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Research Article

ULTRASOUND-ASSISTED EXTRACTION OF FLAXSEED MUCILAGE AND ITS APPLICATION AS A FAT AND EGG REPLACER IN CUPCAKES

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Abstract

The present study was designed to investigate the effects of two extraction techniques—conventional hot water extraction and ultrasound-assisted extraction—were compared for flaxseed mucilage (FM) in terms of yield, composition, and antioxidant activity. The yield by hot water extraction technique was 5.30%, while Ultrasound-assisted extraction (from 10 to 30 min) enhanced the yield from 5.25 to 6.95%. Additionally, carbohydrates content decreased, while protein, phenols and lignan contents increased with longer ultrasound treatment. The extracted FM with ultrasound technique showed higher scavenging ability on DPPH and ABTS radical. This research supports the development of functional, value-added flaxseed products and highlights the potential used of FM as a novel food hydrocolloid, including and its application as fat and egg replacer in cupcakes.

Cupcakes were prepared with varying substitution levels (25, 50, 75, and 100%) from egg and oil, and their sensory, physical, and textural properties were evaluated. Results showed that 25–50% replacement levels provided acceptable quality and consumer acceptability, demonstrating the functional viability of flaxseed mucilage in cupcakes. In general, the replacement of FM (25%) as fat or egg replacement in cupcake formulation showed higher scores in all sensory attributes as compared to the control. Results demonstrated that flaxseed mucilage enhanced the total phenolic content of cupcakes when used as a substitute for fat, eggs, or both.

1. Introduction

Flaxseed is classified as one of the most important functional foods because of its content of polyunsaturated acid (Omega 3), lignan, dietary fiber, phenols compounds, protein and mucilage (Azarpazhooh et al., 2021). Mucilage consists of biologically interesting macromolecules rich in polysaccharides. It dissolves when it comes into contact with water and produces a viscous substance, like gelatin. Mucilage has good functional properties and used as food coatings,

edible films, smart packaging, pharmaceutical gels, encapsulation material and fat replacer (Prajapati et al., 2013; Elsorady, 2016; Mohtarami et al., 2022; Elsorady et al., 2024a,2024b). Mucilage has an important role in food industry because of its emulsifying properties. Flaxseed mucilage (FM) is a valuable hydrocolloid with functional properties similar to those of gum arabic. FM exhibits excellent rheological properties and strong oil- and water-binding capacity. It contains 50–80% carbohydrate, 4–20% protein, and 3–9% ash (Puligundla and Lim, 2022).

FM is widely used in food as a texturing agent due to its thickening properties. It is used as a lubricant for skin surface in cosmetic and pharmaceutical applications. Food contains FM can prevent intestinal inflammation and decreasing blood glucose and blood cholesterol (Fabre et al., 2015). FM is one of the by-products of flaxseed about 3–10%. Its composition depends on variety, cultivation practices, and environmental conditions. FM possesses valuable functional properties—such as water-holding capacity, emulsifying ability, fat replacement, texture modification, stabilization, and interfacial activity—which make it an essential ingredient in diverse food applications, including gluten-free baked goods, plant-based meat and dairy alternatives, salad dressings, edible gels, and emulsions (Chand et al., 2024).The yield, purity, and functional properties of FM depend on extraction method and temperature (Lorenc et al., 2022). The most common method for FM extraction is aqueous extraction which is not considered time-and energy efficient. So, the best emerging technique is sonication due to its simplicity, cost efficiency, and optimum extraction yield with fixed biological properties. Sonication has various advantages such as low temperature and energy requirements, lower extraction time, and improve quality of extracted material. Ultrasonic waves disrupt tissues via physical forces developed during the cavitation process, which accelerate the extraction of organic compounds, contained within the body of plants and enhanced mass transfer of cell contents in a short time (Hromádková et al., 1999, Chand et al., 2024). Several studies have demonstrated the benefits of ultrasonication in extracting bioactive compounds, such as antioxidants and polysaccharide gums, from plant-based sources (Akhtar et al., 2019; Chemat et al., 2020; Kumar et al., 2021; Manzoor et al., 2021).

Cakes are among the most widely consumed bakery products worldwide, appreciated by people from diverse backgrounds. Cakes structure is often made up of gelatinized starch particles enclosed in foam-forming egg white protein. The proteins that give cakes their strength and shape were present in both flour and eggs. The egg's and cake flour's structural strengthening effects are counterbalanced by the sugar and fat ingredients' tenderizing effects (Salem et al., 2024)

Hydrocolloids are proteins or polysaccharides which have functional properties such as thickening, gelling, stabilizing, film forming, dispersing and texture modifying that are successfully used in food products (Elsorady, 2016). FM is used in the food industry to enhance stability, increase viscosity, improve emulsifying ability, and provide desirable rheological and foaming properties (Mueed et al., 2022). Consumers have become more health conscious in recent years. For that, there is a growing trend for rising low-fat foods as consumers are increasing looking to modify their dietary intake to reduce fat (Yang et al., 2007). Fat replacers are commonly used in baked products, offering similar functional properties to fat but with fewer calories (Mohtarami et al., 2022). Egg is one of the most used and consumed ingredients in food applications (Miranda et al., 2015). It is usually used into food to improve sensory, emulsifying, foaming and gelling properties (Abeyrathne et al., 2013; Gallo et al., 2020). However, with the increasing demand for vegan options and food allergies to eggs (Gallo et al., 2020), FM was explored as fat and egg replacer in cupcakes. This study aimed to extract FM using ultrasound-assisted techniques and to evaluate

its effectiveness as a fat and egg replacer in product cupcake by fortified with flaxseed mucilage extraction to become a functional healthy food high in bioactive components

2. Materials and methods

2.1. Materials

Flaxseed meal (Giza12), was obtained as byproduct after oil extraction, was defatted. All chemicals and reagents were obtained from Sigma-Aldrich (St.Louis, USA) and El-Gomhoria Co. for Pharmaceutical (Alexandria, Egypt).

2.2. Methods

2.2.1. Flaxseed mucilage (FM) extraction

FM was extracted by hot water extraction and ultrasound-assisted extraction.

2.2.1.1. Hot water extraction

FM was extracted from flaxseed meal using hot water extraction according to Elsorady et al. (2024a) with minor modification. Flaxseed meal was soaked in distilled water (1:8 w/v) with continuous stirring (2 hr) at 90 °C. Then soaked meal was filtered, centrifuged (4,000 rpm, 10 min). The precipitated gum was lyophilized.

2.2.1.2. Ultrasound treatment

Ultrasound-assisted extraction was conducted according to Mahmod et al. (2023), with minor modifications. Flaxseed meal was sonicated by ultrasonic waves, a SELECTA ultrasonic bath. Flaxseed meal was bottled with distilled water (1:8 w/v) and put into bath filled with distilled water. Flaxseed meal was treated with ultrasound waves for various times (10, 15, 20, 25 and 30 minutes). The soaked mixture was then filtered and centrifuged (4,000 rpm, 10 min). The precipitated mucilage was subsequently lyophilized.

2.2.2. Effect of extraction method on yield and composition of FM

The yields and compositions of the FM samples extracted with different methods were compared.

2.2.2.1 Yield of flaxseed mucilage

Extracted mucilage yield was calculated as follows:

$$\text{Yield (\%)} = (G / S) \times 100 \quad (1)$$

where 'G' is the weight of dried mucilage after extraction from Flaxseed meal and 'S' is Flaxseed meal weight (Elsorady et al., 2024a).

2.2.2.2. Proximate composition of FM

Moisture, protein, fiber, and ash contents were determined according to AOAC (2010). Total carbohydrates were determined by difference. Caloric value (Kcal/ 100g) was calculated from the sum of the percentages of crude protein and total carbohydrates multiplied by a factor of 4 plus the crude fat content multiplied by 9 according to Zambrano et al., (2004).

2.2.2.3. Total phenols content

Using Folin–Ciocalteu reagent, total phenol content was colorimetrically determined at 725 nm as described by Elsorady et al. (2024a).

2.2.2.4. Lignan extraction

Lignan content (%) was determined using the method described by Elsorady et al. (2024a).

2.2.3. Effect of extraction methods on antioxidant activity of FM

2.2.3.1 DPPH scavenging activity

The scavenging ability on 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical was determined according to Elsorady and Abdl Aziz (2011) with minor modifications. The extracted FM by different methods (5, 10, 15, 20, 25, and 30 mg/ml) was dissolved in distilled water (0.5 ml) and mixed with 1 ml of methanolic solution including DPPH radicals, resulting 0.2 mM DPPH as end concentration. The mixture was agitated, and kept in dark at 22 °C, and the absorbance was then estimated at 517 nm against a blank. The scavenging ability was calculated as follows:

Scavenging ability (%) = [(Δ A517 of control – Δ A517 of sample) / Δ A517 of control] (2)

2.2.3.2. ABTS scavenging activity

The scavenging ability on ABTS was determined according to Liang et al. (2017). ABTS (7 mM) and potassium persulfate ($K_2S_2O_8$, 2.45 mM) solutions were mixed and incubated in the dark for 24 hours, then diluted with 10 mM phosphate buffer (pH 7.4) until the

absorbance at 734 nm reached approximately 0.700.of FM {30 µL of various amounts (5, 10, 15, 20, 25, and 30 mg)} were blended with diluted solution of ABTS and absorbance at 734 nm was measured before and after adding FM. The scavenging ability was measured as follows:

Scavenging ability (%) = [(Δ A734 of control – Δ A734 of sample) / Δ A734 of control].

(3)

2.2.4. Preparation of Cupcakes blends

Cupcakes were prepared following the formulations in Table 1, using a procedure adapted from Moiraghi et al. (2013) with slight modifications.. Egg and oil were substituted with FM solution after filtration at ratios 25, 50, 75 and 100 %, all ingredients have been previously equilibrated at room temperature. Then, oil and FM were creamed with sugar using Moulinette machine (Model 320, cod 25, France) for 8 minutes. Next, the rest of the wet ingredients were added followed by dry ingredients and mixed under constant mixing. The dough was transferred to greased baking pans and baked in an oven at 160°C for 35 minutes. After baking, the cupcakes were removed from the pans, left for 1 h at 25°C to cool and then packed in plastic bags to prevent drying. Cupcake evaluation was conducted after 24 hours of storage at room temperature. All treatments were prepared in laboratories Home Economics Department, Faculty of Specific education, Kafrelsheikh Univ., Kafrelsheikh, Egypt.

Table 1. Formulation of Cupcakes with different levels of FM as fat and egg replacers.

Ingredients	**FM as fat replacer**				
	Control	25%	50%	75%	100%
Flour (g)	250	250	250	250	250
Sugar (g)	100	100	100	100	100
Oil (ml)	100	75	50	25	-
FM (ml)	-	25	50	75	100
Egg (g)	150	150	150	150	150
Vanilla (g)	1	1	1	1	1
Baking powder (g)	3	3	3	3	3
Milk (mL)	125	125	125	125	125
Ingredients	**FM as egg replacer**				
	Control	25%	50%	75%	100%
Flour (g)	250	250	250	250	250
Sugar (g)	100	100	100	100	100
Oil (ml)	100	100	100	100	100
Egg (g)	150	112.5	75	37.5	-
FM (ml)	-	37.5	75	112.5	150
Vanilla (g)	1	1	1	1	1

Baking powder (g)	3	3	3	3	3
Milk (mL)	125	125	125	125	125
Ingredients	**FM (No egg and No fat)**				
Flour (g)	250	250			
Sugar (g)	100	100			
Oil (ml)	100	-			
Egg (g)	150	-			
FM (ml)	-	250			
Vanilla (g)	1	1			
Baking powder (g)	3	3			
Milk (mL)	125	125			

2.2.5. Sensory evaluation

Sensory evaluation was conducted on control cupcakes and those containing flaxseed mucilage as fat and egg replacers, using a panel composed of staff and students (both male and female) from the Home Economics Department, Kafrelsheikh University. The panelists evaluated the cupcakes for color, uniformity, softness, odor, taste and overall acceptability. Each sensory attribute was rated on a 5 point hedonic scale (1 = very poor, 2 = poor, 3 = moderate, 4 = good and 5 = very good) (Noshad et al., 2022).

2.2.6. Physical properties and Water Activity

Weight (g), height (cm), volume (cm^3) was measured at room temperature (AACC, 1983). The specific volume was determined as the ratio of volume to weight (Pong et al., 1991). Water activity of cupcakes was determined by a Rotronic Hygro Lab EA10-SCS apparatus (Switzerland). Each sample was analyzed in triplicate.

2.2.7. Proximate composition, caloric value and total phenols of cupcake

Moisture, protein, fibers, and ash were determined according to AOAC, (2010). Total carbohydrates were determined by difference. Caloric value was calculated as the following equation according to Zambrano et al., (2004):

$$\text{Caloric value (Kcal/ 100g)} = 4(P+C) + 9F \quad (3)$$

Where P for proteins, C for carbohydrates and F for fats.

The methanolic extract of cupcakes was prepared as described by Giri et al. (2024) [1 g sample was prepared in 10 ml of methanol and kept for overnight, followed by shaking for 3 h, centrifugation (for 15 min, 10,000 rpm)]. Total phenols content (TPC) of the methanolic extract was determined colorimetrically using Folin-Ciocalteu method (Elsorady 2024a).

2.2.8. Color properties

The colors of cupcakes samples were determined by the colorimeter (Minolta model CR-410, Tokyo, Japan). Results were expressed as lightness-darkness (L), greenness-redness (a), and blueness-yellowness (b) (Mohtarami, 2019).

2.3. Data analysis

Data were illustrated as mean and statistical analysis was performed using ANOVA test by SPSS (V16). The values $P < 0.05$ were considered as statistically significant.

3. Results and discussions

3.1. Effect of extraction method on yield and proximate composition of FM

The extraction yield of FM by hot water extraction (conventional method) was 5.30% (Table 2). According to Safdar et al. (2020), higher temperatures enhance solute solubility and diffusion rates, leading to greater mass transfer and improved extraction efficiency.

The effect of ultrasound extraction time on FM yield is presented in Table 2. Results revealed that increasing ultrasound time significantly affected the yield of FM. Extending ultrasound treatment from 10 to 30 minutes increased the FM yield from 5.25% to 6.95%. The mechanical disruption of cell walls resulting in increased accessibility (Ying et al., 2011).These results agreed with that obtained by Safdar et al. (2020).

Table 2. Effect of extraction method on yield and proximate composition of FM*

Parameters	Hot water extraction	Ultrasonic assisted extraction				
		10 min	15 min	20 min	25 min	30 min
Yield (%)	5.30^{d}	5.25^{d}	5.83^{c}	6.11^{bc}	6.32^{b}	6.95^{a}
Moisture	5.11^{c}	5.56^{ab}	5.51^{b}	5.54^{b}	5.16^{c}	5.76^{a}
Protein	9.52^{d}	8.29^{e}	9.35^{d}	10.84^{c}	11.76^{b}	13.63^{a}
Ash	4.84^{d}	4.78^{d}	5.43^{c}	5.92^{b}	6.23^{ab}	6.47^{a}
Carbohydrates	80.53^{b}	81.37^{a}	79.71^{b}	77.70^{c}	76.25^{c}	74.25^{d}

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

During ultrasound treatment, the ultrasound waves create acoustic cavitation which leads to cell wall rupture and creates pores through them. These lead to high penetration of solvents into the cellular matrix which, in turn, facilitate and accelerate mass transfer (Vinatoru, 2001).

Effect of ultrasonic assisted extraction method on proximate composition of FM was illustrated at Table (2). Carbohydrates were decreased from 81.37 to 74.25% with increment time of extraction up to 30 min. Also, ultrasonic assisted extraction for 10 min increased the carbohydrates content as compared to that extracted with hot water extraction. Also, Safdar et al. (2020) observed that carbohydrates were decreased with with increasing ultrasonic extraction time up to 30 min. Wang et al. (2022) indicated that the carbohydrate content of chia mucilage decreased significantly with extraction time and temperature. It may be related to several compounds (for example, insoluble substances) that could be suspended in the extraction mixture when the cells of the raw material ruptured and caused subsequent low permeation of the solvent (Maran et al., 2013), resulting in higher yield but lower FSG purity with the increasing extraction time. Furthermore, the protein and ash content of mucilage was higher when the extraction time and temperature rose, the proportion of carbohydrates was relatively low. Furthermore, protein was increased from 8.29 to 13.63% with increasing time from 10 min to 30 min. It is related to small molecules of protein than carbohydrates and more intense in deeper layers of flaxseed. Ultrasounds do not promote the release of the protein content but by increasing ultrasonic treatment time, the seeds

begin to become de-structured, and proteins may therefore be more easily extracted (Fabre et al., 2015; Ying et al., 2011). The results were agreed with those obtained by Fabre et al. (2015), Wang et al., (2022). Farahnaky et al. (2013) mentioned that, the microscopic shearing forces generated by ultrasonic wave can damage seed coat, and more protein and ash is released to the aqueous solution as the extraction time increased.

Data also indicated that there is no significant difference in carbohydrates and protein contents between FM extracted by hot water extraction and that extracted by ultrasonic assisted extraction for 15 min. Furthermore, there is significant difference in yield of FM between that extracted by hot water extraction and that extracted by ultrasonic assisted extraction for 15 min.

3.2. Effect of extraction method on bioactive components of FM

Also data indicated that total phenols and lignan contents in FM were increased with increasing time extraction (Table 3). Total phenols and lignan contents of FM prepared with ultrasound extraction for 30 min was significantly higher than that of FM prepared with hot water extraction.

Table 3. Effect of extraction method on bioactive components of FM*

Bioactive components	Hot water extraction	Ultrasonic assisted extraction				
		10 min	15 min	20 min	25 min	30 min
Total phenols (mg GAE/100 g)	19.25^{d}	15.24^{f}	17.62^{e}	19.67^{c}	20.41^{b}	22.30^{a}
Lignans (%)	3.54^{d}	2.35^{f}	2.89^{e}	3.64^{c}	3.93^{b}	4.51^{a}

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

Linseed mucilage (LM) shows good antioxidant quantity as it is rich in phenolic compounds such as ellagic acid, cinnamic acid, caffeic acid, epicatechin, and vanillic acid. This behavior of LM positively impacts the gut as it helps synthesize α-glucosidase inhibitors and lipase, which in turn regulate the metabolism of the GIT tract (Chand et al., 2024). Other supporting studies associated with mucilage antioxidant activity were recorded in okra, quince seed mucilage, and Opuntia ficus-indica [Jouki et al., (2013); Nampuak and Tongkhao, (2020)]. Also, many studied indicated that ultrasound assisted extraction increased the extracted yield of phenols components from plants [Ma et al., (2008); Wang et al., (2008); Taha et al., (2011)]

3.3. Effect of extraction method on Antioxidant activity of FM

Antioxidant activity of FM was determined by DPPH and ABTS tests. The antioxidant of FM can react with free radicals DPPH and change the purple color of this substance to yellow by reducing the stability of free radicals. The ability of DPPH to scavenge free radicals is measured by the amount of color change produced. The results showed that the DPPH scavenging activity of FM extracted by Ultrasonic assisted extraction was higher than those extracted by hot water extraction (Table 4). As shown, DPPH scavenging activity increased with FM concentration increment. It is may be related to bioactive components in FM. Also, it may be related to the ability of polysaccharide to release hydrogen/electrons that elevated with increasing concentration to terminate the chain reaction of the free radicals that could be the possible reason (Wang et al.,

2017). These results agreed with Safdar et al. (2020) and Akhtar et al. (2023).

Table 4. Effect of extraction method on antioxidant activity of FM*

Antioxidant activity	FM (mg/ml)											
	Hot water extraction						Ultrasonic assisted extraction for 30 min					
	5	10	15	20	25	30	5	10	15	20	25	30
DPPH scavenging (%)	**45.22^{l}**	**72.32^{j}**	**75.59^{i}**	**85.42^{f}**	**90.40^{d}**	**92.52^{c}**	**53.31^{k}**	**76.42^{h}**	**78.51^{g}**	**88.31^{e}**	**93.45^{b}**	**96.35^{a}**
ABTS scavenging (%)	**22.52^{l}**	**28.34^{j}**	**32.57^{i}**	**48.36^{f}**	**58.63^{d}**	**63.54^{b}**	**23.21^{k}**	**35.41^{h}**	**40.18^{g}**	**51.33^{e}**	**62.60^{c}**	**68.68^{a}**

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

The ABTS radical scavenging activity is a decolorizing technique that is applicable for water and lipid soluble antioxidant capacity, generally used in natural components (i.e. polysaccharides and polyphenols) (Wang et al., 2017). The scavenging ability was higher in FM extracted by Ultrasonic assisted extraction than that extracted by hot water extraction (Table 4).Date showed that the ABTS scavenging ability was increased by increasing the concentration of FM concentration. These results agreed with Safdar et al. (2020) and Akhtar et al. (2023).

FM has antioxidant activity contra ABTS and DPPH. Whereas the antioxidant activity of FM contra DPPH are primarily effected by the content of hydroxycinnamic acid (mostly p-Coumaric acid), other phenols components are effective contra ABTS, closely related to the content of phenols (Kučka et al., 2024). These results develop the functional and value-added products from flaxseed.

