of *A. eupatoria* extract on SOD, CAT, GST and GPx activities of larvae of *G. mellonella*. (There are no statistical differences between groups.)

LPO is the main event that plays an important role in xenobiotic toxicity (Apaydin *et al.*, 2014). Since MDA is the end product of LPO, increased MDA is an important indicator of LPO (Kara *et al.*, 2016). Cells have various defense mechanisms against oxidative damage, and these enzymatic antioxidants in tissues neutralize the oxidative stress that occurs due to the formation of free radicals (Bas and Pandır, 2016). Therefore, if the antioxidant enzyme activity is insufficient in the cell, the free radical level increases; for this reason, the activity determination of these enzymes is important in the determination of oxidative stress and so toxicity of a substance (Bas *et al.*, 2014). No change in MDA level and antioxidant enzyme activities depending on the dose applied in this study is proof that extract of *A. eupatoria* doesn't cause oxidative stress.

Acetylcholine esterase (AChE), found in tissues, is an enzyme that can hydrolyze acetylcholine. Xenobiotics can also exert their toxic effect by inhibiting AChE. As a result of inhibited AChE, acetylcholine accumulation occurs in the synapses, and therefore continuous stimulation occurs in the cholinergic system (Hazarika *et al.*, 2003). If this enzyme is inhibited, acetylcholine molecules can send the muscles to contract continuously, causing partial or general paralysis. Xenobiotics often work on this principle. No change in AChE activity due to increasing application doses in this study suggests that extract of *A. eupatoria* can be used as a medicinal plant.

4. Conclusions

Based on the study of *A. eupatoria* essential oil for their chemical composition, phenolic content, flavonoid content, and antioxidant activity, it could be concluded that all the studied samples are important sources of biologically active compounds. We can reveal that *A. eupatoria*, which has a high antioxidant effect, will also be a pioneer for toxicological studies. Toxicological tests on medicinal, aromatic plants are limited, and for their safe use, toxicological tests are required. Therefore, we

conducted this research on *G. mellonella* larvae, as a model organism. As a result, it was determined that the applied doses did not affect the parameters observed. These results will also be a beginning study for future toxicological research.

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EVALUATION OF QUALITY PARAMETERS OF PARBOILED AND NON-PARBOILED ZINC BIOFORTIFIED BRRI DHAN84 RICE VARIETY IN BANGLADESH

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ABSTRACT

Zinc deficiency is a global public health problem in developing countries, including Bangladesh, where rice is the primary consumed staple food. To combat zinc deficiency in Bangladesh, BRRI dhan84 (paddy) has been released as a zinc-biofortified and high-yielding rice variety. Therefore, the objective of this study was to evaluate the changes in physicochemical properties, nutritional attributes, and contents of zinc in newly released rice variety after parboiling and cooking using standard methods. Significant differences were noted among different properties. The study revealed that the parboiling method increased fat (2.36%), protein (9.42%), and zinc (2.741%), whereas those not using parboiling methods resulted in 1.72% fat, 6.68% protein and 2.732% zinc. In contrast, other components like fiber, carbohydrates, ash, and iron decreased after parboiling. Physicochemical analysis showed that parboiled rice was found to be slightly higher in length (6.507 mm), broader in width (2.032 mm), lower in solid gruel loss (0.090%), and also higher in head rice yield (80.09%) than non-parboiled rice. A prolonged cooking time of approximately 39.41 minutes was observed in parboiled rice as compared to non-parboiled rice. However, the cooking process negatively affected the nutrient contents of both parboiled and non-parboiled rice than uncooked or raw rice. Zinc content was generally similar between parboiled and non-parboiled rice after cooking (2.264-2.344 mg/100g). The sensory test further revealed that the parboiled rice obtained more overall acceptability, although the rice color was darker than that of non-parboiled rice. The study concluded that, depending on desired physicochemical and nutritional properties, the zinc-biofortified BRRI dhan84 rice variety could be the important option for improving rice grain nutritional quality to overcome the micronutrient problem in Bangladesh.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most common and consumed cereals worldwide, and it is considered a staple food by 50% of the world's population (Liu et al., 2019). A significant proportion (50-85%) of the daily energy requirements of the world's population, especially those living in Asian and African continents, are fulfilled by their daily rice consumption (Zeng et al., 2010). In general, rice contains macronutrients, mainly carbohydrates, protein, and dietary fibers, together with micronutrients, including vitamin A and several B vitamins, and also minerals. It also contains trace minerals, most importantly zinc (Zn), iron (Fe), manganese (Mn), and copper (Cu), and the relative contents of these trace minerals may vary depending on several factors, typically varieties, fertilizer used, soil type, milling technique, environmental condition, and cultivation methods, etc. (Verma & Srivastav, 2017). In particular, the level of Zn (11–16 μg/g) in rice is subjective to the milling process and thus distinctly varied from county to county, causing the low intake of Zn when compared with estimated average requirements (EAR) in a population group (Mayer et al., 2008). According to the World Health Organization (WHO), zinc deficiency is one of the fifth leading etiological causes of illness and diseases in developing countries, ranked eleventh globally (Cakmak, 2008). Globally, about 17% of the population is estimated to be at risk of inadequate dietary zinc intake, and the number is as high as 22% in East and Southeast Asia (Wessells & Brown, 2012). An additional 175 million people globally, including 63 million in South Asia, are expected to become Zn deficient by 2050 (Smith & Myers, 2018). In Bangladesh, numerous people, especially children and pregnant women, suffer from micronutrient deficiencies, particularly zinc insufficiency (Nguyen et al., 2014). Hence, there is a continuous search to improve the deficiency problem of Zn by adopting dietary-based interventions relating to cost-effective management practices such as genetically modified plant breeding and biofortification.

Parboiling is a hydrothermal process that alters the quality and processing behaviors of rice by first soaking the paddy in water to become tender, followed by draining, heating, and or steaming to precook the grain, and finally drying (Bhattacharya, 2011). This plays a vital role in postharvest handling and processing, including storage, milling, cooking, and eating quality (Patindol et al., 2008). During milling, the breakage of parboiled rice is significantly decreased by healing the cracks and chalkiness of rice kernels. Parboiling also improves the nutritional quality of rice, preventing the loss of vitamins and other micronutrients during milling. Cooked parboiled rice is firmer, fluffier, and less sticky than cooked raw rice (Bhattacharya, 2011). Mayer et al. (2008) evaluated the impact of milling on Bangladeshi parboiled rice, reporting 24–39% lower Zn concentration in milled compared to non-milled (brown) parboiled rice. Biswas et al. (2018) reported that Zn concentration in milled parboiled rice was 8–20% lower compared to brown parboiled rice, but it was 13–28% higher than milled non-parboiled rice. In contrast, Denardin et al. (2004) reported 44% lower Zn concentration in milled parboiled rice compared to milled non-parboiled rice without reporting the degree of milling.

HarvestPlus and its partners specifically target these essential micronutrients to improve the nutritional profile of commonly consumed staple crops such as maize, wheat, and rice to address this nutritional gap. (Jena et al., 2018; White & Broadley, 2009). A study conducted in Asia has revealed that biofortified rice varieties with up to 28 micrograms per gram in the milled grain have improved the Zn intake of Zn deficient population groups (Andersson et al., 2017). Thus, this establishes the opportunity for increasing daily Zn intake, particularly when combined with dietary diversity and nutritional education approaches (Singh et al., 2016), which could combat and eradicate deficiency problems of this

micronutrient (Bouis et al., 2011). Though several strategies currently exist to combat micronutrient deficiencies or "hidden hunger" through food fortification, distribution of dietary supplements, and promotion of dietary diversification (Bouis et al., 2011), in many situations, these methods are unsuccessful due to elevated costs, complex logistics, low compliance, and limited access to various foods (Singh et al., 2016). Over the past few decades, Bangladesh Rice Research Institute (BRRI), in collaboration with Harvest Plus, has developed five open-pollinated zinc rice cultivars, such as BRRI dhan62, BRRI dhan64, BRRI dhan72, BRRI dhan74, and BRRI dhan84 in the years of 2013, 2014, 2015, 2015, and 2017 respectively. Among all these varieties, BBRI dhan84 is a newly developed improved variety with a high yield over the other zinc biofortified rice varieties for the Boro season. It was genetically modified through crossing between BRRI dhan29/IR68144//BRRIdhan28///BR11 to get a higher zinc content in polished grain. BRRI dhan84 is a fine-slender-grain with a red pericarp (Kader et al., 2020). This new zinc rice variety offers competitive yield characteristics with market-leading varieties for commercial production. BRRI dhan84 contains the highest zinc level (27.6 ppm) among the other zinc rice varieties. The protein and amylose percentages of BRRI dhan84 are 9.7% and 25.9%, respectively. BRRI dhan84 is a straight type that can be processed in any milling machine, with a milling outturn of 70% and a head rice recovery of 53% (Kader et al., 2020).

Recently, a grain quality characterization and a sensory acceptability analysis were carried out with two varieties of zinc-biofortified rice and local control by Woods et al. (2020). Their results showed that the grain quality properties of rice have an influence on acceptability and that consumers accept zinc-biofortified rice varieties. In another study, Hotz et al. (2015) determined the increase in zinc and iron content in Bangladeshi rice varieties when zinc sulfate and iron–EDTA were added to the soaking water before parboiling, using local parboiling conditions. The results displayed that the addition of 1300 mg zinc L^{-1} increased raw polished rice zinc content from 16.6 to 44.9 mg kg^{-1} and from 12.6 to 32.9 mg kg^{-1} in the open and closed parboiling systems, equivalent to 170% and 161% increases, respectively. Retention of zinc after washing and cooking was 70–81% across all concentrations tested (Hotz et al., 2015).

Numerous researches have already been performed regarding the nutritional properties, functional properties, and other effects of rice and rice bran. However, to the best of our knowledge, no detailed study has been conducted concerning the proximate, functional properties, and cooking qualities of zinc-biofortified rice in Bangladesh. Therefore, the current study is designed to determine the proximate composition, physicochemical properties, and cooking characteristics of parboiled and non-parboiled zinc-biofortified BRRI dhan84 rice so that after consuming this product the deficiency of Zn could be reduced in Bangladesh.

2. Materials and methods

2.1. Rice samples

A newly released zinc biofortified rice (BRRI dhan84) was collected from the regional branch of the Bangladesh Rice Research Institute (BRRI), Rangpur, Bangladesh. After collection, foreign materials were removed from the paddy and separated into two batches. Each batch was individually packed in a polythene bag and stored at room temperature. One batch was processed further as a parboiled rice sample, and the other was considered as a non-parboiled sample.

2.2. Sample Preparation

2.2.1. Parboiling and milling technique of BRRI dhan84

The collected paddy sample was divided into two portions. One portion was used as non-parboiled, and another portion was utilized for parboiling operation. The general workflow of this study is shown in Figure 1. This study used the traditional parboiling method, where paddy was first soaked in normal water (1:4 w/v) for

12 hours at ambient temperature. The water was drained, and then the hydrated paddy was placed in an aluminum pot with water for boiling. Boiling was stopped whenever the paddy seemed to be boiled by visual inspection. Then, the boiled paddy was spread on the floor under the sun for drying. In the case of non-parboiling rice, collected paddy was directly dried under the sun without soaking and boiling.

The dried parboiled and non-parboiled paddy was dehulled using a laboratory dehusking machine (model number- THU35B-3-T). All the dehulled rice was put in sealed polyethylene bags without milling and stored at room temperature for further analysis.

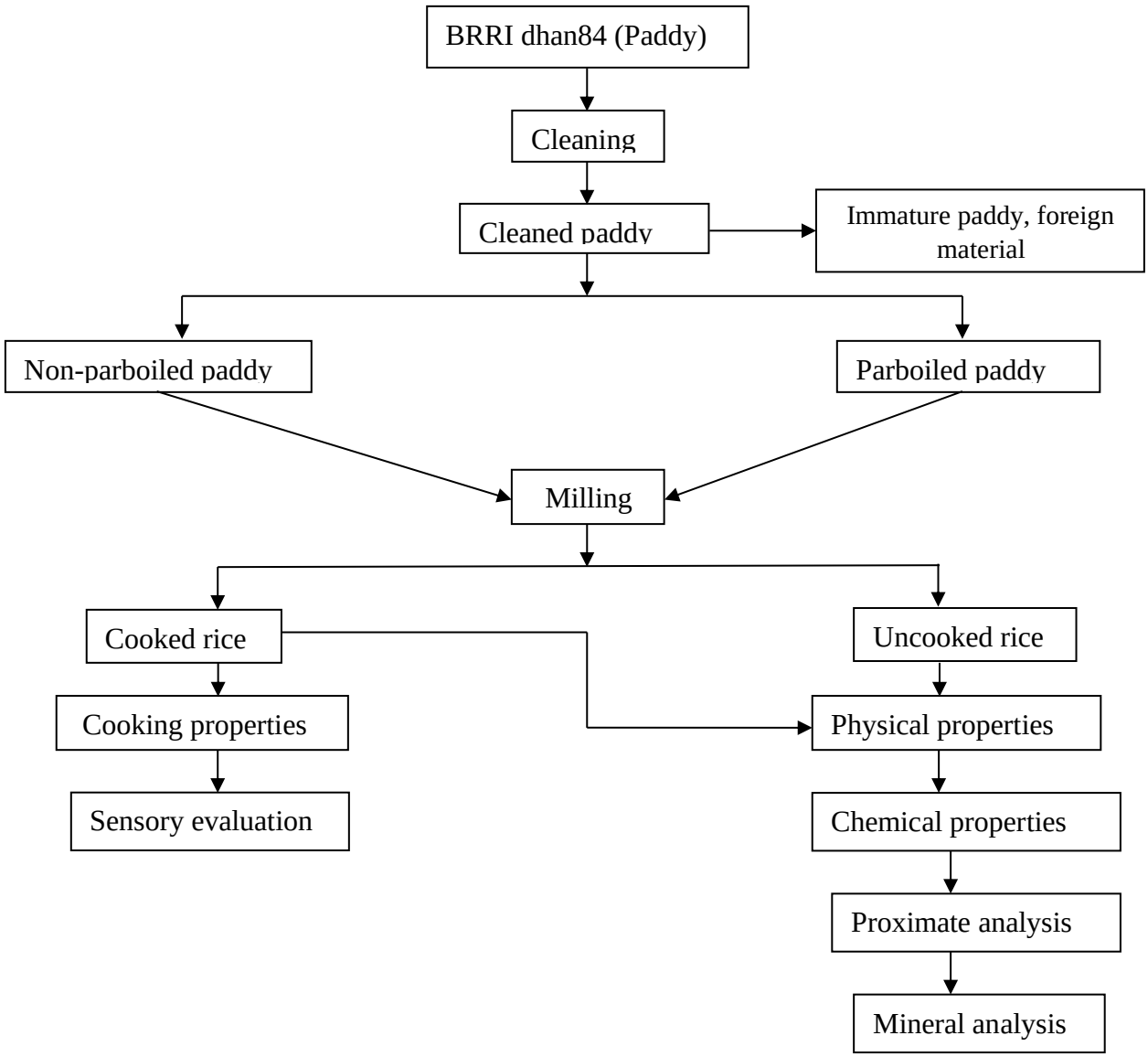

Figure 1. The flowchart of this study design

2.3. Physical properties

2.3.1. Length and width

The length and width were determined manually using a micrometer digital screw

gauge. After de-husking, ten whole rice kernels were randomly chosen from each non-parboiled and parboiled sample. The average length (mm) and width (mm) of these grains were recorded (Fofana et al., 2011).

2.3.2. Length-breath ratio

The length and breadth (at the midpoint) of 10 uncooked head rice kernels were measured using a micrometer digital screw gauge. The length-breath ratio was determined by dividing the cumulative length of 10 rice kernels by the breadth of 10 kernels. A mean of three replicates was taken for this measurement (Sanusi et al., 2017).

2.3.3. Bulk density

First, rice was taken up to the 100 ml mark in a beaker, and then measured the mass of rice grains without the beaker. The recorded mass of the rice grain was divided by the rice volume.

2.3.4. Thousand kernel weights

The 1000 rice kernel was selected randomly and then carefully weighed on a digital scale (Kern 572-30) with an accuracy of 0.001g. The procedure was repeated three times, and average values were taken in grams (Varnamkhasti et al., 2008).

2.3.5. Head rice yield

Head rice was defined as dehusked rice grains with a length of more than three-quarters of the whole grain. The head rice was manually separated from 50 g of cleaned dehusked rice sample. Then, the head rice yield (HRY) was calculated using the method stated by Wazed et al. (2021) and Wazed et al. (2022) as follows:

$$\% \text{ Head rice yield} = \frac{\text{Weight of head rice}}{\text{Weight of milled rice sample}\,(\text{g})} \times 100 \quad (1)$$

2.4. Cooking properties of BRRI dhan84 rice

2.4.1. Cooking time determination

The cooking was conducted using an electric rice cooker (Miyako electric rice cooker) for both samples. 10 g of non-parboiled rice sample was taken in a bowl and rinsed adequately with water before being cooked. Then, the rice kernels were placed in the cooking pot of an electric rice cooker with the rice to water ratio of 1:3 (w/w), and cooking was continued until the electric cooker automatically turned off. Simmering was done for 5 minutes to obtain completely cooked rice. The exact cooking process was followed for parboiled rice.

2.4.2. Elongation ratio and length–breadth ratio

The elongation ratio was measured according to the method described by Sanusi et al. (2017). The length of 10 randomly selected cooked rice was measured, and then the average length of 10 cooked rice was divided by the average length of uncooked rice. This procedure was repeated three times, and average values were taken. The elongation ratio was expressed as follows:

$$\text{Elongation ratio} = \frac{\text{Average length of cooked rice (mm)}}{\text{Average length of uncooked rice (mm)}} \quad (2)$$

2.4.3. Water uptake ratio

10 g of rice were cooked in 30 ml of distilled water for a minimum cooking time on an electric rice cooker. After cooking, the excess water was drained, and the samples were wiped softly by tissue to remove the clinging surface water on the cooked rice. Then, the cooked rice was weighed, and the water uptake ratio was measured by dividing the weight of the rice after cooking by the weight of the rice before cooking (Zohoun et al., 2018).

2.4.4. Gruel solid loss (%)

10 g of whole rice was taken in an aluminum pot with 30 ml water. The rice kernel was cooked in an induction cooker for the predetermined cooking time. After cooking, the gruel was separated from the cooked rice and transferred into a pre-weighed dried petri dish.

The gruel-containing petri dish was placed in an oven at 100°C for 24 hours to remove moisture. The weight of the petri dish was taken after drying (constant weight), and the mean of the three replicates was recorded. Gruel solid loss was calculated as the ratio of the increase in weight of the dish to the weight of the uncooked rice and expressed as follows (Qadir & Wani, 2022).

$$\text{Gruel solid loss (\%)} = \frac{\text{Increase in weight of petri dish (g)}}{\text{Weight of uncooked rice sample (g)}} \times 100 \quad (3)$$

2.5. Chemical properties of BRRI dhan84 rice

2.5.1. Determination of starch

The starch content of rice was determined by the Lane and Eynon method (AOAC, 2000). Approximately 5 g of rice powder was taken in a beaker with 30 ml of water and heated in a water bath at 60°C for 25 min. A total of 100 ml of 95% ethanol was added and stirred for 15 min. It was filtered using Whatman filter paper no. 2, and the residue was soaked for 1 h in a 50% ethanol solution. The residue was then filtered and washed in a 50% ethanol solution for 4 h. The residue was collected in a round bottom flask and then filled with 100 ml of water and 20 ml of 6M HCl. The condenser was fitted to the flask and heated for 2.5 hours. The mixture was then allowed to cool before being neutralized with a 40% NaOH solution. After that, 10 ml of Fehling solution was titrated against a neutralized sample solution in a conical flask. When a copper sulfate-like color appeared, 3 drops of methylene blue indicator were added, and the titration continued. The brick-red color denoted the endpoint. The following formula was used to calculate the starch content:

$$\text{\% Reducing sugar} = \frac{\text{Factor for Fehling's solution} \times \text{dilution}}{\text{Titre value} \times \text{weight of powder}} \times 100 \quad (4)$$

$$\text{\% Starch} = \text{\% reducing sugar} \times 0.9 \quad (5)$$

2.5.2. Determination of amylose content

Amylose content was determined by following the methods as described by Sompong et al. (2011) and Alam et al. (2023). A volumetric flask was filled with 0.02 g of rice powder. Then, 0.2 ml of 95% ethanol was added. After that, 1.8 ml of 1N NaOH was added, followed by distilled water to make a total volume of 20 ml. It was maintained at room temperature for 20 minutes before boiling for 10 minutes at 45°C, and then Whatman filter paper no. 2 was used to filter it. Then, 1 ml of filtrate was transferred to a 50 mL tube, along with 0.2 ml of 1M acetic acid and 0.4 ml of Lugol's solution, and distilled water was added to make a total volume of 20 ml. The mixture was thoroughly mixed and stored at room temperature for 20 minutes. Then, using a spectrophotometer (T80 U/VIS, United Kingdom), the absorbance was measured at 620 nm. The amylose content was determined using a potato amylose standard curve and expressed as g/100 g extract.

2.5.3. Determination of phenolic content

The phenolic content was determined by using the procedure as explained by Saikia et al. (2012) with certain modifications. Precisely, 1 g of rice powder was extracted for 15 minutes with 20 ml of 25% ethanol and filtered through Whatman no. 2 filter paper. 1 ml of filtrate, 1 ml of Folin reagent, and 5 ml of Na_2CO_3 were then transferred to a volumetric flask and stored at room temperature for 1 hour. Then, by using a spectrophotometer (T80 U/VIS, United Kingdom), the absorbance was measured at 765 nm. The phenolic content was expressed in mg GAE/100 g.

2.6. Proximate analysis of non-parboiled and parboiled rice

2.6.1. Determination of moisture content

The moisture content was determined by oven drying as described in the official AOAC (2005) method. Briefly, an empty crucible was

first washed, dried, cooled, and weighed. Then, the exact quantity (5.0 ± 0.001 g) of the rice sample was taken in a crucible and weighed. The crucible was placed inside the oven and dried at 105°C overnight. After drying, the crucible was removed from the oven and cooled in desiccators, and again, weight was taken. After that, the crucible with the sample was again placed in the oven and dried for 30 minutes, then removed from the oven, cooled in desiccators, and weighed. Drying, cooling, and weighing were repeated until two consecutive weights were the same. The same procedure was followed for all samples. Three replications were performed for each sample to reduce bias, and mean moisture content was then calculated as follows:

% Moisture content (wb)=

$$\frac{\text{Initial weight of sample} - \text{Bone dry weight of sample}}{\text{Initial weight of sample}} \times 100 \quad (6)$$

The moisture-free samples were then used to determine the crude protein, lipid, and ash content.

2.6.2. Determination of ash

The total ash content of the sample was determined by the AOAC (2005) method. The oven-dried rice sample was taken in a crucible and left inside a muffle furnace at 550°C for 6 hours. The muffle furnace was turned off, and waited till the temperature reached at 250°C, carefully opened the door and transferred the crucible to a desiccator to avoid losing ash and gaining moisture. After cooling, the weight of the crucible was recorded. The difference between the weight of oven-dried matter and the final weight represented the ash content, expressed in percentage. It was calculated by using the following formula:

$$\% \text{ Ash} = \frac{\text{Weight of crucible with ash}(g) - \text{Weight of empty crucible}(g)}{\text{Weight of sample}(g)} \times 100 \quad (7)$$

2.6.3. Determination of protein

The protein content was determined by the method of AOAC (2005) using the Kjeldhal apparatus. Precisely, 1 g ground rice sample was taken into a volumetric flask with 1g selenium, 0.1 g $CuSO_4$, and 10 g K_2SO_4. Then, 25 ml of concentrated H_2SO_4 was added. After that, the volumetric flask was heated at 100°C for 3 hours and then cooled for 20 minutes at room temperature. After digestion, exactly 300 ml of distilled water and 125 ml of 40% NaOH were added to the volumetric flask. The 250 ml of 4% boric acid solution and 2-3 drops of mixed indicator were taken in a conical flask. The volumetric flask was connected to one end of the condenser, and the conical flask was connected to the other end. The volumetric flask was heated continuously until the conical flask was filled to 150 ml. The conical flask was disconnected and taken for titration against 0.2 N of H_2SO_4 solution. The endpoint was indicated by the orange color. The total nitrogen value was then calculated by using the following formula:

$$\%\text{Nitrogen} = \frac{\text{Titrate value} \times \text{N} \times 0.014 \times 100}{\text{Weight of sample}(g)} \quad (8)$$

Where, % Protein = % Nitrogen × 6.25;

Here, 0.014 = Mili-equivalent weight of N_2.

2.6.4. Determination of fat content

The crude fat content of rice samples was determined using the AOAC (2005) method. Exactly, 1.0 g of sample was taken into the thimble and plugged with cotton. The thimble was attached to the Soxhlet apparatus with a round bottom flask containing 200 ml of petroleum benzene. The petroleum benzene was filled into a weighted conical flask. The fat was extracted for 6 hours. After that, benzene was evaporated until the conical flask was completely dried and then cooled in

desiccators. Then the weight is taken. The fat content was then determined by using the following formula:

$$\% \text{ Fat Content} = [(W_1 - W_2)/W] \times 100 \quad (9)$$

Where, W= Weight of the sample; W_1= Weight of the evaporated flask with sample; W_2= Weight of empty flask.

2.6.5. Determination of fiber content

The fiber content was determined by the AOAC (2005) method with some modifications. Fat-free (2 g) sample was taken in a 500 ml beaker and added 200 ml of 0.255 N H_2SO_4, then boiled for 30 min. The mixture was filtered with a muslin filter cloth, and the residue was washed with hot water until free from acid. Then, the residue was transferred into a beaker, and 0.313 N of 200 ml NaOH was added and boiled for 30 minutes. After that, the mixture was filtered with a muslin filter cloth, and the residue was washed with hot water until free from alkali and then washed with alcohol and diethyl ether. It was then transferred to a crucible and dried overnight at 105°C. The crucible was then heated for 6 hours at 550°C in a muffle furnace. After that, it was allowed to cool before being weighed. The crude fiber was obtained by the following formula:

$$\% \text{ Crude Fiber} = [(W_1 - W_2)/W] \times 100 \quad (10)$$

Where, W= Weight of the sample; W_1= Weight of crucible with sample; W_2= Weight of empty crucible.

2.6.6. Determination of carbohydrate content

The total carbohydrate content of any food product has been calculated by the different method for many years rather than analyzed directly. The carbohydrate content of the developed samples was determined by subtracting the measured protein, fat, fiber, ash, and moisture from 100 (Roshid et al., 2016; Roni et al., 2021; Wazed and Islam, 2021; Alam et al., 2020; Moni et al., 2023). Therefore,

$$\text{Percentage of carbohydrates} = 100 - \{\text{moisture} + \text{protein} + \text{ash} + \text{fat} + \text{fiber}\} \quad (11)$$

2.7. Mineral analysis of BRRI dhan84 rice

Zinc (Zn), iron (Fe), and calcium (Ca) were analyzed by AOAC (2005) method using an atomic absorption spectrophotometer. The digested sample was analyzed for mineral contents by an atomic absorption spectrophotometer. Different electrode lamps were used for each mineral. The equipment was run for standard solutions of each mineral before and during determination to check that it worked properly. The dilution factor for all minerals was 100. To determine Ca, 1.0 ml of lithium oxide solution was added to the original solution to unmask Ca from Mg. The concentrations of minerals were recorded in terms of "ppm" and converted to milligrams (mg) of the minerals by multiplying the ppm with the dilution factor and dividing by 1000, as follows:

Mineral content =

$$\frac{\text{absorbancey}(\text{ppm}) \times \text{dry wt} \times \text{D}}{\text{Wt. of sample} \times 1000} \quad (12)$$

2.8. Sensory evaluation

Twenty experienced but untrained panelists participated in the evaluation of cooked rice samples. They were provided with instructions to taste the rice samples and cleanse their palate between each tasting. The panelists were then asked to rate various sensory attributes of both parboiled and non-parboiled cooked rice, including color, aroma, texture, taste, and overall acceptability. These sensory attributes were evaluated using the Likert Scale, as Edmonson (2005) described.

2.9. Statistical analysis

The experiments were conducted in triplicate, and the results were reported as the mean value and the corresponding standard

deviation. The data were statistically analyzed by paired-samples t-test (physical properties, cooking qualities, and sensory attributes) and one-way ANOVA (chemical properties, proximate compositions, total phenol content, and mineral contents) using Duncan's multiple range tests at 5% significance level using SPSS version 22.

3. Results and discussions

3.1. Physical Properties of non-parboiled and parboiled BRRI dhan84

The physical properties of non-parboiled and parboiled BRRI dhan84 rice are presented in Table 1. The length of parboiled BRRI dhan84 rice decreased significantly compared to non-parboiled, resulting from 6.507 mm and 6.781 mm, respectively. In contrast, the width of parboiled BRRI dhan84 rice experienced a slight increase compared to non-parboiled rice but was statistically insignificant. According to Kurien et al. (1964), parboiling and subsequent drying reduce the length of rice while increasing the breadth for rough and brown rice. This might be related to the hardening of rice grains due to parboiling followed by drying. The length-breath ratio of non-parboiled BRRI dhan84 rice was significantly higher than the parboiled one. According to the classification of rice given by Bhattacharya et al. (1980), based on grain L/B ratio, both samples fall in slender (L/B ratio > 3.0). In bulk density and 1000 kernel weight of BRRI dhan84 rice, there was no significant change between non-parboiled and parboiled. This could be due to the precision of different preprocessing treatments (Oghbaei & Prakash, 2010). The bulk density and 1000 kernel weight of non-parboiled and parboiled rice were 0.902 g/mL and 0.900 g/mL, 18.40 g and 17.80 g, respectively. This is possibly due to the precision of different preprocessing treatments (Oghbaei & Prakash, 2010). Similar results have been reported by Chavan et al. (2018), who studied the effect of parboiling on the Pusa Basmati 1121 rice varieties.

Table 1. Physical properties of uncooked non-parboiled and parboiled BRRI dhan84 rice

Parameters	Uncooked rice			
	Non-parboiled	Parboiled	t-statistics	p-value
Length (mm)	6.781 ± 0.214	6.507 ± 0.223	6.025	0.000*
Width (mm)	1.966 ± 0.195	2.032 ± 0.099	-1.860	0.073
Length/Breath ratio	3.49 ± 0.414	3.21 ± 0.205	3.884	0.001*
Bulk density (g/ml)	0.902 ± 0.130	0.900 ± 0.003	0.294	0.797
1000 kernel weight (gm)	18.40 ± 0.235	17.80 ± 0.036	3.988	0.058
Head Rice Yield (%)	64.39 ± 4.78	80.09 ± 1.96	- 4.369	0.049*

All values are expressed as means ± SD. *Significant ($P \leq 0.05$).

Parboiled BRRI dhan84 rice recorded the highest HRY (80.09%), while non-parboiled rice recorded the lowest (64.39%) (Table 1). This finding indicates that the parboiling method significantly increased ($p < 0.05$) the head rice yield of BRRI dhan84 rice. Head rice yield increased because parboiling enhanced the rice hardness through starch gelatinization, making the grain less prone to breaking during milling (Jagtap et al., 2008). These findings are higher than those of Kader et al. (2020) for non-parboiled rice. The higher HRY or reduction in rice breakage depends on selecting the best soaking temperature and steaming time, using the best parboiling equipment that allows the uniform distribution of heat during soaking and steaming (Ndindeng et al., 2015), and milling with a rubber roll type mill (Bhattacharya, 1969).

3.2. Effect of cooking on non-parboiled and parboiled BRRI dhan84 rice

Cooking properties such as water uptake ratio, grain elongation ratio, gruel solids loss in the cooking water, and optimal cooking time for rice are presented in Table 2. The water uptake ratios of non-parboiled and parboiled rice were not statistically different, demonstrating 2.180 and 2.113, respectively, for non-parboiled and parboiled cooked rice. These findings align with Meresa et al. (2020), who studied the influence of parboiling conditions on the cooking quality of selected rice. Regarding elongation ratio, non-parboiled and parboiled cooked rice yielded 1.1168 and 1.1171, respectively.

Table 2. Cooking properties of non-parboiled and parboiled cooked BRRI dhan84 rice

Parameters	Cooked rice			
	Non-parboiled	Parboiled	t-statistics	p-value
Water uptake ratio	2.180 ± 0.199	2.113 ± 0.035	0.675	0.569
Elongation ratio	1.116 ± 0.078	1.117 ± 0.091	0.044	0.691
Gruel solid loss (%)	1.033 ± 0.056	0.090± 0.010	34.574	0.001*
Cooking time (min)	34.45 ± 0.060	39.41 ± 0.029	41.250	0.001*

All values are expressed as means ± SD. *Significant ($P \leq 0.05$).

Parboiling did not affect the rice elongation ratio. Compared to non-parboiled rice, parboiling significantly reduced gruel solid loss during cooking, with values of 1.033 % and 0.090 %, respectively. This may occur due to the seal of internal cracks and reduced starch solubilization in cooking water due to hydrothermal treatment (Pal et al., 2018) of milled rice and parboiled milled rice among different rice varieties. These findings are also supported by Chavan et al. (2018). Parboiled BRRI dhan84 rice recorded the longest cooking time (39.41 min), while non-parboiled rice recorded the shortest cooking time (34.45 min), as indicated in Table 2. It specifies that cooking time for parboiled rice was significantly longer than for non-parboiled rice. Disorganized cellular structure can enhance the probability of high water absorption during cooking and can contribute to longer cooking time (Thomas et al., 2013). Sareepuang et al. (2008) also argue that the longer cooking time of parboiled rice may be due to strong cohesion between the endosperm cells, which are tightly packed, making starch grains hydrate at a slower rate. Economically, the longer cooking time of parboiled rice involves more combustibles (fuel, gas, or firewood) and, therefore, higher cooking costs. Cooking time also depends on the parboiling process, rice variety, and storage time (Issah et al., 2015).

3.3. Amylose, starch, and total phenol content of uncooked and cooked BRRI dhan84 rice

In terms of cooking and gelling characteristics, the amylose content of rice is one of the most vital parameters for rice quality, which affects the cooking, eating, and pasting characteristics of rice (Asghar et al., 2012). In this study, the amylose content of uncooked non-parboiled and parboiled BRRI dhan84 was 22.37 % and 21.58 %, respectively (Table 3). This amylose percentage was lower than that of Kader et al. (2020), who found a 25.9% amylose content in the same non-parboiled rice. This could be due to the differences in the environmental conditions in which the crop is grown, particularly temperature (Hettiarachchy et al., 1997). The amylose percentage of parboiled BRRI dhan84 rice was significantly lower compared to non-parboiled BRRI dhan84 rice. This reduction may be due to the leaching of amylose molecules into the dissolving water during soaking and consequent steaming during parboiling (Otegbayo et al., 2001). Starch is a major component of rice endosperm. The starch content of uncooked non-parboiled and parboiled BRRI dhan84 rice was 62.10 % and 57.03 %, respectively (Table 3). This reduction in starch after parboiling might be due to

amylose leaching during soaking and steaming and the development of an amylose-lipid complex during steaming (Singh et al., 2006). The total phenolic content (TPC) in uncooked non-parboiled and parboiled BRRI dhan84 rice was found to be 4.44 mg GAE/g and 1.31 mg GAE/g, respectively (Table 3). The parboiling decreased the TPC significantly. Cooking reduced the percentage of starch, amylose, and TPC in both non-parboiled and parboiled BRRI dhan84 rice (Table 3). The non-parboiled and parboiled cooked BRRI dhan84 rice contained significantly lower starch content than that of uncooked rice, with 53.70% and 46.74%, respectively. TPC of both non-parboiled and parboiled reduced from 4.44 to 0.81 mg GAE/g and 1.31 to 0.73 mg GAE/g, respectively. Most of the phenolic content is unstable and easily destroyed in the presence of heat, light, and oxygen, which could be the possible reason for the reduction of TPC after parboiling and followed by cooking (Junior et al., 2010). In another study, Widyasaputra et al. (2020) reported a similar reduction trend for TPC in black rice after parboiling. A reduction of TPC in cooked rice has also been observed by Pal et al. (2018) in previous research. According to Zhang & Hamauzu (2004), the breakdown or conversion of phenolic to other compounds and vapor during cooking is the cause of phenolic losses during cooking.

3.4. Proximate composition of BRRI dhan84 rice

The proximate compositions of uncooked and cooked non-parboiled and parboiled rice of BRRI dhan84 are presented in Table 4.

Table 3. Chemical properties of uncooked and cooked BRRI dhan84 rice

Parameters	Uncooked rice		Cooked rice	
	Non-parboiled	Parboiled	Non-parboiled	Parboiled
Amylose (%)	22.37 ± 0.34^{a}	21.58 ± 0.18^{b}	17.22 ± 0.13^{c}	16.78 ± 0.14^{d}
Starch (%)	62.10 ± 1.67^{a}	57.03 ± 0.85^{b}	53.70 ± 1.77^{c}	46.74 ± 0.95^{d}
Total Phenolic content (mg GAE/g)	4.44 ± 0.14^{a}	1.31 ± 0.01^{b}	0.81 ± 0.27^{c}	0.73 ± 0.01^{c}

All values are expressed as means ± SD. Mean with different superscript letters in the same row indicates a significant difference ($p \leq 0.05$) from each other.

The percentage of ash, fat, protein, and carbohydrates in parboiled rice was found to be higher than that of non-parboiled rice. The highest amount of ash (1.99%) was estimated in parboiled uncooked rice. The finding was higher than the value (1.3%) as presented in the Food Composition Table for Bangladesh (Shaheen *et al.*, 2013), which was attributed to the parboiling process. The parboiling process caused the degradation and diffusion of components from rice bran and husk into rice endosperm, which brought about a significant increase in ash percent (Thammapat et al., 2016). The fat content of parboiled rice samples was also significantly greater than that of non-parboiled rice. This might be explained by the increased temperature and steaming pressure that happens during the parboiling process, which causes the leaching and rupturing of the oil globules (Chukwu et al., 2009). The fat content of the parboiled uncooked sample exceeds the limit of 1.7% set by FAO (1994) for sound conservation and prevention of rice turning rancid during storage. The protein content increased significantly after parboiling. The Food Composition Table for Bangladesh quantified 7.8% protein in parboiled uncooked rice, which

was lower than the current study. The increase in protein content in parboiled rice could be due to the synthesis of some enzymes, which may result in the production of some amino acid during protein synthesis, or it might be due to the endosperm of parboiled rice, which had a good number of proteins as well as parboiled milled rice (Uwaegbute et al., 2000). For protein contents, the current result of non-parboiled and parboiled rice is lower than that of a study, as explained by Kader et al. (2020), reporting 9.7% protein in non-parboiled BRRI dhan84.

The parboiling significantly decreased the carbohydrate of rice also compared to non-parboiling from 77.91% to 75.53%. This could be attributed to the leaching of soluble carbohydrate components during soaking, drying, and molecule rupturing caused by steaming.

Table 4. Proximate composition of non-parboiled-parboiled and uncooked-cooked BRRI dhan84 rice

Parameters	Uncooked rice		Cooked rice	
	Non-parboiled	Parboiled	Non-parboiled	Parboiled
Moisture	10.86±0.115^{c}	9.89±0.370^{d}	52.83±0.577^{a}	50.67±0.289^{b}
Ash	1.98±0.243^{a}	1.99±0.493^{a}	0.61±0.005^{b}	0.52±0.006^{b}
Fat	1.72±0.327^{b}	2.36±0.035^{a}	0.15±0.004^{c}	0.23±0.021^{c}
Protein	6.68±0.050^{b}	9.42±0.91^{a}	4.17±0.085^{d}	4.78±0.131^{c}
Fiber	0.97±0.072^{a}	0.81±0.226^{ab}	0.85±0.031^{ab}	0.69±0.059^{b}
Carbohydrate	77.92±0.083^{a}	75.53±0.625^{b}	41.38±0.624^{d}	43.12±0.377^{c}

All values are expressed as means ± SD. Mean with different superscript letters in the same row indicates a significant difference ($p \leq 0.05$) from each other.

After cooking, both non-parboiled and parboiled samples were found to show a significant decrease in all proximate compositions except moisture content. Similar trends were found by Boora (2015) for Improved Pusa Basmati-I (an Indian rice variety). According to Ebuehi & Oyewole (2007), cooking and soaking in water changed the protein content of Ada and Aroso rice types. Cooking rice denatures the protein, and soaking increases the solubility of specific proteins, resulting in a protein decrease (Ebuehi & Oyewole, 2007). The reduction in fat content caused by cooking might be attributed to chemical and physical changes in fat that occur during heating (Roth & Rock, 1972). The increased moisture level, which also impacts milling quality, may be responsible for reducing carbohydrate content in cooked rice (USA Rice Federation, 2002). In this study, after cooking, the highest amount of carbohydrate (43.12%) was estimated in parboiled rice, which was higher than the value (23.2%) as reported by the Food Composition Table for Bangladesh (Shaheen et al., 2013). Also, parboiled BRRI dhan84 rice retained more fat and protein after cooking than non-parboiled rice. The retention of some nutrients in parboiled rice is because of water-soaking before parboiling, which penetrates the void space and seals the internal cracks of the rice grain. As a result, compared to non-parboiled rice, the leaching of solids into cooking water and solubilization of the kernels decreases significantly during cooking (Luh & Shinlu, 1991).

3.5. Mineral content of BRRI dhan84 rice

Table 5 represents the mineral composition of BRRI dhan84 rice. The calcium content of uncooked parboiled rice was significantly higher than that of non-parboiled BRRI dhan84

rice. These findings align with a previous study on the Nigerian long-grained rice variety IR-8 (Chukwu et al., 2009). Compared to non-parboiling, parboiling significantly reduced the iron content of BRRI dhan84 to 4.871 mg/100g from 5.105 mg/100g and increased the zinc content of rice slightly from 2.732 mg/100g to 2.741 mg/100g. In general, no significant difference in zinc was found between parboiled and non-parboiled uncooked rice. The highest amount of zinc (2.741 mg/100 g) was estimated in parboiled uncooked rice, which was higher than the value (1.90 mg/100 g) as reported by Food Composition Table for Bangladesh (Shaheen et al., 2013), which was expected because these biofortified varieties were bred to have higher zinc concentration compared to other commercial varieties (Taleon et al., 2021). Besides, this slight rise in zinc content in parboiled BRRI dhan84 rice contradicts the findings of Chukwu et al. (2009) for parboiled and non-parboiled rice, which might be attributed to differences in rice varieties, physical and chemical properties of the rice grain and the parboiling method (Taleon et al., 2020). The zinc content was found in this study to be compatible with findings concerning non-parboiled rice (Kader et al., 2020).

After cooking, both parboiled and non-parboiled rice showed a considerable decrease in calcium, iron, and zinc content. This decrease in minerals might be attributed to increased moisture levels and minerals leaching into the cooking water during the cooking process (Adepoju et al., 2012). The finding is consistent with a previous study of non-parboiled and parboiled cooked rice of different rice varieties (Thomas et al., 2016). The Food Composition Table for Bangladesh (Shaheen et al., 2013) quantified 0.58 mg/100 g zinc in parboiled cooked rice, which was lower than the current study. The higher zinc (2.344 mg/100g) found in the biofortified variety BRRI dhan84 for parboiled cooked rice suggests that the additional zinc in this biofortified variety is more concentrated in the endosperm, similar to the findings of Taleon et al. (2020).

Table 5. The mineral content of uncooked and cooked BRRI dhan84 rice

Parameters (mg/100g)	Uncooked		Cooked	
	Non-parboiled	Parboiled	Non-parboiled	Parboiled
Calcium	4.603 ± 0.083^{b}	5.100 ± 0.030^{a}	3.733 ± 0.025^{d}	4.320 ± 0.170^{c}
Iron	5.105 ± 0.034^{a}	4.871 ± 0.535^{b}	4.659 ± 0.717^{c}	4.571 ± 0.061^{c}
Zinc	2.732 ± 0.029^{a}	2.741 ± 0.031^{a}	2.264 ± 0.033^{c}	2.344 ± 0.064^{b}

All values are expressed as means ± SD. Mean with different superscript letters in the same row indicates a significant difference ($p \leq 0.05$) from each other.

3.6. Sensory analysis

The results of the sensory assessment showed that the color, texture, and overall acceptability of cooked parboiled BRRI dhan84 rice were significantly different from the non-parboiled rice (Table 6). The parboiled rice was darker in color than the non-parboiled rice, with values of 2.04 and 3.08, respectively. The panel appreciated the lighter color of non-parboiled rice, although both rice scored a low color preference rating. The degree of color change has been reported to be influenced by soaking temperature, heating duration, and heating and drying temperatures (Bhattacharya, 1985). In another study, it was found that the grain color is associated with the iron content. As the rice is milled, iron content decreases, and there is a slight change in the color of the rice. In the case of aroma and taste, both parboiled and non-parboiled exhibited an almost similar score (Gregorio et al., 2000). However, these scores were in the neutral range of the Likert scale for the taste of rice. Non-parboiled rice secured a significantly higher score in textural properties compared to parboiled rice. They found that parboiled rice

was slightly stiffer. This might be due to the stiffening of parboiled rice after parboiling. Besides, as rice aged, texture was improved because of the modification of interactions among the components of grains (Butt et al., 2008). Although parboiled rice received a higher overall acceptability rating by the panel, cooked parboiled and non-parboiled rice scored almost near the agree and neutral ranges, respectively.

Table 6. Sensory evaluation of cooked non-parboiled and parboiled BRRI dhan84

Parameters	Cooked			
	Non-parboiled	**Parboiled**	**t-statistics**	**p-value**
Color	3.08 ± 0.812	2.04 ± 0.735	-5.099	0.000*
Aroma	3.92 ± 0.702	3.96 ± 0.735	0.214	0.832
Texture	3.92 ± 0.640	2.92 ± 0.862	-4.804	0.000*
Taste	3.04 ± 0.735	3.08 ± 0.640	0.253	0.802
Overall acceptability	3.04 ± 0.790	3.60 ± 0.866	3.219	0.004*

All values are expressed as means ± SD. *Significant ($P < 0.05$).

4. Conclusions

The parboiling process practiced by almost every rice industry in Bangladesh affected the properties of rice in both positive and negative ways. Cooking for rice consumption was the most common method adopted by the people of Bangladesh that, resulted in the lowering of almost all nutritional constituents. The present study was the first to evaluate the physicochemical properties, proximate composition, and cooking characteristics of BRRI dhan84 rice and its changes during parboiling. It can be concluded that parboiling resulted in an increase in mineral, fat, and protein content. In contrast, other constituents were significantly lower compared to raw rice. Parboiling had a positive effect on the physical properties but some negative aspects, especially in terms of cooking time. The cooking process negatively affected all properties except the antioxidant activity, total phenol content (TPC), and moisture content. The results of the current study reflected that the consumption of BRRI dhan84 rice could be extended to society by making people aware of its nutritional constituents and its possible application on different food items.

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EFFECTS OF XANTHAN GUM ON THE QUALITY OF GLUTEN-FREE BORA RICE AND CORN FLOUR NOODLES USING D-OPTIMAL MIXTURE DESIGN APPROACH

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ABSTRACT

Bora rice found in Assam contains a significant amylopectin concentration (i.e., > 95%) and has a waxy, branched polymer, indicating physical durability and resistance to enzymatic action. This category of rice starch hydrates and expands when exposed to cold water, generating sols that contribute to its bio-adhesive characteristics. Due to glutinous nature, it poses a challenge in making extruded products. Now, xanthan gum has the ability to replace the gluten network of bora rice and blended with corn flour to develop noodles by using extrusion technology. The study was based upon the experiments for three independent variables using a D-optimal mixture design (DOMD). Different responses viz. cooking time, cooking loss, swelling power and solubility were studied. Numerical optimization was done and the optimum values were found to be rice flour (82.3%), corn flour (15%), and xanthan gum (2.6%). The developed noodle product was then analyzed for physiochemical, sensory, and storage properties. The recorded readings were swelling index ($37 gg^{-1}$), solubility (36.5%), cooking loss (27%) and cooking time (209 s). The moisture, protein and ash content were found as approximately 2.5%, 30% and 0.213% respectively. Therefore, based on DOMD optimization technique, a good balance between the bora rice, corn flour and xanthan gum led to the development of extruded noodle with characteristic physicochemical property, storage stability and satisfactory sensory quality.

1. Introduction

Rice, also known as *Oryza sativa lour*, is a staple food in the majority of nations (Verma and Srivastava, 2020).It is the most extensively consumed staple meal in the world for a substantial portion of the human population, particularly in Asia and Africa (Marti et al., 2013).Non-glutinous and glutinous rice are two types of rice that differ in their amylose concentration (also termed as non-waxy and waxy or sweet rice) (Belitzet al., 2009) (Marti et al., 2010) (Devi et al., 2020). While eating food containing wheat, rye, or barley, certain individuals with a particular genetic disposition suffer celiac disease or non-tropical sprue. For those with such problems with wheat gluten allergies, using rice instead of wheat to manufacture rice-based extruded items would be effective (Barbiroli et al., 2013). Traditional rice noodles are produced with long-grain rice flour with an amylose concentration that is between intermediate and high (>22 g/100 g),

which is essential for the development of a starch network in rice noodles (Kohlwey, Kendall, &Mohindra, 1995) (Marti et al., 2011).Rice noodles have traditionally been made by a lengthy process of gelatinization, extrusion/slitting, cooking, retrogradation, acid-pickling, drying, packaging, and sterilizing (Li et al.., 2021)**(**Marti &Pagani,2013).Several research has evaluated the effectiveness of noodles prepared from various rice types. Huang et al., (2021) found out that brown rice noodles had 12–19% higher yield than that of white rice noodles but cooking loss rate was 5-10% higher in brown rice noodles. Also, for economic reasons, it is expanded in many nations where wheat is not a significant local crop by adding other flours. Moreover, amylose has been identified in maize noodles as the element responsible for maintaining their textural integrity after cooking.Dexter and Matsuo, (1979**)** showed that in corn blends, the lower the amylose content, the lower the noodle cooking quality. *Xanthomonas campestris* is a bacterium that secretes xanthan gum, an extracellular polysaccharide. It is produced commercially using a fermentation procedure and cold water makes xanthan gum soluble, and solutions show strong pseudoplastic flow and an interaction with galactomannans and glucomannans that works synergistically (Sworn, 2021) (Sozer 2009). Kaur et al., (2015) studied the effects of xanthan gum on noodle-making properties and found that the addition of the gum improved hot paste viscosity and final viscosities while decreasing peak viscosity for mung and corn starches.