Figure 1. Cupcakes processed with FM at different ratios

3.4. Effect of replacement ratios of FM on sensory evaluation of cupcakes

Incorporating flaxseed mucilage as a partial substitute for eggs or fat in cupcakes resulted in noticeable changes in the product's quality attributes (Figure 1). At 25% and 50% replacement levels, the cupcakes maintained a soft crumb, satisfactory volume, and desirable moisture content, closely resembling the control sample. These samples also received favorable scores in sensory evaluation, particularly in taste and texture. However, higher substitution levels (75% and 100%) led to a denser texture and slight flavor deviations, affecting consumer preference. These outcomes suggest that mucilage substitution at moderate levels preserves cupcake quality while enhancing its nutritional profile.

Sensory attributes have important role in assessing product acceptability (Hesarinejad et

al., 2019). The sensory evaluation of cupcakes using FM as fat and egg replacers was evaluated in terms of color, uniformity, softness, odor, taste and overall acceptability (Table 5). Results indicated that cupcakes produced containing FM as fat replacers at 25% and as egg replacers at 25% showed higher scores in all sensory attributes as compared to the control.

Table 5. Effect of replacement ratios of FM on sensory evaluation of cupcakes*

Attributes	**FM as fat replacer**				
	Control	25%	50%	75%	100%
Color	4.25$^{a\,b}$	4.50^{b}	3.75$^{a\,b}$	3.75$^{a\,b}$	3.50^{a}
Uniformity	4.00$^{a\,b}$	4.25^{b}	3.75$^{a\,b}$	3.50$^{a\,b}$	3.25^{a}
Softness	4.00^{a}	4.75^{b}	4.75^{b}	4.75^{b}	4.75^{b}
Odor	4.25^{b}	4.50^{b}	3.75$^{a\,b}$	3.25^{a}	3.25^{a}
Taste	4.50^{c}	4.75^{c}	3.75^{b}	3.25$^{a\,b}$	3.00^{a}
Overall acceptability	4.50$^{b\,c}$	4.75^{c}	3.75$^{a\,b}$	3.75$^{a\,b}$	3.5^{a}
Attributes	**FM as egg replacer**				
	Control	25%	50%	75%	100%
Color	4.25$^{a\,b}$	4.75^{b}	4.00^{a}	4.00^{a}	3.75^{a}
Uniformity	4.00$^{a\,b}$	4.50^{b}	4.25$^{a\,b}$	3.75$^{a\,b}$	3.50^{a}
Softness	4.00^{a}	4.75^{b}	4.75^{b}	4.75^{b}	4.75^{b}
Odor	4.25$^{a\,b}$	4.75^{b}	4.00$^{a\,b}$	3.50^{a}	3.50^{a}
Taste	4.50^{b}	4.75^{b}	4.00$^{a\,b}$	3.50^{a}	3.50^{a}
Overall acceptability	4.50$^{b\,c}$	4.75^{c}	4.00$^{a\,b\,c}$	3.75$^{a\,b}$	3.50^{a}
Attributes	**FM (No egg and No fat)**				
Color	4.25^{a}		3.50^{a}		
Uniformity	4.00^{b}		3.25^{a}		
Softness	4.00^{a}		4.75^{b}		
Odor	4.25^{a}		3.50^{a}		

Taste	4.50 [b]	3.00 [a]
Overall acceptability	4.50 [b]	3.00 [a]

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

The sensory evaluation of cupcakes produced containing FM as fat and egg replacers (No egg- No fat) had lower scores in all sensory attributes as compared to control sample.

After sensory appreciation of the control sample, Cupcakes using FM as Fat replacer (25%) , Cupcakes using FM as egg replacer (25%) and Cupcakes using FM as fat and egg replacers (No egg- No fat) were selected for further analyses to assess some physical properties and chemical composition.

3.5. Effect of replacement ratios egg and oil levels with different levels of FM on some physical properties of Cupcakes

The data regarding height, weight, volume, specific volume, water activity and baking loss weight of cupcakes prepared with FM as fat and egg replacements are illustrated in Table 6. Using FM as egg replacer and both fat and egg replacers enhanced height, weight, volume and specific volume as compared with control sample. Also, results revealed that using FM increased water activity of cupcakes. It may be related to good water holding capacity of FM. Addition of hydrocolloids may increase the air bubble distribution in cupcakes and help to retain that air bubble in the cake during baking (Andrade et al., 2018; Ozkoc and Seyhun, 2015). Hussain et al. (2013) indicated that flaxseed meals improved air retention and enhanced batter aeration.

Table 6. Effect of replacement ratios egg and oil levels with different levels of FM on some physical properties of cupcakes compared to cupcakes (control)*

Cupcakes samples	Height (cm)	Weight (g)	Volume (cm^3)	Specific volume	Water activity
Cupcakes (Control)	3.80^{ab}	50.40^{c}	122.18^{a}	2.42^{a}	0.854^{d}
Cupcakes (75% oil : 25% mucilage as fat replacer)	3.50^{b}	47.33^{d}	112.54^{b}	2.38^{b}	0.875^{c}
Cupcakes (75% egg : 25% mucilage as egg replacer)	3.80^{ab}	53.21^{b}	129.94^{a}	2.44^{a}	0.895^{b}
Cupcakes (Mucilage as no egg and fat replacers)	4.30^{a}	55.80^{a}	138.26^{a}	2.48^{a}	0.905^{a}

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

3.6. Effect of replacement ratios egg and oil levels with different levels of FM on chemical composition of Cupcakes

FM was replaced to cupcakes ingredients as fat and egg replacements. When FM was used as a fat replacer, the fat content decreased by 17.11%, 5.28% as egg replacement, 92.12% as both egg and fat replacements. Similarly, FM increased the carbohydrate content in cupcakes, which improved their nutritional profiles. Results were in accordance with that obtained ay Ahmad et al., (2021). Felisberto et al. (2015)

indicated that moisture is a desirable quality often associated with the softness of a baked product.

Control sample showed lower moisture, carbohydrates contents and higher oil content and caloric value than other samples (Table 7). As expected, significant increases in moisture, carbohydrates contents were obtained in the cupcakes using FM is related to good water-holding property (Chand et al., 2024) and chemical composition of FM (Table 2). Also, FM efficiency as fat and egg replacers in cupcakes was assessed based on caloric values of all formulated cupcakes. The calorie value ranged from 398.03 to 506.25 kcal. The calorie value of cupcakes was decreased by replacing fat and egg with FM. Using FM as fat or egg replacers in cupcakes plays an important role in reducing calories value. It was agreed with that obtained by Ahmad et al., (2021).

Table 7. Effect of replacement ratios egg and oil levels with different levels of FM on chemical composition of Cupcakes (on dry weight)*

Cupcakes samples	Moisture	Crude protein	Fat	Ash	Total Carbohydrates	Caloric value (Kcal/100g)	Reduction of calories (%)
Cupcakes (Control)	35.56^{c}	8.31^{b}	22.73^{a}	1.85^{c}	67.11^{c}	506.25^{a}	-
Cupcakes (75% oil : 25% mucilage as fat replacer)	36.56^{c}	9.66^{a}	18.84^{b}	2.04^{b}	69.46^{b}	486.04^{b}	3.99
Cupcakes (75% egg : 25% mucilage as egg replacer)	38.10^{b}	7.75^{c}	21.53^{a}	1.70^{c}	69.02^{b}	500.85^{a}	1.08
Cupcakes (Mucilage as no egg and fat replacers)	48.49^{a}	9.50^{a}	1.79^{c}	2.73^{a}	85.98^{a}	398.03^{c}	21.38

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

Moisture content was increased with replacing fat and egg with FM. This is due to the water holding property of FM (Mueed et al., 2022). Similar results were reported by Elsorady et al., (2024b) when using FM as bread improver or oil replacers in pan bread. The results were also consistent with the study of Fernandes and Salas-Mellado (2017) when chia mucilage was used as a fat substitute in cake and bread.

Figure 2 illustrates the effect of using FM as a fat or egg replacer on the total phenolic content (TPC) of cupcakes. As the level of FM substitution increased—whether for fat, egg, or

both the TPC of the cupcake samples increased significantly compared to the control. Specifically, the TPC increased by 1.90, 1.98, and 3.92 times in samples using FM as fat, egg, and combined fat-and-egg replacers, respectively. These increases are attributed to the presence of phenolic compounds naturally found in FM. This finding aligns with previous findings by Mohtarami et al. (2022) and Ahmadinia et al. (2023), reinforcing the potential of FM to enhance the benficial nutritional profile of baked products through its bioactive components.

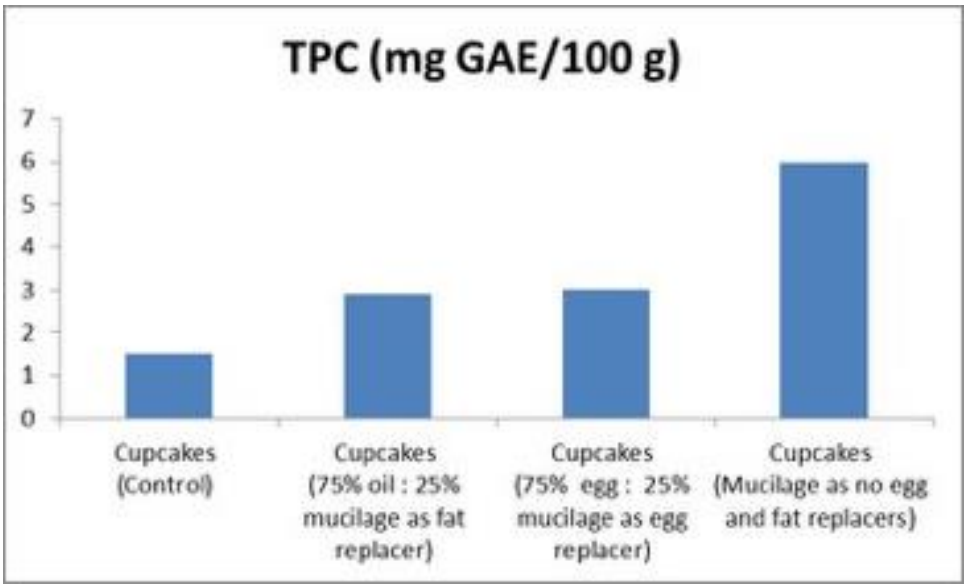

Figure 2. Total phenols content (TPC) in cupcake samples.

3.7. Effect of replacement ratios egg and oil levels with different levels of FM on color of Cupcakes

Color is one of the most important qualitative parameter of bakery product that influences consumer sensory acceptance. It is affected by several factors such as ingredients, baking conditions and reactions that are occurred during baking (Maillard reaction and caramelization) (Ameur et al., 2007). Table (8) presents L, a, and b color values for the crust and crumb of cupcake samples. The results of color analysis of cupcakes using FM as fat and egg replacers showed that using FM significantly increased brightness (L) of cupcakes for crust and crumb, and significantly reduced redness (a) and yellowness (b) for crust and crumb of cupcakes. The increase of L may be related to the lighted color of FM. Salehi (2018) indicated that the increase of balangu seed gum increased the amount of lightness (L*) which may be related to increase in the cake's volume, which makes the internal texture of the cakes brighter. Increasing L* value may be related to retention of moisture by the gums (Lazaridou et al., 2007). Retaining moisture during baking reduces crust changes in bakery products. The lightening of cake was due to changes of crust. Uniform crust more than non-uniform crust enhanced L* value (Lazaridou et al., 2007). Results were in agreement with those obtained by Elsorady et al. (2024b) of using FM as bread improver and fat replacers in pan bread and Mohtarami et al. (2022) as using FM as fat replacer on cookies.

Table 8. Effect of replacement ratios egg and oil levels with different levels of FM on color of Cupcakes*

Cupcakes samples	Crust			Crumb		
	L	a	b	L	a	b
Cupcakes (Control)	55.24^{d}	12.03^{a}	29.10^{a}	68.13^{d}	-0.41^{a}	17.38^{a}
Cupcakes (75% oil : 25% mucilage)	55.83^{c}	11.61^{b}	28.41^{b}	70.72^{c}	-0.46^{b}	17.33^{b}
Cupcakes (75% egg : 25% mucilage)	56.26^{b}	11.40^{c}	27.71^{c}	71.12^{b}	-0.50^{c}	17.23^{c}
Cupcakes (Mucilage as no egg and fat replacers)	57.21^{a}	11.22^{d}	26.14^{d}	72.30^{a}	-0.55^{d}	17.13^{d}

*different superscripts indicate significant differences using Duncan's multiple range tests ($p < 0.05$)

The substitution of flaxseed mucilage for eggs or fat in cupcake formulation represents a deliberate approach to improve both the nutritional and functional characteristics of the final baked product. Flaxseed mucilage possesses inherent emulsifying and water-retention properties, which are critical for replicating the structural and moisture-conserving roles traditionally fulfilled by eggs and fats. In this study, partial replacement at levels of 25–50% preserved essential product qualities such as crumb softness, volume, and moisture balance. These results suggest that flaxseed mucilage can serve as a viable alternative to conventional animal-derived or lipid-rich components, supporting cleaner label formulations without significantly affecting consumer sensory perception.

4. Conclusions

Economic importance of research must be mentioned, with reference to cost of extraction and the cost of laying eggs and fats, in addition to functional importance. It can be concluded that the ultrasound-assisted extraction technique of flaxseed mucilage (FM) provided a higher yield and antioxidant content, along with a shorter extraction time compared to conventional methods. The research demonstrated that FM could effectively replace fat or eggs at a 25% substitution level. Its incorporation improved the sensory attributes of the cupcakes, enhanced moisture content, and reduced both fat and caloric values, supporting the development of healthier baked products and increased total phenolic content (TPC) of cupcakes. Depending on the substitution type, TPC levels increased by 1.90 times (fat replacer), 1.98 times (egg replacer), and 3.92 times (combined fat and egg replacer) relative to the control. This enhancement in phenolic content reflects the bioactive potential of FM and its contribution to the functional value of the final product. The flaxseed mucilage (FM) is reinforced its credibility as a functional food ingredient. Adding flaxseed mucilage to food items like cupcakes may result in many nutritional advantages such as antioxidant properties, healthy fat and other bioactive components. More investigation is required to ascertain the ideal concentration of flaxseed mucilage in various food products for best nutritional advantages as well as to examine the long-term impacts of consuming flaxseed mucilage-enriched food products.

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Research article

TAILORED 3D FOOD PRINTING INK RECIPE WITH ISOLATED SOY PROTEIN, TEMPEH FLOUR AND PROBIOTIC : COMPARISON OF DURIAN SEED FLOUR, SODIUM ALGINAT AND XANTHAN GUM AS A HYDROCOLLOID

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Abstract

This study is a part of the 3DFP product development which is formulated with isolated soybean protein (ISP), defatted tempeh flour (DTF), durian seed flour (DSF), and probiotic. The potential of durian seed flour as an alternative hydrocolloid ingredient for 3D food printing ink will be investigated. The food-inks were printed at different temperatures of 30°C, 40°C and 50°C, then baked at 145°C for 6 min. The printed results were analyzed for printability and microstructure. The rheology of the food ink formulated with DSF measured at different temperatures showed comparable behavior to those formulated with SA and XG. Moreover, the higher the shear rate the higher the shear stress and the lower viscosity of those food-inks. The results also showed that food-ink had lower yield stress at higher temperatures. These results indicate that DSF is a suitable ingredient for food-ink formulation with acceptable printability, comparable to those formulated with SA and XG. Based on the printability and microstructure analysis, food inks printed at 40°C were superior to those printed at 30°C and 50°C. Therefore, DSF is a qualified ingredient that can be used in the formulation of 3DFP food-ink.

1. Introduction

With the constant increase of the global population reaching 8 billion in 2024, sustainable food production to supply nutritious food for each individual is a major Sustainable Development Goal (SDG) and is becoming a major food security challenge to many governments to provide a sustainable food supply. Therefore, various efforts to overcome these problems continue to be explored, one of which is the development of 3D food printing (3DFP) technology which opens up opportunities for the use of various materials in designing food-ink to produce palatable nutraceutical food products with new characteristics that are in accordance with consumer needs from the physical, nutritional, sensory and functional health value aspects. The working principle of 3DFP is to make 3-dimensional food products from edible ink (food-ink) layer by layer according to the design and size made with specific. Computer software and controlled electronically. The widely used 3D food printer is a printer that involves an extrusion process, where food-ink made from food ingredients is inserted into a cylinder and extruded out through a hole or mold with a certain shape (nozzle) with the push of a hydraulic piston (Altiparmak *et al*., 2022). The desired characteristics of food-ink are that it can flow through the nozzle and can solidify after printing (Lee, 2021). Food-ink that is suitable for printing (printable) is a food-ink that has a viscosity and rheology with certain viscoelasticity that has good extrudability and self-supporting stability.

Food materials that have been widely used commercially as food-ink are chocolate, but various studies have shown that protein solutions and materials that contain a lot of hydrogels such as starch and hydrocolloids can also be formulated into 3D printable food-ink (Liu *et al*., 2018; Kewuyemi *et al*., 2022; Ma and Zhang, 2018). One of the potential materials that can be used as food-ink materials is the flour of durian seed. Based on its chemical composition (dry matter), durian seeds contain 40.29% starch and 55.44% gum (Permatasari *et al*., 2021), so it has the potential to produce printable food-ink. One of the largest durian producers in the world is Indonesia. Based on data from the Central Bureau of Statistics Indonesia, durian production in Indonesia reached 1,582,172 tons in 2022 (CBSI, 2023). Durian seeds make up 5-15% of the total weight of durian and are generally discarded as waste (Rozikhin *et al*., 2020), so on an annual basis there are around 79,109 to 237,326 tons of durian seed waste in Indonesia. The use of durian seeds as food ingredients is expected to support a green economy in terms of sustainability while supporting the achievement of the 2030 SDGS goals, namely zero hunger [SDG-2]. The use of durian seed flour (DSF) as a food-ink material has never been studied, even though it has the potential to develop sustainable superfoods using 3DFP technology. In developing sustainable superfoods with 3DFP, DSF needs to be combined with other complementing nutritious ingredients such as functional protein. An economical source of protein that is widely consumed by the community is tempeh. Tempeh is a fermented soybean product that is coined as a superfood owing to its high nutrient density (especially protein and vitamin B12); bioactive compounds that have functional health value such as isoflavones and γ-amino butyric acid (GABA); and its production process that is classified as traditional so it is environmentally friendly, sustainable food product (Romulo and Surya, 2021; Maskar *et al*., 2022). Isolated Soybean Protein (ISP) is also an economical protein source material that has various functional values and can improve the texture and flavor of the final product (Lee, 2015). Other ingredients that can be combined with DSF are probiotics. Probiotic bacteria from local isolates that have been widely used for food product development are *Lactobacillus plantarum* Dad-13 (*L. plantarum* Dad-13). This probiotic bacteria has various health benefits, namely improving digestive tract health, anti-diarrhea, boosting the immune system, facilitating the production of folic acid, and enhancing growth among malnourished children (Kamil *et al*., 2023).

The combination of DSF, tempeh flour, ISP, and *L. plantarum* Dad-13 is expected to produce nutrient-dense tailored printable food-ink in order to develop sustainable food using 3DFP. In this study, potential of durian seed flour as a hydrocolloid alternative ingredient of 3D food printing ink will be investigated.

2. Materials and methods

2.1. Materials

Materials for food-ink formulation were durian seed flour, isolated soy protein, defatted tempeh flour, gelatin, water and probiotic *L. plantarum* Dad-13. Durian seed flour was prepared from durian seed, obtained from a durian fruit processing unit in Surabaya, Indonesia. Durian seeds were washed, sorted, boiled, peeled, cut, sterilized, dried, and crushed into flour and sieved with a Tyler sieve shaker at 70 mesh. Isolated soy protein and gelatin edible grade 200 bloom were purchased from the local market. The defatted tempeh flour was prepared from hygienic tempeh purchased from the local market, then sliced, steam blanched at 90°C for 2 min, dried at 60°C for 8 h, then crushed and sieved at 70 mesh with a Tyler sieve shaker. The tempeh flour was defatted with hexane at room temperature for 2 h, then dried at 60°C for 8 h.

2.2. Methods

2.2.1. Preparation of food-ink and 3D printing

Food-ink was made according to the procedure outlined by Chen et al. (2022). In this research, 0.2% of DSF was mixed with water, 4% DTF, 10% gelatin, 17% ISP, and 0.1% probiotic *L. plantarum* Dad-13, stirred (100 rpm, 5 minutes) and settled (4°C, 24 hours), then heated to 35°C before characterization. For comparison, food-ink was also made in the same way, but the DSF was substituted with 0.5% xanthan gum (XG) and 0.5% sodium alginate (SA). The viscosity and rheology properties of the food-inks were determined before being printed. The 3DFP used in this study is an extrusion-based Foodbot 3D Food Printer, with the following conditions: 2 mm nozzle, 7 mm/s printing speed, and dimensions of 150x150x70 mm. Different printing temperatures were applied (30°C, 40°C and 50°C), then baked at 145°C for 6 min. The printed results were analyzed for printability and microstructure.

2.2.2 Determination of the viscosity and rheology properties

The viscosity and rheology properties of the food-ink were determined by using a rheometer (RM200 CP 4000, Lamy Rheology, France). Flow tests were carried out at different temperatures i.e. 25°C; 35°C and 45°C from the view of the experimental environment, edible condition and sample preparation. The 25°C is around the room temperature, the 35°C is around the body temperature (~37°C), and the 45°C is the temperature to solve the gelatin during the protein paste preparation. Casson model analysis was used for determining yield stress and viscosity.

2.2.3. Printability determination

The printability was determined by observation of the shape and surface of the printed results, and photographed. The baked products were also observed for their shape and surface, and photographed.

2.2.4. Microstructure analysis

Microstructural analysis was performed using SEM (Scanning Electron Microscope, EVO MA 10, Zeiss). The samples were freeze-dried and then coated by using Pb-Au. In the observation process with SEM, the samples were observed at a certain magnification. The results of the particle diameter magnification were analyzed using Image-J software.

2.2.5. Statistical analysis

The obtained data were calculated for the mean and standard deviation.

3. Results and discussions

3.1. Viscosity and rheology properties of food-ink

Food-ink prepared from isolated soy protein, defatted tempeh flour, gelatin and probiotic with 3 (three) different hydrocolloids (DSF, SA, and XG) were determined for the viscosity and rheology properties. According to Lee (2021), viscosity and rheology properties of food-ink affect its capability to flow through the nozzle and can solidify after 3D printing,

thus important to its printability. The viscosity of the food-inks should be low enough to ensure that the material can be smoothly extruded from the smaller nozzles, and high enough to ensure that the food-inks have sufficient mechanical strength to maintain the stability of the printing products (Guo et al., 2022).

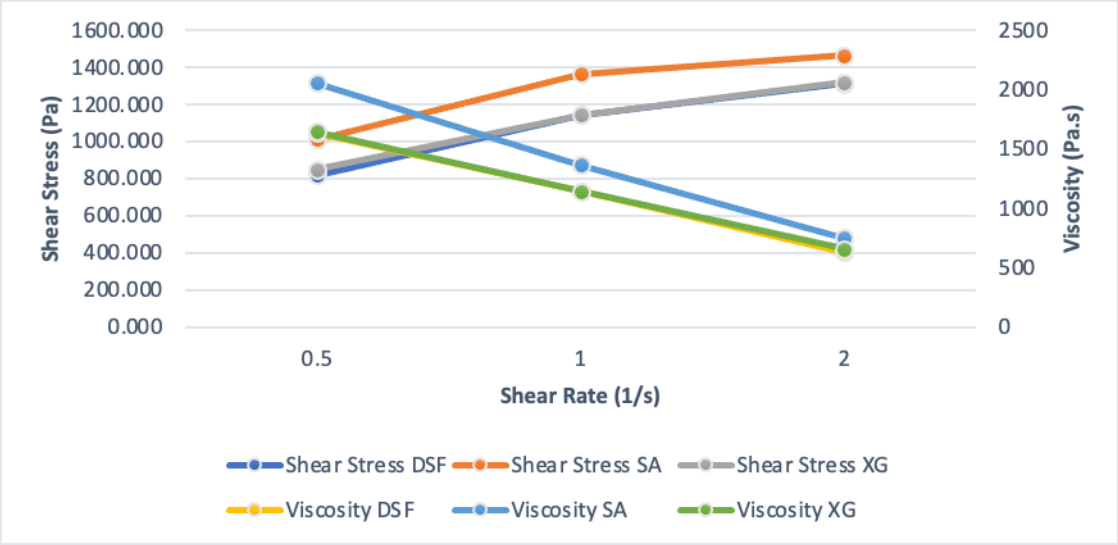

Figure 1A. Viscosity and rheology properties of food-inks determined at 25°C

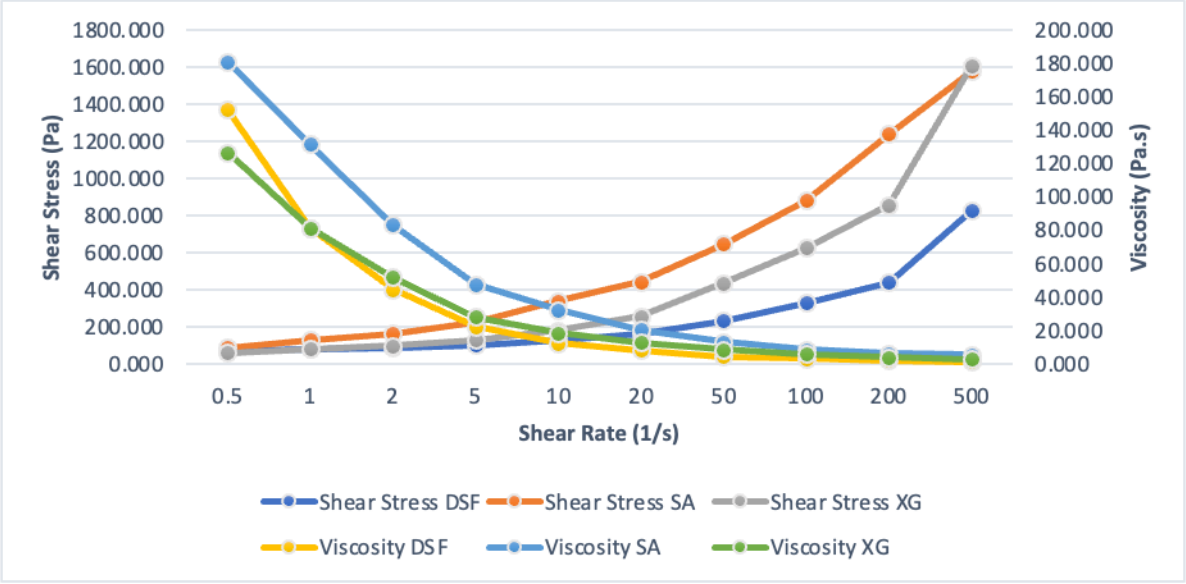

Figure 1B. Viscosity and rheology properties of food-inks determined at 35°C

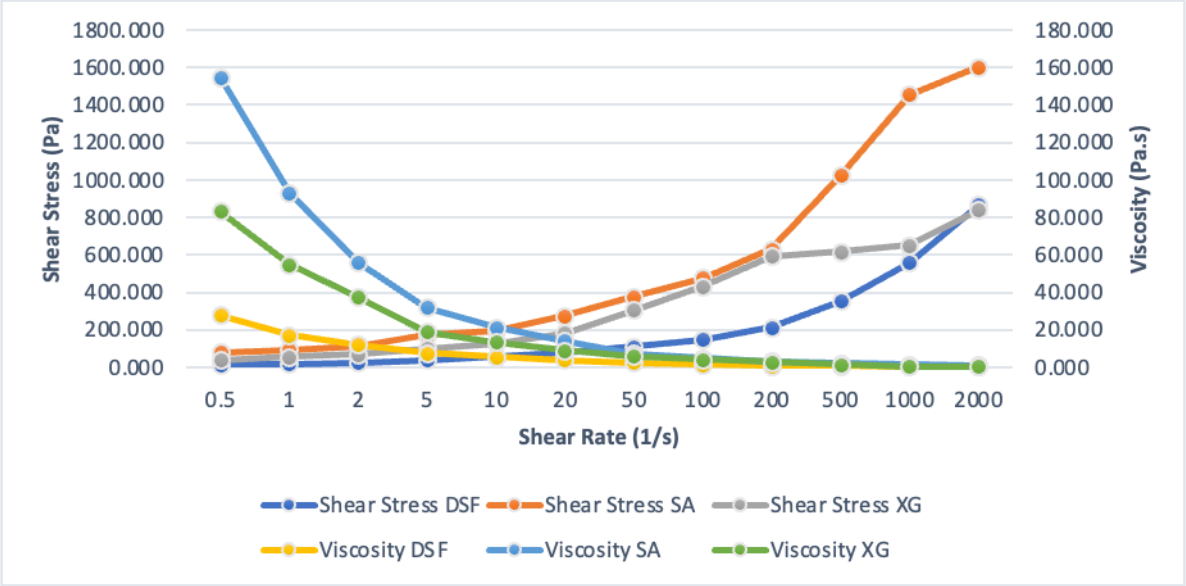

Figure 1c. Viscosity and rheology properties of food-inks determined at 45°C

Table 1. Yield stress of food-ink with different hydrocolloids.

Hydrocolloid	Yield Stress (Pa)		
	25°C	35°C	45°C
DSF	453.45 ± 46.60	78.70 ± 8.34	47.4 ± 0.28
SA	825.45 ± 44.48	124.40 ± 2.83	78.7 ± 0.57
XG	644.3 ± 24.18	74.6 ± 4.53	59.95 ± 4.45

Figure 1 shows the viscosity and shear stress at different shear rates of the three food-inks which were determined at different temperatures of 25°C, 35°C, and 45°C. At 25°C measurement of food-ink with DSF, increasing shear rate, shear stress increased from 816.69 Pa at a shear rate of 0.5/s to become 1,311.18 Pa at a shear rate of 2.0/s and, viscosity decreased from 1,633.37 Pa.s at shear rate 0.5/s to become 630.59 Pa.s at shear rate 2/s. At higher temperatures (35°C and 45°C), a similar phenomenon has been found, increasing the shear rate resulted in increasing shear stress and decreasing viscosity. At 35°C, the shear stress was 73.61 Pa at shear rate of 0.5/s increased to 89.99 Pa at 2/s. The similar trend is also can be observed at °C, the shear stress was 13.54 Pa at shear rate of 0.5/s become 24.61 Pa at 2/s, while the viscosity decreased from 27.72 Pa.s become 12.01 Pa.s. During preliminary research, food-ink with 0.5% DSF concentration could not be printed because the food-ink had too high viscosity. The durian seed contains hydrocolloids in the form of starch and gum, which contribute to the rheology behavior. Amin et al. (2017) reported that durian seed gum had been purified and characterized. The viscosity of 1% durian seed gum solution was 65 mPa.s at a shear rate of 1000/s at 29.8°C. The viscosity of fully hydrated durian seed gum solution decreased as temperature increased.

It was noted that the rheology behavior of food-ink formulated with DSF was similar to those formulated with SA, and XG at the same temperatures. When the shear rate increases, the shear stress decreases and viscosity increases. However, the values of shear stress and viscosity among those three food-inks are different. The shear stress and viscosity values of food-ink formulated with DSF are between the values of those food-inks formulated with SA and XG. For example, shear stress of food-inks formulated with DSF, SA and XG measured at 35°C and 0.5/s were 73.61, 90.46, and 63.37 Pa, respectively. The viscosity values at those conditions were 152.22, 180.93, and 126.75 Pa.s, respectively.

Table 1 shows the yield stress of food-ink with different hydrocolloids. The yield stress values decrease when the temperature increases. The yield stress values of the food-ink formulated with DFS at 25°C, 35°C, and 45°C were 453.45 Pa, 78.70 Pa, and 47.4 Pa, respectively. Those values were comparable to yield stress values of food-ink formulated with pregelatinized corn starch, in a range of 87.5 to 883.2 Pa (Maldonado-Rosas et al., 2022). Based on the dry matter, durian seed contains hydrocolloids in the form of starch (40.29%) and gum (55.44%) (Permatasari et al., 2021), which contribute to the rheology behavior.

3.2. Printability of food-ink

The highly desirable materials for extrusion-based printing are pseudoplastic fluids with shear thinning behavior. A low viscosity indicates strong shear-thinning behavior and materials with this behavior could be easily extruded out due to low viscosity with the application of shear stress (Liu et al., 2018). Figure 2 shows the printing results of the different printing temperatures. Temperature is known to affect the rheological properties of food material and printability of the material. The figures show that at 30°C, less smooth surface of printed results found especially from food-inks formulated with DSF and XG. All three food-inks resulted smooth surface when printed at 40°C and 50°C. Chen et al. (2022) reported the similar finding that the printing temperature (25, 35 and 45°C) affect the printability, higher temperature enhanced texture properties of 3D printed objects of protein-based food-ink formulated with isolated soy protein. However, higher temperature printing can cause the surface of the printed food to widen. We found that all three food-inks resulted widen surface when printed at 50°C. In case of food-ink with DSF that contains starch, temperature of 30°C is not sufficient for interaction of the starch.

According to Liu et al. (2020), with the increase of the 3D printing temperature, the sufficient chain interactions between starch particles led to the formation of a uniform gel structure. Figure 2A shows that food-ink with 0.2% DSF produced good printability at higher temperatures, and was comparable to food-ink formulated with SA and XG (Figure 2B and C). XG and SA are hydrocolloids that are frequently used in the food-ink formulation thus the printability results confirm that DSF can be used as hydrocolloids in 3DFP food-ink formulation.

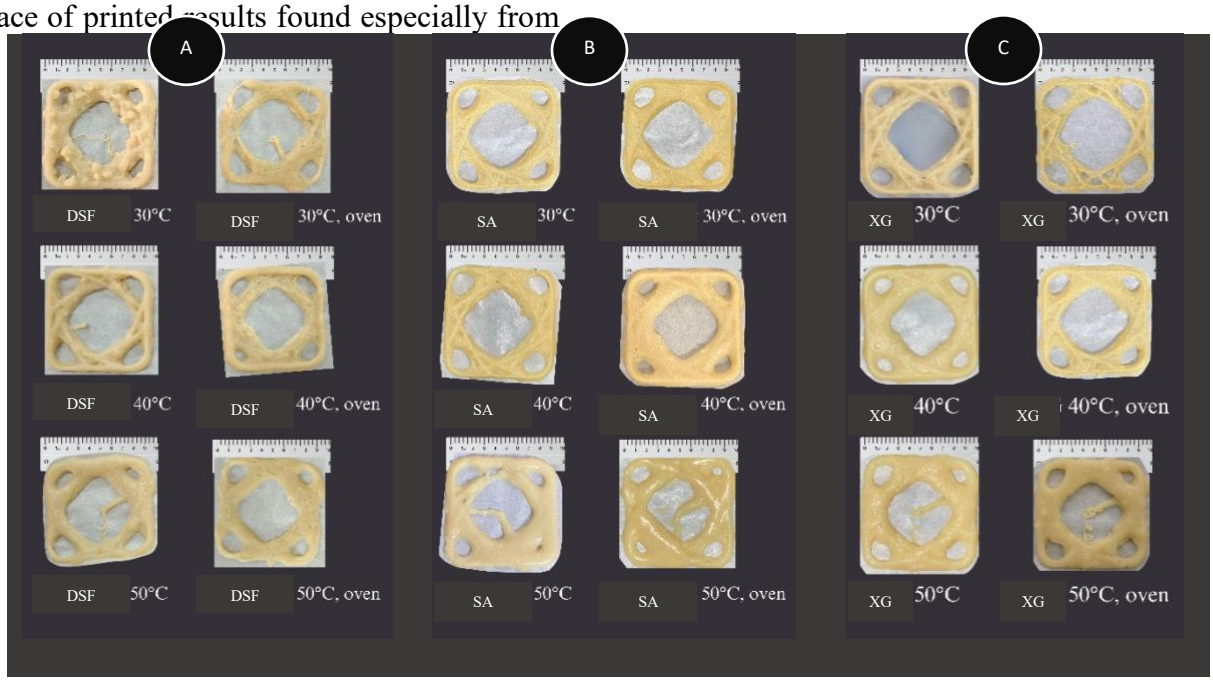

Figure 2. Printed food-ink formulated with DSF(A), SA(B), and XG(C) at different temperatures and its baked products

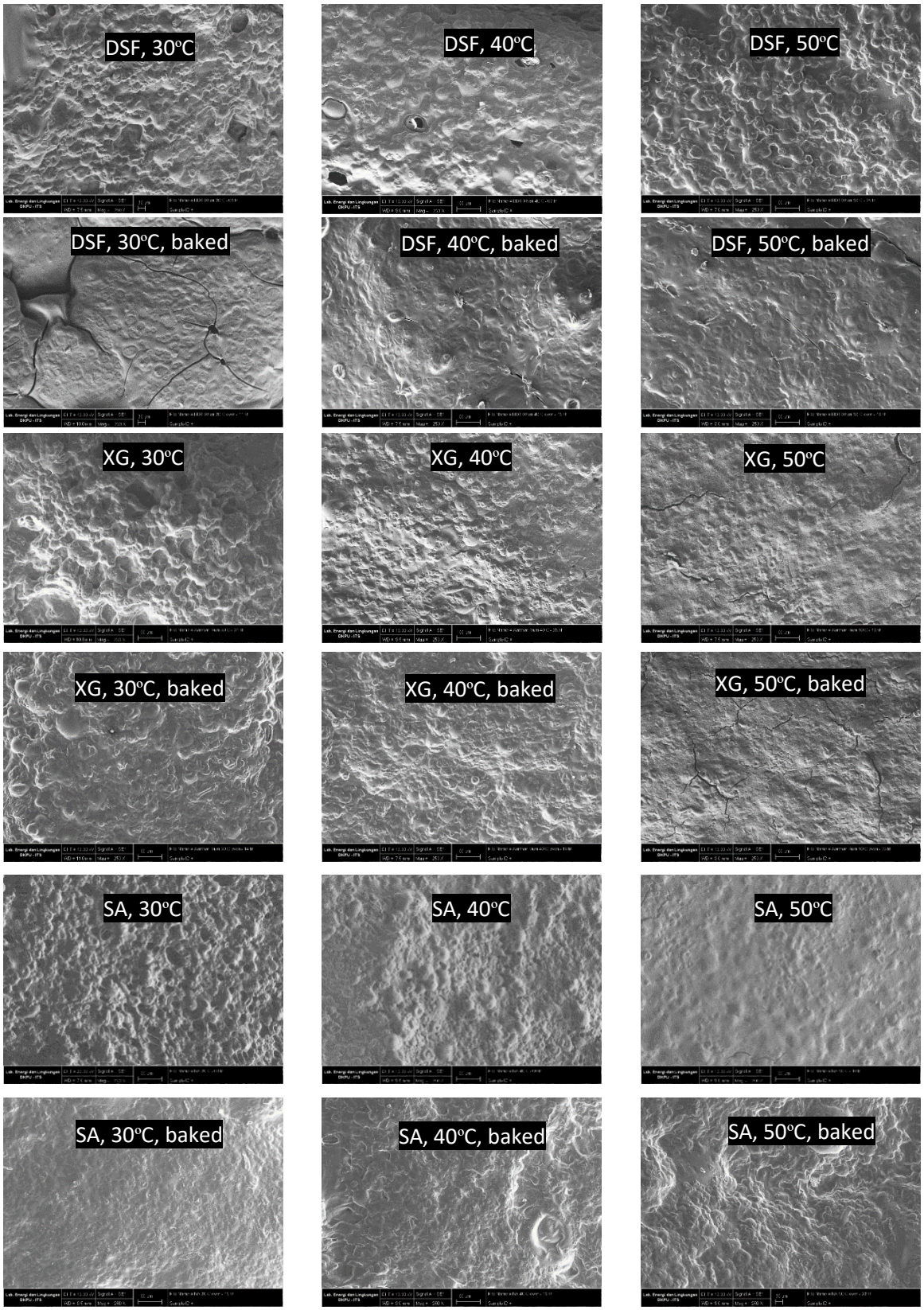

Figure 3. Microstructure of printed food-ink formulated with different hydrocolloids at different temperatures and its baked products

3.3. Microstructure of printed food-ink and baked 3DFP product

SEM was used to observe the microstructure of the printed food-inks formulated with 0.2% DSF with various printing temperatures and compared to food-inks formulated with SA and XG (Figure 3). Temperature is also known to affect the microstructure of food material. Higher printing temperature tends to produce better structure, indicated by fine particle distribution status. This is possibly caused by the gelatinization of hydrocolloids in the food-inks. As the printing temperature increases, the gelatinization process increases. This was further confirmed by the microstructure of baked food-ink that showed a better particle distribution status. The DSF seemed like a cracked structure, which might be caused by partial starch gelatinization. Higher temperature printing showed no cracking in the structure. Other research also showed the microstructure of printed food-ink based on starch-rich materials. Compared to food-inks formulated with SA and XG, the microstructure of food-inks formulated with 0.2% DSF exhibits a coarser particle distribution status. This is possibly caused by different gelatinization profiles of those hydrocolloids and different concentrations of hydrocolloids used. SA and XG used for the food-ink was 0.5% while DSF used was only 0.2%. However, baked food-ink formulated with 0.2% DSF showed a finer particle distribution status than those formulated with SA and XG.

4. Conclusions

The characterization of tailored food-ink formulated with ISP, DTF, DSF, and probiotic *L. plantarum* Dad-13 has been accomplished. DSF produced 3DFP food-ink with viscosity, shear stress values, and SEM results comparable to those formulated with SA and XG. Therefore, DSF is a qualified ingredient that can be used in the formulation of 3DFP food-ink. Further research is needed to confirm the nutritional values and functional properties of this newly tailored food-ink formula.