2. Materials and methods

2.1. Materials

Traditionally grown in Assam, India, Assam Bora rice (*Oryza glutinosalour*) was purchased from a neighborhood market in Tezpur. Gluten-free corn flour was obtained from a seller on the e-commerce website i.eAmazon. Xanthan Gum was purchased from Himedia® Laboratories Pvt. Ltd.

2.2. Methods

2.2.1. Preparation of Noodles

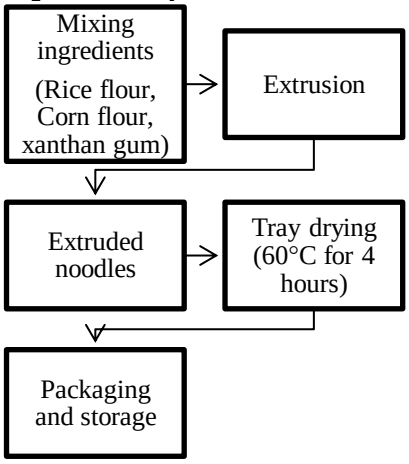

Figure 2.1. Flowchart for the preparation of Rice Noodles

The noodles were developed by using a laboratory model extrusion machine (Mini Dolly Pasta machine, LA MONFERRINA, Italy). On the basis of preliminary trials to obtain well shaped noodles, noodles were prepared by addition of rice flour, corn flour, xanthan gum with 45 ml water addition in 100g. The noodle samples were made by varying the quantity of the three components by using Mixture design in the Design Expert Software. The prepared noodles were then stored in Resealable Zip Lock Cover Pouch bags.

2.2.2. Experimental design for preparation of noodle using D-optimal Mixture Design

The experiment was carried out with various settings using the D-optimal mixture design. The measured response in a combination experiment is presumed to be solely dependent on the relative quantity of ingredients or components present, which typically add to 100%. The present work employed a mixture experiment with three components: Rice Flour (A), Corn Flour(B), Xanthan gum(C). Parameters ranges for D-

optimal mixture design in preparing noodles were prepared and the levels of various input variables were selected as follows: Rice Flour (80-85%), Corn Flour (10-15%) and Xanthan gum (1-5%). The three variables generated 16 formulations of noodles with different composition of each ingredient as shown in Table 2.1. Different responses were carried out on the noodles to select the best combination of input variables which could result in most suitable form of noodles. The examined responses were cooking time, cooking loss, swelling index, solubility.

Traditional Scheffe type models for the mixture variables and response surface models for the design variables are typically combined to create models for interpreting data from mixture designs (Scheffe, 1958). The cubic model was fitted as suggested.

Table 2.1. Experimental design showing different formulation for developing noodles

Std No	A:Rice Flour	B:Corn Flour	C:Xanthan Gum
1	82.449	12.551	5.00
2	82.370	15.000	2.630
3	85.000	13.751	1.249
4	80.002	14.998	5.000
5	83.678	14.735	1.587
6	80.002	14.998	5.000
7	83.575	13.508	2.916
8	82.370	15.000	2.630
9	84.957	11.127	3.915
10	85.000	13.751	1.249
11	81.604	14.146	4.250
12	85.000	10.002	4.998
13	85.000	10.002	4.998
14	83.787	12.214	3.999
15	85.000	12.210	2.790
16	82.449	12.551	5.000

Using a statistical package, computations were carried out, including the selection of experimental points, randomization, analysis of variance, fitting of the models, and graphical displays (Design-Expert Version 7.0). On cooking characteristics and starch qualities, descriptive statistics were used. Analysis of variance (ANOVA) was performed on the data to find variations across formulations that were statistically significant ($p<0.05$).

2.2.3. Characterization of Rice Noodles

2.2.3.1. Proximate Analysis

To measure the moisture and protein of the noodle samples, the
Association of Official Analytical Chemists' standard techniques were employed AOAC (2000).

2.2.3.2. Starch properties

Starch properties such as solubility and swelling power will be determined according to the method of Crosbie et al., (1992)with slight modification. In centrifugal tubes, 0.5g (dwb) of flour samples were combined with 25 ml of water. The mixture was then heated to 85°C and maintained there for 30 min. The samples were centrifuged at 5000 x g for 15 minutes after being cooled to room temperature. It was then kept overnight at 130°C, after that the supernatant was evaporated and weighed. The weight of the dried supernatant to the initial weight of the dry flour is used to calculate solubility. The following formula was used to determine the samples of rice flour's solubility and swelling power.

$$Swelling\ Power\ (gg^{-1}) = \frac{weight\ of\ the\ wet\ sediments}{weight\ of\ dry\ flour} \quad (1)$$

$$Solubility\ (\%) = \frac{Weight\ of\ dried\ supernatant}{Initial\ weight\ of\ dry\ flour} \quad (2)$$

2.2.3.3. Cooking attributes

Cooking Time

Following (ISO, 7304), the ideal cooking period was determined, by removing a long strand of noodle and cutting it using a cutter until the continuous white line visible at the center of the cut section disappears. 25g of noodles were cooked in 300 ml of distilled water that was boiled. A timer was set, and the

product was taken out every 30 seconds to determine how much cooking had taken effect.

Cooking Loss

Each sample, which weighed 25g, was cooked to perfection in 300ml of boiling distilled water. The leftover cooking liquid was collected and dried by evaporation in a 100°C oven for 24 hours. Weighted solids were used to report cooking loss as a proportion of the raw sample.

$$Cooking\ loss = \frac{Weight\ before\ cooking - Weight\ after\ cooking}{Weight\ before\ cooking} * 100 \quad (3)$$

2.2.3.4. Modelling of experimental data

The statistical significance of the response was checked through analysis of Variance (ANOVA) and the coefficient of determination ($R^{2)}$ values were checked.

2.2.3.5. Proximate analysis of optimized noodle sample

Parameters like moisture content, protein content and ash content of the optimized noodle sample were measured following the methods provided by the AOAC (2000).

2.2.3.6. Rheology

On a controlled stress rheometer, dynamic rheological measurements were made. The spacing between the parallel 50 mm-diameter plates on which the rice dough was laid was set to 2 mm. With a constant tension of 2 Pa and 30 °C, a frequency sweep from 0.01 to 10 Hz was carried out. The dough was allowed to rest for 5 minutes to allow residual stresses to release. The applied strain was 1%. By contrasting log plots of the storage modulus (G′) and loss modulus (G″) with frequency, the dough structure was assessed.

2.2.3.7. Sensory evaluation

Panel consisting of 9 judges from the Department of Food Engineering and Technology evaluated the cooked pasta samples. The judges scored the sample in terms of appearance, flavor, color, texture, aroma and overall acceptability using a 9-point hedonic scale where 9 - extremely likely, 8 – like very much, 7-like moderately, 6-like slightly, 5-neither like nor dislike slightly, 4 – dislike slightly, 3 – dislike moderately, 2 – dislike moderately, 2 – dislike very much, 1 – dislike extremely.

2.2.3.8. Effect of storage on moisture content of the optimized noodle sample

The optimized sample's increased moisture content was observed for 15 days at 5-day intervals.

3. Results and discussions

3.1 Moisture and Protein Content

Table 3.1. Moisture and protein content of Rice noodles

Sl No.	Moisture Content (%)	Protein Content (%)
1	2.3	36.0
2	1.2	29.1
3	1.6	26.0
4	2.4	36.8
5	1.5	28.0
6	2.6	36.3
7	1.2	29.0
8	1.7	28.0
9	2.8	26.0
10	1.9	25.0
11	3.4	29.0
12	1.4	35.8
13	2.7	35.8
14	1.3	24.6
15	2.4	34.3
16	2.8	35.9

In the above table, it is seen that the moisture content of the 16 noodle samples lies in the range of 1-3%. Protein content was estimated using the Lowry's method and the range varies from 25-35%

3.2. Starch Properties

The relative strengths of the bonding within the granules are determined by evaluating the

solubility of starches at various temperatures (Schoch, 1964). According to reports, tests for swelling and solubility at an oven temperature of 80°C serve as a decent simulation of the actual process for creating noodles and have been utilized as quick, small-scale ways to forecast the eating quality of wheat and starch noodles (Crosbie et al., 1992).

Table 3.2. The swelling index and solubility for the noodle samples are shown

Sl No.	Swelling Index(gg^{-1})	Solubility *(%)*
1	26.8	24.8
2	37.1	36.0
3	37.9	35.8
4	26.8	24.8
5	37.8	35.8
6	26.8	24.8
7	37.3	36.0
8	37.1	36.0
9	36.0	26.0
10	37.9	35.8
11	25.9	23.0
12	26.5	24.1
13	26.5	24.1
14	36.0	26.0
15	37.2	36.0
16	26.8	24.2

3.3. Cooking Attributes

Table 3.3. Table showing data for the cooking time and cooking loss for the noodle samples

Sl No.	Cooking Time(s)	Cooking Loss *(%)*
1	254	26.1
2	210	27.0
3	180	30.8
4	252	26.2
5	193	30.5
6	250	25.9
7	223	27.0
8	210	27.0
9	235	26.5
10	180	30.8
11	243	26.1
12	248	26.3
13	248	26.3
14	235	26.5
15	218	27.0
16	250	26.1

In the above table, it can be seen that as the xanthan gum content in the product increases the cooking time increases and vice versa. The cooking loss however decreases when the

xanthan gum content in the sample is comparatively more.

3.4. Modelling of experimental data

The experimental design and the corresponding responses are shown in the Table 3.4. The statistical significance of the response was checked through analysis of Variance (ANOVA) and was presented in Table 3.6 – 3.10. The value of determination coefficient (R^2) should be 0.8 and above for the good fit of model (Joglekar&May, 1987). The coefficient of determination (R^2) of all responses in the present study was above 0.8, which implied that the independent variables were highly attributed for the noodle making formulations.

Table 3.4. Experimental design for noodle development

	Factors			Responses			
Run No	**A: Rice Flour(g)**	**B: Corn Flour(g)**	**C: Xanthan Gum(g)**	**Cooking Time(s)**	**Cooking Loss (%)**	**Swelling Index(gg^{-1})**	**Solubility (%)**
1	82.449	12.551	5.00	254	26.1	26.8	24.8
2	82.370	15.000	2.630	210	27.0	37.1	36.0
3	85.000	13.751	1.249	180	30.8	37.9	35.8
4	80.002	14.998	5.000	252	26.2	26.8	24.8
5	83.678	14.735	1.587	193	30.5	37.8	35.8
6	80.002	14.998	5.000	250	25.9	26.8	24.8
7	83.575	13.508	2.916	223	27.0	37.3	36.0
8	82.370	15.000	2.630	210	27.0	37.1	36.0
9	84.957	11.127	3.915	235	26.5	36.0	26.0
10	85.000	13.751	1.249	180	30.8	37.9	35.8
11	81.604	14.146	4.250	243	26.1	25.9	23.0
12	85.000	10.002	4.998	248	26.3	26.5	24.1
13	85.000	10.002	4.998	248	26.3	26.5	24.1
14	83.787	12.214	3.999	235	26.5	36.0	26.0
15	85.000	12.210	2.790	218	27.0	37.2	36.0
16	82.449	12.551	5.000	250	26.1	26.8	24.2

A high R^2 value does not, however, guarantee that the regression model is always a fit one. A decent statistical model should have comparable adjusted and unadjusted R^2 values. A CV more than 10% typically denotes substantial mean value variation and a failure to meaningfully establish an acceptable response model. For the model and parameters to be significant, p values of 0.05 or below must be acquired, but for lack of fit, p values greater than 0.05 ($p>0.05$) must be produced. The cubic model was determined to be the best model for cooking time, cooking loss, and solubility when these rules for physical parameters were followed.

3.4.1. Effect of variables on cooking time

The statistical analysis from Design Expert Version 7.0 software suggested a cubic model as the best model.

Table 3.5. Analysis of Variance Table for cooking time

Source	***Sum of squares***	***df***	***Mean Square***	***F value***	***p-value prob>F***	
Model (cubic)	9916.39	9	1101.82	658.17	<0.0001	significant
Linear Mixture	9713.19	2	4856.59	2901.07	<0.0001	
AB	5.36	1	5.36	3.20	0.1238	
AC	29.22	1	29.22	17.46	0.0058	
BC	36.36	1	36.36	21.72	0.0035	
ABC	0.095	1	0.095	0.057	0.8194	
Residual	10.04	1	1.67			
Lack of fit	0.044	1	0.044	0.022	0.8874	Not significant
R^2	0.9990					
R^2_{adj}	0.9975					
C.V. %	0.57					

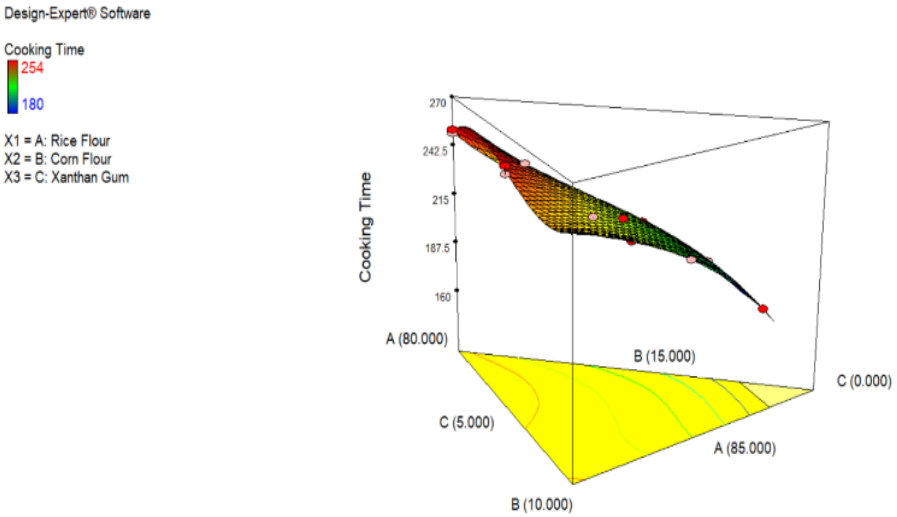

Figure 3.1.3D surface of effects of variables on cooking time

3.4.2. Effect of variables on cooking lossThe statistical analysis from Design Expert Version 7.0 software suggested a cubic model as the best model.

Table 3.6. Analysis of Variance Table for cooking loss

Source	***Sum of squares***	***df***	***Mean Square***	***F value***	***p-value prob>F***	
Model (cubic)	45.76	9	5.08	388.13	<0.0001	significant
Linear Mixture	35.72	2	17.86	1363.37	<0.0001	
AB	6.493E-003	1	6.493E-003	0.50	0.5078	
AC	2.87	1	2.87	219.14	<0.0001	

BC	2.22	1	2.22	169.42	<0.0001	
ABC	0.67	1	0.67	51.09	0.0004	
Residual	0.079	1	0.013			
Lack of fit	0.034	1	0.034	3.73	0.1112	Not significant
R^2	0.9983					
R^2_{adj}	0.9957					
C.V. %	0.42					

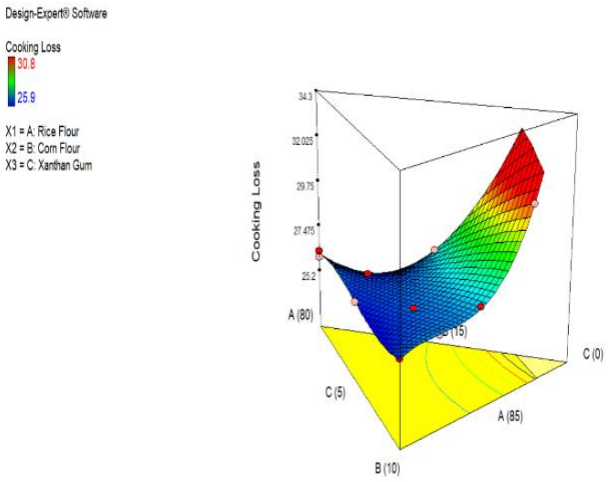

Figure 3.2. 3D surface of effects of variables on cooking loss

3.4.3. Effect of variables The statistical analysis from Design Expert Version 7.0 software suggested a cubic model as the best model.

Table 3.7. Analysis of Variance Table for swelling index

Source	***Sum of squares***	***df***	***Mean Square***	***F value***	***p-value prob>F***	
Model (cubic)	440.93	9	48.99	100.05	<0.0001	significant
Linear Mixture	349.48	2	174.74	356.84	<0.0001	
AB	0.19	1	0.19	0.38	0.5605	
AC	2.42	1	2.42	4.94	0.0680	
BC	2.46	1	2.46	5.03	0.0660	
ABC	0.035	1	0.035	0.072	0.7970	
Residual	2.94	6	0.49			
Lack of fit	2.94	1	2.94			
R^2	0.9934					
R^2_{adj}	0.9835					
C.V. %	2.15					

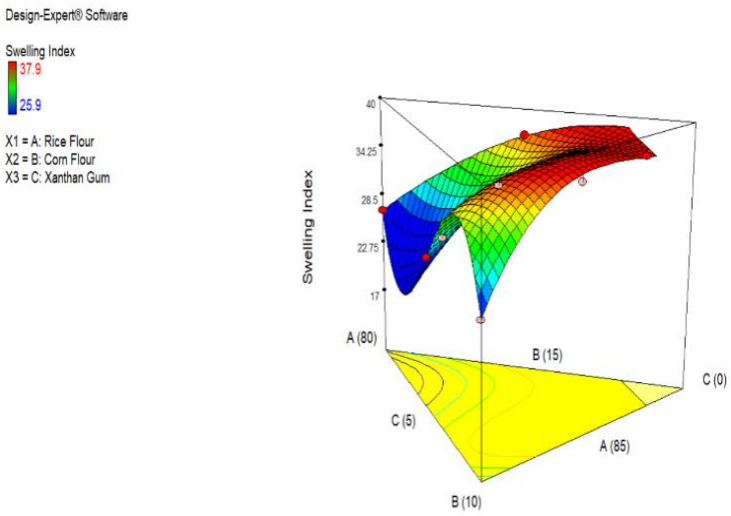

Figure3.3. 3D surface of effects of variables on swelling index

3.4.4. Effect of variables on solubilityThe statistical analysis from Design Expert Version 7.0 software suggested a cubic model as the best model.

Table 3.8. Analysis of Variance Table for solubility

Source	Sum of squares	df	Mean Square	F value	p-value prob>F	
Model (cubic)	505.77	9	56.20	205.36	<0.0001	significant
Linear Mixture	424.87	2	212.44	776.31	<0.0001	
AB	6.705E-004	1	6.705E-004	2.450E-003	0.9621	
AC	70.64	1	70.64	258.15	<0.0001	
BC	60.65	1	60.65	221.62	<0.0001	
ABC	37.33	1	37.33	136.42	<0.0001	
Residual	1.64	6	0.27			
Lack of fit	1.46	1	0.044	0.022	0.8874	significant
R^2	0.9968					
R^2_{adj}	0.9919					
C.V. %	1.77					

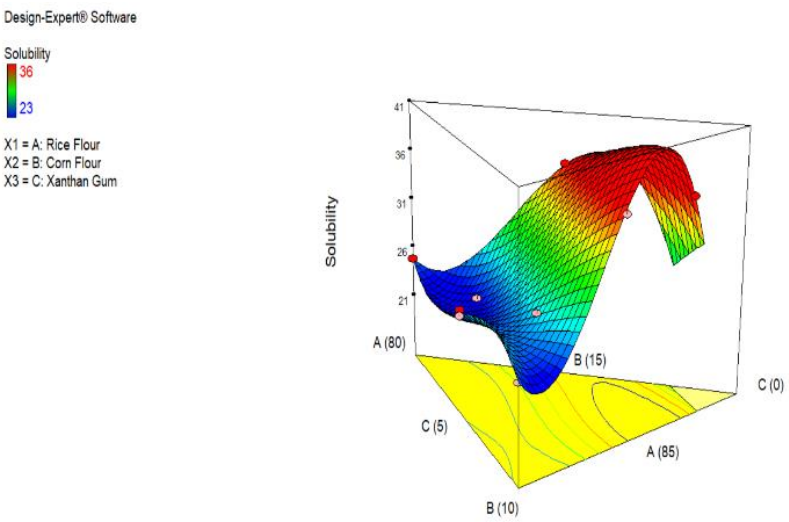

Fig 3.4. 3D surface of effects of variables on solubility

3.4.5. Standardization of Noodle composition

By choosing the responses from cooking time, cooking loss, swelling index, and solubility, the values of the variables were optimized. Based on the observation that the replies directly affected the caliber of the generated noodles, as demonstrated by the corresponding R^2 values, numerical as well as graphical optimization was used. The optimum values were 15% corn flour, 2.6% xanthan gum, and 82.386% rice flour. The standardized combination's validation was completed. The experimental results were discovered to be rather near to the projected results, demonstrating the accuracy of the predicted models.

Table 3.9. Criteria used for the optimization along with predicted value responses

Constraints	Goal	Lower limit	Upper limit	Importance	Standardized Values
Rice Flour	In range	80.0022	85	3	82.386
Corn Flour	In range	10.0018	15	3	15.000
Xanthan Gum	In range	1.24887	5	3	2.614
Cooking time	Minimize	180	254	3	209.776
Cooking loss	Minimize	25.9	30.8	3	27.0488
Swelling index	Maximize	25.9	37.9	3	37.0262
Solubility	Maximize	23	36	3	36

Table 3.10. Validation of the standardized combination

Parameters	*Actual*	*Predicted*
Rice Flour	82.3	82.3
Corn Flour	15.0	15.0
Xanthan Gum	2.6	2.6
Cooking Time	207.5	209.776
Cooking Loss	26.91	27.0488
Swelling Index	36.7	37.0262
Solubility	35.5	36

3.5. Proximate analysis of optimized noodle sample

Table 3.11 summarizes the proximate composition of the optimized noodle sample

Noodle sample	Moisture Db*(%)	Protein (%)	Ash
Optimized	2.5±0.30	29.9±0.97	0.213±0.17

*Values are mean ± SD of three independent analyses (n=3)

A B

Figure 3.5. Optimized (A- Uncooked Noodles) and (B – Cooked Noodles)

3.6. Rheology

Rheological behavior of the optimized sample's dough was studied and compared to that of a plain rice dough, in order to study the effects of addition of corn flour and xanthan gum.

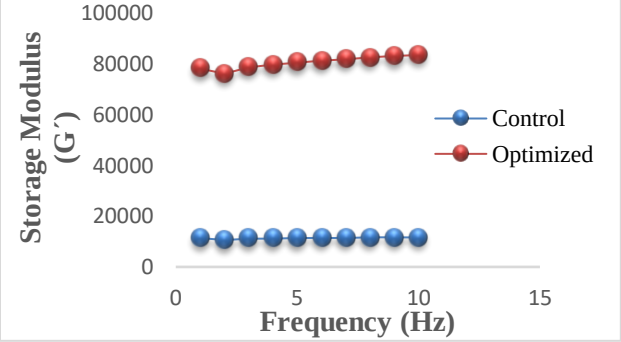

Figure 3.6. Effect of corn flour and xanthan gum incorporation into rice flour on storage modulus of rice dough

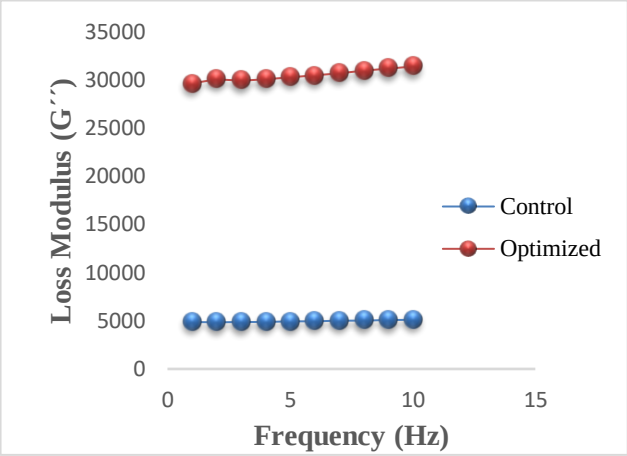

Figure 3.7. Effect of corn flour and xanthan gum incorporation into rice flour on Loss modulus of rice dough

The storage modulus provides information on the degree of structure in a material. It is a representation of the energy held inside the sample's elastic structure. The material can be regarded as predominantly elastic if the storage modulus is larger than the loss modulus and the phase shift is lower than 45ºC. The loss modulus represents the viscous component of the total amount of elasticity. A material is equally elastic and viscous if the phase shift is 45ºc or tanφ=1.

It is considered to be a hard dough if the difference between storage modulus and loss modulus is greater than 1000, and a soft dough if the difference value is less than 1000. The addition of maize flour and xanthan gum enhanced both the elastic modulus (G') and viscous modulus (G''). The rise in modulus value suggests that the dough got stronger.

3.7. Sensory evaluation

The optimized noodle sample was subjected to sensory analysis and there was a total of 20 number of panelists. Each panelist were provided with a sensory analysis sheet and hedonic scale rating was followed. The product received a sensory score of 7.83 and 8.04 respectively for color and aroma.

Table 3.12. Sensory scores of the cooked noodles

Parameters	*Score**
Appearance	7.50 ±0.5
Colour	7.83 ±1.0
Texture	8.33± 0.8
Aroma	8.04 ±1.4
Taste	8.40 ±0.7
Overall acceptability	8.22 ±0.3

*Values are mean SD of three independent analyses (n=9)

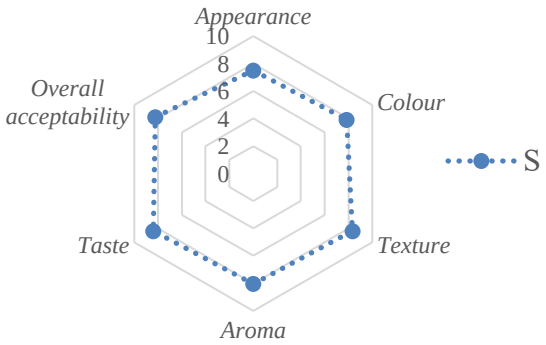

Figure 3.8. Radar graph for sensory data of the noodles

3.8. Effect of storage on moisture content of the optimized noodle sample

The amount of moisture gained by the optimized sample kept in an accelerated shelf-life condition for 15 days is shown in the table below.

Table 3.13. Storage studies of optimizednoodles

No. of days	*Gain of moisture (%)*
0	0
5	1.129
10	6.967
15	7.969

4. Conclusions

From the current research carried out, it can be concluded that the addition of corn flour and xanthan gum in the rice flour alters the rheological behavior, greatly. From the rheological data, it is seen that the dough is hard and possesses viscoelastic nature. Both elastic modulus (G´) and viscous modulus (G´´) were seen to increase with the addition. The magnitude of the modulus increased, which implies that the rice dough became stronger.

The optimized product has a protein content of 30% whereas noodles obtained in the market have a protein content of 11-15%. This considerable amount of protein can be attributed to the presence of both rice flour and corn flour bound together by xanthan gum.

The product has a cooking time of 3-4 minutes and gives off a good aroma thus a sensory score of 8 in average.

However, the cooking loss of 26-27% is a considerable amount of loss of the product and doesn't comply with the acceptable limit (BIS, 1993). The weakening and disruption of the protein-starch matrix is blamed for cooking loss. Disruption of the protein starch matrix may be the cause of an increase in cooking loss. Further research for the stability of the noodles prepared from the Bora Rice Flour can be carried out in the future.

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FERMENTATION POTENTIALS OF BAOBAB (*Adansonia digitata*) PULP POWDER IN THE PRODUCTION OF YOGHURT FROM COW MILK

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ABSTRACT

Yoghurt was produced from fresh cow milk inoculated with baobab pulp powder. Baobab pulp powder was added at 0.34%, 0.52%, 0.69%, and 0.85% respectively, while the control sample (A) was yoghurt produced from fresh cow milk inoculated with regular commercial starter culture. The physical, chemical, microbiological, anti-nutritional and sensory properties of the samples were analysed using standard procedures. The moisture content, crude protein and fat content decreased with the added baobab pulp powder, while the fibre, ash and carbohydrate contents increased. The pH ranged from 4.34 to 4.74, coupled with negligible anti-nutritional composition. The titratable acidity increased with added baobab pulp powder from 0.52-0.69%. Brix and viscosity of treated samples increased respectively from 30.85-40.02 and 200.05-200.14. Total bacterial and fungi counts ranged from 8.65×10^4 to 15.51×10^4 Cfu/mL and 1.12×10^4 to 4.54×10^4 Cfu/mL respectively, with the control sample having the higher loads. The over-all acceptability of the samples were significant ($p>0.05$); sample E (0.85% inoculation) was the most preferred, followed by sample B (0.34% inoculation), while the least accepted sample was the control. Inoculating milk with baobab pulp powder produced yoghurt with improved and acceptable qualities.

1. Introduction

Worldwide, fruit trees are important biological resources in many agro-ecological and forest ecosystems, with economic impact (Rasheed *et al*., 2015). Fruits usually have essential nutrients, antioxidants and health benefits important to humans and animals in all respect. Sometimes, fruits differ from vegetables and other edible agricultural/horticultural requiring pre-treatments, like heating, before being consumed (Rasheed *et al*., 2015; Cernansky, 2015), with the tropics blessed with many nutritious edible varieties (Paull & Duarte, 2011; Sthapit *et al.*, 2012).

Adansonia digitata from the genus *Adansonia* family, *Malvaceae,* is a deciduous tree, with the genus *Adansonia,* being the most common specie, and majorly found in the hot savannahs or sub Saharan Africa. The tree is locally called "ose" in "Yoruba" speaking areas of Nigeria or "kuka" in Hausa language (Stapleton, 2015). Baobab (*Adansonia digitata*) is a typical tropical fruit tree with nutritional and medicinal benefits (Donatien *et al*., 2011); the leaves are used for preparing soup, seeds as thickening agent or fermented and used as flavouring agent, or could be roasted and eaten as snacks (Bvenura & Sirakumar, 2017; Kamatou *et al*., 2011). The fruit pulp is licked or processed into drinks, while the tree bark is used to make rope (Donatien *et al*., 2011). As such, all baobab tree parts are useful; it included food provision, shelter, clothing, and for medicinal purposes.

Bilcke *et al*., 2013 and Afolabi & Popoola, 2005 reported lactic acid fermenting bacteria in baobab fruit pulp. The fruit is an indehiscent large egg-shaped capsule, while the pulp, when dried, hardens, and falls to pieces to look like chunks of powdery dry bread (Kamatou *et al*., 2011; Bvenura & Sirakumar, 2017; Namratha & Sahithi, 2015). The seeds are kidney shaped, hard and black in appearance (Donatien *et al*., 2011), with the pulps high in phytochemicals, like antioxidant, anti-inflammatory, anti-microbial etc. According to Bvenura & Sirakumar, 2017, baobab has about ten times Vit. C contents of orange, and as such, could increase products shelf-life (Donatien *et al*., 2011). Over the years, fermentation process has been improved upon to give varieties of digestible and edible foods that have improved sensory qualities, increased shelf-life, improved nutrition and preservation (Yasmine, 2002).

Yoghurt from Turkish verb, "jugurt", means "curdled or coagulated" (Weerathilake *et al*., 2014; Obi *et* al., 2016). It is a fermented product with many nutritional and health benefits (Fisberg & Rachel, 2015). Its production involved the use of thermophilic organisms, lactobacillus and streptococcal species in the milk fermentation (Marshall, 1993). It was reported that fermenting organisms have symbiotic relationship to produce yoghurt from milk coagulation, with sufficient quantity of lactic acid (Obi *et al.*, 2016; Amanze, 2015). The consumption of dairy products with probiotic bacteria is highly beneficial, all because the high quantities of the organisms in the colon improve intestinal health. Michelle, 2005 as well as Fisberg & Rachel, 2015 were of the opinion that yoghurt supplies high quality proteins, minerals and sufficient quantities of vitamins. The use of starter cultures for yoghurt production helps to give the desired characteristics, and therapeutic benefits. As such, producers do add 2-4 % starter culture for production, but of recent, addition of fruit flavour is trending (Namratha & Sahithi, 2015; Ghadge *et al.*, 2008).

Yoghurts vary in appearance, flavour and ingredients, with its quality and composition influenced by bacterial cultures used (Kim & Ham, 2019). According to Weerathilake *et al.*, 2014, standardized milk sourced is homogenized at about 55-65°C and 15-20 MPa, and pasteurized for 30mins at 80-85°C. It was then cooled to between 40-45°C, incubation temperature for starter culture addition. The fermented milk could be transformed into either set or stirred yoghurt. For set yoghurt, the fermented milk is packed and incubated before chilling and cooling, while for stirred one, the fermented milk is incubated and cooled to 20-25°C before stirring, cooled again and pumped. Both set and stirred yoghurt are normally cold and stored before dispatch. Fisberg & Rachel, 2015, and Weerathilake *et al*., 2014, classified yoghurt on the following bases: Chemical composition based on fat content; physical nature, either solid, semi-solid or fluid; flavour component i.e. on flavour; and post fermentation process, where it was re-classified based on processes after fermentation e.g. enzyme hydrolysis, vitamin fortification, heat treatment etc.

Industrially, yoghurt production is in three stages of mix preparation of physical treatments (homogenization, heat treatment, cooling, de-aeration); fermentation process of inoculating the mix; and harvesting, post-treatment, and packaging etc., with the final product quality being a function of adopted production steps, except for the set-type yoghurt, product flavouring and cup filling after fermentation (Corrieu & Beal, 2016; Weerathilake *et al*., 2014).

Yoghurt nutrient depends on raw milk quality, animal feed, lactation stage, age, and environmental factors such as season or temperature, heat exposure period, exposure to light, and storage conditions etc. (Fisberg & Rachel, 2015; Michelle, 2005). The milk constituents variation during fermentation, strain of bacteria used, milk solids and source, fermentation duration etc. could also determine the final product (Rekha *et al*., 2012; Fisberg & Rachel, 2015).

Baobab fruit pulp is nutrient-dense, but the tree is practically going into extinction in this part of the world. This position informed the

design of this research work, which was aimed at increasing yoghurt consumption, enjoying the high medicinal, nutritional qualities, and particularly, exploiting the potentials of the pulp's fermenting organism. Utilizing the pulp will reduce the dependence on imported fermenting culture; increase the nation's external reserves, creates jobs through baobab planting to ease accessibility, and ultimately reduced yoghurt unit cost. The research aimed at studying the fermentation potentials of baobab (*Adansonia digitata)* pulp powder in yoghurt production.

2. Materials and methods

2.1. Sample Collection

Fresh baobab fruit was sourced from a village at Egbejila, Asa-dam area of Ilorin, Kwara State, while fresh cow milk was sourced from the University of Ilorin dairy farm.

2.2. Extraction of the fruit pulp

The pulp, which was reported to be high in lactic acid fermenting bacterial, according to Afolabi & Popoola, 2005 and Donatien *et al*. (2011), was extraction from the fruit. Fruit was washed, dirt removed, and broken up to remove pulp encrusted seeds, and dried for easy removal during pounding. The pulp was winnowed, properly pounded for smooth textured powder and sieved to remove dirt (Fig. 1).

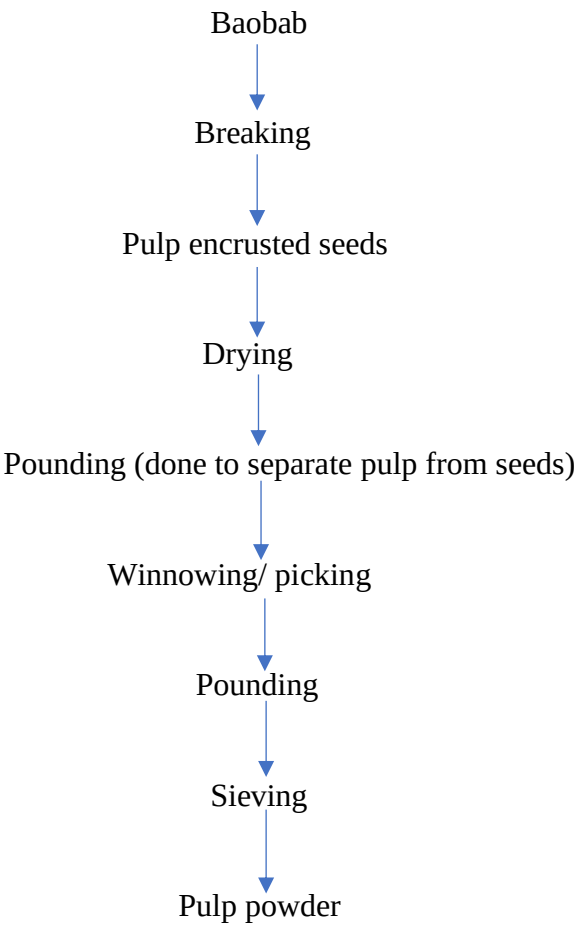

Figure 1. Production of Baobab pulp **(**Afolabi and Popoola, 2005)

2.3. Preparation of the Baobab Fermented Yoghurt

Milk was pasteurized (90ºC) for 3mins, homogenized, cool to 45-46ºC, inoculated with baobab pulp powder (Table 1), fermented for 20-22hrs, and cooled to 7ºC to deactivate fermenting organisms (Fig. 2) (Corrieu and Beal, 2016; Han *et al*., 2012; Abioye *et al*., 2012).

Table 1. Formulation table for the baobab fermented yoghurt

Sample	Fresh Milk (%)	Baobab pulp powder (%)
A	100	0
B	99.66	0.34
C	99.48	0.52
D	99.31	0.69
E	99.15	0.85

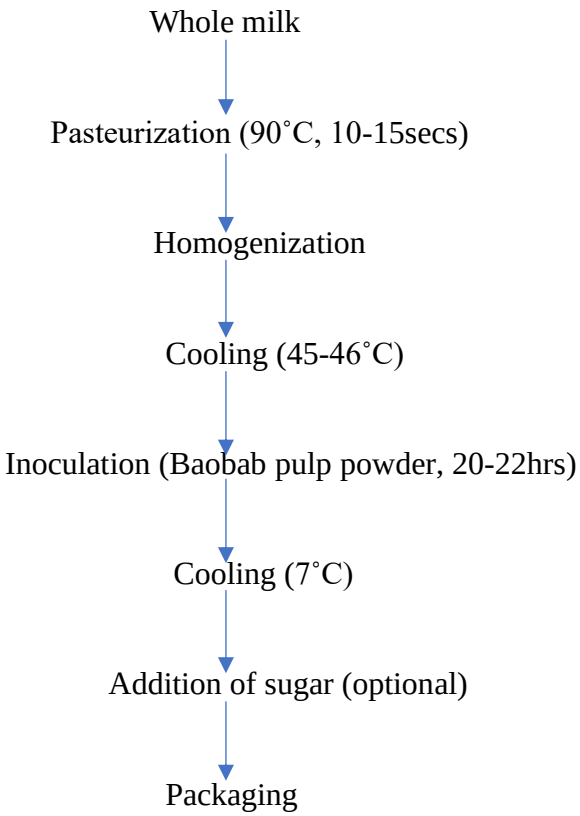

Figure 2. Production of baobab fermented yoghurt **(**Corrieu and Beal, 2016; Han *et al*., 2012)

2.4. Physicochemical Screening of Baobab Fermented yoghurt

Physicochemical properties of samples were analyzed with AOAC (2005) standard methods.

2.4.1. pH measurement

The pH was measured at room temperature (26±2˚C) with a digital pH meter previously calibrated with buffer standards of pH 4 and pH 10.

2.4.2. Total Titratable Acid (TTA)

Titratable acidity was determined by the method of Joseph & Joy, 2011, using phenolphthalein indicator, and end point volume of NaOH used was used to calculate acid percentage.

Titratable acidity: $\frac{Titre\ value \times M \times 90 \times 100}{volume\ of\ sample \times 1000}$

Where M= molar concentration of NaOH

(1)

2.4.3. Measurement of Viscosity

Viscosity was measured with a viscometer model HAAKE Viscosimeter (Mess Technik GmbH) (Brookfield Engineering Laboratories Inc., Stoughton, MA) in mPas (AOAC, 2005).

2.4.4. Measurement of Colour Attributes

The colour attributes (Hunter L*, a* and b* values) of the yoghurt samples was obtained with a colorimeter (Minolta CR 300 Series, Minolta Camera Co., Ltd., Osaka, Japan). The samples were placed on white standard plate, values were taken, and the parameters determined appropriately (Asal *et al*., 2015).

2.4.5. Brix Measurement

Brix value was measured with a refractometer using the method of Amanze, 2015.

2.4.6. Proximate Composition of the Yoghurt Samples

2.4.6.1. Determination of Protein Content

Protein was determined by macro Kjeldahl method. 2 g sample was measured into Kjeldahl digestion flask; 10 g copper sulphate and sodium sulphate added in ratio 5:1, and 25 mL conc. sulphuric acid added to digest at high temperature in a fume cupboard until frothing ceased, with clear light blue. Digest was cooled and diluted with distilled water to 100 mL mark; 10 mL of dilute and 18 mL 40% NaOH were poured into the distillation apparatus. 25 mL of 2% boric acid was added to the receiving flask, with 2 drops of bromocresol green and methyl red mixed indicator. Distillation continued until boric acid turned yellowish green from pink, and then titrated with 0.1N HCl to end point, but the blank with distilled water (AOAC, 2005).

% crude protein=% nitrogen × 6.25

% nitrogen=

$$\frac{(\text{ml standard acid} - \text{ml blank}) \times \text{N of acid} \times 1.4007 \text{sample in gram}}{sample\ in\ grams} \quad (2)$$

2.4.6.2. Determination of Moisture Content

2 g sample was weighed into a dried crucible with known weight, and dried in a controlled oven (105˚C) for 5hrs, cooled in a desiccator and re-weighed (AOAC, 2005).

$$\%moisture\ content\ \frac{W1 - W_{\circ}}{W2} \times 100 \quad (3)$$

Where, W1=initial weight of crucible and dried sample; W2=weight of the sample; $W_{\circ}$= weight of the empty crucible

2.4.6.3. Determination of Ash Content

2 g sample was weighed into a dried crucible, and incinerated to ash in a muffle furnace at 550℃. It was removed, cooled in desiccator, and ash weight determined (kemelo *et al*., 2019).

% ash=

$$\frac{weight\ after\ ashing - weight\ of\ crucible}{weight\ of\ samples} \times 100 \quad (4)$$

2.4.6.4. Determination of Crude fat

5 g sample was properly mixed with 0.88 mL ammonia solution and 10 mL of 95% ethanol. 25 mL diethyl ether was added and vigorously shaken for 1 min. About 25 mL petroleum ether was added, shaken vigorously, and left to stand for 1hr to separate aqueous and organic phases. Fat extract (organic phase) was collected and aqueous phase removed by distillation. Fat extract dried at 100℃ for 30mins, cooled in a desiccator, and fat mass determined (Kemelo *et al*., 2019).

$$\%\ \text{fat} = \frac{\text{weight of extracted fat (g)}}{\text{weight of sample used (g)}} \times 100 \quad (5)$$

2.4.6.5. Determination of Crude Fibre

2 g sample was hydrolysed in a beaker containing 299 mL of 1.25% sulphuric acid and boiled for 30mins; mixture was filtered under vacuum, residue washed with hot distilled water thrice, re-boiled for 30mins with 200 mL of 1.25% of NaOH and filtered. Digested was washed with HCl to neutralize NaOH and distilled thrice with hot distilled water. Residue was poured into a crucible, oven dried (100℃;

2hrs), cooled in a desiccator and re-weighed. Dried residue was incinerated (500℃; 5hrs) to totally burn off carbonaceous matters, cooled and weighed (AOAC, 2005; Friedman & Brandon, 2013).

$$\% \text{ crude fibre} = \frac{\text{loss in weighed (g) after ignition}}{\text{weight of the original sample } (g)} \times 100 \quad (vi)$$

$$= \frac{W1-W2}{W} \times 100 \quad (6)$$

Where: W1= weight of digested sample and crucible before ash; W2=weight of crucible and ash; W=weight of sample used.

2.4.7. Determination of Ascorbic Acid (Vitamin C)

The official method of AOAC, 2005 was combined with that of Kim & Ham, 2019 was adopted, with results reported as mg ascorbic acid/100g. 20 mL sample solution was measured into 250 mL conical flask, with 2 mL oxalic acid, 150 mL distilled water and 1 mL starch indicator, and titrated with 0.005molL^{-1} iodine solution.

2.4.8. Anti-nutrients in the yoghurt sample

2.4.8.1. Determination of Phytate Content

4 g sample was diluted with 100 mL 2% HCl and filtered. Within a conical flask was 25 mL filtrate and 5 mL 0.3% ammonium thiocyanate as indicator; 53.5 mL distilled water added to adjust pH to 3.5, and titrated with ferric chloride solution having 0.00195g iron/mL for brownish yellow colour persisting for 5mins, and phytate (mg/100g) calculated (Kayode *et al*, 2013):

$$\text{Phytate content (mol/Kg)} = \frac{T \times 564.11}{M} \quad (7)$$

Where: T = titre value; M= molar mass of phytate

2.4.8.2. Determination of Cyanide Content

4 g sample was added to mixture of 40 mL distilled water and 2 mL orthophosphoric acid, and left overnight (22-26˚C) to release bound hydrocyanic acid, with extract distilled with a drop of paraffin as antifoaming agent and broken chips as anti-bump. 5 mL distillate, 40 mL distilled water and 0.1g NaOH pellets were transferred into 50 mL volumetric flask and made up to mark with distilled water. 20 mL of solution and 1 mL of 5% KI solution were titrated with 0.01M silver nitrate solution, with distilled water used as blank (Oluwaniyi & Oladipo, 2017).

2.4.8.3. Determination of Oxalate Content

75 mL of 3.0M H_2SO_4 was added to 1 g sample and stirred intermittently with a magnetic stirrer for 1hr and filtered. 25 mL of filtrate was titrated while hot (80^{0}C) with 0.05M $KMnO_4$ solution until a faint pink colour appeared, and persisted for at least 30secs.

$$\text{Oxalates content (mg/100 g)} = \frac{T \times [Vme] \times [DF] \times 2.4 \times 10^2}{ME \times Mf} \quad (8)$$

Where: T = titer of $KMnO_4$,; Vme = Volume-mass equivalent (*i.e* 1 mL of 0.05 M $KMnO_4$ solution is equivalent to 0.00225 g anhydrous oxalic acid); DF = Dilution factor, VT/A; VT = Total volume of filtrate (75 mL),

A = Aliquot used (25 mL); ME = molar equivalent of $KMnO_4$, Mf = Weight of sample used (Kayode *et al*., 2013).

2.4.9. Microbial Analysis of the yoghurt

2.4.9.1. Sterilization of Materials

Wares, inoculating loop, needles, and other required materials were properly sterilized (160˚C) and sanitized to destroy possible contaminants. The environment was sanitized, and work benches wiped with 70% alcohol (Fawole & Oso, 2007).

2.4.9.2. Preparation of Culture Media

Bacterial nutrient agar used has 28 g powder dissolved in 1L distilled water, mixed and heated to dissolve complete. Flask mouth was plugged with cotton wool, wrapped in aluminum foil, sterilized at 121˚C for 15mins, cooled to 45˚C, and poured aseptically into petri-dishes.

2.4.9.3. Total Bacteria Count

1 mL sample was pipetted aseptically into 9 mL sterile distilled water in test tube, using serial dilution of 1mL into 9 mL sterile distilled water, prepared up to $10^{-4.}$. This was plated in duplicate by pouring 1 mL into separate petri-dishes, sterile molten agar added, plate mixed by swirling before solidifying, incubated at 37˚C for 24hrs and colonies counted in cfu g^{-1}.

2.4.9.4. Total Fungi Count

1 mL of sample was pipetted aseptically into 9 mL sterile distilled water in a test tube, using serial dilution of 1 mL into 9 mL sterile distilled

water up to $10^{-4.}$ It was plated in duplicate with sterile molten agar added, and mixed by swirling plate before solidifying. They were incubated (37˚C for 48hrs) and examined for growth, and colonies were counted in cfu g^{-1}.

2.4.10. Sensory Evaluation & Statistical analysis

50 untrained panellists, but regular yoghurt consumers, evaluated samples for taste, appearance, flavour, consistency and overall acceptability with 9-point hedonic scale of excellent (score = 9) to poor (score = 0) (Obi *et al*., 2010), and data computed and analyzed.

3. Results and discussions

3.1. Proximate Composition of the Yoghurt Samples

Table 2 showed the proximate composition of the yoghurt samples. The moisture content, crude fat, crude protein, ash and carbohydrate were significant ($p \geq 0.05$), but not in the crude fibre. Reduced moisture in the treated samples may be due to added baobab pulp powder. Sample E had the highest moisture among the treated samples, but the control sample was the least viscous, though with higher moisture content. The values obtained were similar to that of Obi *et al*., 2016. Crude protein values ranged from 5.87% to 6.26%, with the control having the highest value. The values reduced with increasing quantities of baobab pulp inoculum, perhaps due to its lower protein content when compared to that in animals. Protein value of 2.3% was reported for baobab pulp by Bvenura & Sirakumar, 2017, and Sadiq *et al*., 2009, reported 1.53%. Michelle, 2005, however, reported 6 to 8.6% protein in plain yoghurt, which was similar to our recorded values.

Fat content varies based on yoghurt type, with values of 0.5% in nonfat to about 2% in low-fat sample, and about 3.25% in full fat yoghurt (Mbaeyi-Nwaoha & Ekere, 2014). The fat content was 3.66-4.02%, portraying the samples to be full fat yoghurts, and similar to 3.17% to 3.95% reported by Obi e*t al*., 2016 and Fisberg & Rachel, 2015 for full fat yoghurt. Treated samples fibre content was not significant ($p<0.05$) from one another, but significant to the control, with values similar to that of Amanze & Amanze, 2011 and Michelle, 2005.

Carbohydrate values were between 10.03 and19.21%. Sample D had the highest value, while the control, had the least. The values were similar to 9.41-19.33% reported by Mbaeyi-Nwaoha & Ekere, 2014 for yoghurt from skimmed milk. Ash content usually measures mineral content of samples. The control sample was significantly different from the treated samples, with values (0.87-1.31%) increasing with increased addition of baobab pulp.

Table 2. Proximate Composition of the Yoghurt samples

Sample	Moisture (%)	Protein (%)	Fat (%)	Fibre (%)	Carb. (%)	Ash (%)
A	78.23±0.013^{a}	6.26±0.023^{a}	4.21±0.005^{a}	0.00±0.008^{b}	10.03±0.025^{e}	0.87±0.001^{c}
B	73.44±0.022^{b}	6.12±0.018^{a}	4.02±0.024^{a}	0.07±0.014^{a}	15.44±0.076^{c}	1.10±0.013^{b}
C	72.12±0.021^{c}	6.04±0.036^{a}	3.86±0.036^{b}	0.07±0.000^{a}	17.35±0.065^{b}	1.23±0.054^{a}
D	73.34±0.031^{b}	5.94±0.002^{b}	3.71±0.001^{b}	0.08±0.001^{a}	19.21±0.027^{a}	1.23±0.056^{a}
E	75.03±0.011^{b}	5.87±0.017^{b}	3.66±0.012^{b}	0.09±0.000^{a}	13.84±0.010^{d}	1.31±0.047^{a}

Values are means ± SD. Mean sharing a common superscript letter in a column are not significantly different ($p \geq 0.05$).

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

3.2. Physicochemical Properties of the Yoghurt Samples

Table 3 showed the results of the physicochemical properties of the samples. The samples had lower pH (4.38-4.54), which were not different from 4.5-5.0 reported by Fisberg & Rachel, 2015, but close to 4.34-6.70 reported by Fatiha *et al*., 2016. The low pH values, according to Afolabi & Popoola, 2005, are attributable to the presence of *Lactobacillus acidophilus* and *Streptococcus lactis* in the pulp. Fermented milk lactose gives lactic acid that confirms presence of lactic acid fermenters in baobab pulp (Mataragas *et al*., 2011). Brix values increased with increased sugar content. Brix of baobab inoculated samples was between 40 and 40.02˚Brix, similar to that reported by Afolabi & Popoola, 2005 and Obi *et al*., 2010. Viscosity influence final product quality (Guzel-seydim *et al*., 2005), and is usually affected by the milk composition, heat, starter culture used, and processing method etc. (Dantas, 2016). The treated samples were slightly more viscous, and significant ($p \geq 0.05$) to the control.

Table 3. Physicochemical Properties of the Yoghurt Samples.

Sample	Viscosity (m.p.s)	Titratable Acidity (g/100ml)	pH	Brix (˚)
A	200.05±0.57^{b}	0.43±0.01^{b}	4.74±0.00^{a}	30.85±0.00^{b}
B	200.19±1.29^{a}	0.69±0.00^{a}	4.38±0.00^{a}	40.00±0.00^{a}
C	200.17±0.34^{a}	0.65±0.00^{a}	4.45±0.01^{a}	40.01±0.00^{a}
D	200.17±1.43^{a}	0.66±0.01^{a}	4.51±0.02^{a}	40.01±0.00^{a}
E	200.14±0.00^{a}	0.62±0.00^{a}	4.54±0.01^{a}	40.02±0.00^{a}

Values are means ± SD. Mean sharing a common superscript letter in a column are not significantly different ($p \geq 0.05$).

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

3.3. Ascorbic acid and Colour Parameters of Yoghurt Samples

Table 4 showed the result of ascorbic acid and colour parameters measured. Ascorbic acid content of treated samples was significant ($p > 0.05$) to the control, with the control sample having the least value. High Vit. C content in the treated samples was due to the high Vit. C reported in baobab pulp, and similar to that reported by Taneva & Panayotov, 2019. The high value will improve shelf life and medicinal derivatives because ascorbic acid is an antioxidant, and in foods, could prevent oxidation of free radicals and enhances proper functioning of the immune system.

Table 4. Ascorbic acid content and Colour Parameters of Yoghurt Samples

Sample	Vit. C (mg/100g)	L*	a*	b*
A	8.02±0.01^{b}	48.73±0.12^{b}	1.14±0.14^{a}	7.17±0.10^{d}
B	10.04±0.02^{a}	49.53±0.46^{a}	1.08±0.02^{a}	10.73±0.07^{c}
C	10.02±0.02^{a}	49.94±0.42^{a}	0.27±0.04^{b}	11.43±0.15^{b}
D	10.03±0.01^{a}	49.74±0.34^{a}	0.15±0.03^{c}	12.32±0.02^{a}
E	8.02±0.01^{a}	49.87±0.13^{a}	0.06±0.07^{d}	11.57±0.50^{b}

Values are means ± SD. Mean sharing a common superscript letter in a column are not significantly different ($p \geq 0.05$).

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

The addition of baobab significantly affected colour. Lightness (L*) increased from 48.73 in the control to 49.94 in sample C. High L* value was associated with whiteness (Emmanuel *et al*.,

2019), and this increase, perhaps, may be due to presence of bioactive component in baobab pulp, which aided the breakdown of some milk compounds (Bojana *et al*., 2020). Redness a* was significant (1.14 to 0.06), probably because of carotene presence, while b* values of treated samples were higher than the control, likely due to pulp colour and thickness (Hasim *et al*., 2009).

3.4. Anti-Nutritional Composition of Yoghurt Samples

Cyanide values were not significant ($p>0.05$), but the oxalate and phytate were significant. Analog milk usually has anti-nutrient compounds absent in dairy. Baobab with 2% phytic acid, 10% oxalate according to Bvenura & Sirakumar (2017), could have reduced during the processing regimes.

Table 5. Anti-nutritional Composition of the Yoghurt Samples

Samples	Phytate (mg/100g)	HCN (mg/100g)	Oxalate (mg/100g)
A	0.01^{b}±0.01	0.02^{a}±0.00	0.05^{b}±0.01
B	0.33^{a}±0.32	0.04^{a}±0.23	0.05^{b}±0.02
C	0.43^{a}±0.30	0.02^{a}±0.21	0.06^{b}±0.34
D	0.57^{a}±0.01	0.04^{a}±0.02	0.07^{b}±0.05
E	0.73^{a}±0.32	0.06^{a}±0.12	0.10^{a}±0.01

Values are means ± SD. Mean sharing a common superscript letter in a column are not significantly different ($p\geq0.05$).

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

3.5. Total Bacterial and Fungi Count of the Yoghurt Samples

Table 6 showed the total bacterial and fungal counts of the samples (in Cfu/mL), with significant differences ($p>0.05$) noticed. The control sample had the highest bacterial and fungal loads. Lesser loads noticed in the treated samples may be due to lower pH value and high ascorbic acid contents (an antioxidant) in baobab pulp. Sample D had least bacteria load, and better preserved, which corroborated the report of Afolabi & Popoola, 2005 that the presence of baobab pulp in fermented tempe reduced the growth of spoilage bacteria. Aside sample E, fungi count of treated samples decreased with baobab pulp, likely due to the bioactive components of baobab pulp. Chadare *et al.,* 2009 and Ramadan *et al.,* 1993 had reported the bioactive components to include triterpenoids, flavonoids, phenolic compounds, saponins, β-sitosterol, phytates etc.

Table 6. Total Bacterial and Fungi Count of the Yoghurt Samples

Samples	Total bacterial count (Cfu/ml) (10^4)	Total Fungi count (Cfu/ml) (10^4)
A	15.51 ± 0.16^{a}	4.54 ± 022^{a}
B	11.32 ± 0.12^{b}	1.12 ± 1.11^{c}
C	9.15 ± 0.42^{d}	1.14 ± 1.32^{c}
D	8.65 ± 0.62^{d}	2.47 ± 1.58^{b}
E	10.76 ± 0.57^{c}	2.76 ± 0.06^{b}

Values are means ± SD. Mean sharing a common superscript letter in a column are not significantly different ($p\geq0.05$).

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

3.6. Sensory Evaluation of the Yoghurt Samples

Table 7 showed the sensory scores of the samples, as well as the radar graph of the sensory evaluation. The mouth feel was not significant, but the flavour, appearance, taste, consistency and overall acceptability were significant ($p \geq 0.05$). Baobab pulp had effect on the samples judging by the recorded scores. Control had the best rating for appearance (7.51), while sample E was rated least (6.13). The control was equally rated highest for flavour, but treated samples had less pronounced yoghurt flavour, and as longer as fermentation progressed, baobab pulp masked its effect. Sample E had the lowest flavour (6.00).

Taste of treated samples was not significant ($p > 0.05$), though sample E had highest rating (7.22), and the control, the lowest (6.43); probably due to its lesser ascorbic acid content. For consistency, the rating ranged from 6.48 (control) to 7.57 (sample E). Consistency increased with added baobab pulp, except for sample C, which was comparable to that of Afolabi & Popoola, 2005, as well as Fisberg and Rachel, 2015, who stated that decreased pH of milk medium to about 4.6 could lead to casein coagulation (< 4.6 here). Mouth feel was not significant ($p > 0.05$), but was similar to 5.05-7.80 reported for beetroot flavoured yoghurt by Mbaeyi-Nwaoha & Nwachukwu, 2012. Overall acceptability of the samples was significant ($p > 0.05$). The control was rated least (6.65), and different from some of the treated samples, while samples B and E were rated the best.

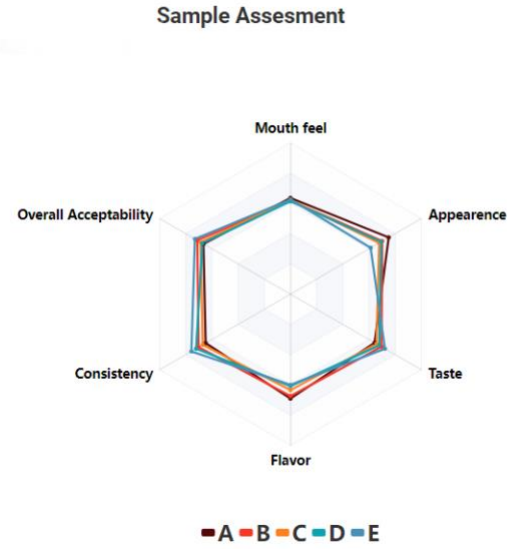

Figure 3: Radar Graph of the Samples Sensory Evaluation

Table 7. Sensory Evaluation of Yoghurt Samples

Sample	Mouth feel	Appearance	Taste	Flavour	Consistency	Overall acceptability
A	$6.36^{a}\pm1.83$	$7.51^{a}\pm1.65$	$6.43^{b}\pm2.04$	$6.91^{a}\pm1.51$	$6.48^{b}\pm1.31$	$6.65^{b}\pm1.56$
B	$6.18^{a}\pm1.25$	$7.01^{a}\pm1.50$	$6.96^{a}\pm1.61$	$6.74^{a}\pm1.74$	$7.02^{a}\pm1.20$	$7.12^{a}\pm1.26$
C	$6.23^{a}\pm0.93$	$6.78^{b}\pm1.45$	$6.57^{b}\pm1.20$	$6.35^{b}\pm1.19$	$6.70^{b}\pm1.10$	$6.87^{b}\pm0.90$
D	$6.14^{a}\pm2.14$	$6.93^{b}\pm1.59$	$6.78^{a}\pm1.78$	$6.08^{c}\pm1.35$	$7.21^{a}\pm1.18$	$6.73^{b}\pm1.13$
E	$6.27^{a}\pm1.33$	$6.13^{c}\pm1.83$	$7.22^{a}\pm1.83$	$6.00^{c}\pm1.75$	$7.57^{a}\pm1.23$	$7.30^{a}\pm1.29$

Mean ± Standard deviation. Mean with different superscripts along the column are significantly different ($p < 0.05$)

Sample key:

A- 100% Cow Milk (Control); **B**- 99.66% Cow Milk; 0.34% (2g) Baobab Pulp Powder; **C**- 99.48% Cow Milk; 0.52% (3g) Baobab Pulp Powder; **D**- 99.31% Cow Milk; 0.69% (4g) Baobab Pulp Powder; **E**- 99.15% Cow Milk; 0.85% (5g) Baobab Pulp Powder

4. Conclusions

From the research results, it could be concluded that the use of baobab pulp powder as inoculum in yoghurt production at 2g and 5g was effective, could be recommended for commercial use. The range of this baobab powder did not affect the overall acceptability of the yoghurt. It however improves taste, consistency and shelf-life due to its acidic nature. It equally reduced production cost, as expensive streptococcus and lactobacillus species for inoculation need not be purchased and stored at extra cost. The use could also increase sales, as consumers in this part of the world are familiar with the plant, but the availability and accessibility of baobab fruit should be seriously looked into.

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DEVELOPMENT OF LYCOPENE CONTENT IN TOMATOES AT COLOUR BREAK AND VARIATION OF IT DURING STORAGE AND PROCESSING

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ABSTRACT

Lycopene is a natural pigment present in tomatoes and is responsible for red colour, Lycopene content in tomatoes is varied based on the variety, climatic condition, soil type and rainfall pattern in cultivated areas and also on the maturity stage of the fruit. So also, it is varied with the stage of harvest, harvesting season, storage condition, storage period and different types of processing. Harvesting at the colour break stage and storing at low temperatures (22^0 C – 30^0C) is favourable for lycopene development rather than storing at high temperatures. Ethylene gas treatment can be used to enhance the lycopene content and it accelerates the ripening process in tomatoes. Processing at high temperatures for long periods and exposure to bright lights causes to decrease the lycopene content.Further, processing of tomatoes increases the lycopene bioavailability and many health benefits according to the results of previous studies. Processing waste of tomato skin and pericarp can be used as a lycopene source for the food industry as well as to the formation of many tablets which are enriched with many vitamins and nutrients. Extracted lycopene can be used as a natural food colorant for artificial colorants in avoiding harmful effects. There is an emerging future trend in preparing many products by increasing the lycopene content in the natural product on the well-being of the consumers.

1. Introduction

Tomato (*Lycopersiconesculentum*) is one of the commonly traded vegetables, cultivated throughout the world. It contains important nutrients such as vitamin A, C, minerals and carotenoids along with lycopene (Eboigbe and Edemevughe, 2019). The most abundant carotenoid in ripe tomatoes is lycopene and it comprises approximately 80% to 90% of all pigments present. (Curl, 1961; Ilahy *et al.*, 2011;Thakur, B.R *et al.*, 1996). Recent researches have revealed that lycopene is a very effective natural antioxidant (Mascio *et al.*,1989). Lycopene is found predominantly in tomatoes and the content of lycopene varies widely among tomato varieties and increases dramatically during ripening (Clinton, 2009; Ajlouni *et al*., 2001). Lycopene content in tomatoes after harvesting has been reported to be dependent upon the ripeness of the fruit at the time of harvesting (Sadler *et al.*, 1990) and also it presents in fresh tomato as trans configuration and due to the occurrence of isomerization and oxidation during tomato processing,a high amount of lycopene gets degraded. This isomerization converts trans isomers into cis isomers. (Shi and Maguer, 2000). It has been suggested that the

bioavailability of cis-lycopene in tomatoes is higher than that of all trans-lycopene (Choksi and Joshi, 2007).

Functional food is defined as foods that provide health benefits beyond basic nutrition by the International Food Information Council (IFIC). Considering the composition and health benefits of tomatoes, they can be categorized as a functional food (Choksi and Joshi, 2007).

2. Chemical structure and properties of Lycopene

Lycopene is a carotenoid found in plant materials possessing antioxidant properties and is responsible for the red coloured pigment in fruits and vegetables. Redness is also affected by the content of lycopene present in the material and tomato can be given as an example (Suwanaruang, 2016; Khairi *et al*., 2015). The Color given by lycopene is matters the functional quality and horticultural performance of tomatoes at the stage of marketing (Ilahy *et al*., 2018).

Lycopene is confined in chloroplasts at the cellular level of tomato fruit and in the early maturity stage of tomato, green chlorophyll is the major pigment presenting in the chloroplast. At the time, when chlorophyll is reduced, lycopene is biosynthesized with different changes in the ultrastructure of the material resulting in the developing of red colour (Shi and Maguer, 2000; Harris, 1970; Khudairi, 1972; Matienco and Yedalty, 1973).

In nature, all lycopene present as trans form and can be isomerized from trans form to cis form at presenting the influence of light, heat or certain chemicals. Trans isomers of lycopene are the predominant isomer type in tomato and represent about 95.4% of total lycopene and they are most thermodynamically stable. During processing and storage of tomatoes, isomerization occurred and thus, a stable form of trans isomers is converted into an unstable form of cis isomers (Shi and Maguer, 2000; Barrett *et al*., 2001).

Chemically lycopene is an acyclic hydrocarbon, an acyclic unsaturated open chain including 13 double bonds, of which 11 are conjugated double bonds arranged linearly and possessing of molecular formula of $C_{40}H_{56}$. Methyl groups are presenting at 1, 5 position while two central methyl groups are at 1,6 position relative to each other. Colour and antioxidant properties are prevailing due to the unique structure and extended system of conjugation (Shi and Maguer, 2000). The antioxidant property given by lycopene is caused by its capacity to sequester singlet oxygen and also the ability to trap peroxyl radicals (Amany *et al*., 2009).

Due to having conjugated unsaturated double bonds in lycopene structure, it is susceptible to deterioration in the presence of light, heat and oxidants. At the industrial applications, adequate consideration should be given when the presence of chemicals, heat and light at the processing of the food matrix which includes lycopene (Shi and Maguer, 2000; Gomes *et al*, 2014). According to the main physical properties of lycopene, molecular weight, melting point, crystal and powder forms and solubility are given in table 01 (Shi and Maguer, 2000).

Table 1. Physical properties of Lycopene

Factor	**Value/ Details**
Molecular Formula	$C_{40}H_{56}$
Molecular weight	536.85 Da
Melting Point	172 – 175 ^{0}C
Crystal form	Long red needle form
Powder Form	Dark reddish brown powder
Solubility	Soluble in Chloroform, hexane, benzene, acetone, petroleum ether Insoluble in water, ethanol and methanol
Sensitivity	Sensitive to light, high temperatures, oxygen and acids

3.Variation of lycopene contents in tomatoes at the field

Lycopene contents in fresh tomatoes at the field are varied mainly on the cultivation regions of the world (Eboigbe and Edemevughe, 2019) and also depending on many internal and external factors such as tomato variety, maturity stage, soil type and environmental conditions. Some tomato varieties at the deep red stage contain more lycopene (15 mg per 100 g of fresh tomatoes) than yellow coloured tomato varieties (0.5 mg per 100 g of fresh tomatoes) (Hart and Scott, 1995; Kotikova *et al.,* 2011).

Lycopene content of high lycopene breeding lines which was grown under open field conditions during the year 2013 -2014 in Tunisia ranged from 98.8 to 280.0 mg/kg fresh weight of tomatoes. Lycopene accumulation potential in tomatoes gets varied due to having differences in temperature and prolonged heat stress might be responsible for less amount of lycopene content in tomatoes at the harvesting stage (Ilahy *et al.,* 2016).

Effect of environmental conditions on colour and lycopene content of tomato at ripening stage was analyzed and reported that lycopene synthesis is inhibited when the temperature of the fruit is exceeding 30 °C (Brandt *et al.,* 2006; Anese, *et al.,* 2002). Best Lycopene development is achieved when the temperature is between 12^0C and 21^0C (Brandt *et al.,* 2006). Further, Lycopene content of tomatoes generally ranging from 7 to 13 mg/100g based on the type of variety, climatic conditions, geographical areas and cultivation techniques (Schierle*et al.*, 1997). The average value of lycopene content varied between 84.1 to 172.9 mg/kg whiles some tomato varieties have particularly high contents of lycopene in many countries (Barrett and Anthon, 2001). Some of the red tomato varieties such as "Flavour top" or "Money marker" contain around 50 mg/kg fw of lycopene when yellow varieties have around 5 mg/kg in fresh weight (Choksi and Joshi, 2007).

Significantly higher lycopene content was observed in glass house grown tomatoes (83.0 mg kg^{-1} f.w) than that growing in the field (59.2 mg kg^{-1} f.w) at a different time of harvesting (Brandt, S.,*et al.*,2003).

Lycopene content in tomatoes has been reported to be dependent upon the stage of maturity (Thompson *et al.*, 2000; Fraser *et al.*, 1994; Sadler *et al.*, 1990) and it was at the immature green stage, mature green stage, colour breaker stage, firm red stage and overripe stage were reported as 25, 10, 370, 4600 and 7050 µg/100 g respectively (Fraser *et al.,* 1994). When maturation was completed, lycopene content was increased from 17 mg/ 100g to 69.98 mg/100g in reddish tomatoes (Kotikova *et al*, 2011).

4. Variation of the lycopene content at the storage

Lycopene content of red colour ripe tomatoes which were stored at room temperature (22^0C) was higher than the tomatoes which were stored at refrigeration temperature (4°C) (Ajlouni*et al.,* 2001). Biosynthesis of lycopene depends upon the temperature range between 12^0C and 32^0C (Leoni,1992) and for optimizing the lycopene content, the suitable temperatures were 16 ^{0}C–18 °C and 26 °C (Turk *et al.*, 1994). A similar conclusion has indicated that lycopene contents were high in ripe tomatoes stored at 20°C for 10 days (Hamauzu *et al.*, 1998).

Tomatoes harvested at the colour break stage and stored at room temperature (22 °C – 23 °C) for 06 days had significantly higher lycopene content than that harvested at the mature green stage, treated with ethylene and stored for 06 days. The lycopene for tomatoes that were stored over 9 days at room temperature (22 °C – 23 °C) are given in table 02 (Thompson *et al.,* 2000). Lycopene biosynthesis was inhibited at temperatures above 30^0C and below 12^0C and stopped at temperatures above 35^0C, even though it was favored at temperatures between 16^0C to 21^0C. Lycopene biosynthesis is also influenced by the light intensity at ripening (Brandt *et al.*, 2006; Leoni,1999; Dumas *et al.*, 2003; Helyes*et al.*, 2003). Studies on temperature effect on

lycopene biosynthesis have shown that temperatures below 12°C strongly inhibited lycopene biosynthesis and temperatures above 35°C stopped this process (Dumas *et al*., 2003). Lycopene content was also found to be influenced by light intensity during ripening (Helyes *et al*., 2003).

Table 2. Lycopene content (mg/100 g) of various tomato cultivars on days 0, 6, 9, and 12 at mature green, breaker, and red ripe stages.

	Maturity stage of the cultivar	Day 0	Day 6	Day 9	Day 12
Agriset	Green	8 (11)	1598^{d} (308)	3744^{cd} (809)	2564^{f} (349)
	Breaker	942^{b} (415)	4574^{b} (1030)	3174^{d} (854)	4276^{bcde} (1152)
	Red	4154^{c} (856)	ND	ND	ND
Solar Set	Green	7 (5)	2502^{cd} (552)	4803^{b} (602)	3638^{def} (620)
	Breaker	1084^{b} (321)	5636^{a} (1008)	4267^{bc} (797)	4489^{abcde} (1380)
	Red	4419^{bc} (742)	ND	ND	ND
Suncoast (og)	Green	8 (9)	3326^{c} (323)	4847^{b} (1786)	4257^{bcde} (842)
	Breaker	1210^{ab} (218)	6207^{a} (1807)	6178^{a} (678)	4571^{abcde} (1694)
	Red	5274^{ab} (998)	ND	ND	ND
FL7692D (og)	Green	7 (6)	2015^{d} (362)	4528^{bc} (630)	3699^{cdef} (1036)
	Breaker	1511^{a} (216)	4589^{b} (917)	4616^{bc} (784)	3077de(1148)
	Red	5560^{a} (597)	ND	ND	ND

$^{a-f}$ The day 0 samples of the four varieties were compared at each maturity stage within the column, while both maturity stages of the day 6, day 9, and day 12 samples were compared within the column. Means

The rate of lycopene synthesis in non-hydroponic tomatoes was higher than the tomatoes grown in the hydroponic cultivar. Tomatoes at a similar degree of maturity were used from two different cultivations such as hydroponic and non-hydroponic. Different samples of tomatoes of two cultivation types were equally divided and stored at room temperature (22°C) and refrigerated temperature (4°C) for 21 days and changes in lycopene contents and colour were measured. Results revealed that the lycopene and colour value was increased with the storage period. Initially, lycopene contents were 36.15±4.17 µg/g fresh weight for both hydroponic and non-hydroponic tomato fruits and it was continuously increased with the storage while recording maximum lycopene content at 14th day of storage at 22^{o}C and reached up to 89.75±4.51 µg/g fresh weight and 115.13±2.08 µg/g fr. wt. for hydroponic and non-hydroponic cultivars, respectively. At 4^{o}C, the increment in lycopene content in non- hydroponic tomatoes was less than the same sample stored at 22^{o}C for the same storage period (Ajlouni *et al*., 2001).

Lycopene content of tomato fruits which were provided with 100% of optimal water supply at the field was less than that of the tomato samples supplied with 50% of optimal water at the field (Brandt *et al*., 2003) and showed variation in lycopene content in tomatoes subjected to water stress compared to no water stress tomatoes at storage (Khairi and Takahashi, 2013) while moderated water stress tomatoes that cultivated in a greenhouse and hydroponically maintained, contains high lycopene (Khairiet al., 2015). Tomatoes were hydroponically grown in a greenhouse and harvested fruit samples were stored in different temperatures such as at 10,15,20,25 and 30^{0}C in cool incubators for 07 days and analyzed for lycopene content and colour variation (table 03). Results revealed that the color of the tomatoes which were stored above 10^{0}C had increased after the storage and the lycopene content of tomatoes had increased at storage when fruits are stored above 20^{0}C (Takahashi *et al.*, 2018).

Table 3. Effect of storage temperatures and duration on lycopene content of the tomato fruits

Storage temperature (^{0}C)	Lycopene content (mg/100 g FW) after storage		
	2 days	4 days	7 days
5	18.4 ±1.2 a	13.8 ±2.1 b	14.5 ±0.7 c
10	17.5 ±1.5 a	16.7 ±1.0 ab	16.8 ±1.7 bc
15	16.4 ±1.7 a	17.2 ±0.9 ab	17.9 ±1.7 abc
20	22.9 ±2.4 a	23.4 ±1.9 a	25.7 ±0.3 a
25	23.6 ±0.8 a	23.6 ±1.8 a	24.3 ±2.4 ab
30	23.3 ±3.7 a	21.9 ±4.1 ab	24.1 ±3.0 ab

* Values are means ± standard deviation (n=4). Different letters indicate a significant difference ($p<0.05$) according to one-way ANOVA followed by Tukey-Kramer test in within columns

Tomato samples were taken for the study from cultivations that maintained irrigation systems with moderate water stress and normal irrigation and the harvested tomatoes were stored in different temperatures (10^0C, 15^0C, 25^0C and 30^0C) until reached to mature stage. Thereafter, the lycopene content was analyzed using a spectrophotometer. The initial lycopene content of water stress tomatoes and normal irrigated tomatoes were 7.024 mg/100g and 6.459 mg/ 100g respectively. Lycopene of water stress tomatoes showed an increasing trend during storage and the highest lycopene content was obtained at 12thday storage at 25 ^{0}C temperature. Lycopene content was increased at a temperature above 15^{0}C for both types of tomatoes obtained from water stress and normal irrigated conditions. After 8th day in storage, lycopene content tends to get reduced. Lycopene content was high for water stress tomatoes than that of no water stress while on storage at all temperatures aforementioned. Test of optimum storage condition for having high lycopene content was done by surface and contour plot analysis and results are shown in table 4 (Khairi *et al.*, 2018).

Table 4. Regression analysis test for optimizing model

	Cons-tant	temperatures	times	Lyco-pene	Temp. Vs Temp.	Times Vs times	Lyco-pene Vs Lyco-pene	Temp. Vs times	Temp. Vs Lyco-pene	Time Vs Lyco-pene
F	0.00	0.785	0.298	0.522	0.496	0.071	0.898	0.555	0.813	0.899

5. Stability of Lycopene during tomato processing

Lycopene bioavailability in unprocessed fresh tomatoes is less than that of processed tomato products. However, different food processing methods caused to improvement of the bioavailability of lycopene by breaking down the cell walls in tomatoes. As a result that, lycopene availability is high in cooked tomatoes than in fresh tomatoes (Shi and Maguer, 2000; Gartner *et al.*, 1997; Barrett and Anthon, 2001; Stahl and Sies, 1992). Lycopene degradation and loss of colour in processed tomato products are occurred due to a number of factors like high-temperature treatment and long duration in storage etc. The main reasons for lycopene degradation during tomato processing are isomerization and oxidation of lycopene (Shi and Maguer, 2000). The heating of tomatoes at high temperatures is not favourable due to the degradation of lycopene content; because temperature negatively affects

the nature and extent of lycopene breakdown. Holding time of tomatoes at high temperature during processing is also seriously affects the degradation of lycopene. The rate of Lycopene loss during heating of tomato juice is high during processing at high temperatures as well as prolonged processing (Table 5).

Table 5. Rate of Lycopene loss of tomato juice during heating

Heating temperature/ ^{0}C	Lycopene loss percentage		
	Heating for 1 min	Heating for 3 min	Heating for 7 min
90	0.6	0.9	1.1
100	0.9	1.4	1.7
110	2.2	3.2	4.4
115	2.7	4.5	7.0
118	3.7	6.0	9.1
121	4.6	7.3	10.6
124	5.5	8.5	12.5
127	6.5	9.9	14.6
130	7.4	11.5	17.1

Source: Miki and Akatsu, 1970.

During vacuum evaporation, a small amount of lycopene loss was noticed. When the tomato was processed at a high temperature within the shortest possible time, it is favorable to retain the quality of the final product particularly lycopene content (Shi and Maguer, 2000). Lycopene bioavailability of tomato paste is higher than of fresh tomato (Gartner *et al*., 1997). Lycopene contents of fresh tomatoes and tomato products drawn from the Romanian market were evaluated and found that fresh tomato contained 12mg/ 100g of lycopene and tomato products like tomato paste, tomato boiled sauce, tomato ketchup and spaghetti sauce contained about 16, 4, 17and 16mg/100g of lycopene respectively (Alda *et al*., 2009).
When tomatoes were boiling for 1 hour with the presence of 1% corn oil, caused to increase the lycopene bioavailability in tomato juice (Cogdell, 1985). By heating the tomatoes in the presence of metallic ions (Cu^{2+}, Fe^{3+}, etc.) or oxygen, lycopene may be partially destroyed (Shi and Maguer, 2000).

Lycopene content of tomato powder varied from 1016.05 to 1181.30 мg/g and total solids and loss is around 8.07% to 20.93% when the preparation of tomato powder using spray drying technology without adding any carrier agent (Goula and Adamopoulos, 2005). Water loss has occurred during the processing of tomato products and it also may be contributed to an increase in the concentration of lycopene content compared to fresh produce (Thompson *et al.,* 2000).

6. Future Developments

Tomato skin, seeds and pericarp tissues are the byproducts that get wasted during the processing of tomato based food products and these skin and outer pericarp tissues contain more than 80% to 90% of the total amount of lycopene in tomato fruit (Shi and Maguer, 2000) and tomato skin contains 12 mg lycopene/ 100 g (wet basis) while whole mature tomato contains 3.4 mg lycopene/100 g (wet basis) (Al-Wandawi*et al.,* 1985). Tomato skin contained more lycopene (53.9 mg/ 100 g) than whole tomato pulp (11mg/100g) on the wet basis (Sharma and Maguer, 1996). Commercial products enriched with lycopene in the pharmaceutical industry are less and there is a high demand for industrial production of lycopene from tomatoes (Shi and Maguer, 2000). Processing waste of tomato skin and pericarp can be used as a lycopene source for the food industry as well as to the formation of many tablets which are enriched with many vitamins and nutrients for the well-being of human health. Therefore more attention should be focused in producing value-added products from lycopene from byproducts of tomato based industries (Shi and Maguer, 2000; Choksi and Joshi, 2007).

The main reason for the degradation of lycopene during food processing and storage is oxidation. Due to that lycopene is needed to be protected from excess heat applications, extremely acidic or basic conditions, exposure to oxygen and high intensity of lights and lipid

degrading enzymes to avoid lycopene from isomerization and oxidation. Application of one of the suitable antioxidants like ethoxyquin, ascorbic acid and sodium acid pyrophosphate may be given positive results (Granado *et al.*, 1992; Clindon *et al.,* 1996; Porrini and Testolin, 1998). Isolation of lycopene from tomatoes could be efficiently carried out by the Super Critical Fluid Extraction (SCF) process (Gomez *et al.,* 2003). Lycopene can be used as a natural food colorant as an alternative to artificial colorants due to avoiding the harmful effects of artificial food colorants (Agarwal and Rao, 1998).

7. Conclusions

Throughout this review, it has been shown that Lycopene is present in tomatoes and responsible for its colour and many bio-active properties. The storage condition and harvesting stage significantly affect the amount of lycopene present in tomatoes. Processing of tomatoes or tomato puree caused to increment of lycopene bioavailability and in extreme heat treatment effect for degradation of lycopene. Immerging improvements in extraction of lycopene from plant materials and processing wastes of tomato skin and seeds and then incorporation of lycopene in the pharmaceutical industry are testing. Super Critical Fluid Extraction has been successfully carried out in the extraction of lycopene from ripe tomatoes

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BASIC QUALITY CRITERIA AND SHELF LIFE OF HOT SMOKED ANTALYA BARB (*CAPOETA ANTALYENSIS*)

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ABSTRACT

In this study, the hot smoking process and storage time of *C. antalyensis* was examined. The moisture, protein, ash, and fat content of fresh raw fish was 76.52±0.43%, 17.29±0.05%, 1.90±0.29%, and 1.73±0.13%, respectively. The changes in the findings of the smoked samples compared to the raw fresh sample were found to be significant ($p<0.5$). During the study, TVB-N, TBA and pH findings changed. The TVB-N value was 35.04±0.77mg/100g on the day 91st, and the TBA value was 9.11±0.65µg MDA/g on the day 70th. The pH values obtained as 6.8 on the day 49th, and 7.0 on the day 91st. An average score of 1.90±0.46 was obtained for the odor criterion on the day 56th. On the day 35th, the Total Plate Count (TPC) was 6.30±0.00 log cfu/g, and the Total Psychrophilic Bacteria count was 6.43±0.15 log cfu/g. The total number of yeast-mold was determined as 7.15±0.15 log cfu/g on the day 42nd. In the analysis of *C. antalyensis*, 27 different fatty acids were determined. Of the saturated fatty acids (SFA), C16:0 had the highest value. It was determined that the species can be processed by the hot smoking method, by nutrient content, and by the sensory taste appreciated.

1.Introduction

Fish, which has rich protein, amino acid, unsaturated fatty acid, and mineral content, is of great importance to healthy and balanced nutrition. In 2013, the world's average fish consumption was 19.8 kg per capita. Per capita consumption was 6.1 kg in the same period in Turkiye. This corresponds to 1.7 kg of seafood-originated protein (FAO, 2018). So, increasing the amount of consumption per capita in our country is a necessity for raising healthy generations.

To make up for the animal-originated protein need, it will be necessary to either make more use of existing sources or find alternative sources. Although known to be found in inland waters, there are many fish species not consumed as human food. These species are of great importance to fulfilling the animal-originated protein deficit.

All fish species die in their natural populations by several effects including predators, natural mortality, and fishing mortality. When they are not caught, fish populations occurring in inland water, complete their life without being used for human use. In addition, recent studies have shown that the number of fish species in those freshwater resources is increasing day by day. So, these fish species should be caught from lakes, dams, and rivers with suitable fishing tools and methods

and should be processed with different processing techniques and presented for consumption. Thus, the per capita fish consumption could be increased.

Capoeta antalyensis (Antalya barb) has not been previously processed by any processing method. After the above explanations, the aim of this study was to investigate the effects of hot smoking processing technology on the quality of *C. antalyensis* and on the storage time. For this purpose, sensory, chemical, and microbiological analyses investigated changes in proximate composition and shelf life.

2. Materials and methods

2.1. Materials

C. antalyensis caught by using gill nets (16x16, 20x20, 25x25, 30x30, 35x35, 40x40 mm mesh) (Özekinci *et al.,* 2003) from Karacaören I Dam (Burdur-Isparta-Turkiye). The fish were iced and carried to the laboratory in an ice-insulated box within 1 h of catching. Approximately 12 kg of fish, which the mean weight of fish was 208.28 ± 39.54 g, were processed with hot smoking technology.

2.2. Methods

2.2.1. Smoking process

The gills and viscera of the fish were removed and cleaned out. Blood, mucus, etc., washed with plenty of icy cold water. The cleaned fish were immersed in an 18% saline solution for 45 minutes. The fish taken out from this solution were mounted to the hanging hooks to be hung in the hot smoking cabinet. It was left hung at room temperature for 20 minutes to remove the water on the fish's surface. The temperatures and times given in Table 1 were applied to the fish under oak shavings in a temperature-controlled mechanical hot smoking cabinet (Bilgin, 2003; İzci, 2004). At the end of the procedure, the fish were allowed to cool to room temperature in the cabinet. The smoked fish was fillet under aseptic conditions. Sufficient samples were reserved for analysis. The remainder fillets were vacuumed packed and placed in the refrigerator (3±1°C).

Table 1. Time and temperature values applied in the smoked process

Time (minutes)	Temp. (°C)
45	30
60	50
60	60
60	70
45	80

2.3. Analysis

Total protein, fat, moisture, ash, TVB-N, TBARS analysis, pH measurement, microbiological analysis, and sensory evaluation were performed to determine the initial values of raw and smoked fish. Sufficient samples were saved for fatty acids analysis (stored at -80°C). TVB-N, TBARS analysis, pH measurement, microbiological analysis, and sensory evaluation were applied to the smoked fish over a weekly period.

2.3.1. Proximate analysis

Moisture (%) was measured with Kern DBS automatic moisture analyser (Günlü, 2007). Ash (%) and total lipid content (%) were determined according to Lovell (1981). Total protein (%) was determined according to the Kjeldahl method (Nx6.25) AOAC (2000) (modified). The carbohydrate (%) value was obtained by calculation according to the following formula.

Carbohydrate (%) = 100- (Moisture + Fat + Protein + Ash) (1)

(Varlık *et al.,* 2007).

2.3.2. Chemical analysis

2.3.2.1 The pH

The pH was measured using a digital pH meter. The previously homogenized fish meat was mixed with distilled water to 1/10 (w/v). The pH was determined by using the pH meter newly calibrated considering the ambient temperature (Erkan and Özden, 2007).

2.3.2.2. TVB-N analysis

TVB-N analysis was conducted by modifying the method of Botta *et al.,,* (1984) (Nicholas, 2003). 50 ml 7.5% TCA (Trichloroacetic acid) was added to 25g of

homogenized fish meat. It was homogenized for 30 seconds in the mixer. The mixture was filtered through coarse filter paper under vacuum using a Buchner funnel. The homogenate obtained was put in the refrigerator until analysis. For analysis, 15 ml homogenate was placed in a distillation tube. Distillation was performed by adding 4 ml of 10% NaOH and 10 ml of distilled water. 15 ml of 4% boric acid and 7-8 drops of Tashiro's Indicator (2:1 0.2% alcohol methyl red: 0.2% alcohol methylene blue) were added to the Erlenmeyer where the distillate would be collected. The >50 ml distillate obtained by the distillation process (3 min) was titrated with 0.25 N HCl. By saving the amount of HCl consumption;

TVB-N (mg /100 g) = [(HCl consumption in titration ml x Normality of HCl x 14,007(Mass Number of Nitrogen) x (67.5 mL / 15 mL))] x (100 g/25 g) calculated with the formula.

2.3.2.3. TBA analysis

TBA analysis was conducted according to Erkan and Özden (2007). The fish meat was put into 50ml centrifuge tubes by weighing 1.9-2.0 g (with a precision of 0.01 g). 100µl of BHT (Butylated hydroxytoluene) solution prepared in ethanol to be 1 g / l was added. Then 25 ml of TCA (Trichloroacetic acid) solution prepared as 50g / l was added. The mixture was mixed with the homogenizer at medium speed and disintegrated. The resulting mixture was filtered through Whatman No: 1 filter paper. 2 ml was taken from the filtrate and transferred to a 20 ml tube. The mouth is tightly closed by adding 2 ml of newly prepared TBA reagent (0.2883g TBA Reagent + 90 ml of Glacial acetic acid + 10 ml of pure water). It was kept in a water bath at 70-80ºC (up to 100ºC to complete color formation) for 40 minutes for the reaction to occur. At this stage, the same process was applied to the blind and standards. After the tubes had cooled down, they were read on the spectrophotometer at 532 nm.

Preparation of the Standard: 50µl of TEP (tetra ethoxy propane) was completed with 50 ml of 0.1 N HCl and heated at 100ºC for 10 minutes. The hydrolysed acetal obtained was taken 2.4 ml and completed to the mark with 100 ml of pure water. This stock standard has 0.1mM MDA (malondialdehyde). Here, the following standards were prepared in order.

Standard 1: 1 ml was taken from the stock standard and completed to 50 ml with pure water. This standard has 0.002mM MDA (0.14406µg MDA / g).

Standard 2: 3 ml was taken from the stock standard and completed to 50 ml with pure water. This standard has 0.006mM MDA (0.43218µg MDA/g).

Standard 3: 5 ml was taken from the stock standard and completed to 50 ml with pure water. This standard has 0.01mM MDA (0.7203µg MDA/g).

Standard 4: 7 ml was taken from the stock standard and completed to 50 ml with pure water. This standard has 0.014mM MDA (1.00842µg MDA/g).

5 ml (or 2 ml, same the sample amount) were taken from these prepared standards. The same amount of TBA solution was added to it. The tubes were then kept in a water bath at 70-80ºC (up to 100ºC to complete color formation) for 40 minutes. After the tubes were cooled, by spectrophotometer was read against the blank at 532nm. The sample results read in the spectrophotometer were calculated using the regression curve equation of the standards, and the TBARS concentrations (µg MDA / ml) were found. These densities were put into the formula below.

TBARS (µg MDA/g) = MDA (µg MDA/ml) x 25 ml/ Sample Weight (g). (2)

2.3.3. Microbiological analysis

The first dilution was prepared with fish meat and peptone water (1/10 w/v). Other dilutions were prepared from this first dilution, respectively. Sowing was performed in Petri dishes using the cast plate method. Total plate count (TPC) was incubated at 30±1 °C for 72 hours by using Plate Count Agar (PCA). Total Number of Psychrophilic Microorganisms (TPA) was incubated at 4±1 °C for 10 days by using PCA. Total *Enterobacteriaceae* was incubated at 30±1 °C for 24 hours by using Violet Red Bile Agar (VRB). The yeast-mold

count was incubated at 22 ± ° C for 4 days by using Potato Dextrose Agar (PDA) (ICMSF 1978; Anonim, 1979; Refai, 1979; Varlık *et al.*, 1993; Anonim, 1994; Arslan *et al.*, 1997). Colonies formed as a result of incubation were counted. 6 log CFU/g limit accepted.

2.3.4. Sensorial analysis

The sensorial analysis was performed completely by using human sensory organs referenced with a hedonic scale. (Huss, 1995; Altuğ and Elmacı, 2005). 10 panellists were informed about the evaluation criteria for evaluation (this was a mixed group, and 7 of the panellists were trained, and 3 were untrained. One of the trained panellists disliked eating freshwater fish). When the products heated in the microwave reached the ambient temperature, they were asked to test in terms of odor, flavor, texture and structure, color and general taste. 10 points were the highest and 0 points were the lowest. The average score of 2 points and below was determined as the limit of deterioration.

2.3.5. Fatty acids analysis

5g of previously homogenized fish meat was weighed and extracted by chloroform/methanol method. Then the chloroform was evaporated to give the oil. 0.01 g of oil was weighed and 2 ml of hexane and 4 ml of 2M KOH were added. The mixture was shaken in the vortex for 2 min. Centrifugation was performed for 10 minutes at 4000 rpm. The upper clear hexane phase was taken into vials and read in the GC (Özoğul *et al.*, 2007).

GC conditions: The device Shimadzu TQ 8040 GC / MS / MS analyzer, and MS detector were used as a detector. The column: TRCN100 (100 m x 0.25 mm x 0.20 µm). Column temperature: 140ºC, Injection temperature: 240ºC, Column temperature program: 6 minutes at 140ºC, 240ºC with an increase of 4ºC/min, and 10 minutes hold time, Split ratio: 1/100, Carrier gas: He, Flow rate 1.18 ml/min., Pressure 274.6kPa, Injection Volume: 1µL. Restek Fame mixture was used. Library: FAME and NIST. MS conditions: Ion source temperature: 200ºC, Interface temperature: 240ºC, Solvent cut time: 7.35 minutes, Scan speed: 1428, Mass range: 41-450 m/z, é: 70 V.

2.3.6. Statistical analysis

The T-test was used to compare proximate composition data, one-way ANOVA was used to determine variance differences, and multiple comparison test DUNCAN was used at a 0.05 significance level to compare groups (Özdamar, 2001).

3. Results and discussions

3.1. Proximate composition

The results obtained from the proximate composition analysis of raw fish and smoked fish are given in Table 2. Moisture content was determined as 76.52 ± 0.43% in raw fish and 66.58 ± 0.08% after smoking. Protein was determined as 17.29 ± 0.05% in raw fish, and 24.46 ± 1.04% after smoking. Ash content in raw fish was 1.90% ± 0.29%, and lipid content was found as 1.73 ± 0.13%. The change of the data obtained after the smoking process with the raw sample in moisture, protein, ash, lipid, and carbohydrate values was found statistically significant ($p \leq 0.05$). After the hot smoking process, a significant decrease was observed in the moisture content with the effect of the applied heat, while an increase was observed in the protein, ash, and lipid content.

Table 2. Chemical composition of raw and smoked samples (**±SE)**

	Moisture (%)	**Protein (%)**	**Ash (%)**	**Lipid (%)**	**CH (%)****
Raw	76.52 ± 0.43 [a]	17.29 ± 0.05[a]	1.90 ± 0.29[a]	1.73 ± 0.13 [a]	2.56
Smoked	66.58 ± 0.08 [b]	24.46 ± 1.04[b]	5.10 ± 0.61[b]	2.91 ± 0.19 [b]	0.94

* Different lower case letters show significant differences ($p \leq 0.05$)

** Obtained by calculation (CH = 100- all others)

3.2. Chemical analysis

TVB-N value, which was determined as 11.01 ± 0.92 mg / 100g in the raw sample, and 18.94 ± 0.15 mg / 100g in the smoked fish, showed a fluctuating change depending on the storage time (Table 3). According to Connell (1980), 15-20 mg N / 100 g TVB-N value in

marine fish shows good quality, and 50 mg N / 100 g of bad quality (Cadun et al., 2005). According to TVB-N content, Kietzmann et al. (1969) fish products are very good up to 25 mg in 100 grams of fish, good up to 30 mg, can be marketed up to 35 mg, over 35 mg has degraded; Ludorf and Meyer (1973) have considered the product containing 35 mg / 100 g TVB-N as marketable and 40 mg / 100 g TVB-N value as degraded (Dokuzlu, 1997). The European Union requests TVB-N analysis in a case of doubt because of the sensory evaluation to determine fish freshness and reports 25, 30, and 35 mg-TVB-N / 100 g as the critical limit for different fish species (Çakli et al., 2006). Findings obtained during storage showed a discontinuous change. The value of 35mg / 100 g for TVB-N was exceeded on the 91st day of storage. It was observed that the findings changed discontinuously of TVB-N obtained in the study where the hot smoking technology was applied in freshwater fish species, in which the samples were obtained by catching from nature (Yanar, 2007), as in our study (Table 7). In some studies given in Table 7, where the fish processed using the smoking technology was obtained by breeding, it was found that this change was continuously and increased tendency from the beginning to the end of storage, but the findings obtained by Bolat et al. (2009) showed a discontinuously change. In none of the studies in Table 7, it was seen that the deterioration limit value was not exceeded in terms of TVB-N, or the study was not continued until the deterioration occurred.

TBA was determined as 1.57 ± 0.27µg MDA /g in raw fish and 1.83 ± 0.03µg MDA / g in smoked fish (Table 3). Classifications were made by various researchers according to the amount of TBA contained in seafood. Schormüller (1968, 1969) reported TBA as less than 3 mg malonaldehyde/kg in excellent quality products, less than 5 mg malonaldehyde/kg in good quality fish, and consumption limit as 7-8 mg malonaldehyde/kg (Cadun et al., 2005). According to Curran et al. (1980), when the TBA value exceeds 4 mg malonaldehyde/kg in fish meat, it starts the rancidity, and the consumption limit value is 8 mg malonaldehyde/kg (Erdem et al., 2005). Kundakçı (1989) reports the maximum limit of consumption for the products containing TBA as 4 mg malonaldehyde/kg. Sinhuber and Yu (1958) stated that TBA value should be 3 mg malonaldehyde/kg and below in good quality products, however products with values between 4-27 mg malonaldehyde/kg can be considered as bad quality (Yapar, 1998).