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Research article

MOZZARELLA CHEESE ENRICHED WITH SPICES: IMPACT ON MUSKY FLAVOUR, NUTRITIONAL PROFILE, TEXTURAL AND MICROSTRUCTURAL PROPERTIES

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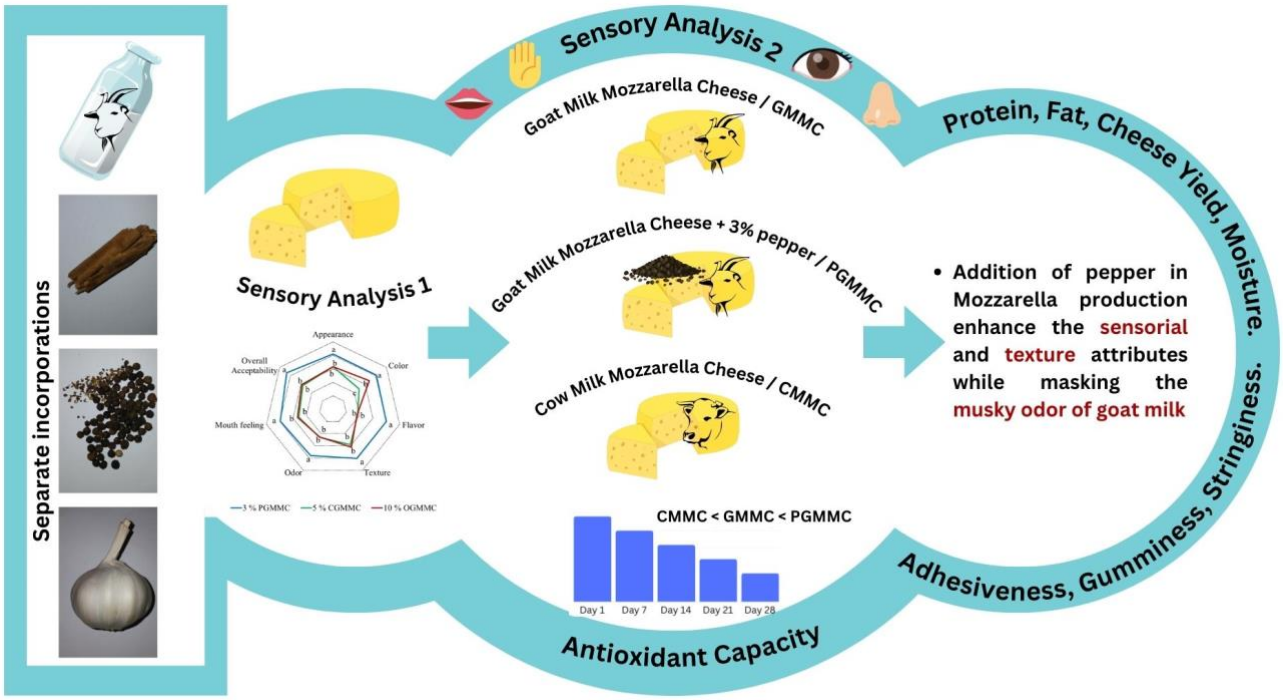
Abstract

Goat milk offers low allergenicity, excellent digestibility, and a rich nutrient profile, but its musky flavour limits consumer acceptance. This study aimed to develop a spice-flavoured goat milk mozzarella cheese to overcome this issue. Preliminary sensory evaluations identified 3% (w/w) pepper (*Piper nigrum*) as the most suitable spice compared to cinnamon (*Cinnamomum verum)* and onion (*Allium cepa*). Selected pepper-incorporated goat milk mozzarella cheese (PGMMC) was developed and compared with conventional goat milk mozzarella cheese (GMMC) and cow milk mozzarella cheese (CMMC) to evaluate flavour masking and product quality. Physicochemical, textural, microstructural, and antioxidant properties were assessed over 28 days of storage. PGMMC showed a higher cheese yield (12.36%), significantly lower fat content (19.94% w/w), and the highest overall acceptance while effectively masking the musky flavour compared to CMMC. PGMMC also exhibited the highest total phenolic content (75.72 mg GAE/100 g), indicating superior antioxidant capacity. Texture analysis revealed GMMC had higher stringiness than PGMMC ($p_{value}<0.05$). Microstructure analysis showed more compact and smooth protein matrices in GMMC and PGMMC compared to CMMC. In conclusion, pepper addition to goat milk mozzarella enhances sensory, textural, and nutritional qualities while successfully masking the undesirable musky flavour, promising an approach to increase consumer appeal.



Graphical abstract

1. Introduction

Goat milk (GM) and cow milk (CM) play a vital role in the dairy industry, where each distinguished by unique attributes such as flavour and nutritional profile. GM and derived dairy products are recognized as healthier foods compared to their CM counterparts due to unique nutritional profiles accompanied by bioactive substances and therapeutic properties (Navamniraj *et al*., 2023). GM contains higher levels of vitamin A and various minerals including potassium, selenium, zinc, calcium, chloride, phosphorus, and copper than CM while GM proteins help to increase the bio-availability of minerals (Lopez-Aliaga *et al*., 2003). Moreover, GM is highly digestible compared to CM which is attributed to the smaller size of fat globules and micelles. Many researchers have claimed that GM has lower allergenic properties thus providing a promising solution for CM allergenicity of infants (Haenlein, 2004). Despite the potential health benefits and functional properties of GM; generally, a lower demand can be observed compared to CM in commercial settings due to the undesirable flavour characteristics. The typical undesirable flavour and aroma of GM is often attributed to the presence of short-chain fatty acids such as caprylic, capric, and caproic acids which limits the further popularization of goat milk among consumers (Posecion *et al*., 2005). Various research efforts have been made to improve the sensory properties of GM and derived dairy products in order to reduce the unpleasant "goaty" flavour (Chen *et al*., 2021).

Production of value-added dairy products such as flavoured or spiced cheese, yoghurt and curd may provide sustainable solutions to overcome such off-flavour properties of fresh goat milk (Kochubei-Lytvynenko *et al*., 2019; Posecion *et al*., 2005).

Cheese made of GM is renowned for its potential health benefits, has garnered increasing attention in recent years. Thus, the present work focuses on the development of a goat milk Mozzarella cheese (GMMC) formulated with spices in attempts to achieve a GMMC that is similarly preferred like cow milk mozzarella cheese (CMMC) alleviating

GM's rancid and musky flavour. The study will help to unveil the opportunities to increase and expand the market of GM offering economic opportunities for farmers and producers, fostering a more sustainable and profitable goat farming and processing industry**.**

2. Materials and methods

2.1. Materials

2.1.1. Sample collection

Cow milk was obtained from jersey-sahiwal cross-breed and goat milk from saanen breed was obtained from the Kottunna Milk Farm situated in the Gampaha district (low country), Sri Lanka. Pepper, cinnamon, and onion powder were purchased from a retail shop at Kiribathgoda, Gampaha district.

2.1.2. Chemical and reagents

All the used chemicals were of analytical reagent grade obtained from the Faculty of Agriculture, Rajarata University of Sri Lanka.

2.2. Methods

2.2.1. Preparation of Mozzarella cheese

Cow milk was obtained from Jersey-Sahiwal crossbred cows while goat milk was obtained from the Saanen breed reared under the same farming conditions in Sri Lanka.

Mozzarella cheese preparation was done following the procedure described by Jana and Tagalpallewar (2017) with several modifications. Pasteurization of milk was done at 72 ℃ for 15 seconds and cooled the milk to 37 ℃. Thermophilic starter culture (CHN-22, Chr.Hansen) with the composition of *Lactococcus cremoris*, *Leuconostoc*, *Lactococcus lactis* subsp. *lactis*, and L*actococcus lactis* subsp. *lactis* biovar diacetylactis was inoculated at a 0.075% w/v rate; afterwards the container was covered with a lid and was kept undisturbed for 45 min at 32 ℃. Then, 0.03% (w/w) of rennet powder containing bovine chymosin (chymosin 100%, 650 International Milk-Clotting Units per gram (IMCU g-1); CHY-MAX) was added to the mixture. For the spiced-flavored mozzarella cheeses, selected spices were added before the addition of rennet powder. The closed container was kept for 60 min at 32 ℃ and cheese curd was cut and allowed to whey drainage for another 30 min at 35-40 ℃. The remaining whey was heated to 80-85 ℃ and the stretching process of cheese in hot whey was practiced for 3–5 min. Then, the cheese was coldly brined (20-23% brine strength) for a sufficient period. Finally, cheese was packed and stored at 4 ℃. Sensory evaluations of Mozzarella cheeses were conducted on the 7th day after preparation.

In the experiment, three types of GMMCs, each with three concentrations, were prepared: PGMMC (Pepper-*Piper nigrum* flavoured GMMC) with pepper concentrations of 3%, 6%, and 9% (w/w), CGMMC (Cinnamon-*Cinnamomum verum* flavoured GMMC) with cinnamon concentrations of 3%, 5%, and 7% (w/w), and OGMMC (*Onion-Allium cepa* flavoured GMMC) with onion concentrations of 4%, 6%, and 10%(w/w). After identifying the best spice-incorporated GMMC, additional samples were prepared, including cow milk mozzarella cheese (CMMC) infused with the selected spice and its optimal concentration. Furthermore, CMMC and goat milk mozzarella cheese (GMMC) without any added spices were produced to evaluate and confirm the best spice-incorporated GMMC.

2.2.2. Sensory analysis

Sensory evaluation was conducted employing thirty (30) untrained panelists. Five (05) separate sensory evaluations were carried out during the study period employing the same panelists for each. The tasting panel consist of 45% male and 55% female with age range of 25-60 years. Five consecutive sensory evaluations were carried out for different response type.

2.2.2.1 Sensory test 1

The first sensory evaluation was designed to determine whether pasteurized GM has any unpleasant rancid and musky flavour compared to pasteurized CM. Duo test - Difference test in sensory evaluation was employed for the test.

2.2.2.2. Sensory test 2

The second sensory evaluation was conducted to determine whether GMMC has reduced the undesirable flavour in GM. Duo

test - Difference test in sensory evaluation was adapted for the test 2.

2.2.2.3. Sensory test 3

In the third sensory assessment, respondents were asked to score sensory properties including appearance, flavour, colour, texture, aroma, and overall acceptability of cheese types according to a 5-point hedonic scale. The same score was not given for two or more samples.

PGMMC with pepper concentrations of 3%, 6%, and 9% (w/w), CGMMC with cinnamon concentrations of 3%, 5%, and 7% (w/w), and OGMMC with onion concentrations of 4%, 6%, and 10% (w/w) were used in preliminary tests in order to find the ideal spice levels.

Then a third sensory evaluation was conducted to select the best spice-flavoured GMMC among the identified best levels of PGMMC, CGMMC, and OGMMC.

2.2.2.4. Sensory test 4

Subsequently, a fourth sensory analysis was done to ascertain the best concentration level of the selected spice to incorporate into GMMC to mask the musky flavour. Friedman test – Descriptive test in sensory evaluation was employed for the test 4.

2.2.2.5 Sensory test 5

The sensory analysis was to determine whether there was a significant flavour difference between CMMC and GMMC when both had been incorporated with the verified optimum level of the selected best spice. Duo test - Difference test in sensory evaluation was adapted in test 5.

2.3. Laboratory analysis of Mozzarella cheese

As the treatments for further analysis, CMMC without spices (control), GMMC without spices, and 3.0% (w/w) PGMMC were used (Figure 1). Protein, fat, cheese yield, microstructure, and textural and microstructural properties were measured on the 7th day of the maturation period. All other physicochemical properties, antioxidant properties and microbiological properties were measured during the storage period of the 1st, 7th, 14th, 21st, and 28th days.

Figure 1. a) 3% Pepper incorporated Goat Milk Mozzarella Cheese; b) Non-spiced Goat Milk Mozzarella Cheese; c) Non-spiced Cow Milk Mozzarella Cheese.

2.3.1.Physiochemical characteristics of Mozzarella cheese

Crude protein and crude fat content were determined by using the Kjeldahl method and the Soxhelt method respectively. The cheese yield was expressed as a percentage of the ratio between the weight of the cheese and of the pasteurized milk sample used at the beginning. The pH of Mozzarella cheese samples was determined at 25˚C by using an electrode immersion pH meter (Thermo Scientific pH 450, Singapore). The titrimetric method was used to determine the titratable acidity, and it

was expressed as the lactic acid percentage (w/w) (Gnanarathna *et al*., 2022). Moisture content of the cheese was measured by the oven dry method; 2-3 grams of cheese were dried in 100 ± 2°C for 24 h and expressed as a percentage by loss of weight for the initial weight of the cheese sample. Physiochemical properties were reported as the average of the three replicates.

2.3.2. Antioxidant assays

The ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) method was used to estimate the total antioxidant capacity (Seneviratne *et al*., 2009). By using spectrophotometry, the absorbance reduction at 734 nm was observed. Trolox was used as the standard for the calibration curve. The antioxidant capacity (AOC) was expressed as (µmol Trolox eq/g) Trolox equivalent antioxidant capacity (TEAC). Antioxidant analysis was conducted for three replicates for each treatment.

2.3.3. Folin-Ciocalteu assay for polyphenols

Folin-Ciocalteu's method was used to determine the total phenolic content (TPC) of prepared Mozzarella cheese samples (Seneviratne *et al*., 2009). Gallic acid was used as the standard for the calibration curve. The TPC was expressed as mg GAE/100g.

2.3.4. Instrumental texture analysis

Textural properties of Mozzarella cheese were evaluated instrumentally using a Brookfield texture analyzer (Texture Pro CT V1.8, USA) as described by Hashim *et al*. (2009). Texture properties including hardness, adhesiveness, cohesiveness, gumminess, and stringiness length were calculated using a software program (Texture Pro software, Brookfield Instruments).

2.3.5. Microbiological quality assessment

Total plate count (TPC), coliform count (CC), and yeast and mould count of mozzarella cheese were assessed using Plate count agar, MacConkey agar, and Potato Dextrose Agar (HIMEDIA, India) at 1st, 7th, 14th, 21st and 28th days respectively, during the storage period.

2.3.6. Microstructure of Mozzarella cheese

Scanning electron microscopic (SEM) images were obtained as the procedure previously described by Jaya (2009) with few modifications. Samples in size of 1 cm × 1 cm × 10 mm were excised from the middle of the Mozzarella cheese samples and were frozen at -18°C. The pre-frozen Mozzarella cheese samples were dried in a laboratory freeze dryer (Alpha 1-4 LS basic, Christ, Germany) for 72 h under the vacuum of 1×10^{-3} kPa and the condenser temperature was -50°C. Once the final moisture content was below 2% (w/w); samples were mounted onto an SEM specimen stub, coated with gold using a sputter coater (Quorum, Germany) at 1×10^{-2} kPa and examined under SEM (ZEISS EVO 18, Germany) operated at 15 kV. Images of the microstructure were obtained in two magnification levels as ×1000 and ×5000.2.2.1 Extraction of phenolic compounds.

2.3. Data analysis

The first, second, and fifth sensory evaluations were tested using the duo-trio test, and 20 responses were the critical value of the test from 30 responses, according to the test table. A Friedman test (alpha = 0.05) was employed to identify whether there were significant differences in cheese types in the third and fourth sensory evaluations.

The experiment design was a complete randomized design (CRD). Cheese yield, fat content, protein content, and textural characteristics were analyzed by the one-way analysis of variance (ANOVA) and mean comparisons were performed by using the Tukey-Kramer HSD test. The rest of the physicochemical parameters and microbiological parameters were analyzed by using the repeated measure ANOVA method and mean comparisons were performed by using the Least square means test. Data was analyzed by SAS software (SAS Institute Inc., Cary, NC, USA.).

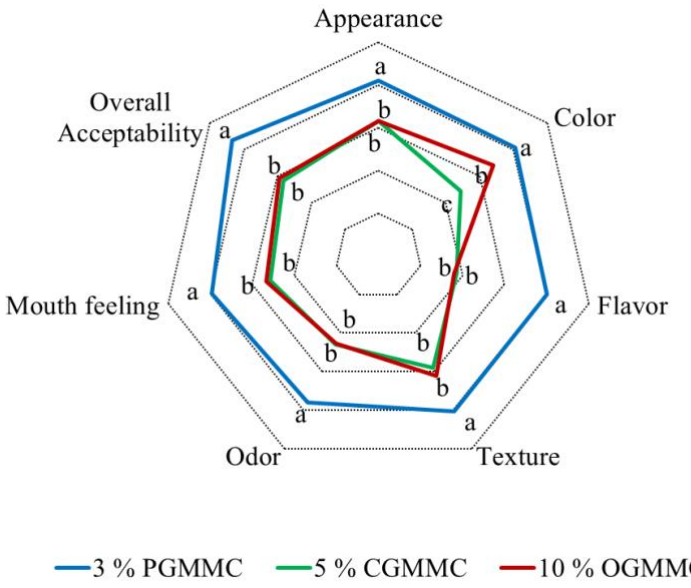


Figure 2. The mean score of tested cheese types for different attributes at third sensory evaluation. Mean scores of an attribute followed by the same letter are not significantly different at $p_{value} < 0.05$. PGMMC: Pepper incorporated Goat Milk Mozzarella Cheese; CGMMC: Cinnamon incorporated Goat Milk Mozzarella Cheese; OGMMC: Onion incorporated Goat Milk Mozzarella Cheese.

3. Results and discussions

3.1. Sensory analysis

The duo test results in the first sensory evaluation demonstrated ($p_{value}<0.05$) that the pasteurized GM sample had a significantly unpleasant musky flavour compared to CM. In the second sensory evaluation, the majority ($p_{value}<0.05$) acknowledged that GMMC had unpleasant musky flavour, but less than pasteurized GM indicating that the Mozzarella cheese production process has contributed to the reduction of musky flavour of GM. According to the results of tested organoleptic properties in the preliminary test of third sensory analysis, the best levels of each PGMMC, CGMMC, and OGMMC were 3%, 5%, and 10% (w/w) respectively. The results of the third sensory analysis indicated that all the tested attributes are statistically significant ($p_{value}<0.05$). The 3% (w/w) PGMMC obtained the most significantly highest rank sums for texture, odour, mouth feel and overall acceptability, establishing it as the most preferred ($p_{value}<0.05$) cheese type (Figure 2). In line with our results, Krumov *et al*. (2010) demonstrated that processed cheese incorporating black pepper exhibited higher sensory performance than the control, suggesting a significant enhancement in sensory quality due to the addition of black pepper. In another study on cottage cheese has shown that, the black pepper addition at level of 1% (w/w) resulted in improved sensory attributes, including flavour, colour, and overall acceptability (Himabindu & Arunkumar, 2017). Further, in agreement with our results, a study conducted on Mudaffara cheese showed that black pepper-added cheese attained the highest scores for taste, odour, and colour, compared to other tested spices even after refrigeration for over four weeks (Ahmed & Foda, 2012). The fifth sensory test results indicated that there was no significant difference between 3% (w/w) pepper-flavoured CMMC and 3% (w/w) PGMMC in preference, suggesting that the 3% PGMMC had no unpleasant musky flavour. Thus, PGMMC added with 3% pepper was used for further evaluation of physiochemical textural and microstructure properties.

3.2. Cheese yield and other physicochemical characteristics of Mozzarella cheese

Both cheese types made of goat milk showed higher ($p_{value}<0.05$) protein content

compared to CMMC (Figure 3(A)). Previous studies have shown that the elevated milk fat content in GM would uphold the enhanced protein recovery in goat milk cheese (Pastorino *et al*., 2003).

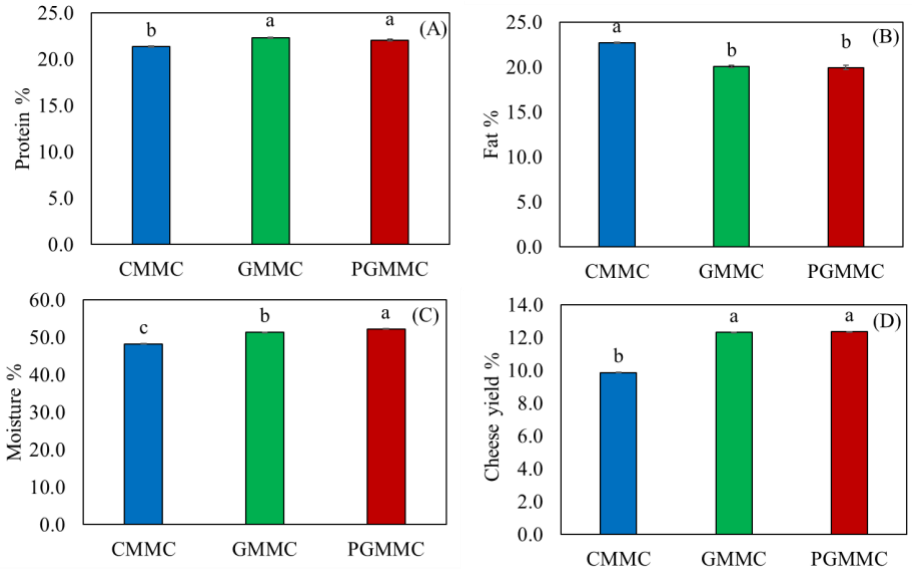


Figure 3. Variation of protein content (A), fat content (B), moisture content (C), and cheese yield (D) in tested cheese types. Means followed by the same letter are not significantly different at α=0.05. CMMC: Cow Milk Mozzarella Cheese, GMMC: Goat Milk Mozzarella Cheese, PGMMC: 3% Pepper incorporated Mozzarella Cheese

As illustrated in Figure 3(B), CMMC displayed the highest fat content (p_{value}<0.05) whereas the fat contents among PGMMC and GMMC were not found to be statistically distinguishable (P > 0.05). The casein-to-protein ratio in milk has been identified as a determinant of fat recovery, but not protein recovery (Pazzola *et al*., 2019). According to Pastorino (2023), higher casein-to-protein ratio is found in CM compared to GM leading to higher fat content in cow milk cheese in accordance with our results. Furthermore, a negative correlation between fat recovery and moisture retention in both GM and CM cheeses was reported by Johnson *et al*. (2001). These findings were also aligned in present study as well; whereas significantly lower fat content and higher moisture content was observed in GMMC than CMMC.

Notably, the PGMMC stands out with the highest (p_{value}<0.05) moisture content (52.25% (w/w)) while GMMC exhibited a significantly higher moisture content (51.35% (w/w)) than CMMC (48.25% (w/w)) (Fig. 2 (C)). Both GMMC and PGMMC had higher (p_{value}<0.05) cheese yields than CMMC (Fig. 2 (D)). Guo *et al*. (2004) found that, fat content, protein content and moisture content of GM have significant positive relationships with the cheese yield. More specifically, a strong correlation has been observed among milk fat content and percentage cheese yield for goat milk cheese (Zeng *et al*., 2007). In agreement with our results, Johnson *et al*. (2001) reported that greater moisture recovery positively correlates with higher cheese yield.

Throughout the storage period, the antioxidant capacities (AOC) of CMMC were found to be higher (p_{value}<0.05) than both GMMCs. This finding is attributed to the fact that cow milk contains a higher number of antioxidant peptide types derived from casein compared to goat milk, which indicates factors that cause variation in the antioxidant capacity of milk among different species (Stobiecka *et al*., 2022). In the present study, PGMMC exhibited consistently higher AOC (p_{value}<0.05) than GMMC, as illustrated in Table 1.

Table 1. Antioxidant capacity (AOC), Total Phenolic content (TPC), pH and titratable acidity of tested cheese types at storage period of 28 days.

Parameter	Day	Mean ± SE		
		CMMC	GMMC	PGMMC
AOC (µmol Trolox eq/g)	1	7.97±0.01^{Aa}	5.30±0.02^{Ca}	6.52±0.02^{Ba}
	7	7.45±0.01^{Ab}	4.89±0.01^{Cb}	5.84±0.01^{Bb}
	14	6.82±0.02^{Ac}	4.75±0.01^{Cc}	5.25±0.02^{Bc}
	21	6.20±0.0^{Ad}	4.14±0.02^{Cd}	4.40±0.02^{Bd}
	28	5.53±0.02^{Ae}	3.10±0.03^{Ce}	3.94±0.01^{Be}
TPC (mg GAE/100 g)	1	55.98±0.17^{Ba}	47.17±0.11^{Ca}	75.72±0.17^{Aa}
	7	46.16±0.17^{Bb}	42.89±0.17^{Cb}	62.39±0.16^{Ab}
	14	41.57±0.17^{Bc}	38.49±0.11^{Cc}	59.56±0.16^{Ac}
	21	38.11±0.11^{Bd}	36.86±0.17^{Cd}	48.05±0.17^{Ad}
	28	34.27±0.17^{Be}	35.34±0.17^{Ce}	40.75±0.11^{Ae}
pH	1	5.92±0.03^{Ca}	6.09±0.01^{Aa}	6.01±0.01^{Ba}
	7	5.63±0.01^{Cb}	5.93±0.01^{Ab}	5.85±0.01^{Bb}
	14	5.52±0.01^{Bc}	5.64±0.01^{Ac}	5.65±0.01^{Ac}
	21	5.43±0.01^{Bd}	5.56±0.01^{Ad}	5.53±0.01^{Ad}
	28	5.32±0.01^{Ce}	5.48±0.01^{Ae}	5.43±0.01^{Be}
Titratable acidity %	1	0.45±0.00^{Be}	0.27±0.00^{Ce}	0.61±0.00^{Ae}
	7	0.55±0.01^{Bd}	0.36±0.00^{Cd}	0.82±0.01^{Ad}
	14	0.78±0.01^{Bc}	0.62±0.00^{Cc}	1.25±0.00^{Ac}
	21	1.03±0.01^{Bb}	0.85±0.01^{Cb}	1.50±0.01^{Ab}
	28	1.27±0.00^{Ba}	1.16±0.00^{Ca}	1.75±0.01^{Aa}

[a] Means in the same row without a common capital letter superscript significantly ($p_{value} < 0.05$) differ for each treatment (cheese type); means in the same column without a common simple letter superscript significantly ($p_{value} < 0.05$) differ among the storage period.

[b]Abbreviations; CMMC: Cow Milk Mozzarella Cheese, GMMC: Goat Milk Mozzarella Cheese, PGMMC: 3% Pepper incorporated Mozzarella Cheese, AOC: Antioxidants capacity, TPC: Total phenolic content, and WHC: Water holding capacity.

The impacts of black pepper on the quality of different cheese types also have consistently reported an increase in antioxidant potential (Ahmed & Foda, 2012). Subsequently, there had been significant ($p_{value}<0.05$) declines in the antioxidant capacities of all three cheese types over the 28-day storage period (Table 1). The total phenolic content (TPC) of PGMMC had the significantly highest values during the storage period. It could be due to black pepper (*Piper nigrum* L.) which is recognized for its rich content of phenolic compounds (Lee *et al*., 2020). Furthermore, CMMC consistently demonstrated higher ($p_{value}<0.05$) TPC than GMMC until the twenty-first day. The TPC of all tested Mozzarella cheese types experienced a significant ($p_{value}<0.05$) decline over the storage period, as indicates in Table 1.

According to Table 1, both GM cheese types consistently exhibited significantly higher pH values compared to the CMMC and there was a significant decline in the pH values of all cheese types over the storage period irrespective of the cheese type. The titratable acidity values of PGMMC over the storage period were the highest ($p_{value}<0.05$) which could have resulted from the higher acid content of pepper (Himabindu & Arunkumar, 2017). CMMC consistently showed higher ($p_{value}<0.05$) titratable acidity than GMMC across the storage period (Table 1). Moreover, a positive gradient in titratable acidity was apparent during the storage for all three cheese types. The activities of starter and non-starter bacteria ferment lactose and metabolize lactic

acid, leading to an increase in acidity over the storage period.

3.3. Textural characteristics of cheese

Instrumental texture properties of cheese act as important attributes to be tested together with sensory properties. As depicted in Figure 4(A), CMMC gave the highest (p_{value}<0.05) mean hardness. In agreement with our observation, Półtorak *et al*. (2015) reported that cheeses that had reduced fat content showed the highest hardness values. Further, the current study results could be attributed to the lower pH and moisture content as the hardness being negatively correlated with moisture content and pH level of cheese (Pastorino *et al*., 2003).

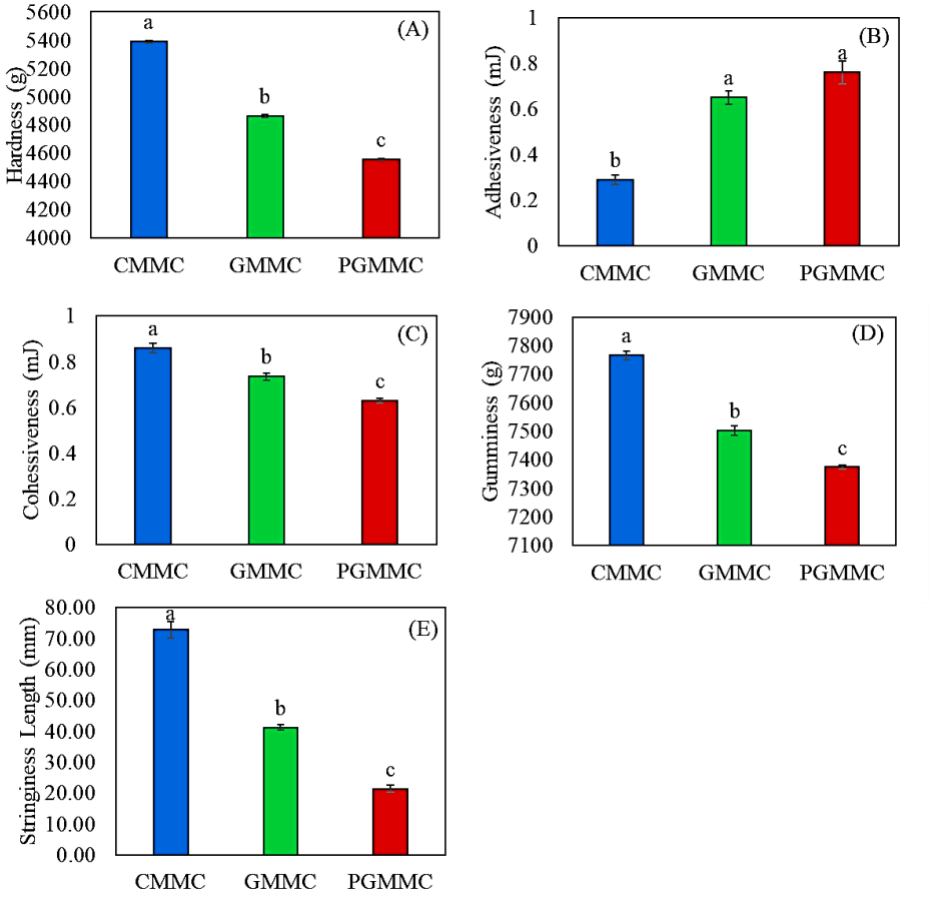


Figure 4. Variation of hardness (A), adhesiveness (B), cohesiveness (C), gumminess (D), and stringiness length (E) in tested cheese types. Means followed by the same letter are not significantly different at p_{value} < 0.05 CMMC: Cow Milk Mozzarella Cheese; GMMC: Goat Milk Mozzarella Cheese; PGMMC: 3 % Pepper incorporated Mozzarella Cheese.

The adhesiveness of both GMMC and PGMMC was significantly higher compared to the adhesiveness of CMMC (Fig. 4B). Moisture content surpassing over 50% has been attributed to the increased adhesiveness of mozzarella cheese which is in line with our results (Kindstedt & Fox, 1993). Yet, the optimum shredability could be obtained with CMMC due to its moderate moisture content (Jana & Tagalpallewar, 2017). Figure 4(C) illustrates both GMMC and PGMMC exhibited (p_{value}<0.05) lower cohesiveness (0.74 mJ and 0.63 mJ) compared to CMMC (0.86 mJ). This is most likely due to small-fat globules in goat milk having interacted more with the casein network, which leads to the weakening of the structure (Logan *et al*., 2017). The highest (p_{value}<0.05) gumminess was found in CMMC while GMMC exhibited higher gumminess than PGMMC (Figure 4D). A review on mozzarella

cheese by Kindstedt (1993) described that gumminess is negatively associated with moisture content, assuring our results of higher moisture content in GMMC than in CMMC.

According to Figure 4(E), CMMC exhibited a significantly greater stringiness length compared to others and GMMC showed higher stringiness length (p_{value}<0.05) than PGMMC. The stringiness is expressed quantitatively as stretched length. Based on the review by Jana and Tagalpalllewar (2017), higher fat content and lower moisture content contribute to increased stringiness length in cheese. Thus, a comparatively higher stretch in CMMC is observed due to the higher fat content and lower moisture content in CMMC compared to both PGMMC and GMMC.

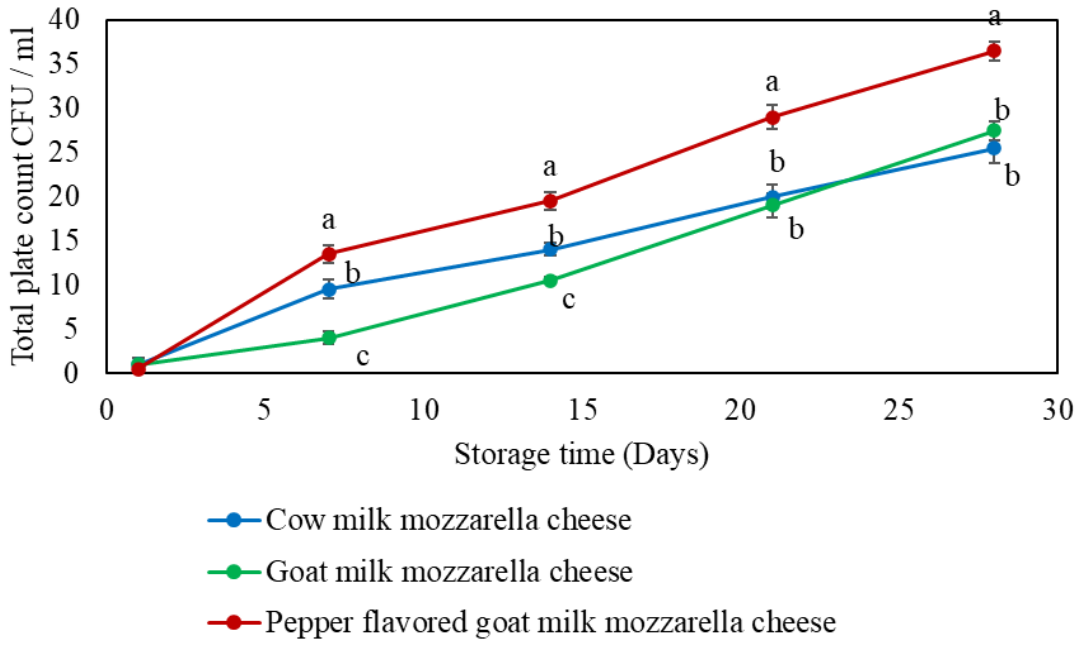


Figure 5. Total Plate Count in tested cheese types during the storage period of 28 days. Means at a storage time followed by the same letter are not significantly different at α=0.05

3.4. Microbiological characteristics of cheese

On the first day of storage, the mean Total plate count (TPC) was 1cfu mL^{-1} in both CMMC and GMMC, while it was 0.5 cfu mL^{-1} in PGMMC. Since the seventh day onwards, PGMMC consistently exhibited higher (p_{value}<0.05) TPC (Figure 5). The CMMC recorded significantly higher TPC values than GMMC before the third week, and on the last day, the GMMC type reported a higher value (p_{value}<0.05). A positive gradient (p_{value}<0.05) is noticeable in all the Mozzarella cheese types throughout the entire storage period (Figure 5). Food safety was ensured with E. coli results, after the 4th week of storage, 1 cfu mL^{-1}, 1 cfu mL^{-1} of and 3 cfu mL^{-1} of E. coli was reported in CMMC, PGMMC and GMMC respectively. According to regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs concern E. coli s an indicator of the degree of hygiene; when the cheese mage with milk and whey which have subjected to heat treatment considered E.coli level as unsatisfactory at >1,000 cfu g^{-1} (Losito *et al.*, 2014). Thus, reported level of the current study is far below than the threshold level.

By the end of the fourth week of storage, the mean count of yeast and mould for each tested cheese type was reached to 2.5 cfu mL^{-1}. PGMMC did not exhibit vulnerability to yeast, mould, or E. coli growth due to the positive impact of the antimicrobial activity of pepper (Himabindu & Arunkumar, 2017; Krumov *et al.*, 2010).

3.5. Microstructure of Mozzarella Cheese

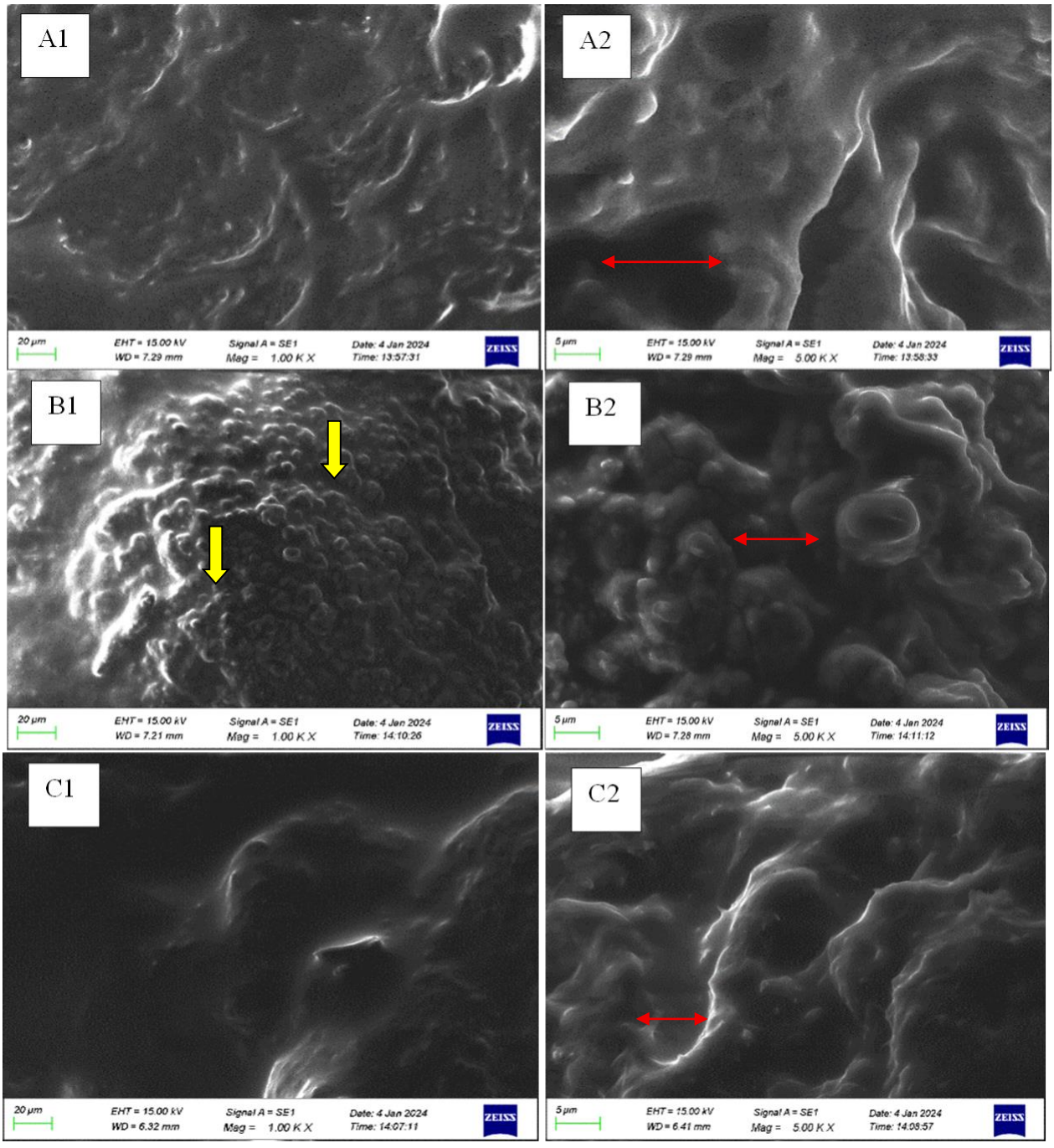


Figure 6. SEMs of CMMC at ×1000 (A1) and ×5000 (A2) magnification, GMMC at ×1000 (B1) and ×5000 (B2) magnification, and PGMMC at ×1000 (C1) and ×5000 (C2) magnification. Yellow colour single head arrows denote the dense protein network and red colour double headed arrow represent the fat globule displaced locations in protein network.

CMMC: Cow Milk Mozzarella Cheese; GMMC: Goat Milk Mozzarella Cheese; PGMMC: 3 % Pepper incorporated Mozzarella Cheese.

A more compact and smooth protein structure was observed in GMMC (Figure 6 B1& B2-yellow colour single headed arrows) compared to CMMC. The protein structure of CMMC (Figure 6 (A1& A2)) appeared less compact than that of GMMC which showed a uniform protein matrix with compressed casein micelles (Cunha *et al*., 2010). Protein matrices of the GMMC and PGMMC had more fat displaced areas compared to protein matrix of CMMC.

The diameter of the displaced fat globules in GM is smaller than that in CM whereas the smaller fat globules facilitate elevated integration into the casein network which was clearly observed through SEM images (Pazzola *et al*., 2019; Weerasingha *et al*., 2022). Similar findings were observed in the microstructure characterization in the present study (red colour double headed arrows represent the variations of the fat globule diameter displaced in the protein matrix of each mozzarella cheese type).

4. Conclusions

There is no difference in flavour between 3% (w/w) PGMMC and 3% (w/w) pepper-flavoured CMMC. Hence, the Mozzarella cheese production process has mitigated the musky flavour characteristic of GM, and pepper flavour has counteracted the residual musky flavour in GMMC. Both GMMC and PGMMC exhibit higher cheese yields compared to CMMC. Compared to CMMC fat contents are lower and protein contents are higher in both GMMC and PGMMC. Nonetheless, there are no significant differences between the PGMMC and GMMC in terms of cheese yield, fat content, or protein content. During the 28-day storage period, PGMMC consistently showed the highest total phenolic content compared to CMMC. When considering the textural properties, adhesiveness is higher in both PGMMC and GMMC compared to CMMC, and cohesiveness, gumminess, and hardness are lower than those of CMMC. The total plate counts of PGMMC would grow significantly higher than those of GMMC and CMMC throughout the 28-day storage period. However, the growth of yeast, mould, and E. coli was not detected in noticeable numbers during the storage period. Based on these finding, it can be concluded that, 3% (w/w) pepper addition would eliminate the musky flavour of the goat milk mozzarella cheese while improving physicochemical, textural and antioxidant properties, and maintaining microbiological safety.

Based on the results, it is evident that GMMC variants exhibit inferior texture properties and this underscores the need for further studies to enhance their texture properties. Additionally, the importance of the development of proper storage and packing methods to extend the shelf life has been pointed out by the research findings. An examination of the impact of the developed product on the nutritional state of the consumer is a timely suggestion because one of its goals that to assist in the mitigation of malnutrition among children, by increasing the consumption of GM.