Table 3. Change of TVB-N, TBA and pH values of raw and smoked samples by the time (**±SE)**

Days	TVB-N (mg /100 g)	TBA (µg MDA/ g)	pH
Raw	11.01±0.92	1.57±0.27	6.84±0.07
0.	18.94±0.15^{b}	1.83±0.03^{a}	6.83±0.08^{d}
7.	23.11±0.34^{d}	3.32±0.24^{b}	6.84±0.04^{d}
14.	21.60±0.42^{c}	4.61±0.19^{bc}	6.77±0.00^{cd}
21.	22.10±0.51^{c}	3.97±0.43^{b}	6.62±0.01^{b}
28.	16.89±0.14^{a}	5.62±0.53^{cd}	6.61±0.01^{b}
35.	25.05±0.80^{e}	6.41±0.70^{d}	6.62±0.00^{b}
42.	21.10±0.08^{c}	6.68±0.31^{d}	6.51±0.00^{a}
49.	21.93±0.64^{c}	3.82±0.22^{b}	6.98±0.00^{e}
56.	30.09±0.80^{f}	3.75±0.84^{b}	6.79±0.01^{cd}
63.	26.05±0.08^{e}	4.26±0.28^{b}	6.75±0.00^{c}
70.	32.19±0.22^{g}	9.11±0.65^{e}	6.58±0.00^{ab}
77.	30.51±0.14^{f}		6.58±0.01^{ab}
84.	33.45±0.22^{g}		6.55±0.01^{ab}
91.	35.04±0.77^{h}		7.31±0.01^{f}

* The difference between the values shown in different letters is significant ($p \leq 0.05$)

It is seen that the findings we obtained in fresh and smoked samples of *C. antalyensis* species showed a discontinuously change. TBA analyses were continued until the samples deteriorated. With the 9.11 ± 0.65 µg MDA/g obtained on the day 70th of storage, it was decided that the samples deteriorated in terms of TBA. As in our study, in some studies where smoking technology was applied to freshwater fish, the TBA value showed a discontinuous change (Özkütük, 2002; Çakli et al., 2006; Bolat et al. 2009), while in other studies a continuous and deteriorative tendency change (İzci, 2004; Yanar, 2007; Salama and Ibrahim, 2012; Fıcıcılar and Genccelep, 2017) (Table 8).

For raw fresh samples of *C. antalyensis* species, the pH value was determined as 6.84 ± 0.07 (Table 3). The pH value (6.83 ± 0.08) determined after the hot smoking process is close to this value and there is no statistically significant difference between them ($p>0.05$). With the progress of time depending on the changes in the fish structure during the storage process, the pH value showed a change in the tendency of decreasing until the day 35th. As of this day, it showed a change in the increase's tendency. The pH, which is frequently used in determining the product freshness, is not sufficient alone to determine the product quality, and it is reported that the results obtained from this should be evaluated together with other analysis methods (Erkan and Özden, 2007; Bilen, 2009). The product is considered being degraded when the pH value is 7 and above in processed seafood (Gülyavuz and Ünlüsayın, 1999), and the consumption limit value is reported to be 6.8-7.0 (Bilgin, 2003). Diaz et al. (2011) report that a pH-related change could not be determined in the deterioration of cooked trout. Garcia-Linares et al. (2004) determined that the fresh salmon and trout pH values were 6.33 and 6.56 respectively. Also, the highest pH value was 6.61 in the sous-vide cooked trout and reported that the changes in pH value obtained are not effective in microorganism behaviors during the storage period.

Although the limit of 6.8 was exceeded with the pH value of 6.98 ± 0.00 obtained on the day 49th measurement, the subsequent measurements showed a discontinuous change. The 7.0 limit value was exceeded with the pH value of 7.31 ± 0.01 on the day 91st measurement. These results show that the pH value alone cannot determine product quality, as expressed by Erkan and Özden (2007), and Bilen (2009). As in our study, the raw fresh fish and smoked sample initial pH values obtained in some studies where the hot smoking process was applied to freshwater fish are high (Diler et al. 2002; İzci 2004; Yanar 2007; Bolat et al. 2009), by an advance of storage time, the pH value has a decreasing tendency first, but then it has an increasing tendency. Similar changes were not seen in other studies examined (Özkütük 2002; Çakli et al. 2006; Salama and Ibrahim 2012; Fıcıcılar and Genccelep 2017).

3.3. Sensorial analysis

Sensorial analysis points at the beginning of the study of smoked fish were 7,90 ± 0,43 for color, 8,00 ± 0,39 for flavor, 8,00 ± 0,45 for odor, 7,90 ± 0,43 for texture structure, 7,90± 0.41 for appearance, and 8.40 ± 0.31 for general acceptance (Table 4). The samples stored in the refrigerator scored below the limit value (2.00) on the day 56th in terms of the odor parameter. The scores of other parameters were also very close to the limit value.

Table 4. Sensorial analysis score (**±SE)**

Days	Color	Flavor	Odor	Texture Structure	Appearance	General Rating
0.	7.90 ± 0.43 ^{d}	8.00± 0.39	8.00±0.45 ^{ef}	7.90±0.43^{ef}	7.90±0.41 ^{e}	8.40±0.31 ^{d}
7.	8.10 ± 0.30 ^{d}	8.20± 0.25	8.65±0.23 ^{f}	8.85±0.17 ^{g}	8.45±0.29 ^{e}	8.60±0.18 ^{d}
14.	6.40 ± 0.31 ^{c}		7.30±0.40^{efg}	7.10±0.31 ^{de}	6.60±0.40 ^{d}	6.80±0.33 ^{c}
21.	5.80±0.39 ^{bc}		5.50±0.58 ^{cd}	6.40±0.52 ^{cd}	6.00±0.37 ^{cd}	6.10±0.46 ^{bc}
28.	4.60±0.37 ^{b}		4.60±0.50 ^{bc}	5.00±0.37 ^{b}	5.00±0.37 ^{c}	5.10±0.41 ^{b}
35.	6.10±0.38 ^{c}		6.70±0.50^{def}	6.50±0.78^{cd}	6.40±0.43 ^{d}	6.40±0.43 ^{c}
42.	6.00±0.50 ^{c}		6.50±0.76^{de}	5.40±0.64^{bc}	6.00±0.45 ^{cd}	6.00±0.45^{bc}
49.	4.60±0.60 ^{b}		4.00±0.60 ^{b}	4.10±0.55^{b}	3.50±0.58 ^{b}	3.80±0.57 ^{a}
56.	2.40±0.34 ^{a}		1.90±0.46 ^{a}	2.20±0.36 ^{a}	2.30±0.37 ^{a}	2.90±0.50 ^{a}

*The difference between the values shown in different letters is significant ($p\leq0.05$)

Although smoking technology is traditionally used in the preservation of fish in many regions of the world, the acceptability of the product depends on its sensory properties (Yanar, 2007; Fıcıcılar and Genccelep, 2017). It is out of the question that a product whose sensory results are not suitable can be offered for consumption and thus marketed (Dokuzlu, 1997). Because if a product acceptable in terms of quality parameters is unacceptable in terms of sensory properties, this product cannot be consumed (Özden et al., 2001). In our study, the sensory scores obtained on the day 7th were higher than the initial values. This case shows that the structural changes that occur in the fish after the hot smoking process have turned into a stable form. For example, the smoke aroma fully integrates with fish meat during storage in the refrigerator. This was seen in all the criteria. In some examined studies, there was a similar change (Bolat et al., 2009; Alçiçek, 2010; Kaba et al., 2013). But there were some studies where this change was not observed (Özkütük, 2002; Çakli et al., 2006; Yanar, 2007). By the progression of the storage period, a relative decrease in sensory scores was detected. These changes depending on time were found statistically significant ($p \leq 0.05$). On the day 56th, the odor criterion was effective in making the sensory deterioration decision for the samples with an average score of 1.90 ± 0.46. On the other hand, the scores obtained for other criteria (color, texture structure, appearance, general rating) were determined above the deterioration limit.

3.4. Microbiological analysis

Total *Enterobacteriaceae* was not observed in the samples during storage in the refrigerator. On the day 35th, the number of TPC was determined as 6.93 ± 0.03 log cfu /g, and the number of TPA was 6.43 ± 0.15 log cfu /g. The total of yeast mold was determined as 6.00 ± 0.05 log cfu /g on the day 28th. In the analysis performed on the 49th storage day, TPC was determined as 7.02 ± 0.02 log cfu /g, TPA 6.84 ± 0.06 log cfu /g, and yeast-mold 6.89 ± 0.01 log cfu /g (Table 5).

Seafood is generally considered as being degraded when it reaches 6-7 log cfu/g microorganisms (ICMSF, 1986; Huss, 1988; Çakli, 2007; Sallam, 2007; Kilinç et al., 2018). In the analysis made on the day 35th, by the obtained a value of 6.30 ± 0.00 log cfu / g the limit value of 6.00 log cfu / g was clearly exceeded. In the analysis made on the day 42nd, the total number of yeast-mold was determined as 7.15 ± 0.15 log cfu / g. It is seen that the product can be consumed up to day 35th in terms of the TPC and the TPA. When tested in terms of yeast-mold count, we can say that the product can be consumed safely until the day 28th.

It is reported that there is no yeast-mold growth in studies that examined smoked trout (Çakli et al., 2006; Bolat et al., 2009). As in our study, in the studies where the fish are supplied by catching from nature, canal catfish (Efiuvwevwere and Ajiboye, 1996), Tench (İzci, 2004), Vimba bream (Diler et al., 2002), yeast-mold development were observed.

Table 5. Microbiological analysis results (**±SE)**

Days	TPC (log kob/g)	TPA (log kob/g)	Enterobacteriaceae (log kob/g)	Mould-Yeast (log kob/g)
Raw	0.54± 0.06^{b}	0.65± 0.05^{a}	<10^{1}	1.04± 0.04^{a}
0.	<10$^{1\ a}$	2.36± 0.25^{b}	<10^{1}	2.72± 0.10^{b}
7.	2.85± 0.00^{c}	2.71± 0.03^{c}	<10^{1}	2.73± 0.03^{b}
14.	4.79± 0.03^{d}	4.00± 0.00^{d}	<10^{1}	4.04± 0.03^{c}
21.	4.95±	5.10±	<10^{1}	5.03±

	0.00^{e}	0.02^{e}		0.01^{d}
28.	5.85± 0.01^{f}	5.91± 0.03^{f}	<10^{1}	6.00± 0.05^{e}
35.	6.93± 0.03^{g}	6.43± 0.15^{g}	<10^{1}	6.30± 0.00^{f}
42.	8.03± 0.00^{ı}	6.77± 0.07^{h}	<10^{1}	7.15± 0.15^{g}
49.	7.02± 0.02^{h}	6.84± 0.06^{h}	<10^{1}	6.89± 0.01^{h}

* The difference between the values shown in different letters is significant ($p \leq 0.05$)

It is grown in trout-controlled conditions and in cold waters. The used fish in studies in which the yeast-mold development was observed, is cyprinid and their habitat is relatively warmer waters. This temperature is also suitable for the survival and development of yeast-mold. In studies that examined the smoked rainbow trout (Arashisar et al., 2004; Bolat et al., 2009; Mutlu 2016) while the development of bacteria, psychrophilic bacteria, and *Enterobacteriaceae* were observed, but it was reported there was no *Enterobacteriaceae* in a study (Çakli et al., 2006).

3.5. Fatty acids analysis

In the analysis of *C. antalyensis* raw sample, 27 different fatty acids were determined (Figure 6). The number of fatty acids without double bonds (SFA) is 8, the number of fatty acids containing one double bond (MUFA) is 7, and the number of fatty acids containing two or more double bonds (PUFA) is 12. Of the double-bonded, 5 fatty acids were determined in the n-3 group, 6 in the n-6 group, 2 in the n-9 group, 1 in the n-11 group, and 1 in the n-12 group (Table 6).

Among fatty acids without double bonds (SFA), C16: 0 had the highest value. C16: 1 value, which was determined as 10.95 ± 0.17% in raw fish, decreased to 6.41 ± 2.52% after the hot smoking process. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) values were higher in smoked fish than in raw fish. Low amounts of C19:0 fatty acids were detected in raw and smoked samples. While the total SFA value determined in the raw sample was 24.62%, this value increased to 25.38% in the smoked samples. On the other hand, in the raw sample, the total value of fatty acids containing one double bond was determined as 31.44%, while it fell to 26.86% in the smoked samples.

While the total value of the n-3 group fatty acids of the raw sample increased in the smoked sample, the opposite result was obtained for the n-6 group fatty acids.

Humans cannot synthesize fatty acids double-bonded containing the 9 C atom and more when counted from the end of the carboxyl molecule. For example, linoleic acid (C18: 2n-6), which is an essential fatty acid, cannot be synthesized from the human body, so it should be taken from the outside (Leaf and Weber, 1988).

In studies conducted, it has been reported that Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), which are among the n-3 group of fatty acids, are found in seafood oils intensely and are a natural source of these fatty acids (Eseceli et al., 2006; Nollet and Toldrá, 2010).

The fat content in fish and the fatty acid composition of these oils may vary depending on the various factors, such as species, individuals, body areas, feeding, fishing season, gender, etc., (Özden et al., 2001; Kaya et al., 2004).

Table 6. Fatty acids detected in the raw and smoked sample (%) (**±SE)**

	Raw	**Smoked**
C4:0	0.17±0.05^{a}	0.56±0.05^{a}
C14:0	1.94±0.07^{a}	1.88±0.97^{a}
C15:0	2.10±0.05^{c}	1.50±0.16^{bc}
C16:0	7.70±0.04^{a}	15.84±1.2^{b}
C16:1	10.95±0.1^{b}	6.41±2.52^{ab}
C17:0	7.92±0.46^{ab}	0.87±0.03^{a}
C17:1	0.53±0.05^{a}	0.88±0.49^{a}
C18:0	2.45±0.01^{a}	4.01±0.95^{a}
C18:1	3.56±0.37^{b}	2.09±0.63^{ab}
C18:1n-9(c)	12.29±4.13^{a}	11.33±1.85^{a}
C18:2n-6(t)	3.10±0.03^{b}	0.15±0.10^{a}
C18:2n-6(c)	2.84±1.72^{a}	3.66±1.34^{a}
C18:3n-6	0.74±0.07^{a}	0.81±0.23^{a}
C18:1n-12	1.13±0.10^{ab}	2.72±2.28^{b}
C18:1n-11	1.15±0.07^{a}	2.51±2.43^{a}
C19:0	2.18±0.17^{b}	0.50±0.00^{a}
C22:5n-3	3.42±0.31^{a}	6.43±0.40^{c}
C20:0	0.16±0.02^{a}	0.22±0.06^{a}
C18:3n-3	11.06±4.18^{b}	6.77±2.67^{ab}
C20:1n-9	1.83±0.23^{ab}	0.92±0.12^{a}
C20:2n-6	0.35±0.02^{ab}	0.25±0.08^{a}
C20:3n-6	0.52±0.34^{a}	0.52±0.07^{a}
C20:3n-3	0.37±0.14^{a}	0.40±0.20^{a}
C20:4n-6	3.31±0.19^{a}	4.06±1.44^{a}
C22:2	0.20±0.11^{a}	0.28±0.00^{a}
C20:5n-3	4.81±0.38^{a}	7.24±0.24^{ab}
C22:6n-3	5.91±0.35^{a}	11.36±3.57^{ab}
Σ SFA	24.62	25.38
Σ MUFA	31.44	26.86
Σ PUFA n-3	25.77	32.48
Σ PUFA n-6	10.86	9.45

*The difference between the values shown in different letters in the same line is significant ($p \leq 0.05$)

Table 7. TVB-N values and variation obtained in some studies where smoked technology is applied to freshwater fish

Study	Species	Supply Area	Initial TVB-N (mgN/100 g)	After Smoking TVB-N (mgN/100 g)	End of Storage TVB-N (mgN/ 100g)	Storage period (day)	Tendency
Yanar 2007	*Clarias gariepinus*	Fisheries	15.47±0.22	17.67±0.81	29.16±1.68	24	Discontinuous
Özkütük 2002	*Oreochromis niloticus*	Breeding		15.02±0.66	22.63±0.23	75	Continous Increase
Bolat et al. 2009	*Oncorhynchus mykiss*	Breeding	14.56±0.70	18.72±0.24	18.53±0.24	30	Discontinuous
Çaklı et all. 2006	*Oncorhynchus mykiss*	Breeding		9.3±0.60	27.90±0.10	40	Continous Increase
Fıcıcılar and Genccelep 2017	*Oncorhynchus mykiss*	Breeding		16.56 ± 0.34	19.74 ± 0.38	21	Continous Increase
İzci 2004	*Tinca tinca*	Fisheries	13.53±0.47	16.33±0.47	26.60±0.81	28	Continous Increase
Salama and Ibrahim 2012	*Ctenopharyngo don idella*	Breeding		9.20± 0.03	22.9± 0.5	30	Continous Increase

Table 8. TBA values and changes obtained in some studies where smoked technology is applied to freshwater fish

Study	Species	Supply Area	Initial TBA (mg mda/ kg)	After Smoking TBA (mg mda/ kg)	End of Storage TBA (mg mda/ kg)	Storage period (day)	Tendency
Yanar, 2007	*Clarias gariepinus*	Fisheries	0.45±0.04	0.84±0.03	2.67±0.62	24	Continous Increase
Özkütük 2002	*Oreochromis niloticus*	Breeding		0.16±0.01	0.11±0.00	75	Discontinuous
Bolat et al. 2009	*Oncorhynchus mykiss*	Breeding		0.85±0.03	0.48±0.07	30	Discontinuous
Çaklı et al. 2006	*Oncorhynchus mykiss*	Breeding		1.4	2.1	40	Discontinuous
Fıcıcılar and Genccelep 2017	*Oncorhynchus mykiss*	Breeding		0.48±0.07	3.65 ± 0.24	21	Continous Increase
İzci 2004	*Tinca tinca*	Fisheries	0.15±0.00	0.32±0.01	3.08±0.01	28	Continous Increase
Salama and Ibrahim 2012	*Ctenopharyngo don idella*	Breeding		0.13± 0.01	1.72± 0.1	30	Continous Increase

4. Conclusions

In this study, applying the hot smoking technology to *C. antalyensis* species and the storage time of the obtained product under refrigerator conditions were studied. It was determined that the species can be processed by hot smoking technology owing especially to the obtained rather high sensory taste score.

Preserving the smoked product in frozen storage instead of a refrigerator and conducting a study to measure the consumer response to a product stored in this way can give up useful results.

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IDENTIFICATION OF THE POTENTIAL BIOACTIVE PEPTIDES IN EDIBLE BIRD'S NEST

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ABSTRACT

The major component in edible bird's nest (EBN) is protein. Thus, it is a potential source of bioactive peptides. Thus, this study aimed to determine the potential bioactive peptides from proteomic profiles of EBN using BIOPEP database. In this study, a proteomic profiling of soluble EBN proteins was carried out using high sensitivity liquid chromatography tandem mass spectrometry. Five proteins were selected as potential precursors for bioactive peptides which were deleted in malignant brain tumors 1, lysyl oxidase 3, acidic mammalian chitinase, NK-lysin and mucin-5AC for further analysis. It was found that the chosen proteins gave six dominant bioactivities which were angiotensin-converting enzyme (ACE) inhibitor, dipeptidyl peptidase-IV (DPP IV) inhibitor, dipeptidyl peptidase-III (DPP III) inhibitor, antioxidative, stimulating and renin inhibitor. Furthermore, the most potential bioactive peptides from soluble EBN proteins were angiotensin-converting enzyme (ACE) inhibitor and dipeptidyl peptidase-IV (DPP IV) inhibitor. Meanwhile for *in silico* proteolysis of EBN proteins using 33 type of enzymes, stem bromelain and pepsin were found to give the highest degree hydrolysis and to produce the highest number of bioactive peptides. Five tripeptides were generated after gastrointestinal digestion simulation for each ACE inhibitory activity, which were IRA, YPG, MKY, IVR and AVL and DPP IV inhibitory peptides that were WRD, WRT, WRS, VPL and APG, respectively. However, all these tripeptides have been reported in previous studies. This study showed that EBN has a promising source of bioactive peptide and *in silico* approach provide better understanding of theoretical and prediction of functional peptides.

1.Introduction

Edible bird nest (EBN) mainly comprises of the salivary gland secretion of several swiftlet species in the genus Aerodramus and Collocalia. These birds are found predominantly in Southeast Asia, e.g., Thailand, Vietnam, Indonesia, Malaysia and Philippines (Marcone, 2005). According to Wu et. al, (2010), swiftlet species that produced EBN belongs to the family Apodidae and genus Aerodamus. *Aerodamus fuciphagus* and *Aerodamus maximus*, commonly known as white-nest and black nest swiftlets respectively, are the two dominant EBN-producing species in Malaysia. EBN

produced by *A. fuciphagus* is more expensive compared to that of *A. maximus* in commercial market (Quek et al., 2015). EBN has been consumed as a Chinese delicacy and traditional Chinese medicine for centuries (Ma & Liu, 2012).

Protein is the major components in EBN which is around 62% protein, followed by carbohydrate, ash and fat (Marcone, 2005). Ma and Liu (2012) reported that medicinal and bioactive activities were only exhibited by hydrolyzed EBN by hydrolytic enzymes. While Syarmila et al. (2018) reported that glycopeptides in EBN in both crude EBN and EBN hydrolysate have functional bioactivities such as antimicrobial, antioxidant and ACE-inhibitory activities. Thus, EBN protein has potential to be used as a health enhancing ingredient in the nutraceutical formulation.

Poor solubility and low in extraction yield have become the major problems for EBN proteomic study (Wong et al., 2018) and several methods were used to enhance the solubility such as centrifugation, sonication, soaking and stewing in hot water. Several proteins have been identified in EBN, including mucin. A previous study showed that EBN was able to promote healthy stomach by preventing gastric caused by pathogens by releasing the fragments from EBN (Kong et al., 2016). Mucin layer has other compounds such as bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, surface-active lipids and protease inhibitors that can make a layer to protect from degradation by gastric acid and pancreatic enzymes (Miner-Williams et al., 2009). As reported by Kong et al. (2016), in addition to mucin, carbonic anhydrase 9, acidic mammalian chitinase-like protein, immunoglobulin, NADH dehydrogenase, proline-rich protein and von Willebrand factors (VWF) were also detected in EBN. Other than mucin, Saengkrajang et. al (2013) reported that there were major essential amino acids found in EBN such as methionine and cysteine. These two amino acids contribute substantially to the maintenance and integrity of cellular systems by influencing cellular redox state and cellular capacity to detoxify toxic compounds, free radicals and reactive oxygen species. Glutamine also was found in EBN that may have benefits in inflammatory conditions such as infections and injuries. Besides these three amino acids, Zukefli et al. (2017) stated that EBN contain serine, valine and glutamic acid which have important role in immune system. However, there are differences in amino acids and nutrient composition content due to seasonal variation and breeding sites (Nur'Aliah et al., 2016).

Besides high in protein, EBN consists of glyconutrient was sialic acid that showed properties of neurological and intellectual advantages in infants as reported by Marcone et al., (2005). The other major glyconutrients include N-acetylgalactosamine (galNAc), N-acetylglucosamine (glcNAc), galactose and fructose. GalNAc is an amino sugar derived from galactose and a prominent precursor for glycosaminoglycans, a major component of joint cartilage that involved in the function of the synapses, the junction between nerve cells and deficiency can cause severe memory problem (Aswir & Nazaimoon, 2011). The EBN has unique structure and the findings on the identification of α 2–3 linked and α 2–6 linked sialoglycoproteins. Sialoglycoproteins that one of glycoproteins are rich in sialic acids and more than 10% were reported to have high biological and medicinal values. From the findings, α 2–3 linked sialoglycoprotein most likely an acidic mammalian chitinase-like protein, and α 2–6 linked sialoglycoprotein was an acidic mammalian chitinase (Zukefli et al., 2017).

Peptides released from dietary proteins by enzymatic hydrolysis have demonstrated bioactivities including, antioxidant, antidiabetic, anti-hypertensive,antithrombotic, immuno-modulating, osteoprotective, antimicrobial, anticarcinogenic and growth-promoting properties (Hall et al., 2018). Enzymatic hydrolysis is one of the most common method to hydrolyse protein other than microbial ferementation (Marciniak et al., 2018) due to cheap, more specific, release biological active,

produce antioxidative peptides and reduce allergenic potential of intact proteins (Singh et al., 2019). Other than that, the enzymatic reactions did not produce residual organic solvents or toxic chemicals. However, the process depends on several factors such as pH, temperature, enzyme, reaction time, enzyme concentration (Marciniak et al., 2018). In order to hydrolyse the protein, there are several enzymes can be chose in order to obtain the optimal results. Plant or microbial enzymes such as ficin, bromelain, papain, alcalase or flavourzyme are commonly employed in the production of protein hydrolysates. Ficin, papain and bromelain are cysteine endopeptidase, whereas alcalase is serine endopeptidase and flavourzyme is a mixture of endopeptidase and exopeptidase (Singh et al., 2019).

Since complete genome or protein database of swiftlet is not currently available, and due to the limitation of homology searching, further research is necessary to confirm the type and profile of proteins present in EBN. However, there are limited numbers of relevant protein sequences deposited in the database. More research is needed at the molecular level to explore the mechanisms behind the biological functions as well as the potential of bioactive peptide content.

In this study, *in silico* approach will be used to determine the potential bioactive peptides from EBN. Thus, proteomic profiles of soluble protein from EBN will be determined using LC-MS/MS method, and the profiles will be further analysed using BIOPEP database to determine the potential bioactive peptides from EBN.

2. Materials and methods

2.1. Materials

One kilogram of raw, cleaned EBN sample was purchased from a swiftlet farmer at Kampung Wa, Dungun, Terengganu, Malaysia. The protocols and method for protein extraction used in this study were performed as described by Kwan & Ismail (2018). Next the EBN solution was hydrolysed using trypsin and analysed using LC-MS/MS to determine its proteomic profiles. PEAKS studio 7.5 software was used to identify the protein.

2.2.Sample collection and protein extraction

First, the raw cleaned EBN was ground into fine powder. Then, 15 mg of the ground sample was dissolved in 1 ml extraction buffer (40 mM Tris-HCl, pH 8.8) and was kept for 20 minutes with occasional vortexing. Then, the sample undergone sonication for 15 minutes using a sonication probe at room temperature. The sample solution was then centrifuged at 12,000 g for 30 minutes, the supernatant was collected and then kept at -35ºC until further analysis.

2.3. Determination of protein content

Bradford assay was carried out to determine the soluble protein content in EBN sample as described by Bradford (1976). Five µL of EBN sample was mixed with 250 uL of Bradford reagent in a 96 well plate. Then the mixture was incubated at room temperature for 15 minutes. A standard curve ranging from 0.01-2 mg/mL was establish using the bovine serum albumin (BSA) by reading the absorbance at 595 nm. The total protein concentration was determined and calculated by comparing the absorbance value obtained for the sample against the standard curve.

2.4. Protein digestion

Protein digestion was carried out according to Kinter & Sherman (2005). The EBN sample was resuspended in 100 µL of 6 M urea, 100 mM Tris buffer. After that, 200 mM dithiothreitol (DTT) was added to the sample and was left at ambient temperature for 1 hour. Then, 200 mM of iodoacetamide was mixed into the sample solution and kept at room temperature for 1 hour, before another 20 µL of 200 mM DTT was added. Next, the sample was diluted with 775 µL of water. The EBN sample was digested with 20 µg of bovine trypsin where the mixture was incubated at 37°C, overnight. The digestion was stopped the next day by adjusting the pH of the mixture to pH less than 6 using concentrated acetic acid. The digested sample was then

concentrated to less than 20 µL using vacuum concentrator.

2.5. LC-MS/MS analysis

The digested EBN sample was reconstituted in 100 µL of 0.1% formic acid in deionized water and filtered using the 0.45 µm regenerated cellulose (RC) membrane syringe filter. Mass spectrometer was performed using the LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, CA, USA) coupled with Easy-nLC II nano-liquid chromatography system. The eluent was sprayed into the mass spectrometer at 2.1 kV (source voltage) at 220°C. Full scan mass analysis was done from m/z 300-2,000 at resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition) with data-dependent MS/MS analyses (ITMS) triggered by the 8 most abundant ions from the parent mass list of predicted peptides with rejection of singly or unassigned charge state. Collision-induced dissociation (CID) was applied as the fragmentation technique. Collision energy was set at 35. The sample was analyzed in duplicate readings.

2.6. Protein identification

PEAKS Studio Version 7.5 (Bioinformatics Solution, Waterloo, Canada) was used to perform de novo sequencing and database matching. UniProt *Avian* (bird) database from October 2019, was used for the database matching. Parameters of analyzing the sample were set according to Kwan & Ismail (2018). Parent mass and precursor mass tolerance was set at 0.1 Da. False detection rate (FDR) <0.1% and significant score (-10logP) for protein >20 was used for protein acceptance. Minimum unique peptide was set at 1.

2.7. Protein sequence evaluation of EBN as precursor for bioactive peptides

The identified EBN proteins in Section 2.4 were selected based on the Uniprot database (https://www.uniprot.org/). The proteins were analysed for potential bioactive peptides using BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) database (accessed on August 8th , 2021) (Dziuba et al., 2004; Iwaniak et al., 2005). The PeptideRanker (Bioware.ucd.ie) was used in order to rank the predicted sequence according to bioactivity. The score presented by PeptideRanker which closer to 1 represent high chances to be bioactive. The toxicity of peptide was predicted by using ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/design.php) while the solubility of the peptide was assessed by the online Innovagen server, available at http://www.innovagen.com/proteomics-tools.

2.8. *In silico* proteolysis to release ACE inhibitory peptides

The most effective proteases to prepare the bioactive peptides with the most dominant bioactivities from EBN, namely ACE inhibitory peptides and dipeptidyl peptidase IV inhibitory peptides, were evaluated using *in silico* proteolysis application in BIOPEP database. All 33 proteases available in BIOPEP were chosen in order to obtain the ACE inhibitory peptides and dipeptidyl peptidase IV inhibitory peptides. The 33 enzymes in BIOPEP are chymotrypsin A (EC 3.4.21.1), trypsin (EC 3.4.21.4), pepsin pH 1.3 (EC 3.4.23.1), proteinase K (EC 3.4.21.67), pancreatic elastase (EC 3.4.21.36), propyl oligopeptidase (EC 3.4.21.26), V8 protease pH 4 (EC 3.4.21.19), thermolysin (EC 3.4.24.27), chymotrypsin C (EC 3.4.21.2), plasmin (EC 3.4.21.7), catepsin G (EC 3.4.21.20), clostripain (EC 3.4.22.8), chymase (EC 3.4.21.39), papain (EC 3.4.22.2), ficin (EC 3.4.22.3), leucocyte elastase II (EC 3.4.21.37), metridin (EC 3.4.21.3), thrombin (EC 3.4.21.5), pancreatic elastase II (EC 3.4.21.71), stem bromelain (EC 3.4.22.32), glutamyl endopeptidase (EC 3.4.21.82), oligopeptidase B (EC 3.4.21.83), calpain 2 (EC 3.4.22.53), glycyl endopeptidase (EC 3.4.22.25), oligopeptidase F, proteinase P1 (EC 3.4.21.9), xaa-pro dipeptidase (EC 3.4.13.9), pepsin pH>2 (EC 3.4.23.1), coccolysin (EC 3.4.24.30), subtilisin (EC 3.4.21.62), chymosin (EC 3.4.23.4), ginger

protease (EC 3.4.22.67) and V-8 protease (EC 3.4.21.9).

3.Results and discussions

3.1. Protein content

It was found that the crude protein content of raw cleaned EBN using Kjeldahl method was 54.30%, which is in similar range as reported by Wong et al. (2018) (52%) and Nur 'Aliah et al. (2016) (56.47-60.63%). This shows that EBN has high protein content (Zukefli et al., 2017). However, determination of soluble protein extracted from EBN using Bradford assay only gave a protein content of 1.45 mg/ml. This may indicate the insolubility of EBN protein in aqueous as well as the inefficiency in extraction method (Zukefli et al., 2017).

3.2. Protein identification

There were five dominant proteins found in edible bird's nest (EBN) consisting of 115 to 1654 amino acid sequences while mass of proteins ranging from 13.232 to 58.700 kDa as stated in Table 1.

Table 1. Protein identification by de novo sequencing using ESI-ion-trap MS/MS

Protein name	UniProt Accession number	Species	Amino acid length	Average mass (kDa)	Protein sequence
Deleted in malignant brain tumors 1	A0A093BE17	*Chaetura pelagica (Chimney swift)*	115	13.232	PYHVDVNQDLFLEAYLHSSDPDLVLFLD TCVASPTPHNVSTVTYDIIRNGCAQDPTY TTYDSPSSYIVRFKFNAFRFVRKSPSVYL KCEFVVCRTYDYHSRCYQGCITTSRSQSS
Lysyl oxidase 3	A0A093BIT2	*Chaetura pelagica (Chimney swift)*	527	58.700	GRIWLDNVNCAGGEKSIGDCKHRGWGN SDCSHEEDAGVICKDERIPGFKDSNVIET EQSQGEEVRLRPVVSGARRLLPVTEGIVE LRYKDGWAQICDQGWDSRNSRVVCGM MGFPAEKKVNRNFYKLASKSQPKQKRR EDVGSKKSWQPLSCTVACQAVLSHAILS HVPCHVSHVPHHVSHIPRHALTRSLQARI RLKGGAKVGEGRVEVLRSSEWGTICDD RWNLQSASVVCRELGFGSAKEALTGAR MGQGTGPIHLNEVQCLGTEKSLWSCPYR NITREDCKHTEDAAVRCNIPYMGYENLV GTVEAAGIRGHSAWGLLVLGTLDDGWT TKEAMVACRQLGLGYSLHAVTETWYW DA SNVTEMVLSGVKCAGHEMSLNHCQHHG TSLNCRKTGTRFAAGVICSETASDLLLHA PLVQETAYIEDRPLHMLYCAAEENCLSSS ARLANWPYGHRRLLRFSSQIHNNGRADF RPKAGRHSWVWHECHRHYHSMDVFTH YDILTPNGTKVAEGHKASFCLEDTECEE
Acidic mammalian chitinase	A0A093BFV9	*Chaetura pelagica (Chimney swift)*	352	39.104	GTANVLTCYFTNWAQYRPGLGKFMPEN IDPFLCNHLIYAFANMNNNEITTYEWND ETLYKSFNGLKNQNRNLKTLLAIGGWNF GTAKFSTMVSTPQNRRTFINSVIRFLRKH NFDGLDLDWECPGSRGSPPQAKTLFTVL VKEMVAAFEQEARQSNRPRLMVTAAVA AGLSTIQAGYEIAELGKYLDYIHVMTYD FHGSGDGRTGENSPLHSGGNPQLSVEYA MKYWRDNGAPAKKLLVGFPTYGRTFTL QNPSNTAVGAPASGPGPAGTYTQEAGLL AYYEICSFLNSGATQAWDAPEDVPYAYK

					GSEWVGYDNVKSFNIKVDWLKKNNFGG AMVWTVDLDDFTGTFCNQG
NK-lysin	A0A0H5A QZ5	*Coturnix japonica (Japanese quail)*	137	14.663	MAAAIIVMMAMGAVLQVVVTEPPHDDQ RDVAAGSPWEQQWQLLQDGSAVWDEG DAMGPGKMKCSACVKLVKKLQKIVGD DPDEEAIGTALGQVCGTKRILKGICRQLG KKLRQQLSDALQDDSDPRSVCTTLGLCK G
Mucin - 5AC	R7VT28	*Columba livia (Rock dove)*	16544	18.104	MGTGGGIRIPLWISILALAFIQIKVQAQDV DPQTKSNYVSPSILQRQKRVPPSSKSQEV TIIPPFQNTLKLKAGNPSHNGRVCSTWGN FHFKTFDGDIFYFPGICNYIFASNCKSPYE DFNIQIRRTMVENATIITNVIMKLDGIVIE LTRGSVLLDGKLVQMPYSHMGVLIEKSN NYLKVSAKLGLTFLWNEEDALLVELDK KYANQTCGLCGDFNGIPISNEFISENTKL TPIQFGNRQKMDGPTEQCDDPIPPTLLVN CSAEFASICETVLTSKAFTSCNVLVNVQD YIETCIQDLCHCDSSMADFCMCNTFAEY SRQCAHAGGQPLNWRTSELCPKLCPFN MQYQECGSPCSDTCSNPERSALCEDHCT DGCVCPPGKLISCSFLIAEAARTYICAGC VPRKECHCTYEGEIYAPGASFSSKCRSCT CTGGEWSCVSQSCLGTCSIEGGSHISTFD EKFYSFFGDCSYVLTKLCDSNEFTVLGDI QKCGLTDTETCLKGIAISLSGGQTNIVIQP SGSVFVNMIYTQLPFSAANVTIFRPSSFFII LQTTFGLQLQVQLVPLMQLFIDLDPSHK GRTCGLCGNFNDMQTDDFKTTSGVIEGT SAAFGNTWKTRADCPDAKNTFENPCTVS IQNDQYAQHWCGLLSDTMGPFAECHST VNPEVYEKNCMFDTCNCEKSEDCMCAA LSSYVRACAAKGVLLTGWRSKACTKYT TLCPKSLKYMDNVDACQPTCRSLSEPDV TCSIKFVPVDGCTCINGTYMDESGKCVP ASSCPCYYKGMPLSSGEVVHDNGVVCT CTYGKLSCIGEKPEPVCVPPMVYIDCGN VTTDVVGAGCQKSCQTLDMECYRTHCV SGCVCPHNQVLDGKGSCIAPEDCPCIHN GNSYSPGESIRVGCNNCTCRNRKWHCSQ EPCLETCSVYGDGHYTTFDGKRFDFEGD CEYVLIQNYCGQQGVNQGTFRVITENIPC GTTGTTCSKSIKVFLGNYELVLSDGHSD VIQRTPGGKMPFQIRSMGIYLVVDTTVG LILMWDKKTSIFIKLSPSFQGNVCGLCGN YDGNGNNDFTTRSQSVVGNVLEFANSW KVSSSCPNASPTKDPCTANPYRKAWAQK QCSIITSEVFAKCHSQVEPNEYYQACVDD ACACDTGGDCECFCTAVAAYAQACNEL DICISWRTPSICPLFCDYYNPQGECEWHY

					KPCGAPCMKTCNNPSGNCLHELRGLEGC YPHCPKNKPYFDEETMTCVSNCGCFENG KNYKPGMQMPSKQNCQSCECTNYGKKC KYDEHECVCVYEGQKYNYEDVIYNTTD GTGGCIVATCGSNGTLQRV VYECPISTTPMTATTFHFSTTPPATTSTEN TSFSVTTTSPAITTSESTTFIPSATKEETTT TTEMTKPVTTSPSTTSLCTKEECYWSMW YDASYPGSGYNDGDFDTIQNIEKKGYKV CDNRKEVQCRAVRFPNTPYPLLEQNITC NKEEGLICYNKDQLPPICYNYKIRFKCCK NVRVPCHTTAAPHTTRISTSTSISSTTPST SPTEESTTPHLQTKHKTTTTSMTQPETQY IHTRTTTQPITQTQTETTTSISMPSVSSTTT HSTTACEPEVCSWSEWIDVDVPSSGPNQ GDFETYQRIRAAGMEVCQHPKEIECQAE DYPEVPIQNVGQVV

Deleted in malignant brain tumors 1 (DMBT1), a scavenger receptor cystine rich (SRCR) (also known as glycoprotein-340 or salivary agglutinin) was found to give the shortest sequence (115). The role of DMBT1 is to innate immunity with several factors of protection such as secretory IgA, surfactant protein D and A, and also binding to the bacteria directly (Müller et al., 2013). Besides that, DMBT1 also binds to bovine and human lactoferrin, an 80 kDa iron binding protein belongs to the transferrin family (Ligtenberg et al., 2010). According to Madsen et al. (2013), DMBT1 plays an important role to innate defense in the gastrointestinal and respiratory tracts against bacteria and viruses through direct interaction with microorganism. It also binds with other microorganisms such as *Helicobacter pylori*, several strains of streptococcus and viruses including Human Immunodeficiency Virus Type 1 (HIV) and influenza A virus as well as taking part in cell differentiation and growth. Other than that, the content of DMBT1 can be a biomarker in diagnosis of acute respiratory distress syndrome (ARDS) and predictions of severity disease (Ren et al., 2016). Lack of DMBT1 was found first in brain tumors, but over-expression of this protein has been discovered in pancreatic cancers (Cheung et al., 2008)

Besides DMBT1, lysyl oxidase 3 protein with 527 amino acids also can be found in EBN. Lysyl oxidase 3 (LOX) is a family of copper-dependent oxido-deaminase capable of modifying the side chains of lysyl residues in collagen and elastin, leading to the spontaneous formation of inter-polypeptide chain cross-links derived from non-reducible aldehyde (Herchenhan et al., 2015). Initially this enzyme catalyse the formation of covalent collagen cross-links that is an essential process for fibril stabilization. Not only collagen and elastin, lysyl oxidase also regulate the interaction between the different substrates rather than modification of its intrinsic enzymatic capacity (Rosell-García et al., 2019). Collagen plays an important role to provide mechanical stability in tissues and structures such as skin, blood vessels, bones and tendons (Herchenhan et al., 2015). This proved that EBN is a bone and chondro-protective agents against osteoporosis and osteoarthritis (Kong et al., 2016) because of LOX present in EBN. Wong et al. (2018) also reported the presence of lysyl oxidase in EBN.

Acidic mammalian chitinase (AMCase) was also found in EBN. This enzyme is characterized by an acidic isoelectric point, Therefore, it is known as acidic mammalian chitinase which is able to degrade artificial chitin-like substrates as well as chitin from crab shell and chitin as present in the fungal cell wall (Boot et al., 2001). According to Wong et al. (2018), AMCase can be found in many species such as human, mouse and bird. Identification of AMCase in EBN in

this study is consistent with the finding by Wong et al. (2018) that AMCase-like was the major protein identified, suggesting its abundance in EBN using shotgun and immunoprecipitated products by anti-EBN antibodies methods. According to Ohno et al. (2016), AMCase can act as a digestive enzyme as well as part of the host defense against chitin-containing pathogens in the mouse gastrointestinal tract and as a protease resistant major glucosidase to produce N-acetylglucosamine. Kong et al. (2016) also reported that acidic mammalian chitinase-like protein was found in EBN that was able to promote healthy stomach by preventing gastric caused pathogens by releasing the fragments from EBN. AMCase protein also discovered by Liu et al. (2012) in EBN using liquid-phase isoelectric focusing matched with two-dimensional electrophoresis technique.

With 137 amino acids sequence and 14.663 kDa mass, NK-lysin is a cationic peptide with antibacterial activity that was originally isolated from porcine intestinal tissue. The protein sequences of NK-lysin are rich in amino acid with positive charges and include maintained cysteine residues that form intrachain disulfide bonds (Wang et al., 2006). It is a member of the saposin-like protein family, and exhibits potent antitumor activity and has antimicrobial activity (Lee et. al, 2014). The antitumor and antimicrobial properties of the NK-lysin protein have the same effect with the other saposin-like proteins that able to form pores in the cell membrane due to its α-helical structure (Zhang et al., 2000). This statement supported by (Lee et al., 2014) that antibacterial activity is found in all peptides corresponding to each helical region of NK-lysin. This findings is similar reported by (Syarmila et. al, 2018) which glycopeptides in EBN both in crude and hydrolysate demonstrated functional bioactivities such as antimicrobial, antioxidant and ACE-inhibitory components.

Last but not least, mucin-5AC is also identified as a major structural protein in crude EBN. Under simulated gastro-intestinal condition, EBN was released peptides high likely to mucin using de novo sequencing (Kong et al., 2016). Wong et al., (2018) also identified mucin-5AC like in EBN by monoclonal antibodies method. Mucin is a family of polydisperse molecules containing high molecular mass and a high proportion of covalently bound oligosaccharide side chains that afford high resistance to the effects of acid and digestive enzymes. Mucin also contains a few compounds that have good functions such as bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, protease inhibitors and surface-active lipids (Miner-Williams et. al, 2009). Thus, structural proteins may enhance the properties in EBN. To date, only this study discovered DMBT1 and NK-lysin proteins in EBN.

3.3. The potential of edible bird's nest as precursor for bioactive peptides

The potential of the five EBN proteins as precursor for bioactive peptides were analyzed using BIOPEP software. BIOPEP gave values of A, ∑A, B and also lists of potential bioactive peptides from each protein sequence. From the BIOPEP analysis, A and B values for major bioactivity was detected from EBN proteins. The A value gave the frequency of encrypted bioactive peptides occurring in a particular protein (Dziuba *et al.* 2004). BIOPEP contains 48 major classes of peptide bioactivity databases. However, BIOPEP analysis of EBN protein sequences showed only 29 subclasses of potential bioactivity were present for EBN. Among the 29 subclasses of bioactivities, 6 of these are the major bioactivities present in EBN protein sequences as stated in Table 2, which were angiotensin I-converting enzyme (ACE) inhibitor, dipeptidyl peptidase (DPP) IV inhibitor, dipeptidyl peptidase (DPP) III inhibitor, antioxidative, stimulating and renin inhibitor. The other 23 bioactivities present in at least any one of the EBN protein sequences were activating ubiquitin-mediated proteolysis, bacterial permease ligand, hypotensive, alpha-glucosidase inhibitor, antiamnestic, antibacterial, antithrombotic,

chemotactic, immunomodulating, immunostimulating, inhibitor, opioid and others.

Table 2. Number of potential bioactive peptides and potential biological activity (B) of identified proteins using BIOPEP

Proteins	UniProt Accession number	Activity					
		ACE inhibitor	Dipeptidyl peptidase IV inhibitor	Dipeptidyl peptidase III inhibitor	Antioxidative	Stimulating	Renin inhibitor
Deleted in malignant brain tumors 1	A0A093BE17	23 (0.0209)	51 (0.0002)	7	10	4	3
Lysyl oxidase 3	A0A093BIT2	108 (0.0093)	161 (0.0004)	24	33	9	7
Acidic mammalian chitinase	A0A093BFV9	94 (0.0198)	141 (0.0005)	22	19	5	9
NK-lysin	A0A0H5AQZ5	42 (0.0157)	53 (0.0003)	3	7	7	-
Mucin -5AC	R7VT28	166 (0.0117)	229 (0.0004)	30	47	11	12

Based on the A values, the predominant bioactivity for all EBN proteins are DPP IV inhibitor and ACE inhibitor. This finding is consistent with previous studies on *in-vitro* hydrolysis of EBN releasing ACE inhibitory and antioxidative activity (Amiza *et al.* 2014; Ghassem *et al.* 2017).

DPP IV inhibitor is one of the main peptides bioactivities in EBN proteins. DPP-IV (EC 3.4.1.4.5), a serine protease cleaves dipeptides of X-Pro or X-Ala from N terminal (Hildebrandt *et al.* 2000). Inhibition of DPP IV activity has a positive effect on type 2 diabetes (Agirbasli & Cavas 2017). Diabetes is a chronic metabolic disorder resulted in high blood sugar levels over a prolonged period. In recent years, diabetes has become one of the leading causes of death worldwide. According to the International Diabetes Federation (IDF), in 2017, about 425 million people were living with diabetes globally. However, synthetic DPP-IV drugs are reported to have some adverse effects such as gastrointestinal adverse effects, allergic reactions, skin-related side effects and musculoskeletal disorders (Liu *et al.* 2019). Many DPP-IV inhibitory peptides have been discovered in various food protein hydrolysates, including milk proteins (hua *et al.* 2011), rice bran (Hatanaka *et al.* 2012) and oat (Bleakley *et al*. 2017).

ACE inhibitor is the other main peptide bioactivity in EBN proteins. ACE plays an important role in regulating blood pressure in the renin angiotensin system (RAS) and kallikrein-kinin system. In RAS, ACE converts angiotensin I to an active vasoconstrictor angiotensin II, resulting in blood pressure increase. ACE activity inhibition is mainly used to prevent hypertension (Shahidi & Zhong 2008). ACE inhibitors such as captopril is widely used as pharmaceutical drugs for treatment of cardiovascular diseases. However, they often cause side effects such as coughing, skin rashes, and taste disturbances (Lee & Hur, 2017). Natural ACE inhibitory peptides are a natural alternative to synthetic drugs. ACE-inhibitory peptides usually contained hydrophobic (proline) and aliphatic amino acids (isoleucine and leucine) at the N-terminal (Lee & Hur, 2017).

BIOPEP analysis shows that the highest value of ∑A value is that of mucin-5AC like

gave the lowest ∑A value with 1.1241. According to Wong *et al.* (2018), mucin-5AC like was one of major protein found in EBN and it consists of polydisperse molecules with high molecular mass and a high proportion of covalently bound oligosaccharide side chains. The function of mucin-5AC like is to protect the delicate epithelial surfaces of the mucosa is primarily due to the polymerization of mucin monomers to form viscoelastic gels (Miner-Williams *et al.*, 2009).

Table 3 shows that taking into consideration both A and B values, the most potent bioactivity in EBN proteins is ACE inhibitor, followed by DPP IV inhibitor. Although DPP IV inhibitor has the highest A value, however, its B value is much lower than that of ACE inhibitor. On the other hand, although ACE inhibitor has lower A value than DPP IV inhibitor, its B value is much higher.