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Research article

DEVELOPMENT OF VEGETABLE SAUSAGE USING NATIVE INGREDIENTS: EFFECT ON PHYSICOCHEMICAL, NUTRITIONAL AND TEXTURAL PROPERTIES

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ABSTRACT

In this work, the changes in the physicochemical, nutritional and textural properties of vegetable sausages prepared from the combination of lentil, rice, oat and carob flours were evaluated. As food hydrocolloids, brea gum and carrageenan (Genuvisco MB 11F) were used. The proximal composition, energy intake, total polyphenols, total dietary fiber, aqueous activity, pH, water retention capacity and textural parameters of each of the samples were determined. The total polyphenol content as well as the total dietary fiber content increased substantially with the addition of carob flour. The amino acid profile was determined for the vegetable sausage made with four types of flour; the composition of essential amino acids meets the amino acid requirements of adults and adolescents. There was significant variation ($p < 0.05$) concerning color among treatments, and the the samples containing carob flour was the darkest colored. The hardness was not different ($p > 0.05$) among the products when carrageenan was replaced by brea gum at the same concentration. However, in the range studied, the products are slightly stickier. The formulation of vegetable sausage incorporating regional ingredients was feasible, generating a product with appropriate textural and nutritional characteristics

1.Introduction

The plant-based diet is a type of diet that promotes care for the environment and reduces the possibility of getting sick from certain diseases.

In a recent work, (Bryant, 2022), 43 studies on the health and environmental sustainability of

products made from vegetable proteins compared to similar products of animal origin were reviewed. In terms of environmental sustainability, the former is more sustainable when greenhouse gas emissions, water use, and land use are analysed. In terms of health, they present a series of benefits, including generally favourable nutritional profiles, which aid weight loss and muscle synthesis, and attention to specific health conditions.

A significant number of current consumers choose food products based on vegetable proteins either by adhering to veganism, vegetarianism or by convictions that attend to the care of the environment and health. New food developments include formulation with alternative proteins such as legumes and cereals. Within the offer of vegetarian foods are meat substitute products. In the development of this type of food, an attempt is made to imitate the flavour, texture and even the size of products made with meat (Ahmad *et al.,* 2022; Bakhsh *et al*., 2022; Corrêa *et al.*, 2023; Kamani *et al.*, 2019; Lyu *et al.*, 2023). Thus, for example, in the production of meatless sausages, hamburgers or nuggets, the food industry uses soy as the main ingredient due to its high protein content, high hydration capacity, amino acid composition and other properties. Soybean products, when used in meat products, show positive effects in terms of improving sensory properties, while ensuring stable production, as well as benefits for large-scale production (Munialo *et al.*, 2022). In addition to soybeans, legumes are another important source of vegetable protein. Legumes consumption has been shown to reduce the risk of cardiovascular disease and diabetes (Papandreu *et al.*, 2019). Other likely sources of plant protein studied include mung beans, peanuts, and peas (Samard and Ryu, 2019). Lentil (*Lens culinaris*) is another legume grain with a high concentration of nutrients. Its proteins are incomplete because they lack methionine, an essential amino acid, but if it is combined with other sources of protein such as rice, which is rich in this amino acid, it forms a protein of high biological value.

Another legume of interest in the development of healthy, which is an autochthonous food in the region of the province of San Luis, is the carob tree. Carob trees belong to the *Prosopis* gender, which in Argentina has 27 species, being 13 are of them endemic. Carob pods are characterised by containing highly amount of soluble sugar (40–50%), proteins (3–4%), and lipids (0.4–0.8%). Moreover, carob flour contains substantial amounts of polyphenols, especially condensed tannins. Polyphenols exhibit a wide range of biological properties, and among these, antioxidant activity is the most known (Mom *et al*., 2020). In addition to the high content of phenolic compounds, carob flour is considered a product that contains a high level of dietary fiber, minerals (Fe, Ca, Na, K, P and S) and vitamins (E, D, C, niacin, B6 and folic acid). In view of its high nutritional value, carob flour has been used as an ingredient in functional or healthy foods (Alvarez Sala J, 2017; Babiker *et al*., 2020; Berk *et al.*, 2017; Mom *et al.*, 2020; Rodriguez *et al*., 2019; Sciammaro *et al*., 2018).

On the other hand, the desirable organoleptic characteristics in a sausage made without meat require the addition of gums or food hydrocolloids. Studies carried out on this subject, (Majzoobi *et al*., 2017), showed that the protein and fat contents of meatless sausages (12.8% protein and 13.3% fat) were like those of common meat sausages (12–14% protein and 13–15% fat). However, certain problems associated with texture, low water retention capacity and dark colouration were found. The incorporation of carrageenan and mannan gum managed to correct some of these deficiencies. These studies support the need to study a new hydrocolloid for food use to improve the textural aspects of this type of product. Brea gum is a natural hydrocolloid obtained from a forest species (*Cercidium praecox*) belonging to the legume family Brea gum is a viscous and uncrystallisable substance, which differs from resins by being soluble in water. It has various properties such as thickener, gelling agent, emulsifier and stabilizer. Physically, it is very similar to gum arabic, so it could be a good

substitute (Bertuzzi *et al*. 2012). Its incorporation into the Argentine Food Code was carried out in 2013 in chapter XVIII, article 1398, section 72.1 as a thickener, stabilizer and emulsifier. Studies on its application in the preparation of edible films, bakery production and microencapsulation of corn oil can be found in the bibliography (Spotti *et al*., 2016; Castel *et al*., 2017; López and Jimenez, 2016; Slavutsky *et al*., 2018).

Based on the above, the hypothesis is raised that the incorporation of carob flour will improve the nutritional properties of the product and the use of brea gum as a new hydrocolloid will allow it to replace an existing additive on the market.

In this work, it is proposed to develop a vegetable sausage, using as sources of vegetable proteins: lentil, rice, oat, and carob flour. The similarities and differences of two natural hydrocolloids: brea gum and carrageenan in the textural properties of the products are also analysed.

2. Materials and methods

2.1. Materials

The raw materials and additives used in the development of the product included: lentil, rice, oat, and carob flour as sources of vegetable protein and fiber; brea gum and carrageenan (CP Kelco, Copenhagen, Denmark) as food hydrocolloids; sunflower oil; spices mix; salt and synthetic casing. The two regional ingredients: *Prosopis* flour and brea gum were provided by the Cooperativa Raíces del Bosque Nativo in the province of San Luis (Argentina). The rest of the mentioned ingredients were purchased in the local trade.

2.2. Experimental design

In the development of the proposed vegetable sausage, an experimental mix design was applied. The response variable will depend on the proportions of the components. The response variables were the protein content of the product and the textural parameters. The controllable factors corresponded to the compositions (percentage wt.) of the vegetable protein sources. Table 1 shows the combinations obtained.

The experiences were repeated using:

- Stage 1: 0.8 %wt. carrageenan (C)
- Stage 2: 0.8 %wt. brea gum (BG)
- Stage 3: 1.5 %wt. (C+BG)

The limits of each of them were established from experience and previous tests.

Analysis of variance (ANOVA) was performed using the free R software with a confidence interval of 95%. The results of the evaluated variables were presented as mean ± standard deviation of three determinations.

Table 1. Experimental mix design

Experience	Lentil flour	Rice flour	Oat flour	Carob flour
1	0.9	0.1	0.0	0.0
2	0.1	0.9	0.0	0.0
3	0.1	0.1	0.8	0.0
4	0.6	0.1	0.0	0.3
5	0.1	0.6	0.0	0.3
6	0.1	0.1	0.5	0.3

2.3. Preparation of Sausages

Sausages were formulated based on 0.8% spices and 0.6% salt, 14% sunflower oil, 42.3% water, a percentage of hydrocolloid and the rest with the mixture of vegetable proteins according to the mix design of Table 1. The ingredients were mixed in a food processor operating at 1500 rpm for 20 minutes. The sausages were filled and later the samples were pasteurized in a water bath at 80 °C for 20 min. Then they were rapidly cooled in cold water (8 °C) until they reached room temperature. The samples were

stored at 4 °C for 24 h before their characterisation.

2.4. Chemical characterisation of sausage formulations

Protein was determined with the AOAC 2001.11 method (6.25 to convert nitrogen content into protein content), fats using AOAC 991.36 method, moisture with the AOAC 945.15 method and ash with AOAC 923.03 method. Carbohydrate content was calculated by difference.

The analyses were carried out in triplicate. The energy value was calculated based on the following calorie contents: 9 kcal·g^{-1} for fats and 4 kcal·g^{-1} for proteins and carbohydrates (FAO, 2003).

The determination of total dietary fiber was carried out according to the AOAC 991.43 standard.

The total phenol content was determined by the Folin-Ciocalteu colorimetric method from the methanolic extract. 50 µL of sample were incubated with 200 µL of the Folin-Ciocalteu reagent. After 1 hour of incubation at room temperature in the dark, sodium carbonate solution (7.5 %w/v) was added and mechanically stirred for 1 hour in the same conditions. The absorbance at 760 nm was measured using an SP Spectrum 2100 UV/SP spectrophotometer. A calibration curve was made with gallic acid (0-100 mg·l^{-1}). The measurement was carried out in triplicate and the total phenolic content was expressed in mg of gallic acid equivalents per 100 g of dry sample.

Amino acid composition was analysed based on ISO 20976–1:2019 (AOAC Method 994.12). Laboratorio Rapela (Buenos Aires, Argentina) performed protein hydrolysis, amino acid analysis and quantification. Individual amino acids were measured and quantified by High Pressure Liquid Chromatography (HPLC).

2.5. Determination of pH and water activity

The pH was determined on ten grams of each sample, grounded in small sizes, mixed with 100 ml of distilled water, homogenizing for 2 min. Then, the pH was measured using a digital pH meter calibrated at room temperature.

The water activity (aw) was measured in triplicate in sausages (at 25°C), using Aqualab equipment. The procedure included filling a chopped sausage sample into a measuring capsule (2/3 of its height), placing it in the measuring part of the apparatus and lasted until the equilibrium was established

2.6. Colour determination

The determination of the measure of the colour of the vegetarian sausage was carried out by recording the L*a*b* coordinates in a ColourTec PCM digital colorimeter. The values of L*(light/dark), a*(red/green) and b*(yellow/blue) were obtained. The measurements were performed at room temperature immediately after the sausages were cut

2.7. Determination of water release

A thin slice of each sample (1 g) was cut and placed between two filter papers (Whatman No. 1) of known weight. It was pressed with 1 kg of weight for 20 min at room temperature. The weight gain of the filter papers was determined gravimetrically using an analytical balance. The weight gain of the filter papers divided by the initial weight of the sausage (1 g) multiplied by 100 is reported as percentage of water release from the samples, which has a negative correlation with the water retention capacity.

2.8. Texture profile analysis (TPA)

The texture properties of the samples were studied using a texture analyser (Texture Analyser, TA Plus, stable Microsystems. Surrey, England). The test was carried out with a blade probe. A test speed of 1 mm·s^{-1} and a cutting distance of 25 mm were used.

3.Results and discussions

3.1. Chemical composition of sausage formulations

Table 2 shows the experimental data of the proximal composition of the tested

formulations. The results for humidity, protein, fat and ashes show that there are significant differences ($p < 0.05$). Protein contents of vegetable sausage ranged between 6.2 and 9.37%. The highest value in protein content responds to the formulation with the highest amount of lentil flour. Other authors, (Corrêa *et al.,* 2023; Marti-Quijal *et al.,* 2019; Priya *et al.,* 2022), have found total protein values that vary between 2.33% to 23% depending on whether they use legume and cereal flours or textured vegetable proteins.The fat medium used was sunflower oil, which has a low saturated fat content.

Table 2. Proximal composition of vegetable sausages

	M1	M2	M3	M4	M5	M6
			Carrageenan (0.8 %)			
Humidity (g·kg^{-1})	479±1^{a}	476±12^{a}	486±6.2^{a}	470±26^{b}	473±20^{b}	481±25^{c}
Proteins (g·kg^{-1})	93±0.2^{a}	77±2.8^{b}	62±0.1^{b}	64±0.1^{b}	63±5.0^{a,b}	66±4.9^{b}
Fat (g·kg^{-1})	117±22^{a}	111±33 ^{b}	113±2^{a,b}	114±2^{a,b}	115±2^{a,b}	118±20^{a}
Ashes (g·kg^{-1})	13±2.4^{a}	11±1.4^{b}	12±1.6^{a,b}	14±5.0^{a}	12±1.8^{a,b}	13±4.7^{a}
Carbohydrates (g·kg^{-1})	297±59	323±68	326±11	337±8.2	335±75	320±76
Energy supply (Kcal·kg^{-1})	2618	2604	2575	2638	2636	2615
Polyphenols (mg·kg^{-1})	82.6	--	--	1344.3	--	--
Total dietary fiber (g·kg^{-1})	63.0±1	--	--	102.0±2	--	--

Mi, where i is the experience number of the experimental design in Table 1. Means within files with different letter (a, b or c) are significantly different ($p < 0.05$).

Table 3. Amino acids profile of vegetable sausage (M6) and the amino acid requirements in different age groups according to FAO/WHO (1985). mgAA*(g protein)$^{-1}$

Amino acids (AA)	(M6)	Infants	Children (2 to 5 years)	Children (10 to 12 years)	Adults
Essential-amino acids					
Leucine	72.89	93	66	44	19
Isoleucine	27.33	46	28	28	13
Lysine	55.81	66	58	44	16
Methionine	9.11	----	----	----	----
Methionine+Cystine	29.61(*)	42	25	22	17
Phenil alanine	46.70	----	----	----	----
Phenil alanine + Tyrosine	82.01	72	63	22	19
Threonine	36.45	43	34	28	9
Valine	36.45	55	35	25	13
Cystine+Cysteine (as Cysteic acid)	20.50	----	----	----	----
Tyrosine	35.31	----	----	----	----
Tryptophan	10.25	17	11	9	5
Total essential amino acids	350.80				
Non essential-amino acids					
Alanine	50.11	----	----	----	----

Arginin	77.45	----	----	----	----
Aspartic acid + Asparagine	112.76	----	----	----	----
Glutamic acid + Glutamine	206.15	----	----	----	----
Glycine	56.95	----	----	----	----
Histidine	27.33	26	19	19	16
Proline	59.23	----	----	----	----
Hydroxiproline	7.97	----	----	----	----
Ornithine	1.14	----	----	----	----
Taurine	1.14	----	----	----	----
Total non essential amino acids	600.23				
Total determined aminoacid	951.03				

(*)Methionine+ Cystine+Cysteine

Recent studies (Domingo *et al.*, 2023), which analyzed the effect of replacing animal fat with vegetable fat (corn, palm oil and margarine) in meat sausages obtained the best results from a sensory and nutritional point of view when using corn oil. As can be seen (Table 2), both the polyphenol content and the total dietary fiber content substantially increase with the addition of carob flour. The fiber content found exceeds values obtained with other high protein formulations based on textured vegetable proteins (Bakhsh *et al.*, 2022).The nutritional value of vegetable sausage is determined not only by its protein content, but also by its amino acid profile. In terms of amino acid composition, legumes protein varies from cereal grain protein because it contains much more lysine and threonine while being deficient in tryptophan and sulphur containing amino acids such as methionine and cysteine. Table 3 shows the amino acid profile of the vegetable sausage made with the four types of flour under study (M6). The same table shows the amino acid requirements in different age groups according to FAO, 2003. From the data, it is observed that the composition of essential amino acids meets the amino acid requirements of adults and adolescents. These age ranges correspond to the potential consumers of this type of products. The combination of cereals and legumes in this formula made it possible to compensate for the deficiencies of each type of flour.

3.2. Determination of pH, water activity (aw), colour and water release

Table 4. Physicochemical properties (aw, pH and L, a, b parameters) and weight losses of sausage samples with 0.8% of carrageenan

Variable	M1	M2	M3	M4	M5	M6
L	52.92 ± 5.57^{a}	61.89 ± 3.32^{b}	65.71 ± 3.48^{c}	19.82 ± 1.92^{d}	18.01 ± 2.94^{d}	43.31 ± 3.75^{e}
a	$3.25\pm1.98^{a,b}$	8.28 ± 2.43^{c}	4.24 ± 3.05^{b}	0.45 ± 0.05^{a}	0.56 ± 0.05^{a}	3.82 ± 1.05^{b}
b	16.80 ± 4.02^{a}	$13.51\pm4.82^{a,b}$	$16.38\pm2.53^{a,b}$	$7.90\pm1.90^{b,c}$	6.93 ± 1.91^{c}	$15.34\pm2.92^{a,b}$
aw (25 °C)	0.97±0.01	0.97±0.01	0.97±0.01	0.96±0.01	0.97±0.00	0.96±0.01
pH	5.85 ± 0.05^{a}	5.73 ± 0.30^{a}	5.85 ± 0.16^{a}	$5.60\pm0.39^{a,b}$	5.33 ± 0.57^{c}	$5.43\pm0.60^{b,c}$
			Weight losses (%)			
Brea gum (BG)	6.43±0.60	4.25±0.60	9.07±0.70	5.40±0.60	5.59±0.10	11.05±0.10
Carrageenan (C)	5.01±0.11	4.01±0.13	8.45±0.11	5.05±0.13	5.04±0.60	10.21±0.20
(BG+C)	6.45±0.20	3.45±0.20	8.01±0.30	5.10±0.20	7.30±0.60	10.35±0.60

Means within files with different letter (a, b, c, d or e) are significantly different ($p < 0.05$).

Table 4 shows the results of aqueous activity, colour parameters and pH for the sausage samples with 0.8% of carrageenan. The luminosity and a* parameters were significantly

modified for the samples containing carob flour, observing a decrease in luminosity and products with a darker coloration. The b* value show a significant difference ($p < 0.05$). Carob flour, has the lowest L*value with high ash content, (Ammar *et al.,* 2022) indicating a significantly darker flour. The results of the sensory analysis of the product (not shown here) indicated that the samples containing carob flour showed a different colour than meat sausage, a particular flavour associated with "regional products", a homogeneous texture and good acceptability.

Table 5. Attributes of the texture profile of vegetarian sausage with different hydrocolloids (mean± deviation standard; n = 3).

Hydrocolloid	M1	M2	M3	M4	M5	M6
			Hardness (N)			
Carrageenan (C)	1.19±0.07	3.26±0.68	2.38±0.25	2.34±0.06	2.73±0.15	2.51±0.07
Brea gum (BG)	3.00±0.48	4.37±0.28	0.83±0.03	1.97±0.14	3.04±0.41	2.04±0.01
BG + C	2.00±0.60	3.01±0.00	0.70±0.00	1.00±0.01	2.59±0.10	1.05±0.05
			Chewiness (mJ)			
Carrageenan (C)	4.07±0.07	0.18±0.08	0.12±0.05	1.04±0.06	0.05±0.00	1.00±0.01
Brea gum (BG)	5.56±0.02	0.01±0.00	1.96±0.01	2.65±0.01	0.10±0.05	1.38±0.01
BG + C	4.02±0.60	0.10±0.00	1.09±0.00	1.50±0.11	0.08±0.00	0.74±0.05
			Stickness (mJ)			
Carrageenan (C)	1.14±0.05	0.10±0.00	1.00±0.05	1.05±0.01	0.14±0.00	0.10±0.01
Brea gum (BG)	2.66±0.30	0.12±0.07	1.49±0.14	2.49±0.27	0.17±0.00	1.00±0.01
BG + C	1.38±0.50	0.23±0.00	1.30±0.00	3.00±0.31	0.23±0.00	0.08±0.00

The water activity values show that it is a very favourable medium for microbial growth and samples should be stored under refrigeration.

The values of water release (Table 4), are statistically significant differences ($p<0.05$) for the flour combination block, but there is no significant effect of the type of hydrocolloid.

The experimental results show that products made with oat flour, (M3 y M6), are those with the lowest water retention. Vazquez *et al.,* (2017), studied the effect of replacing wheat flour with oat flour in bread making and found that mixtures containing oats had a lower water retention capacity.

3.3. Textural analysis

Table 5 shows the TPA parameters of the sausages for sample with the hydrocolloid combinations studied. Since a P-value is less than 0.05, the flour combination factor has a statistically significant effect on hardness, stickness and chewiness with a 95.0% confidence level.

When the experimental data for brea gum are analysed, the R-squared statistic indicates that the models adjusted for the three parameters explain 88.4, 97.9 and 95.6% of the variability of hardness, chewiness and adhesiveness respectively.

Hardness is maximized as the amount of rice flour increases while chewiness and adhesiveness parameters increase with lentil flour concentration.

The analysis of variance of several factors for the hardness parameter showed that there are no statistically significant differences when the type of hydrocolloid is modified. However, both

chewiness and stickiness showed statistically significant differences between carrageenan and brea gum with a 95% confidence level.

Stickiness is a measure of the energy required to overcome the surface attraction of the sausage when chewed. Products made with brea gum are somewhat sticky than when carrageenan is used. Chewyness, which is the product of hardness, cohesiveness and elasticity, represents the work necessary to disintegrate a food until it is ready to be swallowed; this parameter is larger when brea gum is used.

4. Conclusions

The incorporation of two regional raw materials in the formulations of vegetarian sausages produced changes in the physicochemical, textural and nutritional properties. From the nutritional point of view, the presence of carob flour allowed obtaining food with a higher content of polyphenols and fibers. The combination of the four flours studied allowed us to obtain a food that meets the amino acid requirements for adolescents and adults. A statistically significant decrease was observed in the parameters of luminosity and reddish colouration of the samples that contained carob flour. The textural characteristics of the vegetable sausages when brea gum was used as an additive are good. The hardness was not different ($p > 0.05$) among the products when carrageenan was replaced by brea gum at the same concentration. However, in the range studied, the products are slightly stickier.

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Research article

ISOLATION, IDENTIFICATION AND CHARACTERISTICS OF *HEYNDRICKXIA COAGULANS* STRAINS FOR INCLUSION IN PROBIOTIC PREPARATIONS

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Abstract

The inclusion of spore-forming bacteria in probiotic preparations for human and veterinary applications is a new trend in food supplements. The objective of this study was to isolate, identify and characterize the biological activities of two novel strains for the purpose of inclusion in probiotic supplements for animal feed, for food supplements for humans, as well as in the composition of plant protection preparations. The two strains were identified as *Heyndrickxia coagulans* through phenotypic and molecular genetic methods. Both strains were investigated for their total phenolics contents and antioxidant activity (DPPH and FRAP methods). In a series of experiments, the antibacterial and antifungal activity of the biomass and the cell-free supernatant of the cultural medium of *Heyndrickxia coagulans* after the cultivation of the strains in three different cultural media were determined. They showed significant difference in their antioxidant activity ($p>0.05$) and their antimicrobial activity against *Escherichia coli, Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Salmonella enterica* subsp. *enterica* serotype Abony, *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Bacillus cereus, Candida utilis, Saccharomyces cerevisiae, Aspergillus niger, Penicillium chrysogenum, Aspergillus flavus, Fusarium moniliforme*. It was established that one of the strains exhibited clear potential for inclusion in probiotic and plant protection preparations.

1. Introduction

Spore-forming bacteria are used as probiotic additives in animal feed, in human food supplements, and in registered drugs (Casula *et al.*, 2002; Bomko *et al.*, 2016; Cao *et al.*, 2020). Their high heat resistance and ability

to survive as spores in the adverse conditions of the gastrointestinal tract make them suitable as food supplements. This is a new direction in the science of probiotics for human and veterinary purposes (Hong *et al.*, 2005; Cao *et al.*, 2020). Bacterial spores are formed in nature as a means of survival under extreme environmental conditions that could destroy the vegetative bacterial cells (Nicholson *et al.*, 2000). Under harsh conditions, most often depletion of nutrients in the immediate vicinity of the living cell, the bacteria stop their growth and undergo a process of sporulation (Errington, 2003).

One of the most promising spore-forming bacteria with probiotic potential are *Heyndrickxia coagulans* (formerly *Bacillus coagulans*). Their spores tolerate technological processes well, and when they enter the duodenum, they germinate and the resulting vegetative cells populate the intestinal tract of humans and animals. Due to their high biological activity, the spores of this species are included in the composition of pharmaceutical preparations (Hoa *et al.*, 2000; Bomko *et al.*, 2017; Cao *et al.,* 2020).

Extracts derived from bacilli have been shown to have antimicrobial, antifungal, antioxidant, and anticancer activities (Blunt *et al.*, 2018; León *et al.*, 2010; Prazdnova *et al.*, 2015; Velasquez Cardona *et al.*, 2018). Since oxidative stress is directly or indirectly involved in various pathological conditions in humans (Forman and Zhang, 2021), bioproducts derived from *Bacillus* sp. may hold promise as effective agents for the prevention and treatment of chronic diseases by reducing oxidative stress, including cancer (Céspedes *et al.,* 2023). Synthetic antioxidants, which are widely used in industrial applications, are being investigated for their toxicity and carcinogenic effects (Moktan *et al.*, 2008; Thitilertdecha *et al*., 2008). Interest in finding natural antioxidant agents with low cytotoxicity has increased significantly (Thitilertdecha et al., 2008). Plants are mainly used for their production (Teow *et al*., 2007; Erkan *et al*., 2008), but microbial sources have shown potential for the production of natural antioxidants in various fermented products (Sheih *et al*., 2000; Wang *et al*., 2007; Esaki *et al*., 1997; Hirota *et al*., 2000; Ren *et al*., 2006; Yen *et al*., 2003). Kumari *et al*. (2012) also demonstrated that microorganisms can produce antioxidants and these antioxidants act as preservatives in food products.

Bacteria of the former genus *Bacillus* (including *Heyndrickxia coagulans)* exhibit different antimicrobial activities, associated with the formation of more than 200 antibiotic substances (Khochamit *et al*.,2015; Tenea *et al*., 2022), which is why individual strains differ in their antagonistic spectrum (Hoa *et al*., 2000; Tenea *et al*., 2022). This requires the selection of strains with pronounced antimicrobial activity against pathogenic and saprophytic microorganisms.

Bacilli with proven antifungal and antibacterial properties, are a part of biological preparations both for agriculture (as plant protection products, for example, phytosporin and probiotics for animal husbandry - biosporin) and for human purposes (Tenea *et al*., 2022). The activity of these forms depends on the concentration of substances with antimicrobial activity produced by the selected strains during cultivation, as well as on the amount of spores obtained. The type and concentration of substances with antimicrobial activity and the amount of spores depend on the composition of the fermentation medium and the cultivation conditions. In order for a preparation to be used in practice, it is necessary to contain a high concentration of spores of the respective strain (not less than 10^9 cfu/g).

The aim of the present study was to isolate, identify and characterize the biological activities of two *Heyndrickxia coagulans* strains, which are requirements for probiotic bacteria.

2. Materials and methods

2.1. Microorganisms

The subjects of the study are two unidentified bacterial strains, isolated from spontaneously coagulated pasteurized milk. The strains were maintained on nutrient medium with the following composition

(g/dm3): peptone from meat – 10; NaCl – 5; meat extract – 3; glucose – 10; agar-agar – 15, pH 7.5, and subcultured every 60 days.

2.2. Methods

2.2.1 Phenotypic identification

The phenotypic identification was done using API 50 CH and API 50 CHB/E medium (BioMérieux® SA, Marcy-l'Etoile, France) following the manufacturer's manual.

2.2.2 Molecular identification

The molecular identification of the new strains was performed according to the method of Urshev *et al.* (2024).

2.2.3 Cultivation of Heyndrickxia coagulans

The isolates were cultivated in the following nutrient media:

Medium A (g/dm^3): molasses – 20; peptone – 10; corn extract – 3; $CaCl_2$ – 0.22; $MgSO_4$ – 0.11; K_2HPO_4 – 0.24; pH 7.5

Medium B (g/dm^3): malt – 20; corn extract – 3; molasses – 20; CaCl2 – 0.22; $MgSO_4$ – 0.11; K_2HPO_4 – 0.24; pH 7.5

Medium C (g/dm^3): peptone – 10; NaCl – 10; meat extract – 5; $CuCl_2$ – 0.001; $MgSO_4$ – 0.5; pH 7.5

The cultivation of the strains was carried out in 500 cm^3 Erlenmeyer flasks with 100 cm^3 nutrient medium at a temperature of 37°C for 48 hours on a rotary shaker (220 min^{-1}). The nutrient medium was inoculated with 1% (v/v) of 18 h vegetative cell suspension.

2.2.4 Total polyphenolics assay

The content of total polyphenolics in the samples was determined according to the method of Ainsworth and Gillespie (2007) with Folin-Ciocalteu (FC) reagent, briefly - 0.2 mL of the sample was mixed with 1.0 mL of FC reagent and after 30 sec, 0.8 mL of 7.5% Na_2CO_3 was added. After 30 min in the dark, the absorbance was measured at 765 nm. The results are presented as mg gallic acid equivalents (GAE) in 1 mL (mg GAE/mL).

2.2.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The radical scavenging activity of the samples was determined according to a modified method of Dimov *et al.* (2018) as follows: 0.15 mL of the sample was mixed with 2.85 mL of freshly prepared DPPH solution (0.06 mM in 96% ethanol). After 30 minutes in the dark at room temperature (23-25°C) the absorbance was measured at 517 nm. The results are presented as mM Trolox equivalents (TE) in 1 mL (mM TE/mL). For comparison, the synthetic antioxidant butylhydroxytoluene (BHT) was used in concentrations of 0.01-0.1 % (positive control).

2.2.6 FRAP (ferric reducing antioxidant power) assay

The metal reducing activity of the samples was determined according to the following modification of the method of Dimov *et al.* (2018): 0.1 of sample was added to 3 mL of freshly prepared FRAP reagent [0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in a ratio of 10:1:1]. The reaction mixture was incubated for 10 minutes at 37°C in the dark and the absorbance was measured at 593 nm. $FeSO_4$.$7H_2O$ was used to construct a standard line. The results are presented as µmol Fe^{2+} equivalents in 1 mL (µmol Fe^{2+}/mL). For comparison, the synthetic antioxidant BHT was used in concentrations of 0.01-0.1% (positive control).

2.2.7 Determination of antimicrobial activity

To determine the antimicrobial activity of the tested strains against pathogenic and saprophytic microorganisms, biomass in saline (BM) and cell-free supernatant (CFS) were obtained from a 24-hour culture of the strains. The culture medium was subjected to centrifugation at 3500 min^{-1} for 15 min to separate the biomass, after which the cell-free supernatant was separated and filtered through a membrane filter (0.45 µm). The biomass was washed twice with saline and brought to the initial volume with saline.

The antimicrobial activity was tested against *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 25928, *Salmonella enterica* subsp. *enterica* serotype Abony NTCC 6017, *Staphylococcus aureus* ATCC 6538P, *Listeria monocytogenes* ATCC 19111, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 14579, *Candida utilis* ATCC 42402,

Saccharomyces cerevisiae ATCC 9763, *Aspergillus niger* ATCC 1015, *Penicillium chrysogenum* ATCC 28089, *Aspergillus flavus* ATCC 9643, *Fusarium moniliforme* ATCC 38932. Suspensions of each of the test-microorganisms, or their spores for the molds, (10^6-10^7 cfu/cm^3), were prepared and used to inoculate Petri dishes with LBG-agar medium (Composition (g/dm^3): tryptone - 10; yeast extract - 5; NaCl - 10; glucose – 10; agar - 15, pH 7.5). After solidification of the agar, wells (6 mm in diameter) were prepared and 0.06 cm^3 of BM or CFS were pipetted into the wells and the Petri dishes were incubated at 30°C for saprophytic microorganisms or 37°C for pathogenic microorganisms for 24 to 48 h. The antimicrobial activity was determined by measuring the zones of inhibition in mm.

2.3. Data analysis

All experiments were performed with three replications (n=3) and the results were presented as mean values. The obtained results were analysed using Statgraphics Centurion 18 (Statgraphics Technologies, Inc.) by the method of one-way analysis of variance (ANOVA) according to Duncan's test with the probability of accepting the null hypothesis ($p < 0.05$).

3. Results and discussions

Two novel strains were isolated form spontaneously fermented pasteurized milk. Their characterization began with an assessment of the purity of the culture, macroscopic and microscopic morphological control. The colony and cell morphology of the isolates are presented in Tables 1 and 2.

When cultivated on meat extract agar, the isolated strains grew in the form of round to elliptical whitish colonies with lobate edges and a dry texture, which were easily separated from the medium surface. Further microscopic investigations showed that both strains were Gram-positive (Gr (+)), spore-forming rods.

Table 1. Colony morphology of the novel strains

Strain	Shape	Edges	Surface	Elevation	Texture	Colour	Size
M	circular to elliptical	lobate	smooth	raised	dry	whitish	3 – 4 mm medium
BJ	circular to elliptical	lobate	smooth	raised	dry	whitish	3 – 4 mm medium

Table 2. Cell morphology of the novel strains

Strain	Shape	Edges	Spatial arrangement	Motility	Spore formation	Gram staining
M	short fine rods	round-ended	solitary, side-by-side or short chains	yes	yes	Gr (+)
BJ	short fine rods	round-ended	solitary, side-by-side or short chains	yes	yes	Gr (+)

These characteristics necessitated the use of the API 50 CHB/E rapid identification system for representatives of *Bacillaceae.* The ability of the isolated strains to utilize 49 carbon sources included in the system was investigated (Table 3). After processing of the test results with apiweb®, the strains were identified with the corresponding percentage of reliability.

Table 3. Carbohydrate utilization patterns of the tested isolates

Carbohydrate	Strain	
	M	**BJ**
Control	-	-
Glycerol	+	+
Erythriol	-	-
D-arabinose	-	-
L-arabinose	+	+
Ribose	+	+
D-xylose	+	+
L-xylose	-	-
Adonitol	-	-
β-metyl-D-xyloside	-	-
Galactose	+	+
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
L-sorbose	-	-
Rhamnose	-	+
Dulcitol	-	-
Inositol	-	-
Manitol	+	+
Sorbitol	+	+
α-methyl-D-mannoside	+	-
α-methyl-D-glucoside	+	+
N-acetyl-glucosamine	+	-
Amigdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+	+
Saccharose	+	+
Trehalose	+	+
Inulin	+	+
Melezitose	+	+
D-raffinose	+	+
Amidon	+	-
Glycogen	+	+
Xylitol	-	-
β-gentiobiose	+	+
D-turanose	+	-
D-lyxose	-	-
D-tagarose	-	-

D-fuccose	-	-
L-fuccose	-	-
D-arabitol	-	-
L-arabitol	-	-
Gluconate	+	-
2-keto-gluconate	-	-
5-keto-gluconate	-	-
Identification	*Heyndrickxia coagulans*	*Heyndrickxia coagulans*
Reliability, %	93,9	91,8

After the data from Table 3 was processed with the apiweb® software both strains M and BJ were determined as *Heyndrickxia coagulans* with a reliability of 93.9% and 91.8%, respectively. For more accurate species determination, molecular genetic methods were used. The comparative analysis of the 16S rDNA gene sequence confirmed the identification of the strains – strain M was assigned to *Heyndrickxia coagulans* with a percentage of confidence between its nucleotide sequence of the 16S rDNA and the partial sequence of the 16S rDNA of *Heyndrickxia coagulans* DSM 1 = ATCC 7050 of 99%, strain BJ was assigned to *Heyndrickxia coagulans* with a percentage of confidence between its nucleotide sequence and the partial sequence of the 16S rDNA of *Heyndrickxia coagulans* NBRC 12583 of 99 %.

The former genus *Bacillus* is well known for its production of metabolites with antioxidant activity, such as phenolic acids (Safronova *et al*., 2021). Polyphenols exhibit strong antioxidant activity, which is closely related to their high reactivity towards reactive oxygen species. The isolates identified as *H. coagulans* M and *H. coagulans* BJ showed similar phenolic content (Table 4).

Table 4. Antioxidant activity of the *Heyndrickxia coagulans* strains

DPPH, mM TE/cm^3		FRAP, µmol Fe^{2+}/cm^3		Total polyphenols, mg GAE/mL	
H. coagulans M	*H. coagulans* BJ	*H. coagulans* M	*H. coagulans* BJ	*H. coagulans* M	*H. coagulans* BJ
0.38±0.01^a	0.56±0.01^b	0.54±0.02^a	0.80±0.02^b	0.21±0.01^a	0.23±0.02^a

$^{a, b}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows for each method

Most antioxidant compounds were polyphenols, acting as reducing agents (free radical scavengers), metal chelators and singlet oxygen scavengers (Mathew and Abraham, 2006). This was also evidenced by the results form determining the antioxidant activity in the present study. *H. coagulans* BJ showed higher antioxidant activity than *H. coagulans* M, both in the DPPH and FRAP assays (Table 4). Such positive correlations between antioxidant activity and total phenolic content have been demonstrated for a number of foods and beverages - red wines (Vinson and Huntz, 1995), vegetables (Kaur and Kapoor, 2002; Ordoñez *et al*., 2006), grapes, marc, must, wine and juice (Yildirim *et al*., 2005), in some medicinal and aromatic plants (Miliauskas *et al*., 2004). This indicates that polyphenols in our samples can play the role of electron and hydrogen donors.

In order to compare the antioxidant activity the *H. coagulans* strains with the activity of a synthetic antioxidant, the activity of BHT in concentrations of 0.005-0.1 % according to the DPPH and FRAP methods was determined. The results shown in Table 5 indicate that with

increasing the BHT concentration, the antioxidant activity increased proportionally. In the DPPH method, the antioxidant activity values for the two strains ranged within 0.38-0.56 mM TE/cm^3, and at the studied BHT concentrations – within 0.15-0.68mM TE/cm^3, i.e. in this method the results obtained in the present research were comparable to those obtained with the synthetic antioxidant up to a concentration of 0.03%.

Table 5. Antioxidant activity of BHT

Concentration of BHT, %	DPPH, mM TE/mL	FRAP, µmol Fe^{2+}/mL
0.005	0.15±0.00	0.22±0.02
0.01	0.30±0.01	0.48±0.02
0.02	0.46±0.03	0.86±0.03
0.03	0.55±0.03	1.05±0.01
0.04	0.63±0.01	1.35±0.01
0.05	0.67±0.01	1.65±0.02
0.10	0.68±0.02	2.17±0.02

In the FRAP method, BHT showed values ranging between 0.22 µM Fe^{2+}/cm^3 and 2.17 µM Fe^{2+}/cm^3 at the tested concentrations, while our samples had values of 0.54-0.80 µM Fe^{2+}/cm^3, i.e. the extracts showed the ability to donate hydrogen atoms and therefore can serve as free radical "scavengers", acting as primary antioxidants (Chung *et al*., 2006).

In a series of experiments, the antimicrobial activity of the *Heyndrickxia coagulans* strains was studied after their cultivation in three different fermentation media - medium A, medium B and medium C. The influence of the composition of the nutrient medium on the antibacterial and antifungal activity of the biomass and cell-free supernatant was established (Table 6). *H. coagulans* M cultivated on medium A did not exhibit antifungal activity against *Aspergillus flavus* and displayed a less pronounced one against *Penicillium chrysogenum* (Table 6). For the remaining yeasts and molds, a strongly pronounced antifungal activity was observed, which was evidenced by the large diameter of the inhibition zones, ranging from 14 mm to 32 mm. The cell-free supernatant of the strain cultivated in medium A showed antifungal activity against all tested molds, with the diameter of the inhibition zones varying from 10 mm to 32 mm. The biomass of the strain cultivated in medium B also showed lower activity against *Aspergillus flavus*, as well as a less pronounced one against *Penicillium chrysogenum*, where the smallest inhibition zones were established - 9 mm. For all other tested molds and yeasts, a highly pronounced antifungal activity was observed, characterized by inhibition zones ranging from 13 mm to 28 mm. The supernatant obtained from the cultivation of the studied strain in medium B also exhibited high activity against the tested microorganisms, with inhibition zones ranging from 10 mm to 34 mm. Unlike the previous two media, in medium C, a highly pronounced antifungal activity was observed, both in the biomass and in the cell-free supernatant against all tested representatives of molds and yeasts, with the inhibition zones ranging from 12 mm to 37 mm for the different test-microorganisms.

From the data presented in Table 6, it is clear that the biomass of *H. coagulans* M, cultivated in all media exhibited weak antibacterial effect on the growth of *Salmonella enterica* subsp. *enterica* serovar Enteritidis, which was confirmed by the small diameter of the inhibition zones (8 mm). The same result was observed for the activity of the cell-free supernatant against this test-microorganism.

Table 6. Antimicrobial activity of the *Heyndrickxia coagulans* strains

Test-microorganism	*Heyndrickxia coagulans* M						*Heyndrickxia coagulans* BJ					
	Medium A		**Medium B**		**Medium C**		**Medium A**		**Medium B**		**Medium C**	
	BM	CFS	BM	CFS	BM	CFS	BM	CFS	BM	CFS	BM	CFS
***C. utilis* ATCC 42402**	20.33±0.47	20.17±0.24	25.17±0.24	25.33±0.47	37.50±0.41	32.33±0.47	-	-	-	-	-	-
***S. cerevisiae* ATCC 9763**	32.67±0.47	30.17±0.24	28.33±0.47	34.67±0.47	30.67±0.47	24.17±0.24	-	-	-	-	-	-
***A. niger* ATCC 1015**	17.50±0.41	15.17±0.24	13.17±0.24	12.33±0.47	22.17±0.24	22.33±0.47	10.17±0.11	9.17±0.12	9.17±0.45	9,17±0.16	11.17±0.34	9.17±0.29
***A. flavus* ATCC 9643**	-	10.17±0.24	9.17±0.24	10.50±0.41	37.67±0.47	25.67±0.47	9.23±0.22	-	15.24±0.27	10.17±0.33	15.20±0.12	14.11±0.37
***F. moniliforme* ATCC 38932**	28.50±0.41	32.33±0.47	20.33±0.47	33.67±0.47	20.17±0.24	12.17±0.24	9.12±0.34	10.15±0.17	10.14±0.33	9.13±0.42	12.12±0.44	10.17±0.14
***P. chrysogenum* ATCC 28089**	9.17±0.24	10.33±0.47	9.33±0.47	10.17±0.24	15.33±0.47	12.33±0.47	-	-	12.17±0.12	10.15±0.00	10.14±0.22	9.2±0.26
***E. coli* ATCC 25922**	15,33±0,47	17,33±0,47	13,33±0,47	18,50±0,41	13,33±0,47	9,17±0,24	-	-	-	-	-	-
***S. enterica* subsp. *enterica* serovar. Enteritidis ATCC 25928**	8,17±0,24	8,33±0,47	8,17±0,24	8,17±0,24	8,17±0,24	-	-	-	-	-	-	-
***S. enetrica* subsp. *enterica* serotype Abony NTCC 6017**	15,50±0,41	10,33±0,47	9,33±0,47	9,33±0,47	8,33±0,47	-	-	-	-	-	-	-
***S. aureus* ATCC 6538P**	24,67±0,47	24,67±0,47	17,50±0,41	33,67±0,47	32,67±0,47	15,50±0,41	-	-	-	-	-	-
***P. aeruginosa* ATCC 9027**	10,17±0,24	14,67±0,47	13,17±0,24	16,33±0,47	17,50±0,41	9,17±0,24	-	-	-	-	-	-
***L. monocytogenes* ATCC 19111**	30,50±0,41	37,67±0,47	20,17±0,24	33,50±0,41	27,67±0,47	18,33±0,47	-	-	-	-	10.17±0.22	10.17±0.44
***B. cereus* ATCC 14579**	25,17±0,24	28,50±0,41	15,17±0,24	26,33±0,47	17,17±0,24	12,50±0,41	-	-	-	-	-	-

d_{well}=6 mm

The biomass and the cell-free supernatant obtained from the cultivation of *Heyndrickxia coagulans* M on medium A exhibited strong antibacterial activity against the rest of the tested pathogens, with inhibition zones ranging from 10 mm to 37 mm. When the strain was cultivated in the medium B a less pronounced activity of the biomass and the cell-free supernatant against *Salmonella enterica* subsp. *enterica* serotype Abony was observed, with inhibition zones of 9 mm. For all other pathogens, the *H. coagulans* M strain showed high activity with inhibition zones of 13 mm to 33 mm depending on the tested pathogen. The biomass of the strain obtained from its cultivation in medium C also showed weak antimicrobial activity against *Salmonella enterica* subsp. *enterica* serotype Abony, which was confirmed by the small diameter of the inhibition zone – 8 mm. Against all other pathogens, the studied strain showed high antimicrobial activity with inhibition zones ranging from 13 mm to 32 mm. The cell-free supernatant of *H. coagulans* M, obtained by culturing it in medium C, did not show antimicrobial activity against both *Salmonella enterica* subsp. *enterica* strains and exhibited weak activity against *Escherichia coli* and *Pseudomonas aeruginosa* with inhibition zones of 9 mm. For the remaining pathogens, the cell-free supernatant showed highly pronounced antimicrobial activity, with inhibition zones ranging from 12 to 18 mm for the different pathogens.