Table 3. The frequency of occurrence of peptides with a given activity (A) in selected protein sequences

Proteins	**UniProt Accession number**	**Number of Activities**	**∑A**	**A_1**	**A_2**
				ACE Inhibitor	DPP IV Inhibitor
Deleted in malignant brain tumors 1	A0A093BE17	10	1.1653	0.2435	0.5913
Lysyl oxidase 3	A0A093BIT2	17	1.3018	0.4099	0.6262
Acidic mammalian chitinase	A0A093BFV9	17	1.4743	0.4688	0.6705
NK-lysin	A0A0H5AQZ5	16	1.2263	0.4453	0.5036
Mucin -5AC	R7VT28	19	1.1675	0.3482	0.5719

The third highest value of A is given by DPP III activity; however, its B value is lower than renin inhibitor, alpha-glucosidase inhibitor and regulating. DPP III is a member of the M49 family of zinc dependent metallopeptidases and cleaves dipeptides sequentially from the N-terminus of various bioactive peptide substrates (Kumar et al., 2016). Diaz et al., (2018) reported that the main function of DPP III is to hydrolyze peptides ranging from 3 to 10 amino acids in length from their N-terminal in dipeptides and free amino acids and the activity of DPP III enable inhibited by synthetic hermophin-like peptidases in rats and humans. Furthermore, for human DPP III, high resolution crystal structures of the protein in complexes with opioid peptides (Met-and Leu-enkephalin, and endomorphin2), angiotensin-II and the peptide inhibitor have been reported (Kumar et al., 2016). This shows that differences in the binding modes allow a distinction between real substrates and inhibitory peptides. To date, no scientific research has been reported on DPP III in EBN.

Antioxidative is one of the major activities found in EBN but the B value is the lowest among the 10 activities. Ghassem *et al.* (2017) reported from Swiss-Prot and NCBI database found 13 antioxidant peptides sequences from EBN peptides. The peptides have superior antioxidant properties with high ORAC value and acted as a bioavailable free-radical scavenger. Meanwhile, antioxidative protein reported by Ghassem *et al.* (2017) that were ovotransferrin, cyctochrome b and glycosyl-transferase did not show any similarity with the antioxidative proteins in this study. This may suggest that the antioxidant peptides sequences reported by Ghassem *et al.* (2017) were derived from other EBN proteins, different from the types of EBN proteins used in this study.

3.4. *In silico* proteolysis of EBN proteins

Enzymatic hydrolysis is the most common approach to release biologically active peptide (Meng et al., 2018). In the BIOPEP database, there were 33 types of enzymes and all these enzymes were chosen for the *in silico* proteolysis of ACE inhibitory peptides. Most of potential bioactive peptides were dipeptides or tripeptides and the length of bioactive peptides was less than ten amino acids (Huang et al., 2015). Although there were 33 types of proteases in BIOPEP but only 5 proteases have been selected as they demonstrated the action of enzyme to produce the most potent of ACE-inhibitory and possess the highest degree of hydrolysis (DH) as specified in Table 4. According to Hall et al. (2018), DH is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds per unit weight (stated in percentage). The range of DH was between 36.15% to 72.02% for five EBN proteins with five selected enzymes meanwhile pepsin conveyed the highest DH compared to other enzymes that was in range 59.48% to 72.02%. In addition, pepsin also gave the highest value of DH and ACE inhibitory activity in tuna frame protein hydrolysate compared to other enzymes include papain and trypsin (Lee et al., 2010).

Table 4. The predicted efficiency of release of bioactive fragments from selected edible bird's nest protein by *in silico* proteolysis

Protein	Enzymes	DH_t (%)	ACE Inhibitor		DPP IV Inhibitor	
			A_E	W	A_E	W
Deleted in malignant brain tumors 1	Pepsin	59.48	0.0256	0.0966	0.0171	0.1819
	Stem bromelain	52.59	0.0513	0.1936	0.1026	0.1792
	Pancreatic elastase	52.59	0.0342	0.1291	0.0940	0.1642
	Leucocyte elastase II	42.24	0.0342	0.1291	0.0940	0.1642
	Ficin	45.69	0.0513	0.1936	0.0855	0.1493
Lysyl oxidase 3	Pepsin	60.33	0.0522	0.1388	0.1013	0.1703
	Stem bromelain	51.66	0.0350	0.0880	0.0792	0.1332
	Pancreatic elastase	48.71	0.0442	0.1111	0.0902	0.1516
	Leucocyte elastase II	37.82	0.0368	0.0925	0.0681	0.1145
	Ficin	44.65	0.0424	0.1066	0.0663	0.1115
Acidic mammalian chitinase	Pepsin	72.02	0.0663	0.1455	0.0994	0.1544
	Stem bromelain	51.80	0.0387	0.0849	0.0746	0.1159
	Pancreatic elastase	50.69	0.0304	0.0667	0.0801	0.1245
	Leucocyte elastase II	36.57	0.0249	0.0546	0.0691	0.1074
	Ficin	42.11	0.0580	0.1272	0.0663	0.1030
NK-lysin	Pepsin	70.71	0.0496	0.1147	0.0709	0.1470
	Stem bromelain	47.86	0.0709	0.1639	0.0709	0.1470
	Pancreatic elastase	48.57	0.0496	0.1147	0.0496	0.1028
	Leucocyte elastase II	39.29	0.0355	0.0821	0.0213	0.0442
	Ficin	35.00	0.0355	0.0821	0.0355	0.0736
Mucin -5AC	Pepsin	63.91	0.0480	0.1436	0.0767	0.1409
	Stem bromelain	45.11	0.0427	0.1277	0.0603	0.1107
	Pancreatic elastase	47.92	0.0410	0.1226	0.0714	0.1311
	Leucocyte elastase II	37.49	0.0176	0.0526	0.0480	0.0882
	Ficin	36.15	0.0427	0.1277	0.0615	0.1129

Table 4 shows that the degree of hydrolysis (DH) is not proportional to the release of bioactive peptides as in lysyl oxidase 3 protein that has 37.82% of DH with leucocyte elastase II enzyme exhibit A_E and W value of ACE inhibitor 0.0368 and 0.0925 respectively while the same enzyme in NK-lysin protein has 39.29% of DH with A_E and W value 0.0355 and 0.0821 respectively. Most of the enzymes action in the same protein sequence in Table 4 shows

the release frequency (A_E) of ACE inhibitory peptides lower than DPP IV inhibitory peptides and similar action goes to relative release frequency (W) of peptides except for some proteins such as DMBT1 treated by pepsin and NK-lysin treated by leucocyte elastase II also in mucin-5AC treated by pepsin, stem bromelain and ficin.

Every enzyme showed the specific cleavage sites and has different potential to release bioactive peptides from proteins (vu et al., 2019). In this study, pepsin demonstrated the most effective enzyme to produce ACE inhibitory and DPP IV peptides from EBN proteins.

Table 5. Bioactive peptides predicted to be released from edible bird's nest protein based on *in silico* proteolysis

Enzyme	ACE inhibitors	DPP-IV inhibitors
Pepsin	**145**	**239**
	CF (2), HG (1), HK (1), HL (1), HY (1), IA (4), IE (9), IF (4), IG (4), IL (7), 1P (1), IY (5), PG (7), PL (1), PP (1), PG (4), PT (4), RA (3), RF (4), RG (2), RP (1), RY (1), SF (5), SG (7), ST (7), SY (2), VE (7), VF (4), VG (12), VK (5), VM (2), VP (2), VR (1), VY (6), WA (4), WG (4), WL (1), IPY (1), IRA (1)	HA (3), HE (3), HF (2), HL (1), HT (1), HY (2), IA (4), IL (7), IM (1), IN (1), IP (1), IQ (12), IR (2), PA (4), PF (4), PG (7), PK (1), PL (5), PM (1), PN (4), PP (1), PQ (4), PS (2), PT (4), PY (4), RA (2), RG (2), RK (3), RL (1), RM (1), RN (3), RP (1), SF (5), SH (2), SK (3), SL (5), SY (2), VA (8), VD (9), VE (7), VF (4), VG (12), VK (5), VL (19), VM (2), VN (7), VP (2), VQ (7), VR (1), VS (1), VT (12), VY (6), WA (3), WD (4), WG (4), WK (1), WL (1), WN (3), WQ (1), WT (1), WY (2), VPL (1), WRD (1), WRT (1)
Stem bromelain	**175**	**195**
	CF (1), DA (3), DF (6), DG (12), EA (3), EF (2), EG (5), EV (3), HG (1), IA (2), IF (1), IG (3), IL (5), KA (4), KF (3), KG (5), KL (8), KR (2), MG (6)L NH (3), NG (5), PG (7), PL (4), PR (2), PT (5), QG (2), WA (2), WG (3), YA (1), YG (3), YL (2), YV (2), YPG (1)	EG (5), ES (2), ET (6), EV (3), HA (5), HF (2), HR (1), HS (5), HV (2), IA (2), IL (5), IR (5), KA (4), KF (3), KG (5), KR (2), KS (4), KT (5), KV (2), MA (2), MG (6), MV (5), NA (1), NF (3), NF (3), NG (5), NL (1), NR (3), NT (2), NV (9), PA (6), PF (2), PG (7), PL (2), PS (9), PT (5), PV (3), QA (4), QG (2), QL (5), QS (5), QT (5), QV (5), WA (2), WG (3), WR (2), WS (2), WT (2), WV (1), YA (1), YF (1), YG (3), YL (2), YS (3), YT (2), YV (2)
Pancreatic elastase	**116**	**218**
	DA (4), DG (10), DY (2), EA (3), EG (8), EI (3), EV (3), EY (2), FG (3), FY (1), HL (1), HY (2), KA (3), KG (6), KL (10), KY (3), MG (6), NG (3), NY (6), PG (7), PL (5), PT (4), QG (2), RA (4), RG (4), RL (3), RY (1), WA (2), WG (3), WL (1), MKY (1)	EG (8), EI (3), ES (2), ET (7), EV (3), EY (2), FA (4), FL (6), HA (4), HI (1), HL (1), HS (7), HT (1), HV (4), HY (2), KA (3), KG (6), KI (2), KS (5), KT (2), KV (6), KY (3), MA (2), MG (6), MV (5), NG (3), NT (3), NV (9), NY (9), PA (5), PG (7), PI (4), PL (5), PS (9), PT (4), PV (2), PY (5), QA (6), QG (2), QI (4), QL (3), QS (4), QT (5), QV (2), QY (2), RA (4), RG (4), RG (4), RI (4), RL (3), WA (2), WG (3), WI (1), WL (1), WS (3), WT (2), WV (1), WY (1), WRS (1), WRT (1),
Leucocyte elastase II	**65**	**158**
	DA (2), EA (3), EV (2), GA 950, GI (1), GL (6), GS (3), GT (10), GV (3), HL (1), KA (3), KL (6), PL (4), PT (3), RA (3), WA (1), WL (1), YA (3), YL (3), YV (2)	ES (1), ET (7), EV (2), FA (4), FL (6), GA (5), GI (1), GL (6), GV (3), HA (4), HI (1), HL (1), HS (3), HT (1), HV (3), KA (3), KS (5), KT (2), KV (5), MA (2), MV (5), NT (1), NV (6), PA (4), PI (3), PL (4), PS (9), PT (3), PV (2), QA (5), QI (4), QL (2), QS (4), QT (4), QV (3), RA (3), RI (3), RL (3), WA (1), WI (1), WL (1), WS (2), WT (1), WV (1), YA (3), YI (4), YL (3), YS (1), YT (3), YV (2), WRT (1)

Ficin	**123**	**180**
	AF (3), AG (4), AR (3), AY (2), CF (1), DF (5), DG (11), DY (2), EF (1), EG (5), EK (3), IF (2), IG (1), IL (4), IY (2), MG (5), NF (4), NG (7), NK (2), NY (6), PG (7), PH (1), PL (3), PR (1), QG (3), QK (4), TF (7), TG (8), VF (2), VG (4), VK (3), VR (2), VY (2), WG (1), AVL (1), IVR (1)	AF (3), AG (4), AL (3), AS (5), AY (2), EG (5), EK (3), ES (2), IH (2), IL (4), IR (5), MG (5), ML (1), NF (4), NG (7), NH (1), NL (1), NR (3), NY (6), PF (2), PG (7), PH (1), PK (2), PL (3), PS (6), PY (3), QG 93), QL (3), QS (5), TF (7), TG (8), TH (2), TK (3), TL (5), TR (5), TS (9), TY (4), VF (2), VG (4), VK (3), VL (8), VR (2), VS (4), VY (2), WG (1), WH (1), WK (1), WR (3), WS (3), APG (1), VPL (1)

Table 5 shows the predicted of identified ACE inhibitor dipeptides and tripeptides to be released from EBN proteins by proteolysis. Besides that, novel amino acid sequences could be released from the parent peptide during the process of digestion and may exert toxic, allergenic or other potent biological activities when absorbed (Garcia-vaquero et al., 2019). Enzymatic hydrolysis by *in silico* method resulting all the peptides to be compared with the BIOPEP database whether it has been reported or not previously.

After gastrointestinal digestion simulation, there were 10 tripeptides has been released. However, all these 10 tripeptides have been reported previously as ACE and DPP IV inhibitory. From these 10 tripeptides, 5 tripeptides were ACE inhibitor peptides and the remaining were DPP IV peptides. The sequence of IVR that had ACE inhibitory activity property showed the lowest IC_{50} value (0.81 µM) (Rawendra et al., 2014) compared to IPY, IRA and MKY that possess the IC_{50} value 206 µM (Vukic et al., 2017), 1.11 µM and 0.86 µM (Wu et al., 2006) respectively. While the sequence of YPG has been reported to have opioid inhibitory activity in wheat with EC_{50} value 5.4 mM and 1.8 mM for both µ and κ opioid receptor (Garg et al., 2018) and no study was reported the sequence has ACE inhibitory activity to date. An ACE inhibitory peptides that contain amino acid sequences and the activities expressed as the concentration of the necessary to inhibit the activity by 50% (IC_{50}) (Vukic et al., 2017).

Meanwhile for DPP IV inhibitory peptides were showed by the sequence of tripeptides WRD, WRT and WRS that have IC_{50} value 376 µM, 536 µM and 483 µM respectively (Lan et al., 2014). The other tripeptide sequence of APG in dietary proteins that have IC_{50} value 4000 µM (Lacroix & Li-chan, 2012) and IC_{50} value for VPL tripeptide sequence was 15.8 µM (Nongonierma & Fitzgerald, 2014).

Although all the predicted most potential peptides has been reported previously in literature, this results can be a platform for better understanding of protein sequences for searching potential bioactive peptides in food proteins in comparison to traditional approaches conducted in laboratory (Huang et al., 2015). Further *in vitro* and *in vivo* studies is needed to have a better understanding of bioactive peptides from EBN.

4. Conclusions

Profiling of EBN proteomic profiles found 5 proteins as potential precursors for bioactive peptides which were deleted in malignant brain tumors 1, lysyl oxidase 3, acidic mammalian chitinase, NK-lysin and mucin-5AC. BIOPEP analysis of these proteins found that the main bioactivity was those of ACE inhibitory and DPP IV inhibitory activity. Besides, it was predicted that pepsin gave the greatest action in proteolysis of the most EBN proteins with gave the highest degree of hydrolysis and generated the highest DPP IV inhibitory peptides while stem bromelain produced the highest ACE inhibitory peptides. *In silico* proteolysis, a total of 10 tripeptides were discovered to give the most potent of ACE inhibitory and DPP IV activity and sequence of IVR showed the lowest IC_{50} value and YPG was selected to be the novel peptide. This study shows that EBN proteins is

a favorable precursor in producing the bioactive peptides.

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PREVENTION OF MELANOSIS AND QUALITY LOSS OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI)* BY ETHANOL *PERSICARIA ODORATA* EXTRACT DURING FROZEN STORAGE

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ABSTRACT

Ethanolic extract from *Persicaria odorata* leaf was applied to investigate the ability to preserve Pacific white shrimp (*Litopenaeus vannamei*) during frozen storage of 5 days at -21°C in comparison with 1.25% sodium metabisulfite. From evaluating the effects of *P.odorata* extract concentration and immersion time on melanosis formation, shrimps treated with the extract at 1/15 (mg/mL, w/v) in 10 minutes showed the lowest degree of melanosis. Microbiological analyses showed that Pacific white shrimp treated with *P. odorata* extract possessed lower values in total plate count and *Enterobacteriaceae* count compared with the control ($p<0.05$). pH and total volatile base content saw a lower increase in samples treated with *P. odorata* crude extract ($p<0.05$). Freshness loss, protein degradation, and melanosis growth in shrimps with crude extract treatments were impeded. The results show that *P. odorata* extract can be used a potential source of melanosis inhibitors, and natural preservatives for shelf-life extension of Pacific white shrimp during frozen storage.

1.Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is a major cultured shrimp species, constituting 80% of the total shrimp production in the world (Sae-leaw & Benjakul 2018). This crustacean is rich in nutrients such as protein, minerals, amino acids, and fatty acids (Gunalan B et al. 2013), but it is highly perishable and has a limited shelf life (Na et al. 2018). The deterioration of shrimp is mainly associated with chemical, physical and microbial changes (Na et al. 2018), including melanosis (discoloration), the production of unpleasant odor and soft texture (Kim et al. 2020, Kustyawati et al. 2021). Melanosis caused by the activity of tyrosinase during storage, significantly diminishes the shrimp's market value and the consumer's acceptability (Sae-leaw & Benjakul 2018). Off-flavors are reported to be caused by aldehydes, trimethylamine, ammonia, ketones, and sulfur compounds originating from the degradation of proteins and lipids by microorganisms. The softening of meat is also related to protein degradation by microbial spoilage (Kim et al. 2020).

To retard melanosis and maintain the quality of shrimp, 4-hexyl-1,3-benzenediol, sulfiting - based agents, and phosphates have been widely used (Sun et al. 2014). Nevertheless, due to regulatory attention and increasing consumer awareness of the health risks, the use of synthetic compounds is limited since they can result in allergic reactions in some sensitive individuals. Therefore, it is required that novel and safe alternatives should be discovered (Sae-leaw & Benjakul 2018). Many studies on natural

products, especially plant phenolics with antioxidant and antimicrobial activities have been evaluated for that purpose (Nirmal & Benjakul 2009). It is reported that cashew leaf extract (Sae-leaw & Benjakul 2018), grape seed extract (Sun et al. 2014), pomegranate peel extract (Yuan et al. 2016a), green tea extract (Yuan et al. 2016b), Coffea arabica Sediment extract (Phan et al. 2021a) could inhibit melanosis and extend the shelf-life of Pacific white shrimp.

Persicaria odorata, commonly known as Vietnamese coriander, belongs to the family Polygonaceae. It is a tender perennial plant that is traditionally used in traditional Chinese medicine, pharmacy, cosmetics, and Asian cuisine (Ridzuan 2014). This plant is native to Southeast Asia and it grows best in tropical and subtropical areas which are damp and warm. Essential oils obtained from *Persicaria odorata* contain terpenes, aldehydes, alcohols, and fatty acids, and they possess antimicrobial, antioxidant, antitumor, and anti-inflammatory activities (Rebíčková et al. 2020). It is reported that it inhibits *Salmonella choleraesuis* (Fujita et al. 2015), *Enterococcus faecium*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Enterococcus faecalis* (Chansiw et al. 2018).

In a study by Phan (2021b), *P. odorata* extract possessed potent antioxidant and antityrosinase activities and it proved to have the ability to inhibit the growth of black spots and maintain the quality of Pacific white shrimp during cold storage at 2^0C. In this study, we aim to (i) evaluate the effects of plant extract concentration and immersion time on the development of melanosis and (ii) investigate the potentials of *Persicaria odorata* extract to prevent the formation of melanosis (measure the mean gray values of steamed shrimp), microbiological spoilage, physicochemical deterioration during 5 days of frozen storage at -21°C.

2.Materials and methods

2.1.Materials and preparation extract

2.1.1.Chemicals

Sodium metabisulfite (SMS), Ethanol, were obtained from Merck (Darmstadt, Germany). Other chemicals in the study were of analytical grade.

2.1.2.Shrimp collection and treatment

Alive Pacific white shrimps (*L. vannamei*) (with the size of 30-40 shrimps/kg) were purchased from Thu Duc market in Ho Chi Minh City, Viet Nam. The shrimps were kept alive and immediately transported to the laboratory at HCMC University of Technology and Education, Viet Nam within 1 h. The shrimp samples were washed with water. The samples were divided into 3 equal parts, each of which was immersed in the *P. odorata* extract solution, the sodium metabisulfite solution (SMS, 1.25%, w/v), and distilled water (as the control), in the shrimp/solution ratio of 1:2 (w/v) at 4°C for 10 min. After being removed from the solutions, the samples were drained on a sieve at 4°C for 3 min. After that, the shrimps were put in plastic bags and stored at -21 °C for 5 days after, thawing for 30 minutes at room temperature 32 ± 5°C which were analyzed for different assessments: melanosis development, pH changes, total volatile basic nitrogen (TVB-N), shear force, and microbiological analysis

2.1.3.Preparation extraction of P. odorata

The leaves of *P. odorata* bought from Thu Duc Market, Thu Duc District, Ho Chi Minh City, Viet Nam were thoroughly washed under tap water to remove impurities on the surface. The collected material was dried to constant weight in a hot air-blowing oven (Memmert, Germany), and then ground in a blender to obtain a homogenous fine powder.

100 ml of ethanol was mixed with 50 g of dry powder at room temperature for 24h. The mixture was stirred thoroughly and filtered. The filtrate was collected in a flask. The residue was mixed again with ethanol and filtered again, the filtration was repeated one more time. Subsequently, the extract was concentrated in a rotary evaporator (Yamamoto, Japan) to obtain the *P. odorata* crude extract (CE).

2.2.Survey of shrimp storage conditions

Before performing frozen storage, we served shrimp storage conditions, including immersion time and *P. odorata* extract concentration. These experiments were evaluated after 5 days of storage at -2°C and thawing for 30 minutes at room temperature (32ºC ± 2ºC). Gray values were criteria used to evaluate survey storage conditions.

2.2.1.Effect of extract concentration

The effect of *P. odorata* extract concentration on the formation of melanosis of shrimps after 5 days of frozen storage was carried out to find an optimum concentration at which the growth of black spots was minimal. Briefly, shrimps were immersed in the extract solutions at six concentrations of 0, 1/30, 1/25, 1/20, 1/15, and 1/10 mg/mL (*w/v*). After 5 days of storage, the photos of shrimps were carefully taken to calculate the gray mean values that indicate levels of melanosis growth.

2.2.2.Effect of immersion time

Shrimps were dipped into the *P. odorata* solutions (at 1/15 mg/mL, *w/v*) for 3, 5, 10, and 15 minutes, after which they evaluated their gray mean values.

2.3.Assessment of shrimp's quality during frozen storage

Shrimp samples were frozen at -21°C for 5 days and thawed at room temperature (32ºC ± 2ºC) after 30 minutes. pH values, physicochemical and microbiological properties, and melanosis development at 0, 1, 3, and 5 days were assessed during the frozen storage.

2.3.1.Melanosis assessment

All shrimp samples were defrosted at room temperature (32ºC ± 2ºC) for 30 min and then steamed at 100 °C for 3 min to make it easier for sensory evaluation before being photographed using a digital camera (Canon Eos M10, Japan). The change of color in the obtained photographs was analyzed by ImageJ software to calculate mean gray values. The lower mean gray value indicates a higher level of melanosis in the carapace of shrimp (Phan et al. 2021a).

2.3.2.pH evaluation

pH values of shrimps were measured according to the method of Chouljenko et al. (2017). 2 grams of shrimp were homogenized with 20 mL of deionized water in a homogenizer for 1 min (Kinematica AG, CH-6014, Littau/Luzern, Switzerland). Following this, the samples were kept for 5 min at room temperature. The pH values were determined by a pH meter (Sartorious, Edgewood, USA). All measurements were conducted in triplicate.

2.3.3.Microbiological analysis

The microbiological quality of shrimps was evaluated according to the bacterial count method in three aspects: Total plate count (ISO 4833-1:2013), *Enterobacteriaceae* count (ISO 21528-2:2017), and *Pseudomonas aeruginosa* count *(*3347/2001/QĐ-BYT). The colonies formed were counted and expressed as log CFU/g of weight.

2.3.4.Physicochemical analysis

Protein. The protein contents of shrimps were determined according to AOAC Official Method 992.15. All experiments were conducted at Center of Analytical Services and Experimentation (CASE, Ho Chi Minh City, Viet Nam).

Shear force. The shear force of samples during 5 days of storage was tested using the Rheo Tex SD 700II texture analyzer (Sun Scientific, Japan) at Research Institute for Aquaculture No 2 (RIA2, Ho Chi Minh City, Viet Nam) with a cross head speed of 10 mm/s.

Total volatile basic nitrogen (TVB-N). TVB-N was assessed according to the national standard (TCVN 9215-2012) at the Center of Analytical Services and Experimentation (CASE, Ho Chi Minh City, Viet Nam). The total volatile basic nitrogen values were reported as mg N/100 g of shrimp.

2.3.5.Statistical analysis

All results were expressed as mean ± SD. Experimental data were analyzed by the analysis of variance (one-way ANOVA) with Tukey's test ($p < 0.05$). Statistical analyses were conducted using an SPSS package (SPSS 26 for Windows Evaluation Version, IBM Corporation, New York, USA).

3.Results and discussions

3.1.Shrimp storage conditions

3.1.1.Effect of extract concentration

The effect of *P. odorata* extract on the growth of melanosis, as demonstrated by decreased mean gray values of the carapace of shrimps after 5 days of frozen storage is illustrated in Figure 1. Overall, it is clear the mean gray values of shrimp samples rose significantly when the concentrations of extract increased, indicating that the growth of black spots in all shrimp samples was inhibited with the presence of plant extract *(*$p<0.05$*).* As the concentration progressed, the mean gray values increased to reach a peak of 141.68 at the concentration of 1/15 (mg/mL), after which the figure started to decline at 1/10 (mg/mL) ($p<0.05$*).* Therefore, the concentration of crude extract at 1/15 (mg/mL) was chosen in this study to treat all shrimp samples for other experiments. Nirmal et al. (2011) reported that phenolic compounds in the plant extract at a high dose were likely to cross-link the proteinaceous tissues of Pacific white shrimp at which the polyphenoloxidase (PPO) was located, thereby limiting the penetration of these substances to inactivate the activity of PPO.

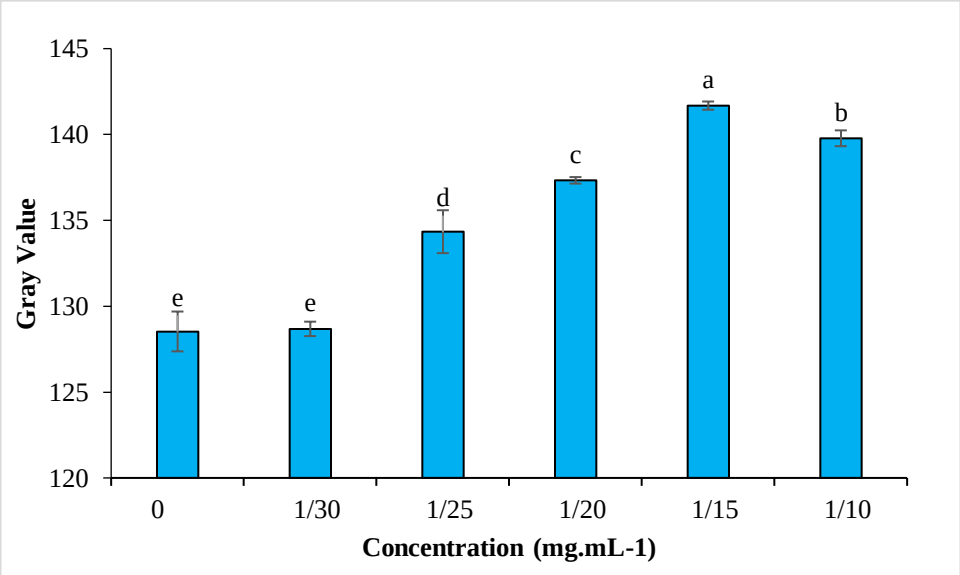


Figure 1. Changes in the mean gray values of *L. vannamei* treated with *P. odorata* extract at different concentrations during frozen storage. Values represent the mean ± standard deviation (n=3). Different lower-case letters represent statistically significant differences ($p<0.05$).

3.1.2.Effect of immersion time

Figure 2 represents the gray mean values of shrimp treated with *P. odorata* extract at different immersion times. As can be seen from the chart, in all samples surveyed, the longer the shrimps were immersed, the more effective the retardation of black spots was. However, there were no statistically significant differences in the gray values among samples treated with the plant extract in 10 and 15 minutes and they showed the highest mean gray values ($p>0.05$). Therefore, in this study, 10 minutes of immersion was chosen to treat shrimps in succeeding experiments.

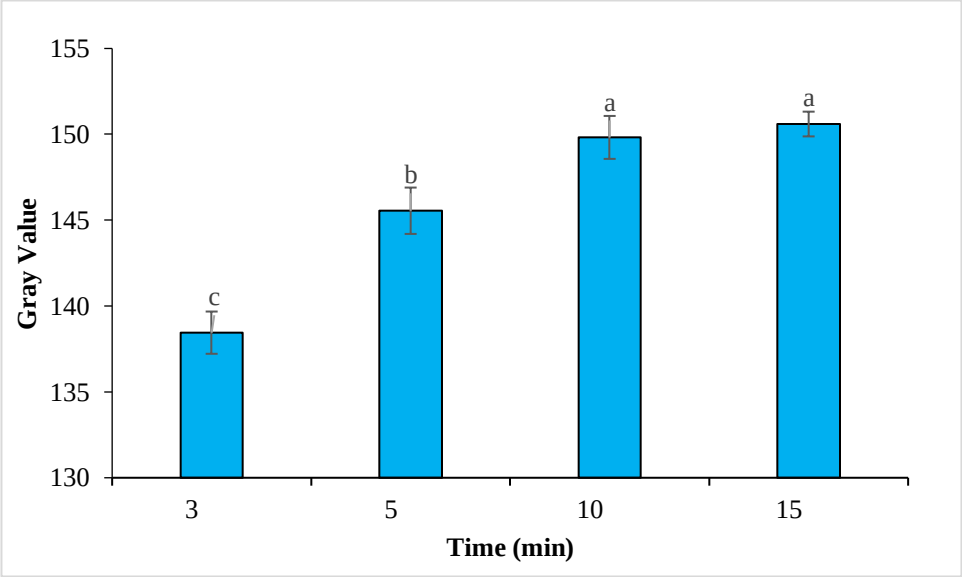


Figure 2. Changes in the mean gray values of *L. vannamei* treated with *P. odorata* extract at storage time during frozen storage. Key: see the caption for Figure 1.

3.2.Shrimp's quality during frozen storage

3.2.1.pH measurement

The changes in pH values of Pacific white shrimp with various treatments: CE, and SMS during frozen storage at -21^{0}C compared with the control (treated with distilled water) are represented in Figure 3. In general, the pH values of all shrimps saw a rise ($p<0.05$) over the period shown, and the figures for the CE treatment batches increased to a lesser extent. After 5 days of frozen storage, a 15% increase was observed in pH values of shrimps without treatments (the control) whilst the increase in shrimps preserved in SMS and CE was lower at 11.2 and 10%, respectively. At day 0, no significant differences were found among pH values from all groups ($p>0.05$). After 1 day of storage, the pH level began to grow and the CE batches had the lowest pH at 6.62, followed by shrimps treated with SMS at 6.71 and the control at 6.89 ($p<0.05$). This pattern was also the case for day 3 and day 5. At the end of the period, pH of the control reached 7.40 while those of the SMS and CE treatment batches were considerably lower at 7.36 and 7.29, respectively. It should be noted that pH change was connected with the accumulative development of basic substances, mainly as a result of either microbial or endogenous enzyme activities (Huang et al. 2012, Nirmal & Benjakul 2009). The lower degree in the pH rise of shrimps immersed in the SMS and CE solutions was consistent with the lower microbial count (Figure 6). In sum, the lower pH changes in the CE treatment batches indicated that the ethanolic extract of *P. odorata* could inhibit endogenous or microbial enzyme activities.

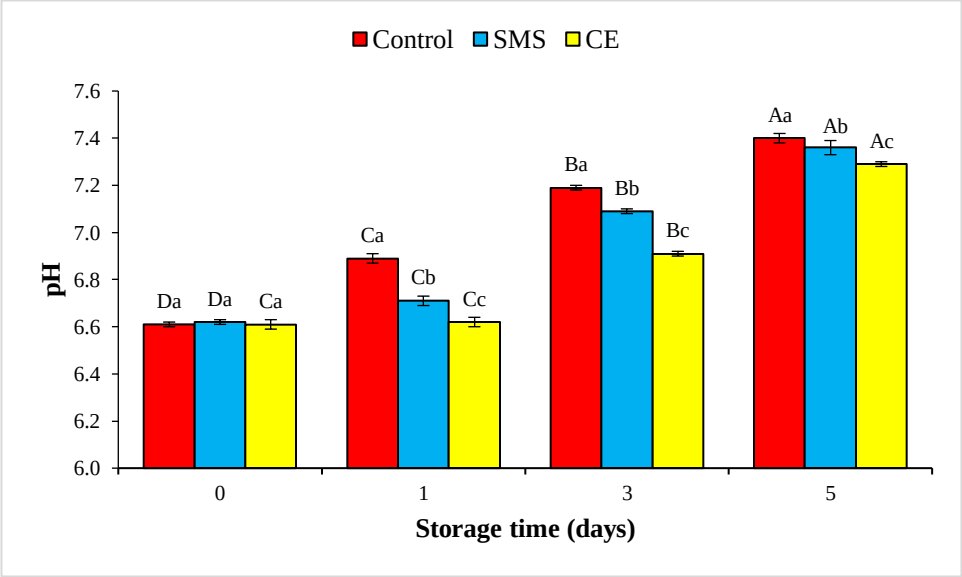


Figure 3. Changes in pH of *L. vannamei* during frozen storage. Values represent the mean ± standard deviation (n=3). Control: no treatment, SMS: samples treated with sodium metabisulfite (1.25%), CE: samples treated with *P. odorata* extract. Different lower-case letters within the same storage time in the same column indicate statistically significant differences ($p < 0.05$). Different uppercase letters within the same treatment in the same column indicate statistically significant differences ($p<0.05$).

3.2.2.Melanosis evaluation

Figure 4 represents the mean gray values and Figure 5 illustrates the photographs of Pacific white shrimp with different treatments during frozen storage. In general, the gray mean values of all samples at all different storage times saw a downward trend, but shrimps preserved in CE and 1.25 wv% SMS solutions decreased to a lesser extent than those treated with distilled water (the control) ($p<0.05$). At day 0 and day 1, all samples had no statistically significant differences in the gray mean values ($p>0.05$). As time progressed, the mean gray values started to decline continuously after 1 day of storage, indicating the formation of melanosis, but there were still no differences in the gray values for the SMS and the CE ($p>0.05$). At day 5, the shrimps treated with CE had the highest mean gray values, followed by samples treated with SMS ($p<0.05$). This result showed that *P. odorata* extract impeded the growth of black spots in shrimps. This finding is consistent with our previous research that this plant extract had high antioxidant and antityrosinase activities (Phan 2021b). According to Kumar et al. 2011, tyrosinase, a copper-containing enzyme, catalyzes the formation of melanin from tyrosine by oxidative processes and phenolics in the plant extract are likely to inactivate this enzyme by interacting with its active site via hydrogen bonding or hydrophobic interactions. In a previous study, by HPLC-EIS-MS analysis, the *P. odorata* extract contained 36 compounds, 22 of which were phenolics (Phan 2021b). Therefore, the presence of phenolic compounds, especially flavonoids, may involve in the antioxidant and tyrosinase inhibition activities and the ability of melanosis inhibition.

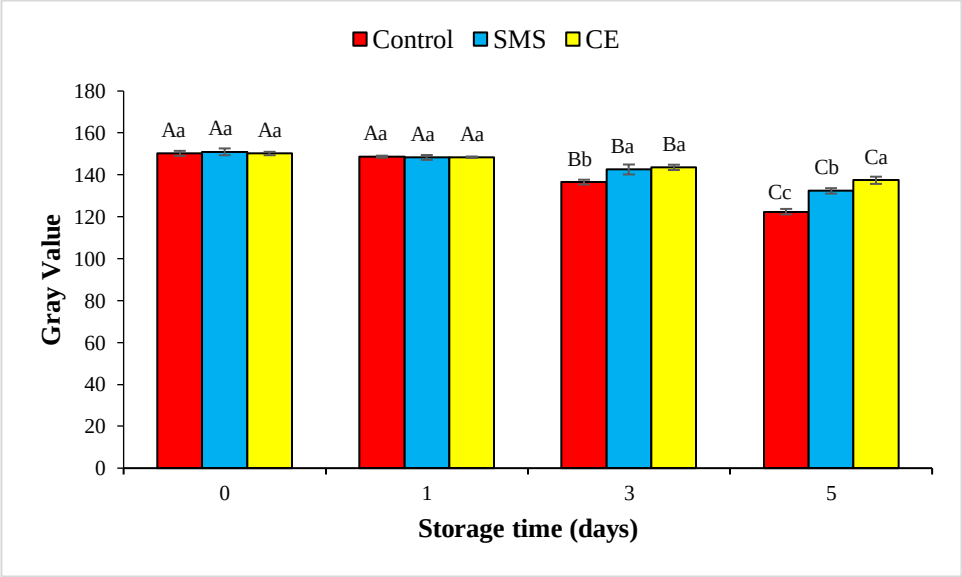


Figure 4. Changes in the mean gray values of the carapace area of *L. vannamei* during frozen storage. Key: see the caption for Figure 3.

Figure 5. Photos of *L. vannamei* without and with different treatments at day 5 of frozen storage, defrosted at room temperature (32ºC ± 2ºC) for 30 min, and steamed for 3 min .

3.2.3.Microbiological analysis

The microbiological spoilage in shrimp samples after 5 days of frozen storage at -21^{0}C is illustrated in Figure 6. It is obvious that at day 0, no significant difference was found in total plate counts (TPC) (Figure 6A) and *Enterobacteriaceae* counts (EBC) (Figure 6B) in all treatment batches ($p>0.05$). In general, after 5 days of frozen storage, the TPC and EBC of all samples were greater than those on day 0 ($p<0.05$). The initial values of TPC in this study ranged from 2.81 to 2.85 Log CFU/g which agreed with the TPC of 2-3 Log CFU/g given by other studies in Pacific white shrimp (*L. vannamei*) (Arancibia et al. 2015) and in pink shrimp (*Parapenaeus longirostris*) (López-Caballero et al. 2002). After 5 days of storage, the TPC of SMS, CE treatment groups were 5.52, 4.95 Log CFU/g, respectively which were smaller than those of the control group (7.02) ($p<0.05$). According to the Viet Nam national standard TCVN 5289: 2006, the acceptable limit of total aerobic microorganisms for frozen aquatic products is 6.0 Log CFU/g. Hence, all microbial counts of shrimps treated with SMS, and CE met the requirements of this standard, whilst the values of the control were higher than this limit.

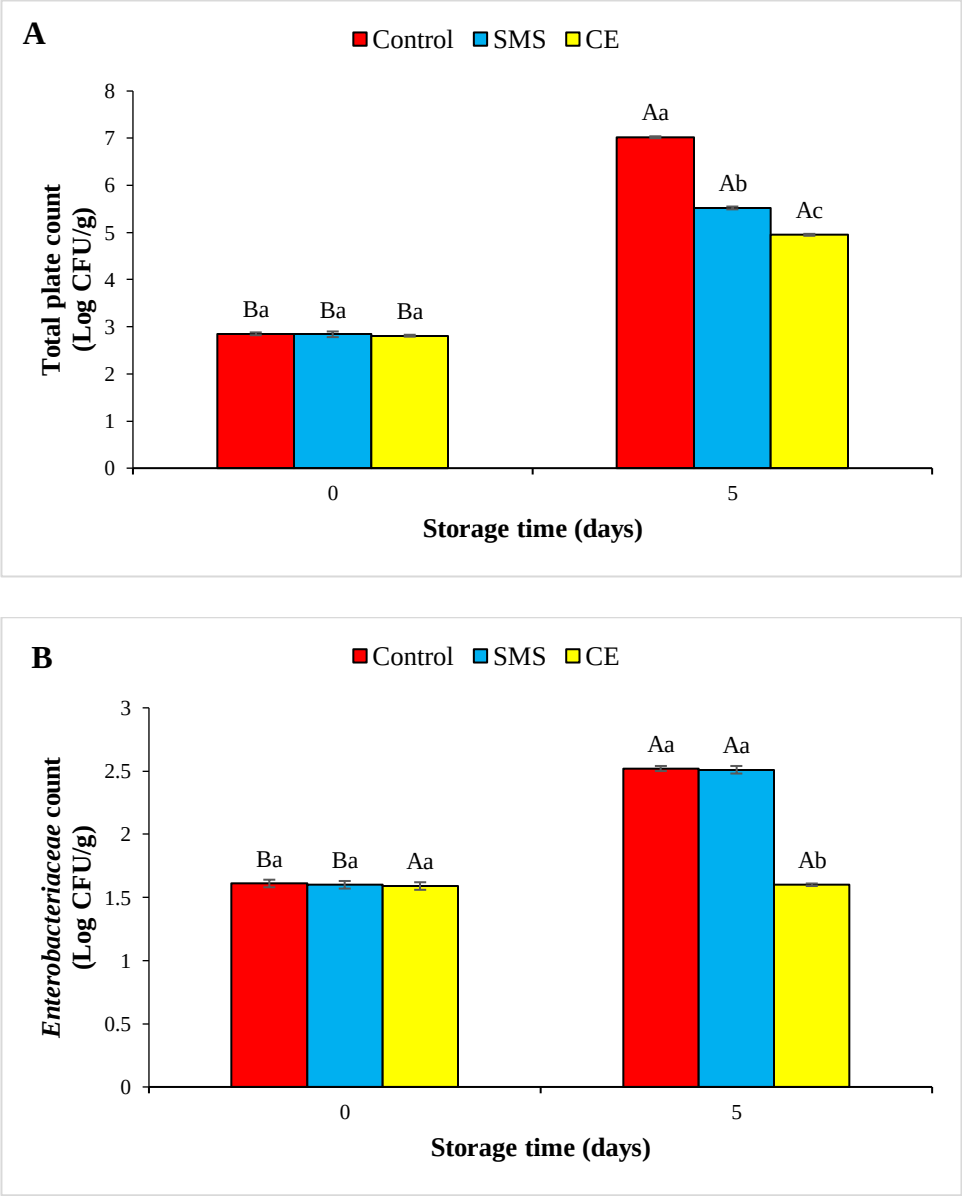


Figure 6. Total plate count (A) and Enterobacteriaceae count (B) of *L. vannamei* during frozen storage. Key: see the caption for Figure 3.

Notably, *Pseudomonas aeruginosa c*ounts of all treatment batches were lower than the detection limit of 1.0 Log CFU/g at both day 0 and day 5 (not illustrated in figures). A study by Shiekh et al. (2019) indicates that *P. aeruginosa* counts of Pacific white shrimp preserved with Chamuang leaf extract were observed in the range of 2.15−2.18 Log CFU/g at day 0 and 5.08-6.82 Log CFU/g at day 10. Nevertheless, counts are dependent on postmortem conditions and the area in which shrimps lived (Huang et al. 2012).

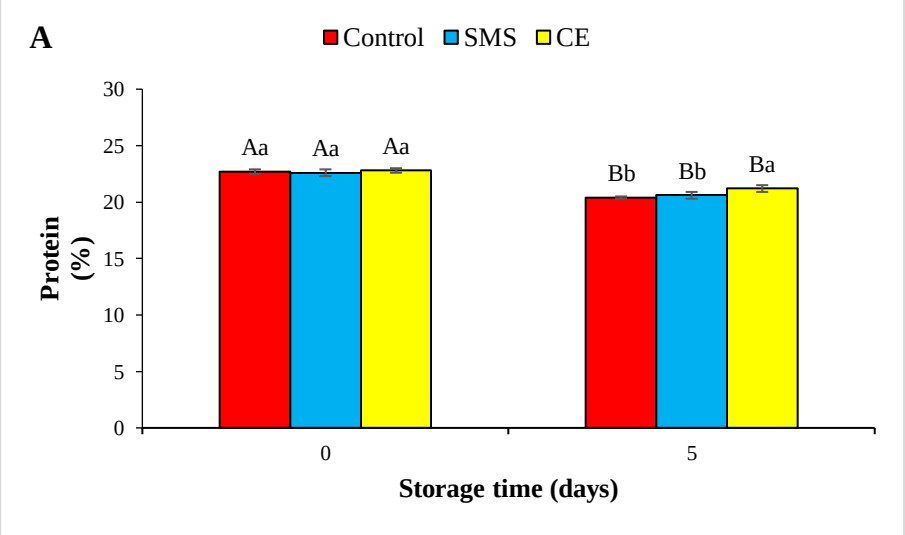
Another noticeable point worth mentioning is that shrimps treated with CE possessed the lowest EBC of 1.60 Log CFU/g in comparison with other treatment batches ($p<0.05$) during frozen storage at -21^{0}C. *Enterobacteriaceae* are a family of Gram-negative bacteria, including a large number of pathogens, namely Salmonella, Escherichia coli, Shigella, Klebsiella, and Yersinia pestis (Rai et al. 2020). Besides,

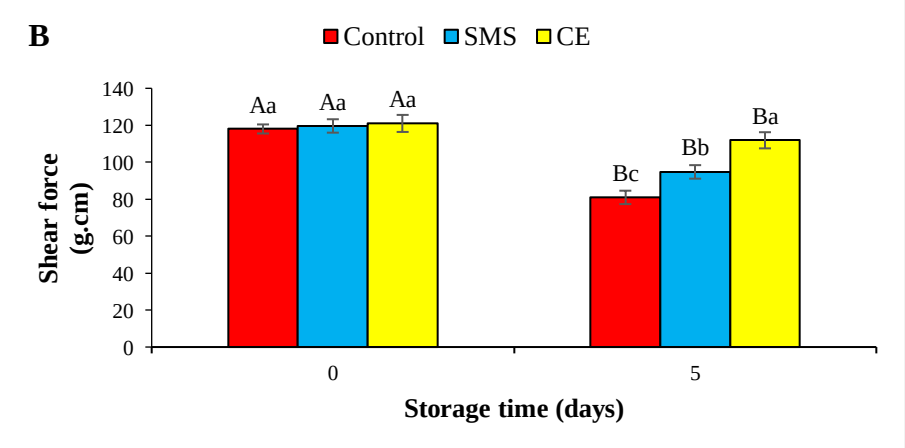
Ibrahim Sallam (2007) noted that the spoilage ability of *Enterobacteriaceae* should be taken into consideration especially for polluted water or lack of frozen post-harvest storage. The results show that the *P. odorata* extract could be used to retard the growth of spoilage microorganisms during frozen storage.

3.2.4.Physicochemical Changes

Physicochemical properties including protein content, shear force, and total volatile basic nitrogen (TVB-N) after 5 days of frozen storage were evaluated and displayed in Figure 7.

According to Figure 7A, the protein content of shrimps immersed in SMS was comparable to that of the control, in the range of 20.4-20.6 ($p>0.05$) and lower than the value of CE ($p<0.05$), suggesting that *P. odorata* extract lowered protein loss and that it could replace SMS in preventing this deterioration. It is believed that the weakening of myofibrils and intramuscular tissues leads to protein loss in shrimps when proteases like cathepsins are released (Pan et al 2019). In literature, there is not much information available on the deterioration of shrimp protein during chilled storage. A study by Kamal et al. (2000) reported that the protein contents declined from 18.46 to 17.05% for giant freshwater prawn (*Macrobrachium rosenbergii*) and 18.06 to 16.85% for tiger shrimp (*Penaeus monodon*) after 10 days of iced storage.





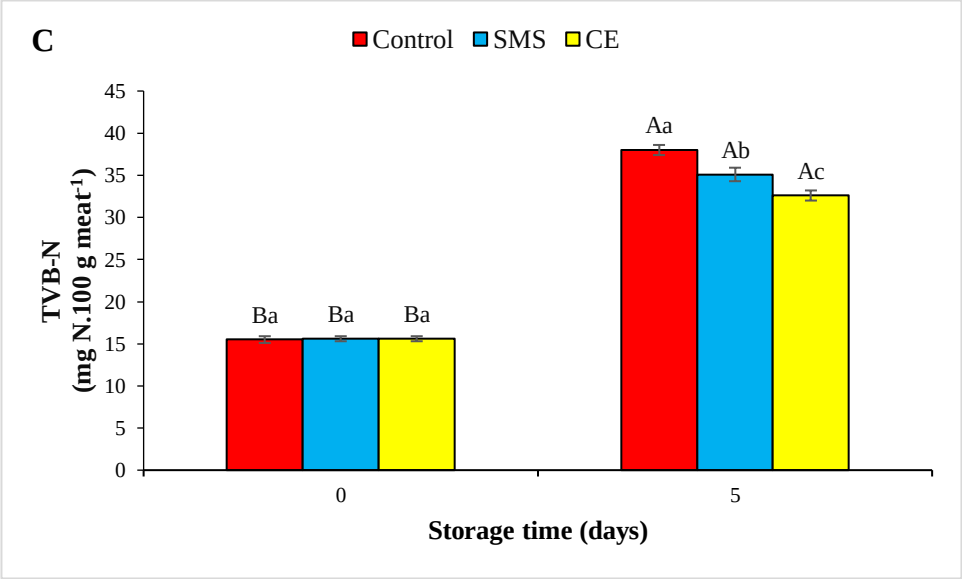


Figure 7. Protein content (A), Shear force (B), and Total volatile base (TVB-N) content (C) of *L. vannamei* during frozen storage. Key: see the caption for Figure 3.

Figure 7B represents that all treatment groups had a similar shear force in the range of 118 - 121 g.cm ($p>0.05$) at day 0. In addition, a higher shear force of shrimps stored with CE was found, compared to those of the SMS and the control after 5 days of frozen storage ($p<0.05$). It is noticeable that the higher shear force of the shrimps in CE treatment batches was in agreement with the lower microbial counts, illustrating that CE inhibited the microbial spoilage and limited collagenase and proteinases which are mainly accountable for the deterioration of muscle protein (Nirmal et al. 2009). The changes in shrimp texture can be caused by many factors: pH, the myofibrillar proteins, and degradation of connective tissues (Yuan et al. 2016a). The texture loss in Pacific white shrimps (*L. vannamei*) was retarded when they were treated with 0.05% catechin and 0.1% catechin (Nirnal et al. 2009) and coated with chitosan and pomegranate peel extract (Yuan et al. 2016a), chitosan-gelatin (Farajzadeh et al. 2016), chitosan-carvacrol (Wang et al. 2018).

Figure 7C shows the values of TVB-N of Pacific white shrimp during frozen storage at -21^{0}C. It is clear that TVB-N contents of all treatment groups were not statistically different at day 0 ($p>0.05$). After 5 days of frozen storage, shrimp treated with SMS, CE showed similar TVB-N values in the range of 32.6 to 35.1 mg N/100g shrimp meat ($p>0.05$) which were smaller than that of the control (38 mg N/100g) ($p<0.05$). It should be noted that *P. odorata* extract could lower the formation of TVB-N and that TVB-N contents were consistent with microbial counts. A delay in the growth of TVB-N was reported when Pacific white shrimp was stored with Chamuang (Garcinia cowa Roxb.) leaf extract in combination with a pulsed electric field (Shiekh et al. 2019), 0.05 or 0.1% chitosan solutions (Nirmal et al. 2009), grape seed extracts (Sun et al. 2014), coated with 1-1.5% O-carboxymethyl chitosan and 1-1.5% chitosan (Huang et al. 2012), chitosan combined with pomegranate peel extract (Yuan et al. 2016a) and green tea extract (Yuan et al. 2016b). Different authors proposed different acceptable limits of TVB-N. To be specific, TVB-N content of 30 mg/100 g shrimp meat was chosen for a quality safety standard by Shiekh et al. (2019) and Huang et al. (2012) while higher limits of 42 mg/100 g of, 40 mg N/100 g was suggested for cephalopods (Altissimi et al. 2018), shrimps (Mendes et al. 2001), respectively; and the European Commission (EC) had a TVB-N limit of 35 mg/100g fish (Asensio et al. 2019). TVB-

N is widely used to evaluate the quality of seafood products and is an indicator of chemical and microbial changes (Huang et al. 2012). The increase of TVB-N content is due to the degradation of nitrogenous substances such as proteins, peptides, amino acids,and nucleotides (Pan et al. 2019).

4.Conclusions

The results of the study indicated that the ethanolic extract of *P. odorata* leaf was effective in preventing melanosis, microbial growth, and physicochemical deterioration of Pacific white shrimp during frozen storage. The samples were soaked at the concentration of 1/15 mg. mL^{-1} for 10 minutes before they were stored at -21°C for 5 days of storage. Furthermore, the efficacy of *P. odorata* extract was greater than that of sodium metabisulfite solution. Therefore, it has a promising potential to replace sulfite additives, which have a large number of adverse health effects, contributing to maintaining food safety in the shrimp industry, and adding value to the economy.

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EXPLORING THE INCORPORATION OF MULBERRY (*MORUS ALBA* L.) INTO FREEZE-DRIED YOGURT FOR ENHANCED NUTRITIONAL VALUE AND QUALITY

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ABSTRACT

Yogurt, popular worldwide, is nutrient-rich with calcium, potassium, protein, B vitamins, and beneficial microorganisms. Mulberries possess medicinal qualities, notably anthocyanins, which have antioxidant, anti-diabetic, and anti-bacterial effects. Though fruit-flavored yogurt is popular, producing it is challenging because of its limited shelf life. However, freeze-drying technology can address this, allowing prolonged storage without compromising its quality. This study evaluated incorporating mulberry into yogurt to enhance its nutritional value and quality while seeking an optimal production process for freeze-dried mulberry yogurt. Mulberry syrup was added in various ratios, demonstrating statistically significant effects on the yogurt's physicochemical properties. Particularly, a 9:1 mulberry syrup to yogurt ratio exhibited a total polyphenol content of 170.84 mg GAE/100 g, marking a 94% increase compared to the yogurt without mulberry syrup. This substantial enhancement signifies an improvement in yogurt efficiency when mulberry is integrated. Similarly, DPPH and ABTS free radical scavenging activities for the 9:1 ratio were observed at 12.22 mgAAE/100g and 22.55 mgAAE/100g, which is an enhancement of 1.89% and 15.99%, respectively, compared to the reference yogurt sample (10:0 ratio). The quality changes in the freeze-dried mulberry yogurt were monitored over a 28-day storage period. The results revealed relatively stable physicochemical properties, microbiological density, and biological activity throughout the storage, highlighting the potential of freeze-drying technology in creating innovative and nutritious yogurt products.

1. Introduction

Yogurt, a dairy product with high nutritional value and delectable taste, is widely consumed across the globe (Sun-Waterhouse et al., 2012). Produced by fermenting milk with lactic acid bacteria, yogurt boasts a tangy flavor and creamy texture (Miller et al., 2006). It is a rich source of essential nutrients that contribute to overall health, including calcium, potassium, protein, vitamin B, and probiotics (Gilliland, 1989; Rizzoli, 2014). Numerous studies have demonstrated the benefits of yogurt consumption, such as improved digestion, reduced cholesterol levels, and prevention of diarrhea (Desobry-Banon et al., 1999). Consequently, there is a growing interest in developing innovative and nutritious yogurt products to cater to consumer needs.

Fruit-flavored yogurt has become a household favorite worldwide, offering a diverse array of fruity flavors while enhancing the product's visual appeal (Ha et al., 2021; Lutchmedial et al., 2004; Sung et al., 2015). One such fruit is the mulberry (*Morus alba* L.), a member of the *Moraceae* family that is globally cultivated and distributed under varied climatic conditions, ranging from tropical to temperate (Yuan & Zhao, 2017). Traditionally employed as a medicinal remedy for colds, liver protection, joint fortification, and blood pressure reduction, mulberries are a rich source of minerals like potassium, manganese, and magnesium (Butt et al., 2008; Yuan & Zhao, 2017; C. Wang et al., 2019). They also rich in components like as anthocyanins, which have antioxidant, anti-diabetic, and anti-microbial properties. Other, compounds such as albafuran, bergaptan, and cyanidin-3-glucosides in mulberries are known for their antioxidant, antibacterial, and anti-inflammatory effects (H. Wang et al., 1997; Grace et al., 2009; C. Wang et al., 2019). In Vietnam, mulberries are primarily processed into wine or jam, highlighting the need to diversify mulberry-based products, which could benefit farmers and food processing enterprises.

However, the production of fruit yogurt presents the challenge of a relatively short shelf life, necessitating low-temperature storage, and transportation (Deshwal et al., 2021). Freeze-drying presents itself as a potential solution to this issue (Valentina et al., 2016). This process involves the removal of water from a product through sublimation, enabling long-term storage at room temperature while preserving the organoleptic, biological, and nutritional properties of the dried product (McDonough et al., 1982; Barbosa et al., 2015; Ermis, 2022). This method could introduce a new innovation: freeze-dried mulberry yogurt enriched with probiotics. This product would combine the high nutrient content of dairy products with biologically active fruit ingredients, such as anthocyanins.

This study aims to evaluate the potential of integrating mulberries into yogurt to augment its nutritional value and quality and devise an effective production process for freeze-dried mulberry yogurt enriched with probiotics. We will analyze the physicochemical properties, sensory value, biological activity, and stability of the yogurt during storage to assess the feasibility of this approach. This investigation presents a unique solution to the challenges associated with fruit yogurt production and has the potential to significantly impact the food industry by contributing to the development of new functional food products.

2. Materials and methods

2.1. Materials

2.1.1.Samples

All ingredients used for yogurt production, such as whole milk powder, saccharose, modified starch, and gelatin, were purchased from a local company in Ho Chi Minh City, Vietnam. The mulberries utilized in this study was procured from a local market, washed with tap water, drained, and blended using a kitchen blender (HR2223/00, Philip, China). The resultant puree was then transferred to a sealed plastic bag and stored at -18°C subsequent use.

2.1.2.Chemicals

The chemicals used in this study included sodium hydroxide (NaOH) 0.1N, which was purchased from Cemaco (Vietnam); methanol (CH_3OH) 99.7%, phenolphthalein, and sodium bicarbonate (Na_2CO_3) 99.5% which were purchased from Xilong (China); Folin-Ciocalteu 2N, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azisbonis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS), and gallic acid which were purchased from Sigma Aldrich (USA). In addition, culture, media like MRS (De Man, Rogosa, and Sharpe) agar and MRS broth were purchased from HiMedia (India).

2.2. Methods

2.2.1.Manufacture of yogurt

This procedure is based on the work by (Golmakani et al., 2021) and our preliminary test. Specifically, the milk mixture comprised 12% whole milk powder, 7.0% sucrose, 1% modified starch, 0.5% gelatin, and 79.5% hot

water at 85 °C. Subsequently, 150 mL of milk was poured into sterilized glass bottles, sealed, and pasteurized at 85 °C for 10 min in a distillation pot (Tomy, SS-325, Japan). Afterward, the milk was cooled down 37 °C and 0.006% of the biomass of commercial probiotic Chr Hansen YC-X11 Thermophilic Yogurt Culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was added to the mixture. Samples were kept in an incubator (DH5000II Faithful, China) for 24 h at 37 °C. The final yogurt reached a pH of 4.33 ± 0.01, a total acid content of 0.75% ± 0.01, and a bacteria density of ~10.3 log CFU/g. Samples were stored in a refrigerator at 4-6 °C, for no more than 24 hours until further experiments were carried out.

2.2.2.Freeze dried yogurt-mulberry procedure

To formulate the yogurt-mulberry blend, yogurt and mulberry syrup were combined in various ratios, specifically: 10:0, 9:1, 8:2, 7:3, 6:4, and 5:5. The prepared mix was poured into a silicone mold (dimensions: 1 x 2 x 1 cm) and frozen at -20 °C for 4 hours, setting the stage for freeze-drying. The dehydration process was executed using an automatic freeze dryer (HR-3, Harvest right, USA), operating at -20 °C with a pressure of 500 mTorr over 24-26 hours in vacuum. After drying, the samples were brought to room temperature, sealed in plastic bags, and stored between 25-27°C. Notably, samples were retained for no more than 24 hours before further analysis.

2.2.3.Physicochemcial properties

The TSS of the samples was measured using a handheld refractometer (0-32%, Alla, France). The total acidity was measured using the titration method with 0.1N NaOH and 1% phenolphthalein as an indicator (Zahid et al., 2022). The data was reported as lactic acid content (%). The pH of the samples was examined using a pH meter (Hanna, HI2210-02, Romania). Prior to evaluation, all FD yogurts were ground and completely dissolved in distilled water. The moisture content and water activity of the samples were measured using a moisture analyzer (MB90, Ohaus, USA) and a water activity meter (Novasina, LabTouch, Switzerland), respectively.

2.2.4.Color measurement

The color properties of samples such as L*, a*, and b* were measured by using a handheld chroma meter (Konica Minolta, CR-400, Japan). In this context, L* indicated lightness/darkness, a* indicated greenness/redness, and b* indicated blueness/yellowness. The different colors between the freeze-dried yogurt with and without mulberry was also determined following the equation (1) as described by (Tuyen et al., 2015)

$$\Delta E = \sqrt{(L_1^* - L_2^*)^2 + (a_1^* - a_2^*)^2 + (b_1^* - b_2^*)^2} \quad (1)$$

Where: L_1*, a_1*, and b_1* are the parameters of sample without mulberry and L_2*, a_2*, and b_2* are the parameter of samples that were mixed with mulberry.

2.2.5.Total anthocyanins content (TAC)

The total anthocyanin content was determined using the method by (Lee et al., 2005). Initially, to ascertain the appropriate dilution factor, the test portion was diluted using a pH 1.0 buffer until the absorbance at 520 nm fell within the linear range of the spectrophotometer. Once this dilution factor was established, the test sample was prepared in two different dilutions: one using a pH 1.0 buffer and the other using a pH 4.5 buffer. The absorbance of each of these test portions, diluted with the respective buffers, was measured at two distinct wavelengths, namely 520 nm and 700 nm. A blank cell filled with distilled water was used as a comparative reference for these diluted test portions. All absorbance measurements were conducted within a time frame of 20 to 50 minutes post-preparation.

Following the measurements, the anthocyanin pigment concentration was calculated and expressed in cyanidin-3-glucoside (cyd-3-glu) equivalents. The formula used for this calculation is as follows (2):

$$C = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \quad (2)$$

Where:

A: (A520nm – A700nm) pH 1.0 – (A520nm – A700nm) pH 4.5;

MW (molecular weight): 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu);

DF: the previously determined dilution factor;

l: pathlength in cm; and

ε: 26,900, which is the molar extinction coefficient in L x mol^{-1} x cm^{-1} for cyd-3-glu;

10^3: factor for conversion from g to mg.

2.2.6.Total phenolic content (TPC)

The analysis was conducted according to the method of (Lim et al., 2007), with slight modifications. In a glass tube, the diluted solution (0.3 mL) was thoroughly mixed with 10% Folin–Ciocalteu solution of 1.5 mL. The mixture was then left at room temperature for 5 minutes in dark conditions. Next, the mixture was vortexed with a 7.5% Na_2CO_3 solution of 1.2 mL and incubated at room temperature for 30 minutes in a dark condition. Finally, the sample the absorbance of the sample was then measured using a spectrophotometer (Thermo Scientific, Evolution 60S, USA) at a wavelength of 765 nm. Gallic acid, with the concentration ranges from 0 µg/mL to 70 µg/mL, was used for the standard curve, and the total phenolic content was expressed as mg gallic acid equivalents per 100 grams of dried sample (mg GAE/100g dw). The blank contains acidified methanol and reagent.

2.2.7.Antioxidant activity using DPPH reagent

The analysis followed (Phuong et al., 2020), with slight modifications. Firstly, the DPPH working solution was prepared by dissolving 3.94 mg of DPPH in 100 mL methanol and kept in the dark at -20 ℃ for further use. Then, the 0.1 mL diluted solution was thoroughly mixed with 2 mL DPPH working solution. The mixture was then left at room temperature for 30 minutes in a dark condition. Finally, the sample the absorbance of the sample was subsequently measured using a spectrophotometer (Thermo Scientific, Evolution 60S, USA) at a wavelength of 517 nm. Ascorbic acid with various concentrations from 0 µg/mL to 100 µg/mL was used as a standard. The antioxidant activity of yogurt samples was expressed as mg ascorbic acid equivalent per 100 g dry matter (mg AAE/100g dw). The acidified methanol was used as a blank sample.

2.2.8.Antioxidant activity using ABTS reagent

The analysis was conducted as described by (Phuong et al., 2020), with slight modifications. Firstly, the ABTS stock solution was prepared by mixing an equal amount of 7 mM ABTS solution (10 distilled water containing 0.0384 g ABTS) and 2.45 mM potassium persulfate solution (10 mL distilled water containing 0.0066 g kali persulfate) and left at room temperature for 12 hours. The stock solution was then diluted with distilled water to reach the OD value of 0.7 (± 0.02) by using a spectrophotometer at a wavelength of 734 nm. The ABTS working solution was prepared 15 minutes before use. In a glass tube, the diluted solution (0.1 mL) was combined with 3.2 mL ABTS working solution. The mixture was then left at room temperature for 5 minutes in dark conditions. Finally, the sample the absorbance of the sample was then measured using a spectrophotometer (Thermo Scientific, Evolution 60S, USA) at a wavelength of 734 nm. The total phenolic content was calculated based on the standard curve of ascorbic acid (ranging 0-50 µg/mL). The data was expressed with a unit as ascorbic acid equivalent/100g dry matter (mg AAE/100g dw). The blank contains acidified methanol.

2.2.9.Viable cell count

One gram of the sample was diluted in a 1:10 ratio with sterilized and then spread on MRS agar plates. The dishes were incubated for 48 hours at 37 ℃ in an incubator. The number of viable cells was quantified as log colony-forming units per gram (log CFU/g) (Barbosa et al., 2015).

2.2.10. Sensorial evaluation

The preference for freeze-dried yogurts was scored by 20 trained panelists that comprising ten males and ten females, aged between 20 and 30 years. The selected panelists consumed yogurt at least two times a week and were free of allergic dairy products. The samples were

presented on a white ceramic plate and coded with randomly coded with three-digit numbers. The assessor asked to rinse their mouths odorless water between sampling each product. The sample's preference was described using a 7 points hedonic scale, herein 1: dislike very much and 7: like very much (Peryam & Pilgrim, 1957). The sensory characteristics, including color, aroma, flavor, and texture, were evaluated in this study.

2.2.11.Statistical analysis

The data obtained from the various experiments are presented as mean ± standard deviation. All data processing, calculations, and graphical representations were conducted using Microsoft Excel 2019. To determine the statistical significance of the experimental results, a one-way Analysis of Variance (ANOVA) followed by the Least Significant Difference (LSD) test was performed. These analyses were executed using STATGRAPHICS Centurion XV software, and statistical significance was established at a p-value of less than 0.05.

3. Results and discussions

3.1.Physicochemical properties and bioactive compounds of fresh mulberry fruit

The physicochemical properties and bioactive compounds of fresh mulberry fruit are detailed in Table 1. These attributes include encompass moisture content, water activity, total sugar content, and total acidity, which are crucial to understanding the unique properties of the mulberry fruit. The sweetness of the fruit is measured by total sugar content and its tartness by total acidity. Likewise, the freshness and shelf-life assessed by moisture content and water activity. The study also examines additional parameters, such as total dissolved solids, ash content, and pH values. These correlate with the findings of previous research on mulberries, validating our results (Saensouk et al., 2022; Sangteerakij et al., 2023). The color of mulberry fruit, ranging from burgundy to black, has been quantified precisely using L*, a*, and b* values, serving as indicators of ripeness (Table 1 and Figure 1). This color analysis is in line with the findings of (Sangteerakij et al., 2023). The documents study reports a remarkable total polyphenol content of 1562.2 mg GAE/100 g in mulberry fruit, echoing earlier studies. The observed is greater than the 10.3 mg GAE/100 g found by (Liu et al., 2009), but falls short of the 2570 mg GAE/100 g reported by (Bae & Suh, 2007). Our mulberry samples display a notably higher anthocyanin content of 261.53 mg/g, exceeding the 65.23 mg/g noted by (Sangteerakij et al., 2023). The variance in these findings might stem from differences in analytical methodologies, fruit quality, variety, environmental factors, climatic conditions, and ripeness at harvest (Chen et al., 2022; Saensouk et al., 2022). Furthermore, our evaluation of the mulberry's antioxidant capacity using DPPH and ABTS free radical neutralizing assays yielded values of 74.45 and 88.04 mg AAE/100 g, respectively, supporting Chen et al., (2022) and Saensouk et al., (2022) description of mulberry's potent antioxidant characteristics. The fruit's antioxidant capacity originates mainly from polyphenols, anthocyanins, and flavonoids, and includes riboflavin (vitamin B2), niacin (vitamin B3), and ascorbic acid (vitamin C) (Okatan et al., 2016). In summary, mulberries boast numerous health and nutritional benefits attributable to their high content of phenolic compounds, total flavonoids, anthocyanins, and antioxidant activity (Chen et al., 2022). It's important to note, however, that these compounds' concentrations can fluctuate based on the fruit's ripeness, highlighting the importance of a thorough evaluation of the raw material to ensure the best processing results (Punthi & Jomduang, 2021). Considering mulberries have a relatively low sugar content, pre-processing adjustments may be useful to fine-tune the final product's flavor.

Table 1. Physicochemical properties and bioactive compound of mulberry fruits

Mulberry fruits	Parameters
Physicochemical properties	
Moisture content (%)	77,34±0,06
Water activity (aw)	0,97±0,01
Total sugar content (%)	8,1±0,10
Total acidity (%)	0,42±0,02
Total Soluble Solids (˚Brix)	9,8±0,1
Ash (%)	2,81±0,2
pH	3,84±0,01
Color values	
L*	24,45±0,12
a*	4,96±0,30
b*	-1,31±0,1
Bioactive compound	
TPC (mg GAE/100 g dw)	1562,2 ± 55,19
TAC (mg/L)	261,53 ±2,83
DPPH (mg AAE/100 g dw)	74,45±2,87
ABTS (mg AAE/100 g dw)	88,04±0,06

Values are expressed as mean ± standard deviation.

Figure 1. The mulberry fruits fully ripened stage.

3.2.Effect of mulberry syrup in different proportions on physicochemical properties and bioactive compound of freeze-dried mulberry yogurt

The physicochemical analysis detailed in Table 2 demonstrates that variations in the percentage of mulberry syrup addition do not significantly influence the moisture content and water activity in freeze-dried yogurt. These attributes, namely moisture content and water activity, are crucial indicators for assessing potential microbial growth and the overall stability of dried products (Tapia et al., 2020). Research establishes that a water activity threshold below 0.60, along with a moisture content less than 5.00%, prevents bacterial growth, auto-oxidation, hydrolysis, and enzyme activity, thus ensuring that dried foods can be stored at room temperature without the risk of spoilage (Bosnea et al., 2017). No statistical difference ($p<0.05$) was observed in these two parameters among the various freeze-dried yogurt samples. After freeze-drying, moisture values ranged between 2.08-4.33%, remaining below the 5.00% threshold, while the water activity values hovered around 0.30, well below the 0.60 limit. This suggests that the freeze-dried yogurt samples remained relatively stable. Unlike moisture content and water activity, the color, total acidity (TA), and total soluble solids (TSS) values displayed statistically significant changes ($p<0.05$) with alterations in mulberry syrup addition. The total acidity decreased, while the total dissolved solids increased a steadily increased with the increasing rate of mulberry syrup addition. The sample with the

most mulberry had the lowest TA value at 2.28% and the highest TSS at 93.06%. In contrast, the yogurt sample without added mulberry syrup recorded the highest TA at 3.23% and the lowest TSS at 78.66%.

Furthermore, the addition rates of mulberry syrup significantly impact both the biological and antioxidant activities of the product. As outlined in Table 3, the total polyphenol content (TPC), total anthocyanin content (TAC), and antioxidant activity against DPPH and ABTS free radicals tend to rise with increasing amounts of mulberry syrup. There exist statistically significant differences ($p<0.05$) between samples as the mulberry syrup proportion increases. Based on the addition ratios from 10:0 to 5:5 of mulberry syrup in yogurt samples, the total polyphenol content ranges from 170.84 to 213.30 mg GAE/100 g DM, while the total anthocyanin content varies between 63.24 and 78.91 mg/L. The antioxidant activities of DPPH and ABTS follow the trends seen in TPC and TAC, showing a similar increase as the mulberry syrup ratio increases from 10:0 to 5:5.

Mulberries also contain valuable components like anthocyanins, known for their antioxidant, anti-diabetic, and anti-microbial properties. Other notable compounds in mulberries, such as albafuran, bergaptan, and cyanidin-3-glucosides, have been recognized for their antioxidant properties (Wang et al., 2019). Thus, the incorporation of mulberry syrup enhances polyphenol and anthocyanin contents and improves the yogurt's texture. Prior studies have shown that the addition enhance antioxidant content in yogurt. For instance, an increase in TPC value of 4.17, 5.63, 7.63, and 9.63 mg GAE/100 g was observed corresponding to the addition of 0%, 1%, 3%, and 5% apple pomace in yogurt (Jovanović et al., 2020). Another study revealed that yogurt with 20% dates had a total polyphenol content of 37.00 mg GAE/100 g, a value 4.3 times higher than that of unsupplemented yogurt (Arfaoui et al., 2020).

Table 2. Physicochemical properties of yogurt samples mixed with mulberry syrup

Yogurt: Mulberry syrup (w/w)	Moisture content	Water activity	Total soluble solids (TSS %)	Total acidity (TA %)	pH
5:5	$4,33^{a}\pm 0,11$	$0,35^{a}\pm 0,01$	$91.33^{a} \pm 0.29$	$2,28^{e} \pm 0,02$	$3,38^{f} \pm 0,01$
6:4	$3,95^{b}\pm 0,31$	$0,35^{a}\pm 0,01$	$89.97^{b} \pm 0.06$	$2,47^{d} \pm 0,04$	$3,42^{e} \pm 0,01$
7:3	$3,6^{bc}\pm 0,24$	$0,33^{a}\pm 0,01$	$89.32^{c} \pm 0.28$	$2,54^{d} \pm 0,2$	$3,64^{d} \pm 0,01$
8:2	$3,45^{bc}\pm 0,52$	$0,33^{a}\pm 0,01$	$83.83^{d} \pm 0.29$	$2,68^{c} \pm 0,04$	$3,58^{c} \pm 0,01$
9:1	$3,39^{bc}\pm 0,64$	$0,30^{a}\pm 0,10$	$80.33^{e} \pm 0.58$	$3,00^{b} \pm 0,02$	$3,72^{b} \pm 0,01$
10:0	$2,08^{c}\pm 0,46$	$0,22^{a}\pm 0,01$	$77.67^{f} \pm 0.29$	$3,23^{a} \pm 0,01$	$3,88^{a} \pm 0,01$

Values are expressed as mean ± standard deviation. The digits in the same column indicate a statistically significant difference ($p<0.05$).

Table 3. Bioactive compound of yogurt samples mixed with mulberry syrup

Yogurt: Mulberry syrup (w/w)	TPC (mg GAE/100g)	TAC (mg/L)	DPPH (mgAAE/100g)	ABTS (mgAAE/100g)
5:5	$213,30^{a} \pm 1,63$	$78,91^{a} \pm 0,24$	$15,39^{a} \pm 1,17$	$22,55^{a} \pm 0,23$
6:4	$200,89^{b} \pm 0,72$	$75,09^{b} \pm 0,38$	$15,62^{a} \pm 0,26$	$21,44^{b} \pm 0,18$
7:3	$197,34^{b} \pm 1,16$	$70,64^{c} \pm 0,48$	$14,25^{ab} \pm 0,05$	$20,22^{c} \pm 0,10$
8:2	$186,14^{c} \pm 1,43$	$66,84^{d} \pm 0,65$	$13,16^{bc} \pm 0,07$	$19,76^{c} \pm 0,12$
9:1	$170,84^{d} \pm 4,75$	$63,42^{e} \pm 0,95$	$12,22^{c} \pm 0,52$	$18,98^{d} \pm 0,01$
10:0	$76,29^{e} \pm 1,00$	$0,00^{f} \pm 0,00$	$10,33^{d} \pm 0,25$	$2,99^{e} \pm 0,59$

Values are expressed as mean ± standard deviation. The digits in the same column indicate a statistically significant difference ($p<0.05$).

3.3.Effect of mulberry syrup in different proportions on the color of freeze-dried mulberry yogurt

Table 4 presents the colorimetric analysis of mulberry yogurt produced with varying ratios of mulberry syrup. The results indicate that the L* (lightness), a* (red-green component), and b* (yellow-blue component) values tend to increase with decreasing amounts of mulberry syrup. The sample supplemented with 50% mulberry displayed the lowest L*, a*, and b* values, representing the darkest purple-red color among the surveyed samples. Conversely, the sample without mulberry (ratio 10:0) exhibited the highest L* and b* values, showcasing the typical ivory-white color characteristic of yogurt. A statistically significant difference ($p<0.05$) was observed in the color difference (ΔE) between mulberry-supplemented samples and those without mulberry (control samples). The ΔE value demonstrated a decreasing trend with the reduction in the rate of mulberry addition. However, all ΔE values exceeded 3.00, indicating that the color difference between samples is discernible to the naked eye (Figure 2). Anthocyanin compounds found in mulberries, such as Cyanidin-3-glucoside, Delphinidin-3-glucoside, and Petunidin-3-glucoside, provide the fruit with its characteristic deep purple-red color (Yawadio & Morita, 2007). These pigments are also known for their antioxidant properties, with potential benefits in reducing the risk of cancer and cardiovascular diseases (Grace et al., 2009; Wang et al., 2019). Consequently, the addition of mulberry syrup has a direct impact on the color of freeze-dried mulberry yogurt, with higher ratios giving a darker purple-red hue, reminiscent of mulberries.

Table 4. Color change between samples of mulberry yogurt freeze-dried with different ratios

Yogurt: Mulberry syrup (w/w)	Color			ΔE
	L*	a*	b*	
5:5	49,630^{d}± 1,620	10,037^{e}± 0,521	-2,290^{d} ± 0,171	48,774^{ab}± 1,437
6:4	49,263^{d}± 1,006	18,050d± 1,091	-1,557^{c} ± 0,023	50,669^{a}± 1,158
7:3	53,167^{c}± 0.597	21,703^{c}± 0,836	-0,650^{b} ± 0,300	48,447^{ab}± 1,051
8:2	55,013^{c}± 1.786	24,777^{b} ± 0,682	-0,373^{b} ± 0,188	48,130^{ab}± 2,064
9:1	59,940^{b}± 0,288	28,373^{a} ± 0,242	-0,347^{b} ± 0,170	45,960^{b}± 0,346
10:0	96,017^{a}± 0,386	2.030^{f} ± 0,035	10,470^{a} ± 0,156	-

Values are expressed as mean ± standard deviation. The digits in the same column indicate a statistically significant difference ($p<0.05$).

Figure 2. The visual appearance of freeze-dried mulberry yogurt at different ratios

3.4.Sensory evaluation of freeze-dried mulberry yogurt

The sensory evaluation of the freeze-dried mulberry yogurt uncovered nuanced preferences related to color, aroma, taste, and texture. The 8:2 mulberry syrup to yogurt ratio achieved the highest score in terms of color (6.15 out of 7.00 points), whereas the 5:5 ratio scored the lowest at 2.55 out of 7.00 points (Table 5). Incorporating mulberry syrup enhanced the aroma, adding compounds like esters, aldehydes, ketones, and terpenes, among others (Calín-Sánchez et al., 2013; Mostafa et al., 2022) to the inherent volatile organic acids in yogurt. The yogurt sample without mulberry syrup was the least preferred, earning 3.2 points,

while the 8:2 and 9:1 samples attained the highest ratings (~5.00 out of 7.00 points). Notably, the 5:5 ratio detracted from taste preferences due to its overpowering sweetness and less crisp texture, evident in the sensory scores of 2.55 and 2.00 points out of 7.00, respectively. In opposition, the 9:1 sample earned considerable appreciation for both taste and texture, garnering 6.10 and 6.45 points out of 7.00, respectively. This balance of sweet and sour was linked tothe sublimation drying process, which increases the concentration of lactic acid in the yogurt, thus enhancing its acidity (Fellows, 2017). The porosity of the samples was inconsistent, rising from the 10:0 to 8:2 ratio and subsequently reducing towards the 5:5 ratio. This change is tied to the physical properties of the ingredients and their interactions during fermentation and drying (Dai et al., 2021). The carbohydrates and fibers in mulberries, including sugars, cellulose, hemicellulose, pectin, and lignin, seemed to bolster the yogurt's structure post-drying (Lin & Tang, 2007), esulting in a firmer and more pleasing texture. In contrast, products devoid of mulberry dissolved swiftly in the mouth, and those with excessive mulberry came across as overly hard and less crunchy. Notably, the 9:1 yogurt to mulberry ratio garnered a high sensory evaluation score across most categories, highlighting its positive effect on overall product quality. Consequently, the 9:1 ratio was selected for further exploration.

Table 5. Sensory evaluation of samples of freeze-dried mulberry yogurt

Yogurt: Mulberry syrup (w/w)	**Sensory Evaluation**				
	Color	**Odor**	**Taste**	**Crispness**	**Overall acceptance**
5:5	$2,55^{c}\pm 0,4$	$4,00^{c}\pm 0,72$	$2,55^{d}\pm 0,88$	$2,00^{e}\pm 0,64$	$2,95^{d}\pm 0,60$
6:4	$3,90^{b}\pm 0,85$	$4,95^{b}\pm 0,6$	$3,90^{c}\pm 1,07$	$2,70^{d}\pm 0,65$	$3,60^{c}\pm 0,59$
7:3	$5,50^{c}\pm 0,67$	$5,95^{a}\pm 0,51$	$4,95^{b}\pm 0,82$	$4,35^{c}\pm 0,74$	$4,65^{b}\pm 0,74$
8:2	$6,15^{a}\pm 0,67$	$5,30^{b}\pm 0,65$	$6,20^{a}\pm 0,69$	$5,75^{b}\pm 0,63$	$6,00^{a}\pm 0,64$
9:1	$5,85^{a}\pm 0,74$	$5,45^{ab}\pm 0,68$	$6,10^{a}\pm 0,64$	$5,75^{b}\pm 0,63$	$6,25^{a}\pm 0,0,78$
10:0	$3,40^{b}\pm 0,94$	$3,20^{d}\pm 0,95$	$3,70^{c}\pm 1,12$	$6,45^{a}\pm 0,60$	$4,15^{bc}\pm 0,81$

The values represent the average sensory scores given by the panelists, with higher scores indicating a more positive evaluation. The letters after the values denote statistical significance at $p< 0.05$.

3.5.Evaluation of the change of mulberry yogurt freeze-dried during storage

Upon examining the impact of storage time on the physicochemical properties, microbiological activity, and biological performance of freeze-dried mulberry yogurt, distinct shifts were observed. The moisture content and water activity of the product rose progressively with storage duration, with these changes being statistically significant ($p<0.05$) as shown in Table 6. The moisture content ranged from 2.42% at the outset and reached

3.76% after 28 days of storage, while water activity values saw a slight increase, moving from 0.28 to 0.29. Even though these increases were moderate, they align with expected changes in dried goods (namely, moisture less than 5.00% and a_w less than 0.60). Alongside, L* values declined, while b* values rose, culminating in a statistically significant ($p<0.05$) increase in ΔE. This suggests the product's color shifted towards a darker red-violet hue by the end of 28 days. This color deviation became perceptible after the 21st day, with ΔE values surpassing 3.00. An uptick in moisture content and exposure to light during storage might have triggered oxidation reactions affecting the product's color (Suh et al., 2004). Simultaneously, modest variations in the synthesis of polyphenol content and antioxidant activity emerged, as depicted in Table 6. The initial total polyphenol content of 170.84 mg GAE/100 g dwindled to 161.14 mg GAE/100 g over 28 days. In parallel, the antioxidant activity measured by DPPH and ABTS assays witnessed a slight descent, suggesting a minor decline possibly due to the reduced polyphenol content. In the microbiological realm, a non-significant decrease in beneficial bacterial density was observed, declining from 10.72 log CFU/g to 9.66 log CFU/g over the 28-day span, as illustrated in Figure 3. Such a drop in the count of live microorganisms and biological activity is consistent with findings from previous studies on freeze-dried products (Liu et al., 2015; Ha et al., 2021; Emteborg et al., 2022). Despite this, freeze-dried mulberry yogurt maintained relatively stable physicochemical, microbiological, and antioxidant properties over the 28-day observation period, reinforcing the product's resilience during storage.

Table 6. Effect of storage time on physicochemical properties, microbiological and biological activities of freeze-dried mulberry yogurt.

Parameters	**Storage time (day)**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Physicochemical properties					
Moisture Content (%)	$2,42^{d} \pm 0,02$	$2,7^{cd} \pm 0,02$	$2,93^{bc} \pm 0,02$	$3,1^{b} \pm 0,01$	$3,76^{a} \pm 0,02$
Water Activity	$0,28^{e} \pm 0,01$	$0,28^{b} \pm 0,01$	$0,28^{c} \pm 0,01$	$0,29^{b} \pm 0,01$	$0,29^{a} \pm 0,01$
ΔE	–	$1,50^{d} \pm 0,06$	$2,90^{c} \pm 0,08$	$4,20^{b} \pm 0,01$	$5,45^{a} \pm 0,10$
Bioactive compound					
TPC (mg GAE/100g)	$170,84^{a} \pm 4,75$	$164,85^{b} \pm 0,45$	$163,97^{b} \pm 0,36$	$163,02^{b} \pm 0,15$	$161,14^{b} \pm 0,34$
TAC (mg/L)	$63,42^{a} \pm 0,95$	$63,80^{a} \pm 0,18$	$63,05^{ab} \pm 0,15$	$62,03^{bc} \pm 0,16$	$61,41^{c} \pm 0,27$
DPPH (mgAAE/100g)	$12,22^{a} \pm 0,52$	$12,07^{a} \pm 0,07$	$11,73^{ab} \pm 0,06$	$11,35^{bc} \pm 0,11$	$10,86^{c} \pm 0,05$
ABTS (mgAAE/100g)	$18,98^{a} \pm 0,01$	$18,66^{b} \pm 0,01$	$18,30^{c} \pm 0,01$	$18,06^{d} \pm 0,01$	$17,78^{e} \pm 0,01$

Values are expressed as mean ± standard deviation. The digits in the same column indicate a statistically significant difference ($p<0.05$).

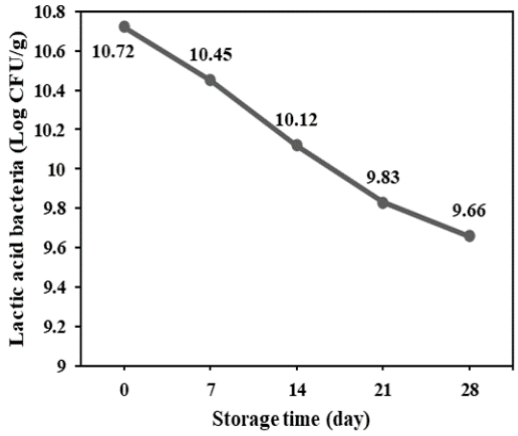

Figure 3. Changes of lactic acid bacteria in freeze-dried mulberry yogurt during storage

4. Conclusions

In conclusion, this study revealed that the addition rate of mulberry syrup and the preservation process are pivotal factors impacting the changes in physicochemical, microbiological, antioxidant, and sensory attributes of freeze-dried mulberry yogurt. Incorporating mulberry syrup at a 9:1 ratio concentration yielded optimal physicochemical properties, such as 3.39% moisture, an aw of 0.3, L* of 59,940, a* of 28,373, TA of 3%, and TSS of 81. Concurrently, total polyphenol content reached 170,84 mg GAE/100 g, while antioxidant activity against DPPH and ABTS free radicals was noted at 12.22 and 18.98 mg AAE/100 g, respectively. This ratio also obtained the highest sensory scores and preference rating, falling between 5.0 and 6.0 out of 7.0 points. Following 28 days of storage at room temperature (25-28℃), humidity, a_w, and ΔE exhibited increases by 3.76%, 0.29, and 5.45, respectively. Microbial density, TA, TSS, TPC, and antioxidant activity indicators, however, remained relatively stable, underscoring the product's overall stability during the storage period.

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CHARACTERISTICS OF YOGHURT ENRICHED WITH PROTEIN HYDROLYSATE FROM PARROTFISH (*CHLORURUS SORDIDUS*) HEAD

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ABSTRACT

Production of parrotfish (*Chlorurus sordidus*) fillets has increased significantly in Indonesia. The processing only uses fish meat, thus producing plenty of by-products. Fish by-products contain valuable protein that can still be processed into fish protein hydrolysate (FPH). FPH can be used to improve yoghurt's physicochemical and organoleptic. This study aimed to determine the effect of protein hydrolysate from parrotfish head addition on the characteristics of yoghurt. The FPH concentrations used were 0%, 0.1%, 0.15%, 0.2%, 0.25 and 3%. This research was conducted by an experimental method using a completely randomized design with six treatments and five replications. The results showed that the variation of FPH concentration significantly affected the pH, total acid, viscosity, syneresis, water holding capacity, colour L*, a*, b*, appearance, aroma, and taste of yoghurt. However, the addition of FPH did not significantly affect the a* colour and texture of yoghurt. The best treatment was observed from the addition of 0.15% FPH with a viscosity value of 2.432 N•s/m^2, syneresis 49.67%, WHC 37.4%, pH 4.36, TTA 0.76%, colour L* 74.55, colour a* -3.76, colour b* 8.2, taste score 5.39, aroma score 5.33, texture score 5.23, and appearance score 5.8.

1. Introduction

Parrotfish (*Chlorurus sordidus*) is one of the Indonesian export commodities with high economic value (Harms-Tuohy, 2021). The total export value of Indonesian parrotfish exceeds $200M, with China as the primary export destination (Tridge, 2021). Most parrotfish caught are processed into fillet for consumption as it has smooth and soft meat fibres. Nevertheless, the high consumption of parrotfish produces many fish processing by-products (Silovs, 2018). Fish processing makes more than 60% of by-products, which are rich in protein but susceptible to microbial spoilage and must be handled immediately. In addition, bone, head, skin, offal, and other by-products can be converted to valuable products such as fish protein hydrolysate (Benjakul *et al.*, 2014). Many researchers have reported fish protein hydrolysate production from various fish by-products, such as the head, offal, and skin of black scabbard fish (*Aphanopus carbo*) and lemurus, parrotfish fish head, by-products of Siamese catfish, and salmon bones (Batista *et al.*, 2010; Prihanto *et al.*, 2019; Thuy *et al.*, 2015; Idowu *et al.*, 2018). Prihanto *et al.*, (2019) mentioned that the protein, fat, moisture content of parrotfish were 20.37%, 3.92% and 71.68, respectively.

Protein hydrolysate is obtained from protein cleavage into peptides (He *et al.*, 2013). The total essential amino acids of FPH parrotfish heads were up to 41.69%. The difference in amino acid composition between hydrolysates

depends on differences in enzyme specificity and hydrolysis conditions (Prihanto *et al.*, 2019). The hydrolysis process can be divided into two methods: chemical (acid and base) and biochemical. Biochemical/enzymatic hydrolysis may occur using proteolytic enzymes naturally present in fish tissue (autolysis) (Petrova *et al.*, 2018). Previous studies reported the production of protein hydrolysate using the enzymatic method on 'sardines' by-products with pepsin, carp by-products with papain, and yellowfin tuna with alcalase (Benhabiles *et al.,* 2012; Saputra and Nurhayati, 2016; Siddik *et al.*, 2020). The use of flavourzyme as an enzyme that accelerates the hydrolysis process has been carried out in the production of protein hydrolysates of Atlantic salmon, carp, and scad (Kristinsson and Rasco, 2000; Dong *et al.,* 2008; Thiansilakul *et al.,* 2007).

Fish protein hydrolysates (FPH) are rich in amino acids or peptides, have good functional properties and offer fortification material to provide high-protein food. It has a balanced amino acid profile, easy to digest and absorb, and contains bioactive peptides (Chalamaiah *et al.,* 2012). In addition, fish protein hydrolysate may improve the characteristics of food products (Asare *et al.,* 2018). For example, Chen *et al.,* (2018) reported using chickpea flour to enhance the quality of yoghurt. Yoghurt fortified with microcapsules of bigeye fish protein hydrolysate (*Ilisha megaloptera*) was also reported by Jamshidi *et al.,* (2019).

Yoghurt is a dairy product rich in protein, lactose, water-soluble minerals, and vitamins (Ozturkoglu-Budak *et al.,* 2016). Yoghurt can be thick, slightly thick, or liquid in texture. The weakness of yoghurt products is that the binding power of water and whey molecules in casein gel is relatively weak. Therefore, yoghurt with a pH value of 4.7 -5.0 usually has poor quality, resulting in low solubility, low viscosity, and increased syneresis, which affects the final quality of yoghurt products (Annisa and Radiati, 2018). Numerous publications related to yoghurt enrichment and improvement have been carried out, including the addition of salmon oil, nuts, mushrooms white oyster, and spirulina (Estrada *et al.,* 2011; Ozturkoglu-Budak *et al.,* 2016; Anissa and Radiati, 2018; Barkallah *et al.*, 2017). Nevertheless, research on improving yoghurt products added with protein hydrolysate from fish by-products is still limited. Therefore, it was necessary to conduct this study to determine the effect of adding Parrotfish protein hydrolysate (*Chlorurus sordidus*) to yoghurt products.

2. Materials and Methods

2.1. Materials

In this study, the protein hydrolysate was made of the Parrotfish fish (*Chlorurus sordidus*) heads obtained from PT. Alam Jaya, Surabaya, East Java. The yoghurt used was Biokul Plain Stirred Yoghurt (DIAMOND). The materials used for the research were 0.2 N NaOH (German Merck, Grade AR/PA) and Flavorzyme 200,000 AU/g (Novozymes, Novo Alle, DK-2880 Bagsvaerd, Denmark). The equipment used was a digital pH meter (Model PH-9, Biobase, China), viscometer (Model LP-74, High-Performance Rotational Viscometer, Gilson, USA), centrifuge (Model PLC-05, Taiwan), and colour reader (Colorimeter CHN Spec CS-10, China). All materials used in this experiment were of analytical grade and were purchased from Merck (Darmstadt, Germany, USA).

2.2. FPH Preparation

Preparation of Parrotfish protein hydrolysate (*Chlorurus sordidus*) referred to the method of He *et al.* (2013) with some modifications. FPH production was carried out enzymatically using flavourzyme. Briefly, 150 g of finely chopped parrotfish head was mixed with 300 mL of distilled water (1:2). The pH of the sample was adjusted to neutral (pH 7) by adding 0.2 N NaOH solution, then conditioned at 50°C for approximately five minutes.

Flavourzyme (0.2 AU/g) was added as a catalyst to the reaction mixture. The samples were hydrolyzed by incubating at 50°C using an incubator shaker for 0, 3, 6, and 12 hours at 150 rpm. Afterwards, the sample was heated at 90-95 °C for 10 minutes for enzyme inactivation. The next stage was the recovery stage. Samples

were centrifuged at 4,500 rpm for 30 minutes to separate layers of different fractions, such as lipid layers, light lipoproteins, soluble proteins, fine particles, and coarse particles. The layer of soluble proteins was separated and would be tested for the degree of hydrolysis (DH). FPH samples with the highest DH value were then dried to obtain FPH in powder using a spray dryer (BUCHI B-290, Labortechnik AG, Flawil, Switzerland) with an inlet temperature of 180 °C, an output temperature of 100 °C, and a flow rate of 40%. The process of making FPH powder from parrot fish head by-product is described in Figure 1. FPH powder was used for the fortification of commercial yoghurt products with various concentrations (A= 0%, B= 0.10%, C= 0.15%, D= 0.2%, E= 0.25%, and F= 0.3%) and subjected to further characterization.

2.3. Degree of Hydrolysis (DH)

The Kjeldahl method was used to measure %N in determining the DH value, according to Hoyle and Merritt (1994). The soluble protein fraction sample was divided into two parts. The first sample (A) was analyzed directly to calculate %N. 2 mL of the second sample (B) was mixed with 2 mL of 20% TCA, and then centrifuged at 4000 rpm for 20 minutes at room temperature. The supernatant collected was used to calculate %N. Finally, the value of the degree of hydrolysis was calculated using the following formula:

$$\text{DH (\%)} = \frac{\text{\% Nitrogen B}}{\text{\% Nitrogen A}} \times 100 \quad (1)$$

2.4. Analysis Method

2.4.1. pH test

The pH test was carried out using a pH meter based on AOAC (2005). pH test was performed using 10 mL of FPH yoghurt as the sample.

2.4.2. Total titratable acid test

A total titratable acid test was done by referring to Nielsen (2017). Briefly, a total of 10 mL of yoghurt samples was put in a measuring flask and diluted with distilled water. After dilution, 5 mL of the sample was taken and put into a 100 mL Erlenmeyer. Three drops of 1% phenolphthalein indicator were then added. The yoghurt sample was titrated with 0.1 N NaOH until the colour changed to pink. The formula for calculating total titratable acid (%) was as follows:

$$\text{TTA (\%)} = \frac{\text{V NaOH} \times \text{N NaOH} \times \text{MW organic acid}}{\text{V sample}} \times 100 \quad (2)$$

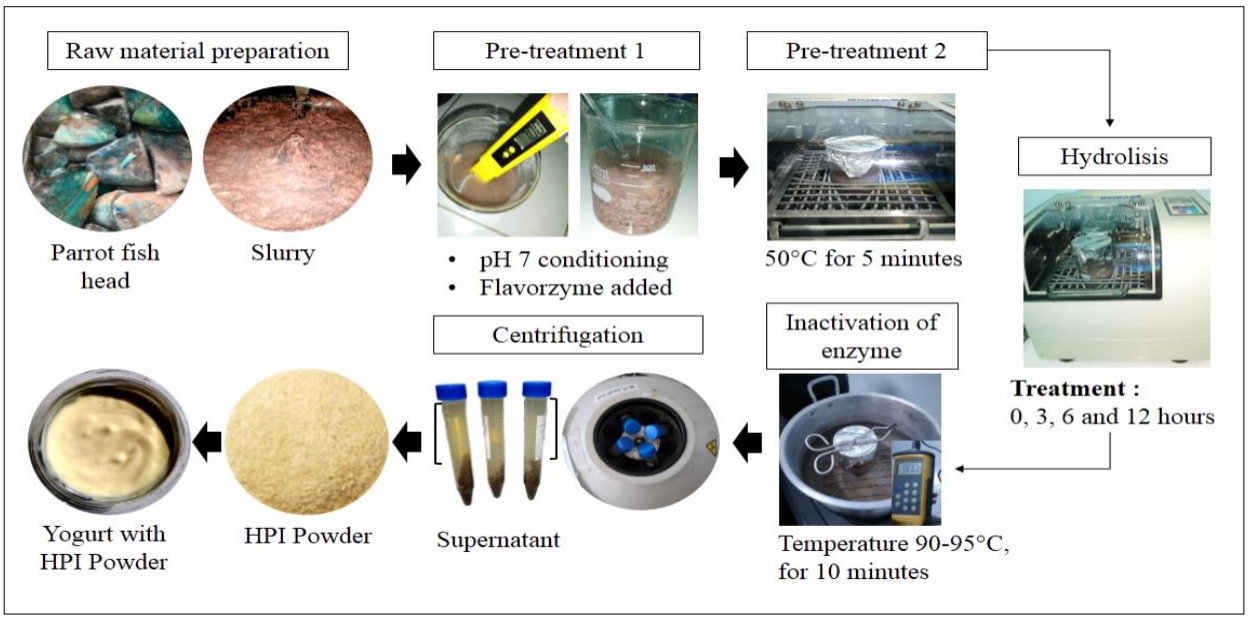

Figure 1. FPH preparation and fortification of yoghurt

2.4.3. Viscosity test

This test aimed to determine the level of yoghurt viscosity using a viscometer (Model LP74, High-Performance Rotational Viscometer) (Usmiati *et al.,* 2022). First, 100 mL of FPH yoghurt was placed into the tester glass. Next, the viscometer spindle was lowered until the yoghurt touched the 'spindle's edge. Finally, the speed was set to 20 rpm. The viscosity level was recorded as a number shown on the viscometer.

2.4.4. Syneresis test

The syneresis test was conducted based on Varnaite *et al.* (2022). This test was carried out by centrifuging a 15 mL yoghurt sample at 2,000 rpm for 20 minutes. After centrifugation, the supernatant was weighed and calculated using the following formula:

$$\text{Syneresis (\%)} = \frac{\text{Supernatant Weight (g)}}{\text{Sample Weight (g)}} \times 100$$

2.4.5. Water holding capacity test

Water holding capacity test referred to Wang *et al.* (2022) with modification. 10 mL of the sample was homogenized using a vortex at room temperature for one minute, then centrifuged at 3.000 rpm for 30 minutes. The pellet or sample residue was weighed. The formula for calculating the water holding capacity (WHC) is as follows:

$$\text{WHC (\%)} = \frac{\text{Weight of sample before centrifugation}}{\text{Weight of sample after centrifugation}} \times 100 \quad (3)$$

2.4.6. Colour test L, a*, b**

Colour testing was carried out using a colour reader (Colorimeter CHN Spec CS-10) (Murda *et al.,* 2021). Measurements were made by placing a yoghurt sample in front of the colour reader sensor. Set the reading button to L*(lightness), a*(redness), and b*(yellowness), and press the target button. The results would come from the reading of the tool and then be recorded.

2.4.7. Organoleptic test

This test used the Hedonic Scale Scoring method, expressing 'panellists' preference level (Putri *et al.,* 2018). This method used a numerical scale, with a score of 1 to 7 (1 = dislike very much, 2 = dislike, 3 = somewhat dislike, 4 = neutral/ordinary, 5 = somewhat like, 6 = like, 7 = very much like) (Lestari and Susilawati, 2015). The test parameters included appearance (colour), aroma (specific aroma of fish, aroma of fermented milk, sour aroma), texture (thickness), and taste (typical taste of fish, taste of milk, sour taste of yoghurt, and sweet taste of yoghurt).

2.5. Data analysis

All data were analyzed by using SPSS Statistical software. The data obtained were analyzed through a one-way analysis of variance (ANOVA), followed by DMRT (Duncan Multiple Range Test) significance level of α=0.05. Data were presented as the mean from the results of five independent experiments ± SD. The organoleptic test data were analyzed using the Kruskal Wallis (Non-Parametric) test to determine the best treatment using the effective index method (De Garmo, Sullivan and Canada, 1984).

3. Results and Discussions

3.1. Degree of Hydrolysis (DH) of FPH

The results showed that the degree of hydrolysis of Parrotfish fish protein hydrolysate ranged from 2.13 to 36.51% (Figure 2). The highest average was obtained at the incubation or hydrolysis duration of 12 hours at 36.51 ± 0.7%. The FPH with the highest degree of hydrolysis result was used for further analysis.

3.2. Physical Characteristics of Enriched Yogurt

The results showed that the addition of hydrolyzed parrot fish protein powder had a significant effect ($p<0.05$) on the viscosity, syneresis, Water Holding Capacity (WHC) and colour values of the yoghurt product enriched with FPH parrotfish (*Chlorurus sordidus*). The physical characteristics of FPH-enriched yoghurt are presented in Table 1.

3.3. Chemical Characteristics of Enriched Yogurt

The results showed that the addition of parrotfish protein hydrolysate powder had a

significant effect ($p<0.05$) on the pH and TTA values of yoghurt (Table 2).

3.4. Sensory Characteristics of Enriched Yogurt

The addition of FPH Parrotfish powder had a significant effect ($p<0.05$) on the appearance, aroma, and taste values but had no significant effect ($p>0.05$) on the texture value (Table 3). The appearance of yogurt with the addition of FPH Parrotfish powder for each treatment can be seen in Figure 3.

3.5. Discussion

Based on the results, the longer the incubation time, the higher the degree of hydrolysis. A longer hydrolysis process increases the number of peptides or small proteins (Hau *et al.*, 2018). The degrees of hydrolysis (DH) of protein could be influenced by the type of protease used, enzyme concentration, temperature, pH, and hydrolysis time (Restiani, 2017). Slizyte *et al.* (2016) mentioned that the peptide bond cleavage was more active at a longer incubation time and produced large amounts of essential amino acids dissolved in TCA. In addition, large amounts of amino acids were also associated with a longer contact time between the catalytic enzyme and fish substrate at optimal enzyme conditions (Ariyani *et al.*, 2017).

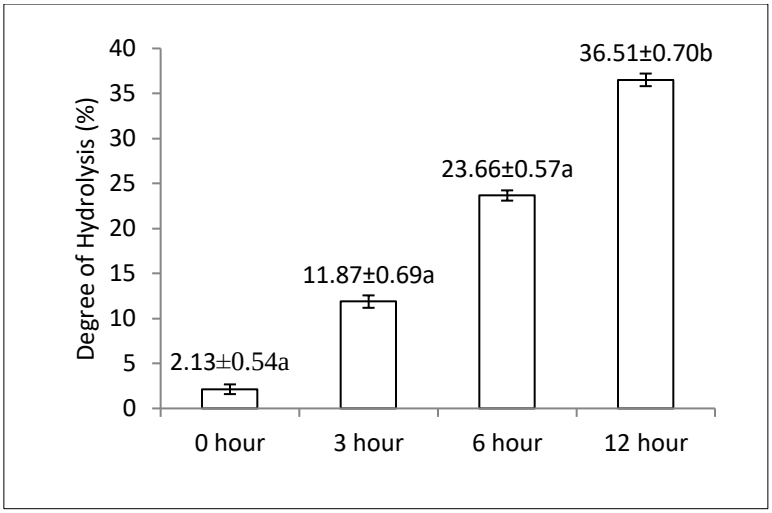

Figure 2. Degree of hydrolysis of FPH from parrotfish head at various incubation times.

Table 1. The physical characteristics of yoghurt enriched with FPH parrotfish

Code	Physical characteristics					
	Viscosity ($N•s/m^2$)	Syneresis (%)	WHC (%)	Colour		
				L*	a*	b*
A	$2.231±6.658^{a}$	$53.53±0.030^{d}$	$34.50±1.353^{a}$	$86.18±0.272^{c}$	-3.13±0.206	$7.06±0.071^{a}$
B	$2.330±3.215^{ab}$	$52.80±0.557^{d}$	$34.57±1.002^{a}$	$75.61±2.199^{b}$	-4.46±1.991	$7.79±0.636^{ab}$
C	$2.432±4.509^{c}$	$49.67±1.528^{c}$	$37.40±0.700^{b}$	$74.55±3.006^{b}$	-3.76±0.485	$8.20±0.478^{abc}$
D	$2.389±78.409^{b}$	$44.13±1.343^{b}$	$41.33±1.201^{c}$	$72.64±2.507^{b}$	-4.78±0.326	$8.81±0.082^{bc}$
E	$2.487±10.263^{d}$	$45.63±1.069^{b}$	$43.17±0.513^{d}$	$68.88±0.742^{a}$	-5.60±0.522	$9.53±0.631^{cd}$
F	$2.524±12.097^{e}$	$30.60±0.964^{a}$	$43.50±0.436^{d}$	$66.63±1.106^{a}$	-5.47±0.979	$10.86±1.557^{d}$

Note: FPH concentration; A=0%, B=0.1%, C=0.15%, D=0.2%, E=0.25, F=3%. WHC= Water Holding Capacity. L*(lightness), a*(redness), b*(yellowness). Data were presented as mean±S.D. Different letters showed a significant difference between treatments.

Table 2. The Chemical characteristics of yoghurt enriched with FPH parrotfish

Code	Chemical Parameters	
	pH	TTA
A	4.31±0.015^{a}	0.78±0.030^{c}
B	4.33±0.015^{ab}	0.77±0.015^{c}
C	4.36±0.010^{b}	0.76±0.015^{bc}
D	4.40±0.010^{c}	0.71±0.061^{ab}
E	4.45±0.030^{d}	0.69±0.010^{a}
F	4.50±0.015^{e}	0.67±0.015^{a}

Note: FPH concentration; A=0%, B=0.1%, C=0.15%, D=0.2%, E=0.25, F=3%. TTA= Total Titratable Acid. Data were presented as mean±S.D. Different letters showed a significant difference between treatments.

Table 3. Organoleptic of yoghurt enriched with FPH parrotfish

Code	Organoleptic Test			
	Appearance	Aroma	Flavour	Texture
A	5.78±0.185^{ab}	5.47±0.188^{bc}	5.63±0.119^{c}	5.10±0.135^{a}
B	5.67±0.035^{ab}	5.59±0.051^{c}	5.27±0.142^{b}	5.09±0.150^{a}
C	5.8±0.030^{b}	5.33±0.035^{b}	5.39±0.051 ^{c}	5.23±0.115^{a}
D	6.03±0.035^{c}	5.21±0.101^{b}	5.52±0.068^{c}	5.14±0.191^{a}
E	5.54±0.103^{a}	5.13±0.035^{b}	5.06±0.081^{b}	5.06±0.511^{a}
F	5.28±0.017^{a}	4.76±0.081^{a}	4.61±0.185^{a}	5.26±0.103^{a}

Note: FPH concentration; A=0%, B=0.1%, C=0.15%, D=0.2%, E=0.25, F=3%. Data were presented as mean±S.D.

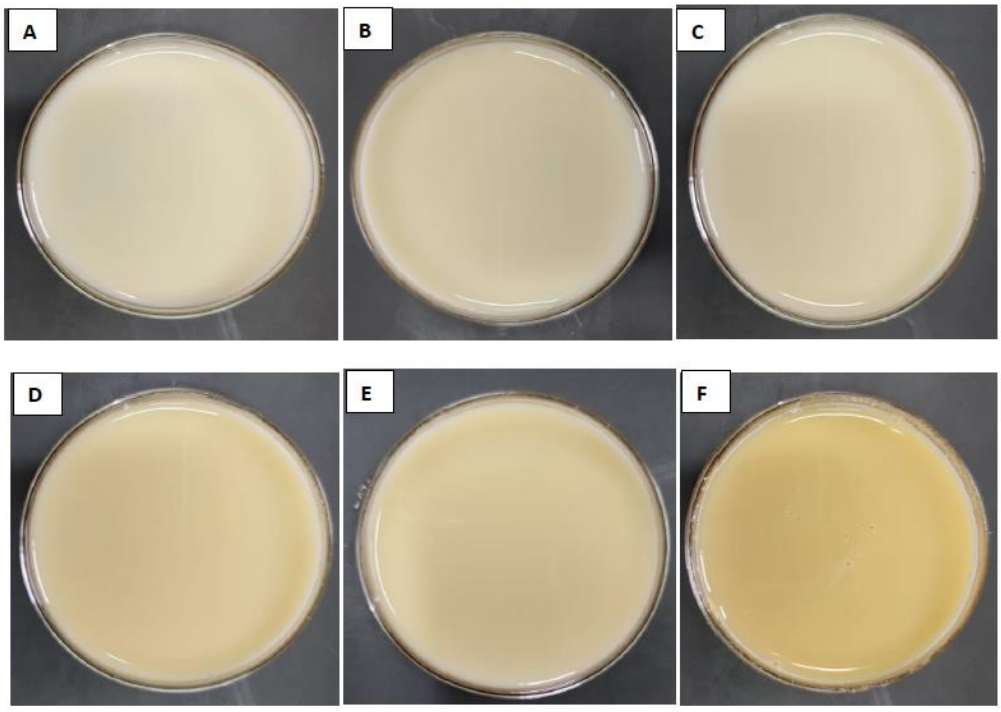

Figure 3. Yoghurt enriched with various concentrations of parrotfish FPH

The viscosity of yoghurt enriched with various concentrations of Parrotfish FPH ranged from 2.231 to 2.524 (N•s/m^2). The highest viscosity was obtained in sample F, with the

parrotfish FPH concentration of 0.3%. The average viscosity of yoghurt in this study was similar to the results of a previous study by Chen *et al.* (2018) at 2.230– 2.900 ($N•s/m^2$). Based on the results, adding Parrotfish protein hydrolysate powder can increase the viscosity of yoghurt. It might be due to the increase in total solids in yoghurt, so the texture becomes more compact and thicker. As explained by Unnikrishnan *et al.* (2019) and Dibyanti *et al.* (2014), the increase in viscosity was directly proportional to the amount of protein and the total solids contained, and the ability to bind water (WHC). Yoghurt viscosity increased due to the formation of lumps or curds of fat globules due to the acidic atmosphere and low temperature. Sakul *et al.* (2020) state that these clots result from denatured proteins, so coagulation occurs in milk proteins. Hau *et al.* (2020) showed that the viscosity of mayonnaise fortified with yellowfin tuna protein hydrolysate was higher than the control. It might be due to the interaction between lipophilic and hydrophobic groups (peptide chains) with proteins in mayonnaise (Unnikrishnan *et al.,* 2019). Enhancing yoghurt with fibre enrichment components increased water retention ability and thickness (Marand *et al.,* 2020).

The results showed that the syneresis of yoghurt with the addition of various concentrations of FPH Parrotfish ranged from 30.6 to 53.53%. The lowest value was obtained in sample F (0.3% FPH). The average syneresis of yoghurt in this study was similar to the results of previous studies by Kwon *et al.* (2019) (30.40% – 36.47%) and Ahmed *et al.* (2021) (48% - 53%). Syneresis occurs because changes in the structure of the associated protein network result in a weak binding to curd protein so that it is released to the surface of yoghurt (Bahrami *et al.,* 2013). The results showed that Parrotfish protein hydrolysate powder added to yoghurt could reduce syneresis. It may be due to an increase in protein, fat and total solids levels, thereby improving the microstructure of yoghurt. In line with the statement of Delikanli and Ozkan (2016), yoghurt with protein enrichment can increase the number of bonds between proteins, resulting in a denser network due to an increase in solids in the protein matrix in the yoghurt gel microstructure. Ahmed *et al.* (2021) stated that yoghurt added with Argel leaf extract showed an interaction of Argel leaf content (polyphenols) with yoghurt protein, which made the yoghurt gel matrix firmer and increased the defence against curd release.

The range of water holding capacity (%) of yoghurt with the addition of various concentrations of FPH Parrotfish was from 34.50 to 43.50. The highest WHC was obtained in sample F, with a concentration of 0.3%. Similarly, Ozturkoglu-Budak *et al.* (2016) reported the average water-holding capacity of yoghurt, ranging from 35.57% to 48.97%. WHC is defined as the ability of a food component to bind water. In this study, it was found that adding Parrotfish protein hydrolysate powder could increase the water-binding capacity of yoghurt. It might be because fish protein hydrolysate contains peptide side chains with hydrophilic groups that are polar or can bind to water. Nurdiani *et al.,* (2016) and Benjakul *et al.* (2014), explained that the high solubility of fish protein hydrolysate was due to the opening of protein molecules during hydrolysis to produce peptides with amino and carboxyl groups. Lima *et al.* (2021) stated that ionic and dipole interactions relate to water retention or holding capacity. This might be due to the 'protein's hydrolyzed amino acid profile, which has a dominant hydrophilic amino acid (57.4%) and a negatively charged amino acid (64.4%). In addition, there might be a contribution from hydrogen bonding and dipole-dipole interactions. Bahrami *et al.* (2013) stated that the increase in water-holding capacity in yoghurt caused by hydrocolloids occurred in two ways: physically and chemically. Physically, water was trapped in increasing the protein 'network's density. At the same time, chemically, the hydrophilic nature of hydrocolloids facilitated the association of proteins with water molecules, thereby increasing the water-holding capacity of the gel.

The Commission Internationale de l'Eclairage (CIE) determined the colour

measurement using L*, a*, b* codes. The value of L* (brightness) was ranged from 100 (white) to 0 (black). The a* value indicated a reddish (+) or greenish (-) colour, while colour b* indicated a yellowish (+) or bluish (-) (Wrolstad and Smith 2017). The results showed that the colour L* value of yoghurt with the addition of various concentrations of FPH Parrotfish ranged from 66.63 to 86.18. The lowest L* value was obtained in sample F (0.3% FPH). The average L* value in this study was not significantly different from the average L* colour of yoghurt in the study of Tamjidi *et al.* (2012), which was 89.75 – 91.50. The colour a* of yoghurt was obtained between 3.13 and -5.60. The lowest a* value was obtained in sample E (0.25% FPH). This study's average a* value was similar to other studies, i.e., -1.69 to -2.94 (Tamjidi *et al.*, 2012) and -4.61 to -5.14 (Sjaznar *et al.,* 2018). The value b* of yoghurt was obtained between 7.06 and 10.86. The lowest value was obtained in sample F. The average b* value in this study was similar to Raikos *et al.* (2018) of 7.03 – 7.76.

The colour of yoghurt with the addition of fish protein hydrolysate powder gives a darker, greenish, and yellowish colour. It may be influenced by genetics or the pigment possessed by the fish used as raw material. According to Madora *et al.* (2016), the colour of yoghurt decreased in brightness when carrot powder was added, as carrot powder contains carotenoid pigments, resulting in a darker colour in yoghurt. The greenish colour of yoghurt in this study could be derived from the turquoise pigment in the skin of the parrotfish head. Taheri *et al.* (2013) explained that the yellowish colour comes from fish protein hydrolysate powder because it contains higher haemoglobin, myoglobin, and brown pigment. The pigment resulted from aldol condensation of carboxyl groups produced from lipid oxidation in the reaction of protein groups.

The pH range of yoghurt with the addition of various concentrations of FPH Parrotfish was 4.31 to 4.5. The highest pH was obtained in sample F, with an FPH Parrotfish concentration of 0.3%. These results were still within the quality standard of yoghurt according to SNI 01-2981-1992, which is in the range of 4.1 – 4.5. The increased pH of yoghurt might be influenced by the addition of various concentrations of FPH Parrotfish, which has a pH of 7 or neutral. According to Unnikrishnan *et al.* (2019), the product will experience an increase in pH if protein hydrolysate is added. In general, the pH of a protein hydrolysate is around 6.24 or close to neutral. A decrease in total acid might also cause an increase in yoghurt pH. Hermiastuti *et al.* (2013) stated that the amino acids in fish protein hydrolysate are neutral in pH. Amino acids with a dipolar structure contain one carboxyl group (negatively charged, acidic) and one amino group (positively charged, primary). Throughout the storage period, a consistent reduction in pH levels was observed across all yoghurt variants, predominantly attributable to the generation of microbial metabolites (Lima *et al.*, 2021).

TTA (%) of yoghurt with the addition of various concentrations of FPH Parrotfish ranged from 0.67 to 0.78. The lowest TTA value was obtained in sample F, with an FPH Parrotfish concentration of 0.3%. The results were still within the quality standard of yoghurt acid according to SNI 01-2981-1992, which is 0.5 - 2.0%. The decrease in TTA as the FPH was added might be due to the decline in the number of hydrogen ions in yoghurt (Anissa and Radiati, 2018).

The yoghurt appearance score with the addition of various concentrations of FPH Parrotfish ranged from 5.28 to 6.03. The highest value was obtained in sample D, with an FPH Parrotfish concentration of 0.2%. The highest hedonic score was 6.03, which means that the panellists liked the appearance of yoghurt with the addition of 0.2% Parrotfish protein hydrolysate powder or accepted by consumers. Panellists prefer ivory white or milky white colour given by FPH Parrotfish powder to yoghurt. The lowest F score was because the panellists thought the yoghurt colour was too yellow. In this study, FPH Parrotfish powder's brown-yellow colour was caused by the spray-dry method in the production process.

The score of yoghurt aroma ranged from 4.76 to 5.59. The highest average was obtained in sample B with the FPH parrotfish concentration of 0.1%. It means that the panellists preferred the smell of yoghurt with the least addition of FPH parrotfish powder (0.1%). The higher the FPH parrotfish powder concentration and the yoghurt's aroma will be pungent. Tamjidi *et al.* (2012) stated that panellists preferred yoghurt with the slightest fishy aroma or still smells typical of yoghurt. According to Junianto *et al.* (2019), the fishy smell in FPH was caused by chemical compounds such as dipeptides and other amine compounds. Adding FPH Parrotfish powder caused a fishy aroma that was not strong in yoghurt. The weak fishy odour detected by the panellists was probably due to the low-fat content in fish raw materials (Bernadeta, 2012).

The yoghurt taste score was from 4.61 to 5.52. The highest score was obtained in sample D with the FPH Parrotfish concentration of 0.2%. In this study, yoghurt with FPH Parrotfish powder had a characteristic sour taste even though it had a slightly bitter taste at higher FPH powder concentrations. The atmosphere or sour taste of yoghurt was due to the lactose metabolism by lactic acid bacteria, where the role of *Lactobacillus bulgaricus* gave a sharp, distinctive flavour to yoghurt. According to Benjakul *et al.* (2014), 'FPH's bitter taste was caused by the formation of peptides containing hydrophobic groups in the amino acid chain. Compounds that could cause a bitter taste include valine, glutamic acid, polyphosphate, and glycine. The level of bitterness in fish protein hydrolysate was significantly influenced by the type of protease enzyme added during hydrolysis. Flavorzyme could minimize bitterness in fish protein hydrolysate by removing terminal hydrophobic amino acids (Dauksas *et al.*, 2008).

The yoghurt flavour score with the addition of various concentrations of FPH Parrotfish ranged from 5.06 to 5.26. The highest value was obtained in sample F, with a concentration of 0.3%. The change in texture was due to the loss of water or fat content, the breakdown of emulsion, and the hydrolysis of proteins and carbohydrates. Yoghurt would be thicker if more FPH Parrotfish powder was added. Asare *et al.* (2018) explained that texture was influenced by the proximate content (fat, protein, moisture, and carbohydrates) of fish protein hydrolysate.

The results of the optimization calculation showed that the best treatment was the addition of 0.15% parrotfish FPH. Generally, the quality of yoghurt produced with a 0.15% concentration of fish protein hydrolysate powder met the established standards for yoghurt. According to the Indonesian National Standard (SNI, 01-2981-1992), the properties of yoghurt should meet the following criteria: pH 4.1-4.5, TTA 0.5-2.0%, and sensory properties typical of fermented milk.

4. Conclusions

The addition of FPH Parrotfish (*Chlorurus sordidus*) powder with different concentrations significantly affected the characteristics of yoghurt (pH, total acid, viscosity, syneresis, water holding capacity, L* colour, b* colour, hedonic parameters appearance, aroma, and taste). The best treatment for all parameters was the 0.15% concentration of Parrotfish fish protein hydrolysate.

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THE EFFECT OF SODIUM ALGINATE ON THE STORAGE LIFE OF SOUR CHERRY FRUITS

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ABSTRACT

The article shows the effect of sodium alginate solution on the quality and duration of storage of cherry fruits. For research purposes, cherry fruits of the Alfa variety were selected. The selected fruits were washed with water, immersed in sodium alginate solutions according to the options with 3% and 5%. The processed fruits were dried for 30 minutes by blowing air created artificially by a fan, packed in polyethylene bags and stored at a temperature of 0±0.5°C and a relative humidity of 95±1%. By the content of dry soluble substances, ascorbic acid, tanning and coloring substances, antioxidant activity. Using Harrington's method, the optimal concentration of sodium alginate solution for pre-treatment of cherry fruits before storage was determined - 5%. A technological scheme for storing cherry fruits treated with sodium alginate solution has been developed.

1.Introduction

Edible films and coatings are classified by type of material. The components used for the preparation of edible films are classified into three categories: hydrocolloids, which include proteins, cellulose derivatives, polysaccharides, kappa-2-carrageenan, alginates, pectins, starch; lipids including fatty acids, acylglycerol, waxes and composites containing lipids and hydrocolloid components (Maftoonazad & Badii, 2009; Nayik et al., 2015; Lopez-Rubio et al., 2017; Pereira, 2017).

Polysaccharides are long-polymer biopolymers formed from mono- or disaccharide repeating units connected by glycosidic bonds. Being hydrophilic in nature, polysaccharide films create a barrier to carbon dioxide and oxygen and thereby delay fruit respiration and ripening. On the other hand, their polarity determines a weak
barrier to both water vapor and general sensitivity to moisture.

Alginate is a polysaccharide used as an effective biopolymer film or coating component due to its characteristic colloidal properties such as thickening, gelling, film forming and emulsion stabilizer (Hassan et al., 2018).

Polysaccharide alginate coatings reduce moisture loss in garlic, extend shelf life and preserve freshness of sliced pineapple (Radev & Dimitrov, 2017), strawberry (Li et al., 2017; Aitboulahsen et al., 2018), nectarine (Chiabrando & Giacalone, 2013) pistachios (Hashemi et al., 2020), cherries (Zapata et al., 2017). Mixing sodium alginate with agar-agar in water as a common solvent resulted in two-component edible films (Po Huo et al., 2015).

According to G. Giacalone, V. Chiabrando (2013), J. Alonso, R. Alique (2004), the use of biodegradable films was beneficial for the preservation of cherry and sweet cherry quality by delaying color changes, loss of firmness and acidity.

Based on polysaccharides, sodium alginate, carbomethylcellulose and glycerin, a film-forming coating has been developed for the mechanical protection of the meat surface, as well as the creation of favorable conditions for

the development of lactic acid bacteria (Kishenya, 2016).

Analysis of the use of edible coatings in various food products shows that they effectively preserve their quality (color, appearance, texture, moisture loss, etc.) during storage.

Despite the great advantages of using edible coatings, the commercial application of this technology in a wide range is still very limited. Improving the water resistance, mechanical and barrier properties of biopolymer films still needs improvement. In addition, a key issue is the acceptability of coverage to consumers, the ultimate cost. There is also a large question regarding the effects of coatings and films on fruit metabolites (Nayik et al., 2015).

Future research can be directed in several directions:

- study of the influence of edible coatings on fresh fruits;
- study of carefully researched fresh fruits with new edible coatings applied to the surface;
- characteristics of physical and chemical solutions for coatings;
- characteristics of films, their gas and vapor permeability;
- study of metabolic reactions, in particular breathing (Radev & Dimitrov, 2017; Vasylyshyna, 2018; 2022)

The purpose of the study was to evaluate the effect of post-harvest treatment of sodium alginate solution on the quality of cherry fruits during storage.

2. Materials and methods

For this purpose, cherry fruits of the Alfa variety were selected during 2016−2018 years at the research station of pomology named after L.P. Simyrenko 2-3 days before the consumer stage of ripeness. They were sorted by size, shape and color. The selected fruits were washed with water. After that, the cherry fruits were immersed in sodium alginate solutions for 1−2 minutes to ensure the uniformity of the coating according to the options: without treatment (control) and treated with sodium alginate solutions of 3 and 5% concentration.

Fruits treated with sodium alginate were dried for 30 minutes by blowing air created artificially by a fan at 25°C. They were packed in polyethylene bags 0.5 kg and stored at a temperature of 0±0.5°C and a relative humidity of 95±1%.

To prepare the solution, sodium alginate was dissolved in distilled water at a temperature of 45°C with a concentration of 3 and 5%. After cooling, 10% glycerol was added to the solution.

2.1.Analytical methods.

Soluble substances – refractometer (PAL-3 (ATAGO), Japan). The content of *titrated acids* was determined by titration with a solution of 0.1 M NaOH.DSTU 4957:2008.

Ascorbic acid was determined using the modified Tillman's method. Ascorbic acid was titrated with 2.6-dichloroindophenol under acid conditions (Naichenko, 2001). *Tanning and coloring substances* - by Neubauer and Leventhal (Naichenko, 2001), titrated with potassium permanganate (0.1n $KMnO_4$).

Antioxidant activity - by FRAP (Khasanov et al, 2004). Measurements were performed on the millivoltmeter (MP 511 Lab pH Meter "Ulab", China) (mV). FRAP values were expressed as mmol 100g of dry matter, as mean value ± standard deviation (N = 3 replicates).

Fruit weight loss. At the end of storage was noted natural weight loss mass by weighting. The boxes of fruit were weighted initially and before and after sampling at each evaluation date. Weight loss was expressed as percentage loss of original weight. Criteria at the end of fruit storage − weight loss no more than 6%.

Fruits were homogeneous in degree of maturity, not overripe and no smaller than 16 mm in size. The number of fruits without a peduncle and with healed damages was not higher than 4%. The fruits were of the same size, color and shape without damages (average diameter is about 15.51 ±0.05mm) (DSTU 8325:2015). Tasting rating on a 5-point scale.

To generalize the research results, the generalized Harrington function (Koltunov & Belinska, 2010; Hayova, 2019) was used, which is the geometric mean of the desirability function.

$$D = \sqrt[q]{d1 \cdot d2 \cdot \dots dq} \quad (1)$$

tod_1, d_2, d_q– is the desired level (desirability function of the 1st, 2nd, etc. optimization parameter); q is the number of optimization parameters.

Dependency (1) allows you to replace several optimization parameters with one.

The following one-sided restrictions were imposed on the optimization parameters: the content of dry soluble substances (y_1); of titrated acids (y_2); vitamin C (y_3); tannins and dyes (y_4); antioxidant activity (y_5); output of commercial products (y_6); mass loss (y_7); tasting evaluation (y_8).

In the case of one-sided restrictions on the optimization parameters, the desirability function has the form:

$$di = (\exp(-exp(-yi)) \quad (2)$$

Where y' is some dimensionless quantity related to the optimization parameter and linear dependence:

$$y = b0^{\cdot} + b1y\iota \quad (3)$$

to b_0, b_1 – are coefficients that can be determined if, for two values of the optimization parameters y_i, the corresponding values of the desirability function (d) are set.

3. Results and discussions

Table 1 shows the existing values of cherry fruits of Alfa varieties before and after storage.

Table 2 shows the value of the coded scale, which was taken from 3 to 6 and from 0 to − 0.5, which correspond to certain values for the content of dry soluble substances, etc., which are set intuitively. For example, on the coded scale, a value of 0.89 corresponds to 0.94 in terms of the content of dry soluble substances.

Based on the complex coding matrix of table 2, we set natural responses to the coded scale and calculate the desirability functions based on it. For example: for cherry fruits of the Alpha variety, after treatment with a 3% solution of sodium alginate (the first feedback is the content of dry soluble substances, we find that:

$$y = 1+(1-1)/((0.82-0.94)*(0.89-0.94)) \quad (4)$$

From here, according to the formula, $d_i = (\exp(-\exp(-y_i'))$ the responses of the conversion on the scale of desirability will be equal to 0.351 and 0.704 of Tables 3 and 4. From here we find the generalized feedback D, which gives an estimate of the scale of desirability.

Because on the coding scale from 0 to −1.5 – "bad" from 0.77 to 1.5 − "good" from 1.5 to 3 "very good". According to the content of dry soluble substances, these values are in the range of 0.56−0.97; of titrated acids − 0.46−0.89; vitamin C - 5.4-6.8; tannins and dyes − 0.09−0.91; antioxidant activity - 4.0-8.3; yield of marketable products - 87.9-92.3, mass loss - 1.5-3.8; change in the tasting score - by 0.2-0.4 points.

Table 1. Changes in the quality of cherry fruits of the Alfa variety, pre-treated with polysaccharide compositions during storage

Indicator	Type of processing		
	No processing (control)	3% solution sodium alginate	5% solution sodium alginate
Dry soluble substances* (%): to storage	15.89±0.2	15.89±0.2	15.89±0.1
after storage	14.48±0.2	15.00±0.1	15.30±0.2
losses	1.41	0.89	0.59
Titrated acids* (%): before storage	1.74±0.02	1.74±0.01	1.74±0.02
after storage	0.84±0.02	0.90±0.03	0.94±0.02
losses	0.90	0.84	0.80
Vitamin C* (mg/100 g): before storage	19.05±0.01	19.05±0.02	19.05±0.01
after storage	12.2±0.01	12.4±0.02	13.00±0.01

losses	6.85	6.65	6.05
Tanning and coloring substances * (%): to storage	0.85±0.01	0.85±0.01	0.85±0.01
after storage	0.72±0.02	0.78±0.02	0.80±0.01
losses	0.13	0.07	0.05
Antioxidant activity* (mmol/dm^3): to storage	28±2.0	28±2.0	28±1.0
after storage	17±2.0	21±1.0	22±1.0
losses	11	7	6
Product yield*,%	85.7±1.0	87.8±1.0	91.3±1.0
Mass loss*,%	4.2±0.5	3.8±0.3	3.4±0.2
Tasting evaluation (points) *: to storage	4.8±1.0	4.8±1.0	4.8±1.0
after storage	3.8±1.0	4.5±1.0	5.0±1.0
losses	1.0	0.3	0.2

*Values are displayed as the mean ± standard deviation (SR) of the three replications (P <0.05).

Table 2. Limit values of natural responses, displayed in a coded scale

Response	Boundary values of natural responses, *y*								
	−1.5	−1.0	−0.48	0	0.77	1.00	1.50	2.00	3.00
Loss of dry soluble substances	1.46	1.34	1.16	1.07	0.97	0.94	0.82	0.73	0.56
Loss of titrated acids	1.2	1.1	1.01	0.92	0.89	0.78	0.68	0.56	0.46
Loss of vitamin C	8.2	8	7.5	7.2	6.8	6.5	6.3	6	5.4
Loss of tannins and dyes	0.17	0.15	0.13	0.11	0.91	0.72	0.46	0.24	0.09
Loss of antioxidant activity	13.4	12.3	11.2	9.1	8.3	7.3	6.3	5	4
Output of commodity products	85.1	85.4	85.8	86.4	87.9	88.5	89.8	91.7	92.3
Loss of mass	6.3	5.7	5.1	4.4	3.8	3.2	2.8	2.1	1.5
Tasting assessment	1.24	1.04	0.84	0.64	0.4	0.35	0.3	0.25	0.2

Table 3. Reviews of the conversion on a scale of desirability

Conversion feedback for metrics	Type of processing		
	No processing (control)	3% solution sodium alginate	5% solution sodium alginate
Dry soluble substances	0.223	0.351	0.095
Titrated acids	0.972	0.899	0.493
Vitamin C	0.189	0.176	0.049
Tanning and coloring substances	0.171	0.064	0.037
Antioxidant activity	0.189	0.099	0.042
Output of commodity products	0.210	0.185	0.514
Loss of mass	0.479	0.109	0.008
Tasting assessment	0.449	0.001	0.001

According to the obtained calculations, we find that the treatment with a 5% solution of sodium alginate was the most effective for the fruits of cherry varieties of Alfa.

Reducing the concentration of sodium alginate to 3% or the complete absence of treatment turned out to be less effective - good and satisfactory, respectively.

Table 4. Transformed feedback and generalized feedback on the scale of desirability

Converted feedback for metrics, *d*	Type of processing		
	No processing (control)	3% solution sodium alginate	5% solution sodium alginate
Dry soluble substances	0.799	0.704	0.909
Titrated acids	0.378	0.407	0.611
Vitamin C	0.828	0.839	0.952
Tanning and coloring substances	0.843	0.938	0.963
Antioxidant activity	0.827	0.906	0.959
Output of commodity products	0.811	0.832	0.986
Loss of mass	0.619	0.896	0.993
Tasting assessment	0.638	0.999	0.999

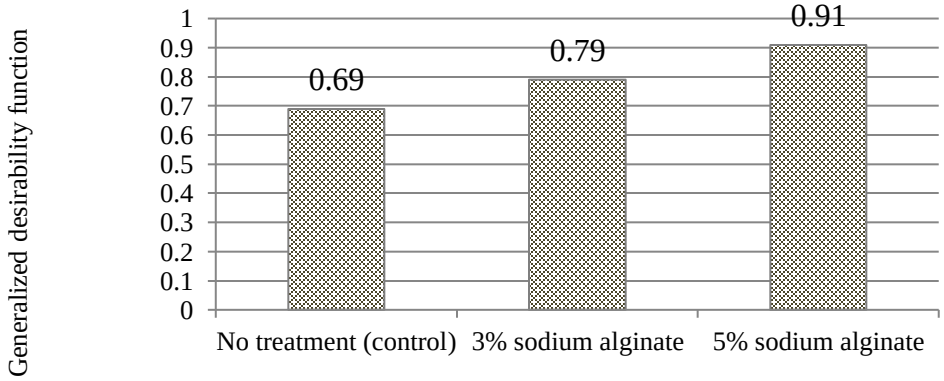


Figure 1. Ranking of pretreatment with sodium alginate on cherry fruits of Alfa varieties in order of decreasing value of the generalized desirability function

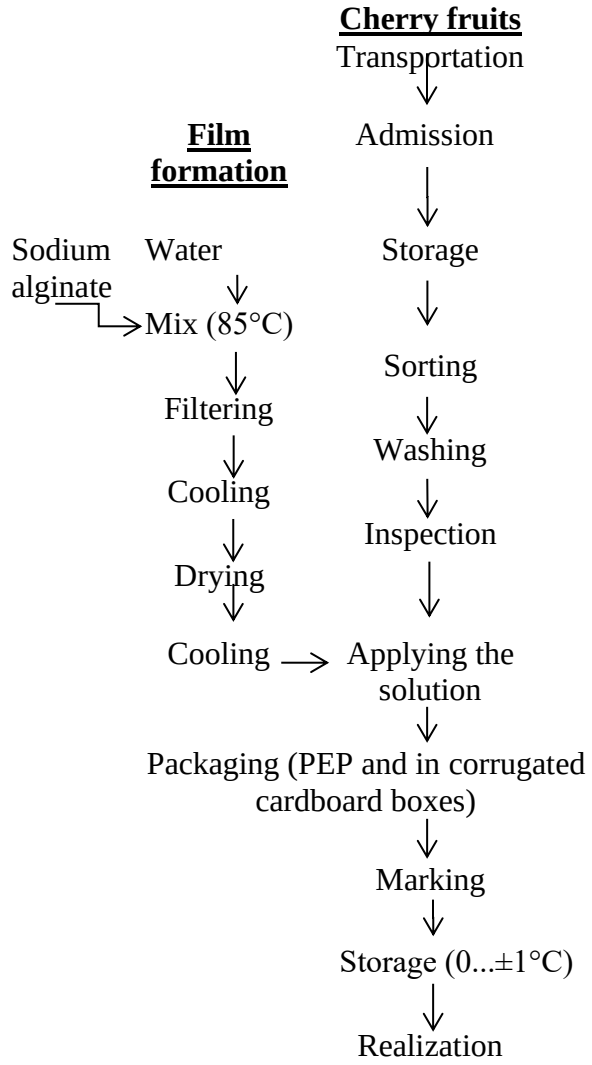
The ranking of samples in order of decreasing value of the generalized function is presented in Figure 1.

According to the ranking indicator, the most suitable for storage were the cherry fruits of the Alfa variety treated with a 5% solution of sodium alginate.

So, according to the technological scheme, after preliminary processing of cherry fruits, transportation, reception, storage in the garden (Figure 2), after unloading and receiving raw materials, sorting by quality, removing crumpled, damaged by agricultural pests, unripe and overripe fruits, the fruits are inspected, washed, and sodium alginate solution is applied.

To prepare a 5% solution, sodium alginate is dissolved in water and placed in a container for processing cherry fruits, after which they are

removed, dried, packed in boxes with a capacity of 5 kg and stored at a temperature of 0...±1°C and air humidity of 95± 1% up to 30 days.



Scheme 1. Technological scheme of storage of cherry fruits treated with sodium alginate solution

4. Conclusions.

Therefore, by carrying out research on the determination of commercial, physico-chemical indicators of cherry fruits of the common Alfa varieties pre-treated with sodium alginate solution during storage using the Harrington method, it was possible to establish the optimal concentration of sodium alginate solution - 5%. A technological scheme for preliminary processing of cherry fruits before storage has been developed.

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QUALITY EVALUATION AND SENSORY PROPERTIES OF *AGIDI* PRODUCED FROM BLENDS OF MAIZE (*ZEA MAYS*) AND PIGEON PEA (*CAJANUS CAJAN*)

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ABSTRACT

Agidi is a traditional fermented starchy food which is smooth-textured, semi-solid (gel-like) with creamy glassy white color cooked from wet-milled and wet-sieved maize paste. It is rich in carbohydrate but low in protein resulting in protein-energy malnutrition. Pigeon pea is a legume with rich source of protein (20-24%), essential amino acids (Lysine, methionine, tryptophan) and fibers. It has remarkable nutritional profile and health benefits. It is an underutilized crop from the family of Leguminosae (*Fabaceae*) and a good alternative for improving the protein content and nutritional value of carbohydrate dense food products. The influence of 25 %pigeon pea substitution with maize in the processing of 'agidi' was studied producing five samples coded A (100% Maize (control), B (75% Maize and 25% Pigeon pea), C (50% Maize and 50% pigeon pea), D (25%Maize and 75% Pigeon pea), E (100% Pigeon pea) and analyzing the nutritional quality and sensory properties in order to further exploit the functionality and acceptability of 'agidi'. Comparing control (100% maize) and sample E (100 % pigeon pea), significant ($p<0.05$) differences were observed in terms of the nutritional quality. Sample E has a smaller values of moisture (9.55 %), viscosity (150 Cps,) with a higher protein content (19.50 %), calcium (212.50 mg/100g), potassium (285.50 mg/100g), Iron (1.16 mg/100g), saponin (0.207 mg/g), a slightly decreased sour taste, flavor and mouth-feel intensity with the addition of pigeon pea, and with an important nutritional intake (ash: 0.75%, protein: 19.50%, crude fiber: 13.70%). Hence, combining pigeon pea with maize in the processing of 'agidi' at the substitution level up to 25% did not vary much from the control (100% maize) and was highly accepted by the consumers.

1. Introduction

Malnutrition is a medical and a social disorder often as a result of poverty and ignorance. Factors that contribute to malnutrition and poor nutrition outcomes are mainly due to production and consumption pattern. In Nigeria, the underlying causes of malnutrition include poverty, inadequate food production, inadequate food intake and improper combination of available indigenous foods due to ignorance. One of the fundamental ways of ensuring food security entail developing and improving food preparation, processing and preservation technologies (Oguche *et al.*, 2017).

In underdeveloped country like Nigeria cereals are the major staples consumed. Cereals

are good source of digestible starch, non-starch polysaccharides and second–class protein due to their deficiency in essential amino acids - lysine and tryptophan (Stadimayr *et al*., 2012). In Nigeria maize is mostly used in the production of *agidi*. This product has different names in different localities such as *eko* (Yoruba), *akasan* (Benin), *komu* (Hausa) and *agidi* (Igbo).

*Agidi*is a traditional fermented starchy food which is smooth-textured, semi-solid (gell-like) with creamy glassy white colour cooked from wet-milled and wet-sieved maize paste (Kolawole *et al.,* 2020). It is rich in carbohydrate but low in protein resulting in protein-energy malnutrition (Stadimayr *et al.*, 2012). It is usually consumed by infants and adults with stew, vegetables, bean cake or bean pudding or with spices and seasonings added to improve its taste while rich ones may contain minced meat (Akpapunam *et al.,* 2019).

Pigeon pea is a legume with rich source of protein (20-24%), essential amino acids (Lysine, methionine, tryptophan) and fibres. It has remarkable nutritional profile and health benefits. It is an underutilized crop from the family of Leguminosae (*Fabaceae*) and a good alternative for improving the protein content and nutritional value of carbohydrate based food products.

Pigeon pea gives similar maize slurry texture adequate for *agidi* purpose and therefore pigeon pea can be used to improve the protein content and other nutritional values in *agidi*. It can also be used instead of maize in the production of *agidi* since it also contains some appreciable amount of carbohydrate and some starch granules which are required for the formation of a semi solid gel like texture product like *agidi*.

A good number of researches has been conducted on fortification of *agidi* using soy bean flour but no research work has been carried out with pigeon pea in *agidi* production.

This study is aimed at production and evaluation of *agidi* from maize, blends of maize and pigeon pea and pigeon pea only.

2.Materials and methods

2.1. Materials

The raw materials include the following: Maize (*Zea mays*), Pigeon pea (*anus cajan*), Water and Uma leaves (*Thaumatococcusdaniellii*). All the raw materials were procured from Ogige market (a local market in Nsukka, Enugu State).

2.2.1. Samples

Sample Blends: 100:00, 75:25, 50:50, 25:75 and 00:100 for maize and pigeon pea respectively.

2.2.2. Preparation of Agidi samples

The *agidi* samples were prepared following the method of Nkama *et al.* (2000). Maize grains and pigeon pea seeds were cleaned to remove stones, dirt, and other foreign bodies that may affect the quality of the final product. Two kilograms (2kg) each of the cleaned grains were steeped in 4000ml of clean tap water in a stainless bowl for 48 hours at room temperature. The soaking water being replaced at 12 hours intervals. The fermented grains were rinsed with clean water and wet-milled in attrition machine. The milled paste was wet sieved using a double clean muslin cloth and residue discarded. The filtrate was allowed to sediment for 24 hours during which fermentation set in. It was decanted and a fine slurry was obtained. The slurry (500 ml) was cooked with water (750 ml) at 80 ^{o}C for 30 minutes while stirring continuously until a thick gel like structure is formed. It is then packaged hot in 'Uma' leaves or a polyethylene bag and allowed to solidify taking the shape of the packaging material used. It cooled, for 30minutes and stored in refrigerator for further analysis (Ogeihor *et al*., 2005)

2.2.3. Proximate Analysis

The proximate analysis (moisture, ash, protein, fat, fibreand carbohydrate) of the agidi samples were determined using the official method of analysis of the Association of Official Analytical chemist (AOAC, 2010). Energy was calculated using Atwater conversion factor (fat × 9 + carbohydrate × 4 + protein × 4 kcal/100g)

2.2.4. Mineral Content

Calcium, Potassium and Iron Analysis were determined according to AOAC (2010)

2.2.5. Physical Analysis of the slurry and agidi

The pH was determined using a pH meter as described by AOAC (2010).

2.2.6. *Determination of Titratable Acidity*

The titratable acidity was determined using the method described by AOAC (2010). Ten (10ml) of each sample blend slurry was pipetted into a conical flask and 25ml of distilled water was added. Two milliliter (200ml) of 0.1M NaOH was poured into a burette and titrated against the sample in the flask using 3 drops of phenolphthalein as indicator. It was titrated until a pink colouration was observed and the corresponding burette reading taken in the equation below:

Titratable acidity (g/100g) = Titre value × M × 90 × 100

Volume of the sample × 100

Where M = Molar concentration of NaOH.

2.2.7. Determination of Viscosity

The viscosity of the slurry was determined using the method described by Bolaji*et al.* (2014). The viscosity of different blend slurry samples were measured in duplicate at ambient temperature using a digital rotational Brookfield viscometer. Their reading was taken after 1 min rotation at speed (100rpm). Spindle number #6 was used for all the measurement. A 100ml beaker was used for the measurement with the viscometer guard leg on. The samples were poured into the beaker to reach a level that covers the immersion groove on the spindle shaft. All viscosity measurements were carried out immediately on blend slurry.

2.2.8. Texture Determination

The texture of the *agidi* sample blends were determined using a universal penetrometer as described by Rosenthal (2015). The weighted cone of the penetrometer was allowed to come in contact with the food material thereby penetrating its surface under its own weight. The distance that it penetrates was then recorded.

2.2.9. Phytochemical Properties (Tannin, Phytate and Saponin) Determination

The tannin content was determined by Folin-Denis calorimetric method described by AOAC (2010). The phytate content was determined by the method described by AOAC (2010) and saponin content was determined by Brunner (1984).

2.2.10. Sensory Evaluation

Sensory evaluation was carried out on the samples using 20 panelists (semi trained consumers) to test on the sensory attributes of the *agidi* from blends of maize and pigeon pea. The panelists were asked to indicate their preference using a 9-point Hedonic scale for colour, taste, mouthfeel, aftertaste, flavour, texture, firmness, smoothness, consistency, appearance and overall acceptability. This was used to determine the sensory acceptability of the sample (Iwe, 2002).

2.2.11. Statistical Analysis and Experimental Design

Data obtained was subjected to one-way analysis of variance (ANOVA) using completely randomized design. Means were separated using Duncan's new multiple range test (DNMRT). Significance was accepted at ($p< 0.05$) as described by Steel and Torrie (1980).

3. Results and discussions

3.1. Proximate composition and energy value of *Agidi*

The proximate composition and energy value of *agidi* samples were presented in Table 1. The moisture content increased with the substitution of 25% maize with pigeon pea to give moisture content of 14.05 % in sample B. Further addition of the pigeon pea in samples C, D and E led to decrease in the moisture contents to 13.70, 10.15 and 9.55 % respectively. Similar decrease in moisture content values was reported by Oguche *et al*. (2017) on soy-fortified *agidi* with moisture content of 9.26 % for 100% maize *agidi* and 8.83 – 8.65 % for the soy-fortified *agidi*. High moisture content of *agidi* is

due to its processing method involving soaking and cooking. From the result the moisture content of *agidi* samples predispose them to rapid spoilage as moisture content is an important factor in determining the shelf-life of foods. (Oguche *et al*., 2017). Hence the need for quick consumption of *agidi* as soon as possible (Kolawole *et al*., 2020).

Protein content ranged from 8.755 to 19.50 % with sample E (100% pigeon pea) having the highest ($p<0.05$) protein content (19.50 %) and the control (100% maize) had the least protein content (8.75%) which increased subsequently with the addition of pigeon pea. Conversely, the carbohydrate content of the *agidi* blends decreased considerably from 68 % in the control (100% maize) to 68, 60.25, 57.75, 60.25 and 55.30 % for B, C, D and E respectively due to the addition of pigeon pea.The increase in protein, content of the *agidi* samples was due to the high level of protein, in pigeon pea. Pigeon pea is a legume known for its rich protein value and is incorporated into cereal products to improve their protein quality (Obizoba and Oganah, 2008). This shows the superior nutritional properties of pigeon pea over maize for the production of *agidi* and also their mutual supplementation effect. This increase in the protein value compared favorably with the report by Ikya *et al*., 2013).

A little increase in protein content made micro-structure become more homogenous and had reduced moisture loss, but continuous addition of protein weakens the structure and water vapour permeability is increased (Basiak *et al*., 2016). The ash, crude fibre and fat contents of the *agidi* ranged from (0.27 - 0.75 %), (10.20 - 13.70 %) and (0.65 - 1.20 %) respectively. This increase with addition of pigeon pea is due to the fact that pigeon pea is a rich source of fibre and minerals (Staughton, 2020).

Table 1. Proximate composition and energy value of *Agidi* samples from blends of maize and pigeon

Proximate Parameters	**A**	**B**	**C**	**D**	**E**
Moisture %)	$11.34^{b}\pm0.21$	$14.05^{a}\pm0.21$	$13.70^{a}\pm0.28$	$10.15^{c}\pm0.21$	$9.55^{d}\pm0.07$
Ash (%)	$0.25^{c}\pm0.07$	$0.35^{bc}\pm0.07$	$0.35^{bc}\pm0.07$	$0.45^{b}\pm0.07$	$0.75^{a}\pm0.07$
Crude Fibre (%)	$10.20^{d}\pm0.14$	$10.60^{d}\pm0.14$	$11.50^{c}\pm0.14$	$12.35^{b}\pm0.21$	$13.70^{a}\pm0.14$
Fat (%)	$0.65^{d}\pm0.07$	$0.75^{cd}\pm0.07$	$0.95^{bc}\pm0.07$	$1.05^{ab}\pm0.07$	$1.20^{a}\pm0.14$
Crude Protein (%)	$8.75^{b}\pm2.47$	$14.00^{ab}\pm0.00$	$15.75^{a}\pm2.47$	$15.75^{a}\pm2.47$	$19.50^{a}\pm2.82$
Carbohydrate (%)	$68.80^{a}\pm2.86$	$60.25^{b}\pm0.21$	$57.75^{b}\pm2.91$	$60.25^{b}\pm1.90$	$55.30^{b}\pm2.54$
Energy Value (kcal)	$315.45^{a}\pm0.63$	$303.75^{c}\pm0.21$	$302.55^{c}\pm0.49$	$313.45^{a}\pm1.62$	$310.00^{b}\pm0.14$

Values are mean ± standard deviation of duplicate readings. Values on the same column with different superscripts are significantly different at $p<0.05$.

A = 100% Maize (Control),B = 75% Maize and 25% Pigeon pea, C = 50% Maize and 50% Pigeon pea, D = 25% Maize and 75% Pigeon pea, E = 100% Pigeon pea

The steady increase in ash, fibre and fat contents with inclusion of pigeon pea is in agreement with Ikya *et al*. (2013) and Kolawole *et al*. (2020) who also reported a steady increase in the ash, fibre and fat contents due to fortification of *agidi* with a legume. Presence of ash is an indication of mineral content in the food. Minerals are required for proper

composition and maintenance of body fluids including blood, nerves, tissues, bones, teeth and muscles. Fiber maintain healthy weight, reduce risk of Type-2 diabetes, lower the chances of heart disease, maintain healthy gut/digestive system, and reduce risk of certain cancers and aid detoxification (Dreisbach, 2021). A small amount of fat is an essential part of a healthy and balanced diet. Fat is a source of essential fatty acids which the body cannot make, by itself and it helps the body absorb vitamin A, D and E (NHS, 2020).

The caloric value of the *agidi* samples ranged from 305.55 to 315.45 kcal with the control sample (A: 100% maize) having the highest caloric value of 315.45 kcal as compared to other sample blends 303.75, 302.55, and 310.00 kcal for B, C and E respectively. The control (100% maize) varied significantly ($p<0.05$) with the sample blends except for sample D. Higher energy or caloric value in the *agidi* samples are expected because energy is a function of protein, fat and carbohydrate contents (Oguche *et al.*, 2017). The *agidi* samples have high protein (i.e blends with more pigeon pea) and carbohydrate content (blends with more maize). This is an indication that *agidi* produced from blends of pigeon pea or only pigeon pea is a good source of energy just like the control (A: 100% maize).

3.2. Micronutrient composition of *Agidi*from blends of maize and pigeon pea

The mineral contents of *agidi* made from maize and pigeon pea were represented in Table 2 and it showed that there were significant differences ($p<0.05$) between the blends and the control.

Table 2. Mineral Content of *Agidi* Samples from Blends of Maize and Pigeon pea

Sample	Calcium (mg/100g)	Potassium (mg/100g)	Iron (mg/g)
A	$122.50^{e}\pm3.53$	$202.00^{e}\pm2.82$	$0.94^{d}\pm0.01$
B	$147.50^{d}\pm3.53$	$215.50^{d}\pm0.70$	$0.98^{c}\pm0.00$
C	$180.00^{c}\pm0.00$	$240.00^{c}\pm0.00$	$1.04^{b}\pm0.00$
D	$197.50^{b}\pm3.53$	$271.50^{b}\pm2.12$	1.14a±0.00
E	$212.50^{a}\pm3.53$	$285.50^{a}\pm0.70$	$1.16^{a}\pm0.01$

Values are mean ± standard deviation of duplicate readings. Values on the same column with different superscripts are significantly different at $p<0.05$.

A = 100% Maize (Control), B = 75% Maize and 25% Pigeon pea, C = 50% Maize and 50% Pigeon pea, D = 25% Maize and 75% Pigeon pea, E = 100% Pigeon pea

The calcium content of the *agidi* ranged between 122.50 to 212.50 mg/100g and it varied significantly ($p<0.05$). The control sample (A: 100% maize) has the least calcium content of 122.50 mg/100g which increased with the addition of pigeon pea. Pigeon pea contain appreciable amount of calcium (Saxena *et al.*, 2010). Calcium plays a significant role in blood clotting, nerve transmission, muscle contraction and also in bone health (Peters and Martini, 2010; Elinge *et al.*, 2012). According to Sexana *et al.* (2010), pigeon pea contains appreciable amount of iron which explains why sample E have the highest iron content.

Potassium content ranged between 202 to 285.50 mg/100g with the control (A: 100% maize) having the least potassium content of 202 mg/100g but increased moderately with the addition of pigeon pea (215.50, 240.00, 271.50, 285.50 mg/100g for B, C, D and E respectively). Sufficient amounts of potassium in the body can increase iron utilization and control hypertension through diuretics and for individuals who experience uncontrolled

potassium excretion via body fluids (Elinge *et al*., 2012).

The iron content of the *agidi* samples ranged between 0.94 to 1.16 mg/g. The control (A: 100% maize) had the least iron content of 0.94 mg/g which slightly increased with the addition of pigeon pea. The high potassium content is due to the fact that pigeon is a rich source of potassium (Staughton, 2020). Iron plays a significant role in producing red blood cells in all body organs, including in the brain, and supporting haemoglobin synthesis in developing foetuses and young children (Georgieff *et al*., 2019).

3.3. Physicochemical properties of *Agidi* from blends of maize and pigeon pea

The physicochemical properties for the blends of maize and pigeon pea slurry and *agidi* sample texture was represented in Table 3. The pH values of all the slurries samples were low and acidic. It ranged between 3.57 to 4.36 with the control (A: 100% maize) having the least pH (3.57) which increased slightly with the addition of pigeon pea to 4.04, 3.87, 4.29, 4.36 for B, C, D and E respectively. The low acidic pH value obtained was as a result of fermentation process involved in *agidi* slurry production and is responsible for reduction or elimination of bacterial pathogens under fermentation conditions (Adegbehingbe *et al*., 2017). It is also attributed to the proliferation of microbial biomass particularly lactic acid bacteria and yeast capable of utilizing free sugar to produce organic acid.

The *agidi* slurry samples had low titratable acidity (TTA) ranging between 0.023 to 0.071 g/100g and varied significantly ($p<0.05$). Titratable acidity decreased with the addition of pigeon pea to 0.023, 0.038, 0.038, 0.038 g/100g for B, C, D and E respectively. The higher the titratable acidity, the stronger the fermentation flavor (Cauvain, 2015). It also indicates that the product will have better keeping quality because acidity can prevent or delay quick growth of spoilage microbes while low titratable acidity ensures proper absorption of mineral elements (Kuyunya *et al.*, 2011). Similar increase in the pH values and decrease in the titratable acidity values compared favourably with that reported by Zakari *et al*., (2010) during the addition of a legume *agidi* production.

The viscosity of the slurry prepared from the various proportion of the blends varied significantly ($p < 0.05$). The viscosity ranged within 150-300 cps with the sample E (100% pigeon pea) having the least viscosity of 150 cps. The viscosity of the *agidi* decreased with the increasing addition of pigeon pea. Viscosity affected the texture, firmness, smoothness, consistency and appearance of the *agidi* which are very importance qualities as they influence its acceptance by the consumers. The difference observed in the viscosities of the *agidi* sample blends slurry may be attributed to the different rate of water absorption capacity of the starch granules of the maize and pigeon pea (Ragaee and Abdel-Aal, 2006). Also maize cereal contains more starch granules than pigeon pea legume.

The texture of the *agidi* samples varied significantly ($p<0.05$) with sample C (50% maize and 50% pigeon pea) ranking the highest in texture (240.75mm) while other samples had 240.00, 239.75, 238.50 and 238.25mm for B, D, A, and E respectively. Texture is a very important quality factor in *agidi* production as it influences the consumers' acceptance of the food. Fermentation processes play important role in the textural properties of *agidi* or ogi. The addition of some materials or fortification during *agidi* production influences its rheological properties such as viscosity, swelling/gelation capacity, texture, etc. It may either increase or decrease the rheological properties (Barbradozier, 2013).

Table 3. Physicochemical properties of *Agidi* samples from blends of maize and pigeon pea

Sample	pH	TTA(g/100g)	Viscosity (cps)	Texture (mm)
A	3.57^{c}±0.00	0.071^{a}±0.00	300^{a}±0.00	238.50^{b}±1.41
B	4.04^{b}±0.07	0.023^{c}±0.00	195^{b}±7.07	240.00^{ab}±0.00
C	3.87^{c}±0.01	0.038^{b}±0.00	175^{bc}±21.21	240.75^{a}±0.35
D	4.29^{a}±0.02	0.038^{b}±0.00	160^{c}±14.14	239.75^{ab}±0.35
E	4.36^{a}±0.00	0.038^{b}±0.00	150^{c}±0.00	238.25^{b}±1.06

Values are mean ± standard deviation of duplicate readings. Values on the same column with different superscripts are significantly different at $p<0.05$.
A = 100% Maize (Control), B = 75% Maize and 25% Pigeon pea, C = 50% Maize and 50% Pigeon pea, D = 25% Maize and 75% Pigeon pea, E = 100% Pigeon pea

3.4. Phytochemical composition of *Agidi* from blends of maize and pigeon pea

Phytochemicals are anti-nutrients which have the capacity of decreasing the digestibility and palatability of protein because they form insoluble complex with them. They also cause deleterious effect on human health (Mbata *et al*., 2009). The phytochemical composition of *agidi*from blends of maize and pigeon pea are represented in Table 4. For all the *agidi* samples analyzed, there was low phytochemical contents which differed significantly ($p<0.05$) in all the parameters. Naturally, legumes including pigeon pea are said to contain some anti-nutrients (e.g.; tannin, polyphenols, phytate, oxalate, etc.) which limits their use and have deleterious effect on human health although most of these anti-nutrients are reduced or eliminate during some processing techniques like soaking, dehulling, sprouting, cooking, fermentation, etc (Sheel *et al.*, 2011). Soaking and fermentation are processing steps in *agidi* production which accounts for the massive reduction in the phytochemical contents of *agidi* as compared to the raw pigeon pea legume which has up to 6.88 mg/g of tannin. Tannin content ranged between 0.074 mg/g and 0.178 mg/g it slightly increased with the addition of pigeon pea to 0.088, 0.112, 0.155 and 0.178 mg/g for B, C, D and E respectively. Tannins are naturally occurring plant polyphenols. Their main characteristics is to bind and precipitate protein thereby interfering in its digestion and absorption. The lethal dose of tannin is 0.7-0.9 mg/100g (Pikuda and Illelaboye, 2013) and the tannin contents of the *agidi* sample blends gotten are far lower than the lethal dose. The tannin values compared favorably with those reported by Uchechukwu *et al*. (2017).

Table 4. Phytochemical composition of the *Agidi* samples made from blends of maize and pigeon pea

Samples	**Tannin (mg/g)**	**Phytate (mg/g)**	**Saponin (mg/g)**
A	0.074^{e}±0.00	0.133^{e}±0.00	0.137^{e}±0.00
B	0.088^{d}±0.00	0.151^{d}±0.00	0.148^{d}±0.00
C	0.112^{c}±0.00	0.185^{c}±0.00	0.170^{c}±0.00
D	0.155^{b}±0.00	0.237^{b}±0.00	0.192^{b}±0.00
E	0.178^{a}±0.00	0.251^{a}±0.00	0.207^{a}±0.00

Values are mean ± standard deviation of duplicate readings. Values on the same column with different superscripts are significantly different at $p<0.05$.A = 100% Maize (Control), B = 75% Maize and 25% Pigeon pea, C = 50% Maize and 50% Pigeon pea, D = 25% Maize and 75% Pigeon pea, E = 100% Pigeon pea

The phytate content ranged between 0.133 mg/g and 0.251 mg/g and varied significantly ($p<0.05$) between the control and the other sample blends. It increased slightly with the addition of pigeon pea to 0.151, 0.185, 0.237, and 0.251 mg/g for B, C, D and E respectively. The increase in the phytate content based on the addition of pigeon pea is as a result of its high content of phytate in pigeon pea of about 5.01 and 12.7 mg/g (Sheel *et al*., 2011). Saponin contents, varied significantly ($p<0.05$) the control (A: 100% maize) had the least saponin content (0.137 mg/g) as compares to the rest sample blends B, C, D and E for 0.148, 0.170, 0.192 and 0.207 mg/g respectively which later increased slightly with the addition of pigeon pea.

The increase in the phytate content based on the addition of pigeon pea is as a result of its high content in pigeon pea of about 5.01 and 12.7 mg/g (Sheel *et al*., 2011). Phytate is one of the anti-nutrients present in pigeon pea and which is known to form complexes with iron, zinc, calcium and magnesium thereby making them less available and thus inadequate in food samples especially for children. It is known that 10-50mg phytate per 100g will not cause a negative effect on the absorption of zinc and iron (Pikuda and Illelaboye, 2013). Although the phytate level increased with the addition of pigeon pea, it also decreased mainly due to the processing steps involved in *agidi* production (such as soaking, fermentation, cooking) and the values gotten are below the safe consumption range above. The phytate values also compared favorably with those reported by Uchechukwu *et al*. (2017).

Pigeon pea is a rich source of saponin up to 21.6 - 34.9 mg/g (Sexana *et al*., 2010). Saponins are good sources of antioxidants which are beneficial to human in the functioning of several organs and in treating various diseases. Ingestion of saponin has been linked with a decrease in the overall blood cholesterol (Owheruo *et al*., 2018).

3.5. Sensory characteristics

The results from the sensory evaluation for the *agidi* made from blends of maize and pigeon pea are represented in Figure 1. It showed the responses of 20 panelists that carried out the sensory evaluation. The results as shown in Table 5 indicated that all the samples were not rejected by the panelists as revealed by the rating of the mean scores. Significant differences at ($p<0.05$) were found to exist between the control (Sample A) and the other blends of the *agidi* samples in all the parameters tested.

Based on the appearance and color on the spider plot (Figure1), the control differed significantly and it was most preferred compared to the other sample blends. Both control and 25% inclusion of pigeon pea gave the same white color. But the inclusion of about 50% of pigeon pea resulted in the development of a grayish color while the use of only pigeon pea resulted in a brownish color. *Agidi* is normally known to be white or off-white in colour depending on the grain color, e.g.; *agidi* prepared from yellow maize gives a yellow colour and that prepared from white maize gives a white or off white color. Therefore, the color of the grain used generally affected the color and appearance of the *agidi.*

For the texture, firmness, smoothness and consistency, the control (A: 100% maize) was most preferred and it differed significantly from the other samples based on the rating of the mean scores. The control (A: 100% maize) had a firm and smooth texture with a thick consistency even at the inclusion of 25% pigeon pea (sample B), but at the inclusion of pigeon pea from about 50% and above (for sample C, D and E), it gave a smooth and moderately thick texture with a slightly watery consistency which needed not much pressure to break. Texture is of an important quality factor in *agidi* production as it influences the consumers' acceptance of the food. The addition of some materials for fortification during *agidi* production influences its texture and consistency either by decreasing or increasing it. Fermentation process and

duration also affect the texture of *agidi*, the longer the fermentation process the lesser or weaker the textural properties (Barbradozier, 2013). Similar effect in the texture and consistency was also reported by Kolawole *et al.* (2020) due to the addition of soybean and orange fleshed sweet potato in the production of *agidi*.

Agidi samples generally had a sour taste. The flavor, mouth-feel and aftertaste of the control (100% maize) were most preferred among the sample blends. The sour taste, flavor and mouth-feel however slightly decreased in intensity with the addition of pigeon pea. *Agidi* generally had a sour taste and flavor which is very common in fermented foods. Also, the higher the acidity during the fermentation process the stronger the fermentation flavor and sour taste (Cauvain, 2015). The least accepted were D and C because of their firmness and aftertaste.

In terms of overall acceptance, the control (Sample A) ranked the highest based on the parameters surveyed, followed by B and E based on the appearance, taste, flavor and aftertaste.

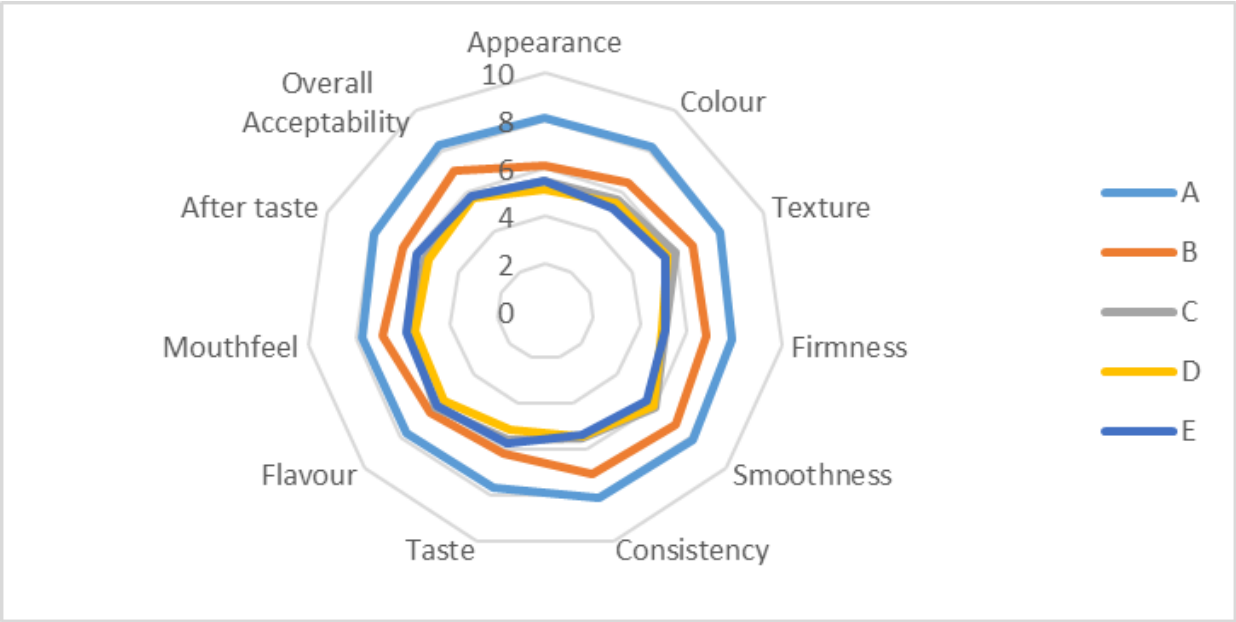

Figure 1. Spider diagram of sensory evaluation of *agidi* made from blends of maize and pigeon pea

(The spider plot represents mean sensory analysis scores for the five treatment of *agidi* made from blends of maize and pigeon pea. Scores are based on 9- point hedonic scale like extremely, 9; like very much, 8; like moderately, 7; like; slightly, 6; neither like or dislike, 5; dislike slightly, 4; dislike moderately, 3; dislike very much, 2; dislike extremely, 1.; number of panelists (n)=20. A = 100% Maize (Control), B = 75% Maize and 25% Pigeon pea, C = 50% Maize and 50% Pigeon pea, D = 25% Maize and 75% Pigeon pea, E = 100% Pigeon pea)

4. Conclusions

The result of this study has shown that *agidi* made from 100% maize is of a low nutritional value although it gave a better texture and appearance which are important quality factors that influences consumers' acceptance.

The protein, crude fibre, ash and fat contents increased with the addition of pigeon pea. Similar increment was also noticed for the mineral (Ca, K, Fe) contents based on the addition of pigeon pea. Therefore, the combination of maize and pigeon pea in the production of *agidi* resulted in a product of higher nutritional status although, its viscosity and acidity decreased with the addition of pigeon pea.

Hence, combining pigeon pea with maize in the processing of 'agidi' at the substitution level up to 25% did not vary much from the control

(100% maize) and was highly accepted by the consumers.

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APPENDIX

Plate 1. 'Agidi' Sample A (100% maize)

Plate 2. 'Agidi' Sample B (75% Maize and 25% Pigeon pea)

Plate 3. 'Agidi' Sample C (25% Maize and 75% Pigeon pea)

Plate 4. 'Agidi' Sample D (50% Maize and 50% Pigeon pea)

Plate 5. 'Agidi Sample E (100% Pigeon pea)

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THE RIPENING CONDITION EFFECT ON THE MAIN TEXTURAL PROPERTIES OF CASCAVAL CHEESE

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ABSTRACT

The textural characteristics represent a significant property used to distinguish various cheese types and are considered by the consumer as a determinant factor of acceptability and overall quality and preferences. Consequently, the main idea of the current research was to study the textural properties of Cascaval cheese during different ripening conditions and in accordance with the main chemical composition. The highest hardness under load was recorded by the unpacked ripened Cascaval samples, which also recorded the highest values for total mechanical work. The recoverable energy determined as the work of elastic deformation ranges from 0.19 to $0.31 \cdot 10^{-3}$ Joules, the highest values registered and the most resilient samples being represented by the ripened unpacked Cascaval. According to Pearson correlation results the modulus of deformability, hardness under load, total mechanical work, relaxation, and relaxation work positively influenced the TPA gumminess ($p < 0.01$, $p < 0.05$). It was also found that the texture parameters resulting from the application of the stress-relaxation method showed more correlations with the chemical composition of the tested samples compared to the TPA method, therefore the stress-relaxation method can be a more powerful tool in the evaluation of Cascaval cheese texture characteristics.

1. Introduction

Milk products inclusive cheeses, some of the oldest dairy products, represent an important group of foods largely consumed worldwide (Giosuè et al., 2022; Lei and Sun, 2019) due to the significant amounts of vital nutrients (valuable proteins, bioactive peptides, essential fatty acids, calcium, phosphorus, and vitamins like vitamin B12, vitamin A or vitamin D), (Roobab et al., 2021), and also of the probiotics content (Hernández et al., 2022).

According to Miller study (Miller et al., 2022) the mean cheese consumption reported globally was 8 g per day with a variation from 1 to 34 g per day, with the highest value of 34 g registered in Eastern-Central Europe and Central Asia, especially in the United Kingdom, France, and Turkey.

Among the categories of cheeses consumed in Romania, fresh cheeses, and cottage cheeses (cow or sheep) are preferred to the detriment of Cascaval cheese, which is more expensive. In 2019 cheese consumption from cow's milk was about 0.5 Kg per month, while sheep's cheese consumption was only 0.26 Kg per month, and the consumption of Cascaval cheese was 0.15 Kg per month (Petre and Drăghici, 2021).

The evaluation of cheeses' quality characteristics is critical for both consumers and the industry and when discussing the quality of cheeses, reference is made to the chemical composition, flavor, appearance, and physical properties represented by cheeses' sensory,

textural, and rheological characteristics (Lei and Sun, 2019).

Food texture is a complicated attribute and according to ISO 11036, (ISO 11036:2020) represents all the mechanical, geometrical, and surface characteristics of a food product, being evaluated as a response of the food to a given constraint. The instrumental texture evaluation is categorized as an objective method of analysis and in comparison with the sensory evaluation offers faster and more accurate results (Guiné, 2022). The sensory evaluation is time-consuming and requires a much longer effort, is influenced by a lot of factors and its constancy and repeatability are tough to guarantee (Liu et al., 2019).

The textural characteristics represent a significant property used to distinguish various cheese types and are considered by the consumer as a determinant factor of acceptability and overall quality and preferences (Allen Foegeding et al., 2003). For texture analysis, the compression tests such as texture profile analysis (TPA) are usually used and textural parameters like hardness, springiness, adhesiveness, gumminess, chewiness, and cohesiveness are determined by the food response (Giha et al., 2021; Chen and Opara, 2013). The majority of cheese varieties are thought to be soft-solid material comprising networks composed of proteins, fats, and water; their textural characteristics are associated with the network composition, structure, and interactions between molecules within the network (Foegeding and Drake, 2007).

The Cascaval cheese, known also as Kashkaval in the Balkans (Nikolova et al., 2022), Kasseri in Greece, and Kashar in Turkey (Ozturk et al., 2022) is particularly popular in Eastern Europe and falls into the semi-hard pasta-filata (stretched curd) cheese category produced from cows milk, sheep milk, or a mixture of both (Gherghina et al., 2021). The production process implies curd stretching and kneading in hot water to obtain desired texture characteristics (plastic elastic consistency) and a fiber-like structure (Talevski et al., 2017). Furthermore, when it comes to Cascaval production, maturation is a critical and complex process that emphasizes the textural characteristics and the specific flavor (Andronoiu et al., 2015). Generally, the maturation process of cheese takes place under carefully controlled environmental conditions. Depending on the type of cheese, these parameters may vary in temperature, relative humidity, and time, which may have a distinct impact on the cheese's biochemical modification (Di Cagno et al., 2007).

This variety of pasta-filata cheese has been studied by other authors as well regarding the sensory properties developed at different maturation temperatures (Ivanova et al., 2021), the effect of raw and pasteurized sheep's milk on microbiological quality (Pappa et al., 2019; Samelis et al., 2019), the temperature influences on lipid hydrolysis and fat oxidation (Ivanova et al., 2020), the effect of temperature storage (Ivanov et al., 2021) and maturation temperature (Ivanov et al., 2018) on Kashkaval texture.

Due to the importance of the textural properties of cheeses for both consumers and processors, this study focused on the evaluation of instrumental texture characteristics of different Cascaval cheeses based on double compression and stress-relaxation methods in accordance with the chemical composition.

2. Materials and methods

The Cascaval samples were produced from raw cow milk (3.5 % fat) following the traditional technology (Andronoiu et al., 2015), which involves in the first phase the coagulation with rennet (Fromase® 50 Chr.Hansen) and ripening the cheese curd up to a pH of 5.0±0.1, followed by the cheese curd scalding in hot water, 73±2°C with 10 ±1% NaCl and manual kneading and stretching. After the forms removing the cheese samples were dried and divided into three categories (Fig. 1); the first one was directly analyzed (fresh Cascaval - A), the second category was ripened directly for sixty days without being packed (ripened Cascaval - B), and the third category was vacuumed in polypropylene (PP) and ripened also for sixty days (packaged Cascaval - C).

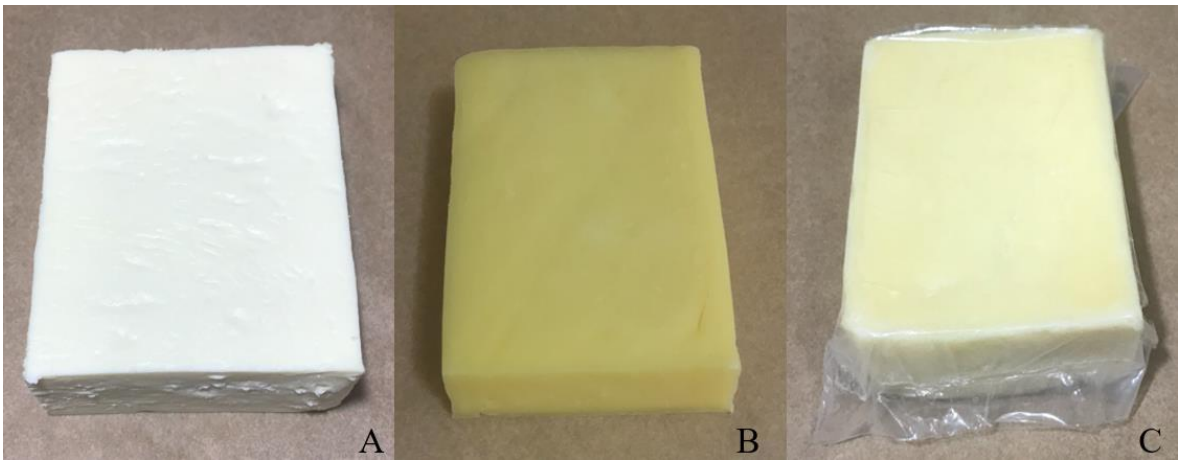

Figure 1. The appearance of fresh Cascaval cheese (A), ripened without packaging Cascaval cheese (B) and ripened in packaging Cascaval cheese (C).

2.1. Physicochemical analysis.

The fat content of Cascaval samples was determined by ISO 3433 (ISO 3433:2008), the protein content was measured by the Kjeldahl method (ISO 17837:2008), the moisture content was assessed by the oven drying to constant mass at 102±2 °C, and the dry matter was also determined according to ISO 5534 (ISO 5534:2004). The titratable acidity expressed as % lactic acid was measured using NaOH and phenolphthalein (Horwitz, 2010)**.** All used reagents were analytical grade from Sigma Aldrich (Germany) and the analyses were made in triplicate.

2.2. Textural analyses.

The stress relaxation method is not so frequently used in the textural evaluation of food products compared to Texture Profile Analysis (TPA) which is widely used. Regarding the Cascaval cheese, there are no reported studies about the evaluation of texture properties through stress relaxation tests. The textural evaluation of Cascaval samples was made with a texture meter from Mark 10 (ESM 301), the used load cell had 100 Newtons (Mark 10 Corporation, USA). The Cascaval samples had cubic shapes with sides of 2 cm (Nyamakwere et al., 2022; Pappa et al., 2020) and were tested by a stress relaxation test and a TPA using a flat probe with a 5 cm diameter. The cheese sample compression rate was established at 30% (Gutt et al., 2014) and relaxation time was set at 10 minutes (t), (Peleg and Pollak, 1982) for the stress relaxation test and 50% for the TPA (Nyamakwere et al., 2022). The loading/unloading was set at 10 mm/min, the trigger force was set as 0.2 N. The output data (load, time, and deformation) for both tests were registered by the MESUREgauge software (Mark 10 Corporation, USA) with a reading rate of 8 points per second (pps). The MESUREgauge software and the resulting stress-deformation curves (Fig. 2) were the basis for the determination of textural properties like hardness under load (Hs, N/mm) calculated as the ratio of the maximum load and the corresponding deformation, total mechanical work (Wt, J) represented by the total area under the loading stress-relaxation curves, elastic mechanical work (We, J) represented by the area under the unloading curves, the loading modulus of deformability (E_L, Pa), relaxation (R, N/s, Eq. 1). The relaxation work (Wr, N·s) was represented by the area under the relaxation curves (Gutt et al., 2014; Zimbru et al., 2020a).

$$R = (F_i - F_f)/t \quad (1)$$

Where F_i and F_f are the initial and final load of relaxation and t represents the time of relaxation in seconds.

The TPA primary parameters (hardness-H, adhesiveness-A, springiness-S, cohesiveness-C,

viscosity-V) and secondary parameters (gumminess-G, chewiness-Ch, resilience-R) parameters were also calculated from the travel-load curves with MESUREgauge software (Mark 10 Corporation, USA), (Zimbru et al., 2020b; Pădureț et al., 2017).

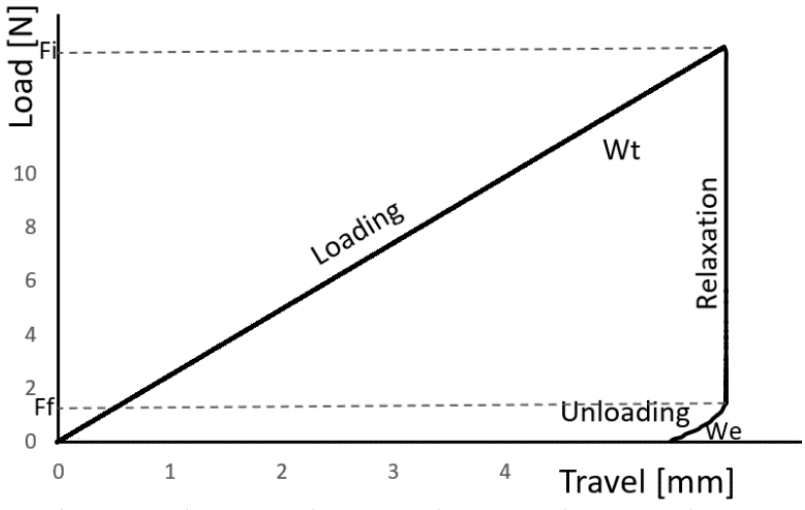

Figure 2. Loading - unloading curve of cheese during the stress-relaxation test.

2.3. Statistical analysis. For the sample's differentiation, the analysis of variance ANOVA (α=0.05) was conducted using Statgraphics Centurion XVI software and multiple comparisons of means using Fisher's least significant difference (LSD) at the 0.95 confidence level. The Pearson correlation was performed with SPSS 16 (SPSS Inc. Chicago, IL).

3. Results and discussions

3.1. Physicochemical analysis.

Figure 3 shows the physicochemical characteristics of Cascaval cheese analyzed in this study. The fat content, protein, and moisture/dry matter are the main composition of cheeses, in addition, cheese proteins are valuable since they are almost completely digestible, and according to Fox 2004 (Fox et al., 2004) the cheeses protein content is influenced negatively by the fat content. The protein content of Cascaval samples ranges between 23.1% for fresh analyzed samples – A category and 24.5% for unpacked ripened Cascaval samples – B category, while the fat content varied from 41.2% registered for fresh analyzed samples – A category to 48.5% for unpacked ripened Cascaval samples – B category. For both fat and protein content, the packaged ripened Cascaval – C category presented intermediate values of 42.9% and 23.6%. The moisture content values are an important factor in cheese classification and according to McSweeney 2017, (McSweeney et al., 2017) the cheeses evaluated belong to the semihard category, with 43% - 55% moisture, (A category – 52.86% and C category – 45.1%) and hard category, with moisture content less than 42%, (B category – 41.3%). The dry matter content of Cascaval samples increased during ripening, the highest values were measured for B category, unpacked ripened Cascaval samples, (58.7%) due to the water evaporation and rind formation; similar observation was reported also by Andronoiu (Andronoiu et al., 2015). The cheese's moisture and fat content are two important factors of great importance that influence the texture parameters of cheeses (Gunasekaran and Ak, 2002) furthermore, other studies (Nogueira et al., 2021) have shown that vacuum-packaging provides a bitter taste to Canastra artisanal cheeses, while unpackaged cheeses show a dry and brittle texture. The titratable acidity results, expressed as lactic acid, were close to those

reported by Tumbarski (Tumbarski et al., 2021) and the highest values were measured for the C category Cascaval samples that were vacuumed in a polypropylene bag and ripened for sixty days.

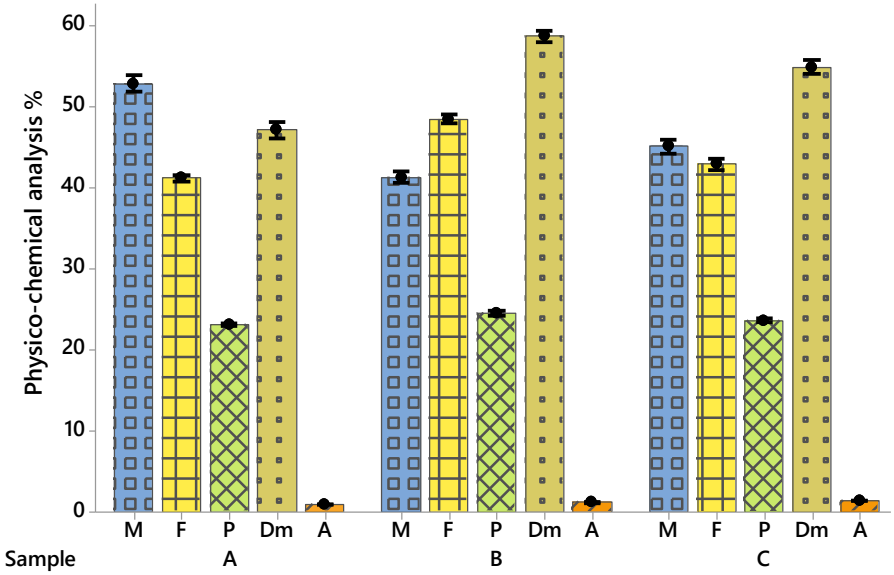

Figure 3. Physicochemical results of Cascaval samples: A-fresh Cascaval, B-unpacked ripened Cascaval, C- packaged ripened Cascaval, M-moisture, F-fat, P-protein, Dm-dry matter, A-titratable acidity

3.2. Textural evaluation.

According to Foegeding 2003 (Allen Foegeding et al., 2003), instrumental texture properties differentiate many kinds of cheese and are taken into account by consumers when determining a cheese's overall quality and preference.

Figure 4 presents the results of the applied stress-relaxation method, and it can be noticed that the analyzed cheese samples were found to exhibit viscous and elastic comportment, specifically viscoelastic solid comportment, with a decrease in stress with time. Table 1 shows the textural parameters measured by the stress relaxation method. The hardness under load (Hs) of Cascaval samples varied from 2.39 N/mm to 3.23 N/mm, the highest values being recorded by the maturated unpacked, B category Cascaval, which also recorded the highest values for total mechanical work (Wt). The measured load values were close to those reported by Darnay 2019, (Darnay et al., 2019) for semi-hard goat cheese. The recoverable energy used for the cheese sample deformation is represented by the work of elastic deformation (We), which ranges from 0.19 to $0.31 \cdot 10^{-3}$ Joules, the highest values registered, and the most resilient samples being represented by the B-unpacked ripened Cascaval. The loading modulus of deformability (E_L) describes the material stiffness and was determined as the slope of the loading stress-strain curves, which is related also to the hardness texture parameter (Serna Cock et al., 2012). The data demonstrate that for Cascaval samples with high hardness and high values of total mechanical work, the modulus of deformability was higher. It can be observed that the modulus of deformability values increased during maturation time, with a high value registered for unpacked ripened Cascaval samples (B-161.5 KPa); the sample differentiation was done at a level of $p < 0.05$. Similarly, Watkinson 2001 (Watkinson et al., 2001) reported an increase in the modulus of deformability for Cheddar and Gouda cheese and this was associated mostly with the moisture decrease and partial triglyceride crystallization. As regards the relaxation and relaxation work results it can be noticed that the values are close to each other, the lower values being measured

for unripened group samples (A), whereas the highest values were measured for maturated unpacked samples (B); the obtained results were in the same range to those previously stated for fresh meat (0.0113 – 0.044 N/s) (Gutt et al., 2014). In addition to the ones mentioned, Table 1 also presents the results of Cascaval TPA.

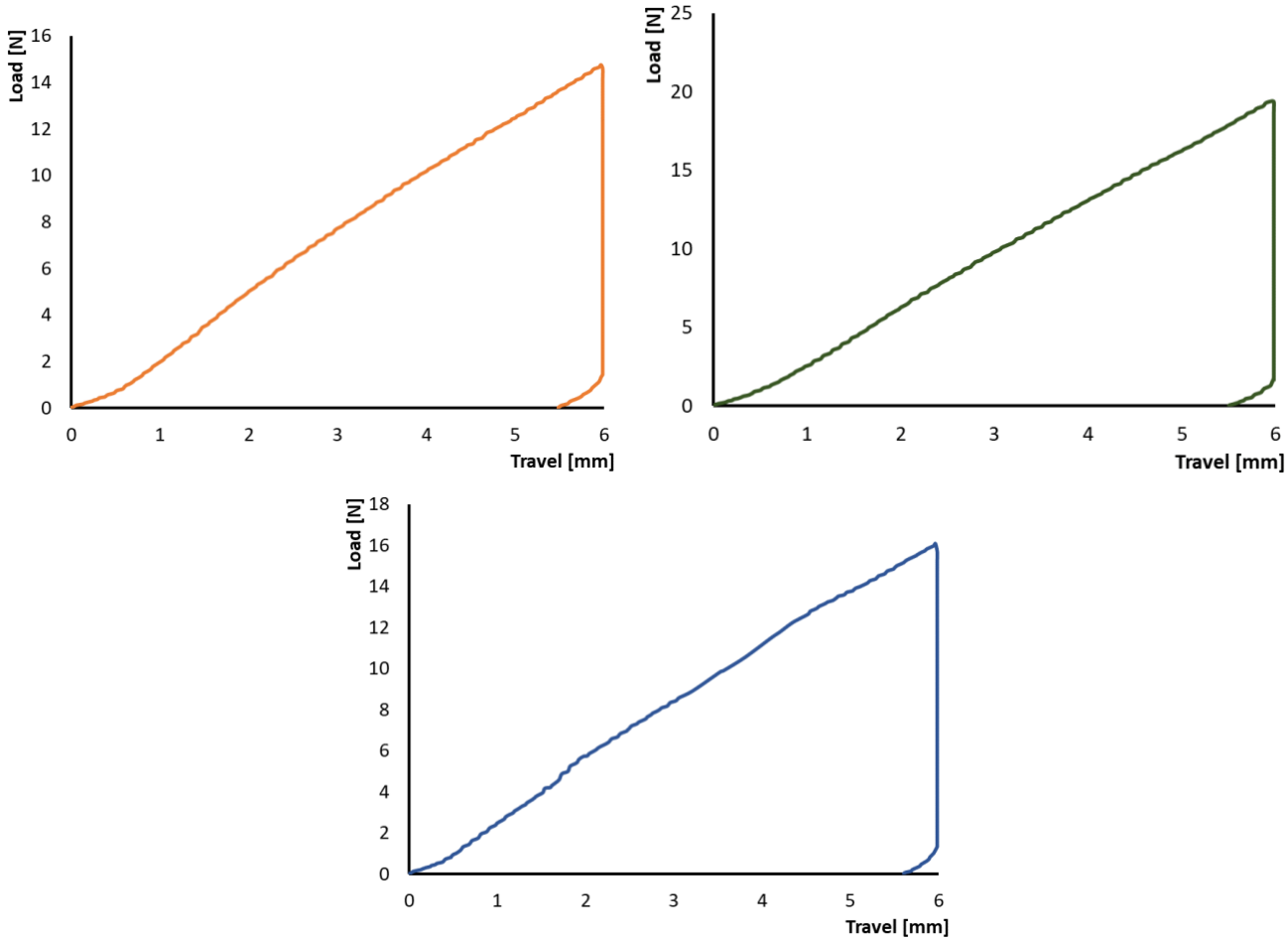

Figure 4. Stress-relaxation curves of Cascaval samples: A-fresh Cascaval (—), B-unpacked ripened Cascaval (—), C- packaged ripened Cascaval (—).

Table 1. The textural properties of Cascaval samples.

Sample		A	B	C
Stress-relaxation texture parameters				
Hardness under load - Hs	[N/mm]	2.39b (0.16)	3.23a (0.17)	2.68b (0.13)
Total mechanical work - Wt	[$\cdot 10^{-3}$ J]	42.53b (2.58)	54.36a (4.58)	47.32b (2.91)
Elastic mechanical work - We	[$\cdot 10^{-3}$ J]	0.28a (0.10)	0.31a (0.09)	0.19a (0.08)
Modulus of deformability – E_L	[KPa]	123b (3.85)	161.5a (8.23)	134b (5.89)
Relaxation - R	[N/s]	0.022c (0.001)	0.029a (0.001)	0.025b (0.001)

Relaxation work - Wr	[N·s]	2035c (12)	2576a (22)	2133b (19)
TPA texture parameters				
Hardness	[N]	16.48b (1.08)	19.74a (0.42)	18.7a (0.32)
Viscosity	[N]	0.32c (0.10)	0.80a (0.08)	0.64b (0.05)
Cohesiveness	[%]	47.65a (3.01)	47.68a (2.21)	44.57a (2.87)
Adhesiveness	[mJ]	0.48b (0.10)	1.05a (0.21)	1.06a (0.23)
Springiness	[%]	75.35a (2.58)	67.20b (1.98)	63.98b (1.58)
Gumminess	[N]	7.85b (0.58)	9.41a (0.34)	8.33b (0.51)
Chewiness	[N]	5.91ab (0.48)	5.33b (0.19)	6.32a (0.28)
Resilience	-	0.24a (0.02)	0.23ab (0.01)	0.21b (0.01)

Mean values and standard deviations. Different letters (a–c) in a row show significant differences between samples ($p < 0.05$) evaluated with One way ANOVA test.

The hardness, viscosity, adhesiveness, and gumminess texture parameters of ripened Cascaval samples showed higher values compared to fresh ones. Also, other authors (Pappa et al., 2020) reported higher hardness values (22-24 N) for sheep–goat milk Kashkaval cheese using the same sample dimensions with the difference that the sample was compressed to 60% of its original height, this aspect could explain the slight difference between values. The ANOVA statistical analysis highlighted differences in hardness, which varied from 16.48 to 19.74 N, at a level of $p<0.05$; the ripened Cascaval samples, B and C categories belonged to the same statistical group. This variation could be due to the moisture decrease, a recent study reported by Nyamakwere 2022 (Nyamakwere et al., 2022) also supports that cheese hardness values increase as the moisture content decreases. The TPA viscosity, calculated as the negative force of the TPA curve, ranged between 0.32 N for fresh Cascaval (A-category) to 0.80 N for unpacked ripened Cascaval (B-category), whereas the packaged ripened Cascaval samples (C-category) showed middle values; the statistical analysis differentiates the samples at $p<0.05$. The recording of the TPA in load versus travel made it possible to calculate the Cascaval adhesiveness as energy expressed in millijoules, according to Bourne 2002 (Bourne, 2002) and ISO 11036:2020 (ISO 11036:2020, n.d.). The analyzed ripened Cascaval samples displayed a higher adhesiveness compared to the fresh ones, there were no differences between unpacked or packaged ripened samples. Considering the statistical analysis results it can be observed that the cohesiveness values of Cascaval cannot be used in sample differentiation; the obtained results belong to the same statistical group. Among the secondary texture parameters, gumminess and chewiness are more complex properties and are directly influenced by the cohesiveness values. Furthermore, cheese chewiness is influenced by the softness of the cheese body and also by the structure of the casein network (Tudoreanu and Dumitrean, 2009). The unpacked ripened Cascaval (B-samples) presented the highest gumminess 9.41 N, followed by packaged ripened Cascaval 8.33 N (C-samples), while fresh Cascaval samples (A-category) had the lowest result 7.85 N. The

springiness decreased after sixty days of ripening, from 75.35% to 63.98%, similar to the results reported by (Fuentes et al., 2015) for vacuumed pasta-filata Mexican cheese. According to Tudoreanu and Dumitrean, 2009 (Tudoreanu and Dumitrean, 2009) the decrease in Cascaval springiness involves the proteolytic breakdown of the protein matrix, leading to a creamier dairy product. In the case of textural parameters, resilience can be defined as the ability of the tested sample to return to its initial position/shape (Zimbru et al., 2020b). The resilience of the tested Cascaval samples were close to each other, ranging between 0.21 to 0.24, with the fresh samples (A category) showing a higher capacity to return to their initial position/shape than ripened ones.

3.3. Pearson correlation

Between physicochemical characteristics of Cascaval samples, stress-relaxation, and TPA texture parameters was performed, and a strong positive correlation ($p < 0.01$) was recorded between protein content and hardness under load, whereas the dry matter content influences, at the same level, the TPA hardness and viscosity results, and consequently these textural parameters are both negatively influenced by the moisture content ($p < 0.01$). The influence of protein content on Kashkaval hardness was also reported by Pappa 2020, (Pappa et al., 2020). Another positive correlation ($p < 0.05$) was observed between fat and modulus of deformability, hardness under load, relaxation work, and gumminess, and also between protein content and total mechanical work, modulus of deformability, relaxation, and gumminess. It could be observed that the modulus of deformability increased with both fat and protein content.

According to Pearson correlation results the stress-relaxation and TPA texture parameters of Cascaval samples were connected between them. The modulus of deformability, hardness under load, total mechanical work, relaxation, and relaxation work positively influenced the TPA gumminess ($p < 0.01$, $p < 0.05$). Based on the Pearson statistical analysis, it was found that the texture parameters resulting from the application of the stress-relaxation method showed more correlations with the chemical composition of the tested samples compared to the TPA method, therefore the stress-relaxation method can be a more powerful tool in the evaluation of Cascaval cheese texture characteristics.

4. Conclusions

The main composition of analyzed Cascaval samples was influenced by the ripening condition, both fat and protein content of the packaged ripened Cascaval presented intermediate values. The dry matter content of Cascaval samples increases during ripening, the highest values were measured for unpacked ripened Cascaval samples, due to the water evaporation and rind formation. In terms of textural evaluation, it could be observed that the Cascaval modulus of deformability, hardness under load, TPA hardness, and viscosity increased during the ripening period, whereas springiness decreased after sixty days of ripening. It could also be observed that the modulus of deformability increased with both fat and protein content, while the relaxation work was influenced only by fat content. The data also showed that for Cascaval samples with high hardness and high values of total mechanical work, the modulus of deformability was higher.

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