Other authors also report on the antimicrobial activity of *Bacillus coagulans* (*Heyndrickxia coagulans*). Mazhar *et al*. (2024) found that novel strain exhibited activity against multiple fungal and bacterial oral, gastrointestinal, skin and UTI pathogens, including *S. enteritidis, E. coli* and *S. aureus,* which were also inhibited by our newly isolated strain *H. coagulans* M. In another study 31 *B. coagulans* strains were effective against *B. cereus* and *S. aureus* (Kim *et al*., 2020). Abdhul *et al* (2015) demonstrated that *Bacillus coagulans* BDU3 was active against the pathogens *Staphylococcus aureus, Enterococcus* sp. and *Bacillus cereus*.

The results presented in Table 6 show that the second strain – *Heyndrickxia coagulans* BJ exhibited much weaker antimicrobial activity, which was strongly influenced by the composition of the nutrient medium. The lowest activity was observed after cultivation in the medium A. The biomass and the cell-free supernatant demonstrated antifungal activity against *A. niger* and *F. moniliforme* with the inhibition zone diameters being in the range of 9 mm to 10 mm. Against *A. flavus* antifungal activity was observed only for the biomass with an inhibition zone of 9 mm. *Heyndrickxia coagulans* BJ did not exhibit activity against *Candida utilis*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum*. The cultivation in the other two fermentation media led to activity against *A.niger, A.flavus, F.moniliforme* and *P. chrysogenum* with antifungal activity exhibited by both the biomass and the supernatant with inhibition zones in the range of 9 mm to 15 mm. This could be explained by the higher protein content in these media, which is a building block of substances with antimicrobial activity.

As for the antibacterial activity of *Heyndrickxia coagulans* BJ, the strain exhibited almost no effect on the pathogenic microorganisms. It was absent against all test microorganisms when the strain was cultivated in a medium with molasses. In a medium with malt, the strain demonstrated activity only against *S. aureus* and only by the biomass with an inhibition zone of 13 mm. When cultivating *Heyndrickxia coagulans* BJ in medium C, antibacterial activity of the biomass and supernatant was observed only against *L. monocytogenes* with inhibition zones of 10 mm.

According to Ostad *et al*. (2024), no antimicrobial activity of the *B. coagulans* supernatant was detected when applying the agar well diffusion method, but MIC results showed that different concentrations of *B. coagulans* supernatant significantly inhibited the growth of *E. coli, S. typhi, S. flexneri* and *B. cereus*. It is possible that the antibacterial substance produced by our strain did not diffuse well in the agar plate, or that its

concentration was too low to be detected with this method.

4. Conclusions

The present study identified and characterised two bacterial isolates as *Heyndrickxia coagulans.* The two strains exhibited significant antioxidant activity. It was demonstrated that *H. coagulans* M had highly pronounced antibacterial and antifungal activity. It was established that *H. coagulans* M demonstrated higher antimicrobial activity when growing in a medium with molasses, followed by a medium with malt and meat extract medium. Further investigation into the nature of the antimicrobial substances and their safety for humans and animals would allow for the inclusion of the novel strains into probiotic supplements.

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Research article

ACTIVITIES OF CMC-ASE AND β-GLUCOSIDASE IN CELLULOLYTIC BACTERIA FROM BUFFALO RUMEN GROWN ON CARBOXYMETHYL CELLULOSE

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Abstract

Cellulase is an enzyme widely used in the feed and food sectors. This study aims to examine the CMC- ase and β- glucosidase activity of cellulolytic bacteria in degrading cellulose content in CMC substrate and determine the optimum incubation period to produce cellulase enzymes from buffalo rumen bacteria. This study used a factorial Completely Randomized Design (CRD) consisting of 2 factors and 4 replications. The first factor is the type of bacterial isolate consisting of 2 types, namely ST6 and ST8. The second factor is the difference in the incubation period consisting of 5 treatments (T0: 0 days, T1: 3 days, T2: 6 days, T3: 9 days, and T4: 12 days) on the use of 1% CMC (Carboxy Methyl Cellulose) substrate. The data were analyzed using variance (ANOVA) and further tested using Duncan's Multiple Range Test (DMRT). The results of data analysis showed that CMC-ase activity was influenced by the incubation period with the best incubation period at 9 days (T3), and there was a significant interaction between the type of isolate and the incubation period. The activity of the β- glucosidase was influenced by the type of isolate and the incubation period, with the best results at an incubation period of 6 days. The interaction between the isolate type and the incubation period significantly affected the activity of the β- glucosidase.

1. Introduction

Cellulases are key enzymes with widespread applications in various industries, including animal feed, agriculture, paper and pulp, winemaking and brewing, food processing, olive oil extraction, carotenoid extraction, pharmaceuticals, and pollution treatment (Artono et al., 2023; Mrudula et al.,

2011; Retnoningtyas et al., 2013). Their significance extends to the bio-conversion of agricultural waste into valuable products like fermentable sugars and bioethanol (Al-baarri et al., 2018; Damayanti et al., 2020; Paramita et al., 2021). Cellulase enzymes hydrolyze cellulose by breaking the β-1,4 glycosidic bonds in cellulose, cellodextrins, cellobiose, and other cellulose derivatives, converting them into simple sugars or glucose (Mrudula et al., 2011; Wibawanti et al., 2021). Typically, cellulase can be derived from various microorganisms, but bacteria are particularly advantageous due to their rapid growth, which shortens production time (Alam et al., 2004).

Among cellulolytic microorganisms, cellulolytic bacteria hold notable promise for efficient cellulose degradation in fields such as waste reduction, biofuel production, and sustainable agriculture. These bacteria, capable of breaking down cellulose complexes into oligosaccharides and glucose can be sourced from the rumen fluid of ruminant livestock, including buffalo (Gharechahi et al., 2023). Cellulolytic bacteria are more easily isolated from the digestive tract of livestock because the digestive system of animals, such as ruminants, supports cellulose fermentation by microbes in the rumen (Kong et al., 2012). Cellulose is broken down into sugars, which are fermented into short-chain fatty acids (SCFA) as an energy source. In contrast, humans cannot directly digest cellulose, which is only fermented by microbes in the large intestine in smaller amounts, making the isolation of cellulolytic bacteria in humans more difficult (Kamil et al., 2021). Buffalo rumen fluid is preferred due to its higher cellulolytic bacterial content and greater cellulolytic activity than cow rumen fluid, as Pradhan (1994) reported; buffalo rumen fluid contains 6.86 x 10^8 CFU/ml cellulolytic bacteria compared to 2.58 x 10^8 CFU/ml in cow rumen fluid. A cellulose-rich environment, coupled with higher bacterial populations, drives more efficient enzyme production, potentially enhancing the effectiveness of cellulase-based technologies (Borthakur et al., 2024)

The production of cellulase enzymes often employs media containing Carboxymethyl Cellulose (CMC) as a substrate, which serves as a primary carbon and energy source without containing reducing sugars or lignin, thereby enhancing enzymatic activity (Doan et al., 2024). Enzyme activity is also influenced by factors like the length of incubation, where optimal incubation periods can maximize hydrolysis efficiency (Meilany et al., 2020). However, studies exploring the conditions for β-glucosidase activity in cellulolytic bacteria from buffalo rumen are limited (Borthakur et al., 2024b; Murtius et al., 2022; Wonoputri et al., 2018). Consequently, optimizing the incubation period for cellulase activity in these bacteria could reveal new opportunities for enhancing cellulose degradation. By advancing the practical applications of cellulases in biofuel production and sustainable waste management, this study could provide valuable insights for developing more efficient, cellulose-based biotechnologies.

2. Materials and methods

2.1.Tools and materials

The tools used in this study include thermoses, gauze, cloth, CO_2 tubes, test tubes, erlenmeyer flasks, test tube racks, butyl rubber, cotton, petri dishes, pH meters, vortex, magnetic stirrers , analytical scales, spatulas, stirring rods, aluminum foil, plastic wrap , bunsen, ose needles, microtubes, anaerobic jars, centrifuges, water bath, incubator, autoclave, scissors, UV-Vis spectrophotometer, measuring cylinder, micropipette, and measuring pipette.

The materials used in this study included cellulolytic bacterial isolates ST6 and ST8, which were isolated from buffalo rumen fluid in Kudus, Central Java, Indonesia. Selection of bacterial isolates based on the highest CMC-ase activity of the 8 isolates that were successfully isolated. Other materials are aquabidest, aquades, alcohol, Nutrient Broth (NB), Carboxyl Methyl Cellulose (CMC), agar, $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCl_2.2H_2O$, *2*-Ortho-Nitrophenyl-β-D-glucopyranoside (NPG), 3,5-dinitrosalicylic acid (DNS), glucose, NaOH, DNS reagent, and standard glucose solution.

2.2.Method

The research design used in this study was a factorial, Completely Randomized Design (CRD) consisting of 2 factors and 4 replications. The first factor is the type of isolate, consist of 2 types, namely ST6 and ST8. The second factor is the difference in incubation time consisting of 5 treatments on the use of 1% CMC substrate, namely:

T_0 : zero day incubation
T_1 : three days incubation
T_2 : six days incubation
T_3 : nine days incubation
T_4 : twelve days incubation

2.3.Research Procedures

2.3.1.CMC fermentation with different incubation times

The materials used in the manufacture of liquid treatment media are $NaNO_3$ 0.75 g, K_2HPO_4 0.1875 g, $MgSO_4.7H_2O$ 0.0075 g, $MnSO_4.7H_2O$ 0.0075 g, $FeSO_4.7H_2O$ 0.0075 g, $CaCl_2.2H_2O$ 1.875 g, aquabidest 375 ml, CMC 3.75 g which are divided into 24 test tubes. First, the weighed materials are mixed until homogeneous then measured and adjusted the pH until the pH value becomes neutral. The homogeneous media is then put into a tube as much as 15 ml in each test tube that already contains 0.15 g of CMC. After that, the media is sterilized using an autoclave at a temperature of 121^0 C with a pressure of 1 atm for 20 minutes. The next step is when the media is warm, isolates ST6 and ST8 are inoculated as much as 0.1 ml into a test tube, each inoculated isolate is repeated four times. After that, all the tubes are flowed with CO_2 and then tightly closed so that the conditions in the tube are anaerobic. Furthermore, all the tubes that have been tightly closed are stored in an incubator at a temperature of 39° C for 0, 3, 6, 9 and 12 days. After the incubation process is complete, then harvesting is carried out by cold centrifugation at a speed of 3000 g for 20 - 30 minutes. The filtrate obtained (as a crude enzyme extract) is put into a 1.5 ml microtube and stored in the freezer for enzyme activity testing.

2.3.2.DNS reagent preparation

The DNS reagent was made by weighing 1 gram of 3,5- dinitrosalicylic acid (DNS) which was then dissolved in 20 ml of 2 N NaOH solution with 50 ml of distilled water, then 30 grams of K-Na tartrate were added and homogenized using a magnetic stirrer . Finally, distilled water was added until the final volume of the solution was 100 ml.

2.3.3.Preparation of glucose standard curve

A standard curve was made to determine the concentration of reducing sugar by making a glucose stock solution, namely 1 gram of glucose dissolved in 100 ml of aquabidest, which means that 1 ml of stock solution contains 10 mg of glucose. Then a dilution series was made with concentrations of 0, 50, 100, 150, 200, 250, and 300 ppm by inserting 0.1 ml of 1 mg/ml glucose solution into a test tube and then adding 1.9 ml of aquades as listed in Table 1. Next, 2 ml of DNS solution (3.5- Dinitro salicylic acid) was added and homogenized.

Table 1. Concentration of Glucose Solution in Making Glucose Standards

Final Concentration		Aquadest (ml)	Standard stock solution of glucose added (ml)
ppm	mg/ml		
0	0.00	2.00	0.00
50	0.05	1.90	0.10
100	0.10	1.80	0.20
150	0.15	1.70	0.30
200	0.20	1.60	0.40
250	0.25	1.50	0.50
300	0.30	1.40	0.60

The resulting mixture solution was then incubated at 100° C for 15 minutes. After the incubation process was complete, it was cooled and the absorbance was measured using a spectrophotometer with a wavelength of 540 nm. The standard curve was made 3 times. The absorbance obtained from the measurement results was processed using Microsoft Excel with the absorbance value as the x-axis and the concentration value as the y-axis, until the reaction equation and regression were obtained.

2.3.4.CMC-ase activity test

Cellulase enzyme measurement was carried out by measuring the reducing sugar content of 3 groups of test tubes consisting of samples, controls, and blanks, the method of analysis referred to Yogyaswari (Yogyaswari et al., 2016) with modification. In the sample, 1 ml of crude extract enzyme was added with 1 ml of 1% CMC solution, then homogenized using a vortex and incubated at room temperature for 60 minutes. A CMC level of 1% was used because it is the best for testing CMC-ase activity and is in accordance with the reference.

After that, 2 ml of DNS and incubated in a water bath at 100° C for 10 minutes and cooled. In control, 1 ml of 1% CMC solution was added with 2 ml of DNS and added with 1 ml of crude extract enzyme, then vortexed and incubated in a water bath at a temperature of 100° C for 10 minutes. Control tubes contain substrate and enzyme but are immediately incubated at a temperature of 100° C to stop the enzyme reaction.

In the blank, 1 ml of 1% CMC solution was added with 2 ml of DNS and 1 ml of aquadest, then vortexed and incubated in a water bath at 100° C for 10 minutes. After cooling, the absorbance was measured in the three tubes using a spectrophotometer with a wavelength of 540 nm. The concentration of reducing sugar can be generated using the formula (Sample Absorbance – Blank Absorbance) – (Control Absorbance – Blank Absorbance) (Wijanarka et al ., 2016). The determination of cellulase activity value can be calculated using the following equation:

Cellulase activity (U/ml) = reducing sugar concentration x DF x 1000MW of flucose x t (1)

Information :
DF: Enzyme dilution factor
MW of glucose : Molecular weight of glucose (180.18 g/mol)
t : Incubation time

Table 2. Concentration of NPG Solution in Making Standard NPG Solution Graphs

Solution Concentration		H_2O Sterile (ml)	NPG Standard Stock Solution (ml)
µg/ml	mg/ml		
0	0,000	5	0.00
20	0.02	4.9	0.10
40	0.04	4.8	0.20
60	0.06	4.7	0.30
80	0.08	4.6	0.40
100	0.1	4.5	0.50
120	0.12	4.4	0.60
140	0.14	4.3	0.70
160	0.16	4.2	0.80
180	0.18	4.1	0.90
200	0.20	4.0	1.00

2.3.5.Making standard graph of NPG solution

The standard graph of NPG solution was made using a stock solution of 2-Ortho-Nitrophenyl-β-D-glucopyranoside (NPG). First, a solution with a concentration of 1 mg/ml was made by dissolving 10 mg of NPG into 10 ml of H_2O . Then a dilution series was made with concentrations of 0, 20, 40, 60, 80, 100, 120,

140, 160, 180 and 200 µg/ml by adding 0.1 ml of NPG solution with a concentration of 1 mg/ml and adding 4.9 ml of sterile H_2O as listed in Table 2.

2.3.6.β-Glucosidase enzyme activity test

The materials used in the β-glucosidase enzyme activity test include: 0.1 M acetate buffer solution at pH 4.8 (200 ml of 0.2 M acetic acid solution plus 300 ml of 0.1 M sodium acetate solution, then dissolved to 1000 ml of H_2O), glycine NaOH solution at pH 10.5 (1.675 g of glycine plus 0.91 g of NaOH then dissolved to 500 ml of H_2O), NPG solution (1 mg of 2-ortho-nitrophenyl-β-D-glucopyranoside dissolved with 1 ml of H_2O). All tubes were incubated at 37° C for 60 minutes. Furthermore, the enzymatic reaction was stopped by adding 4 ml of glycine NaOH pH 10.6. Then, a standard graph is made using an ortho-nitrophenol solution with a concentration of 0 - 200 µg/ml in a sample of 2 ml and read on a spectrophotometer with a wavelength of 425 nm. The calculation of enzyme activity (U / ml) can be calculated using the formula:

Enzyme Activity (U/ml) =[C x 10 x DF]T x BM o-nitrofenol (2)

Information :
C: Concentration of *o* -nitrophenol
T : Incubation time
DF : Dilution factor
BM *o* -nitrophenol : 139.11

3. Results and discussions

Tables 4 and 5 present the CMC-ase and β-glucosidase activity tests of ST6 and ST8 isolates isolated from buffalo rumen fluid and grown on CMC substrate.

Table 4. Effect of Incubation Time on CMC- ase Activity Produced by ST 6 and ST 8 Bacterial Isolates (U/ml)

Incubation Period	Isolate Type	
	ST 6	ST 8
T0	0.4644±0.2754 ^{p}	0.7516±0.1075 ^{qr}
T1	0.4849±0.0460 ^{p}	0.8840±0.3169 ^{r}
T2	0.7758±0.0717 ^{qr}	0.5707±0.0714 ^{pq}
T3	1.9153±0.2033 ^{t}	1.6057±0.1376 ^{s}
T4	0.8112±0.0510 ^{qr}	0.7068±0.0485 ^{pqr}

Note: Different superscript ^{pqrst} indicates significant difference ($p<0.05$). T0: 0 days incubation, T1: 3 days incubation, T2: 6 days incubation, T3: 9 days incubation, T4: 12 days incubation.

The results in Table 4 show that the incubation period significantly affects CMC-ase activity for both bacterial isolates, with peak activity observed at 9 days (T3). Specifically, isolate ST6 reached the highest CMC-ase activity at 1.9153 U/ml, and ST8 at 1.6057 U/ml, indicating that 9 days is the optimal incubation period for maximizing enzyme production. This suggests that adjusting incubation time can enhance cellulase yield, which is valuable for industrial applications where enzyme efficiency is crucial. Lower activities at other incubation times highlight the importance of time optimization in achieving effective cellulolytic activity.

Analysis of variance showed no significant effect from different types of isolates. This is thought to be because both isolates produce enzymes with relatively the same ability because they are grown on the same substrate, namely CMC, which has a pure cellulose content. According to Damayanti et al., (2020) and Thielemans et al., (2023) Cellulose hydrolysis can take place very slowly because cellulose has a dense structure in the crystalline part, which is difficult to degrade so both isolates cannot work optimally on the substrate.

The difference in incubation time significantly affects the activity of the CMC-ase, presumably because CMC has fine particles. Torrado et al., (2014) stated that smaller particle sizes will increase glucose from cellulose hydrolysis. According to Yeh et al. (2010), based on kinetic studies, the hydrolysis

of cellulose into glucose runs faster and produces more glucose at smaller particle sizes than larger particle sizes. Hidayat, (2013) also stated that the smaller the particle size, the more efficient the hydrolysis process will be because the enzyme is more easily in contact with the substrate. Differences in incubation time also provide different opportunities for bacteria to grow, produce enzymes and hydrolyze substrates. Increasing enzyme concentration will increase its activity (Murtius et al., 2022).

There is a significant interaction between the type of isolate and the incubation period. The interaction between the type of bacterial isolate and the incubation period is thought to occur because, at the specified incubation time, the two bacterial isolates have different interaction rates with the substrate. Wang et al., (2022) stated that incubation is the time required for the enzyme to bind to the substrate; a short incubation time will result in low enzyme activity because the time to interact is short, which results in the interaction not taking place as a whole so that the resulting product becomes small.

Enzyme activity in ST6 bacterial isolates showed an increasing trend in activity and reached the highest activity on day 9 (T3). The increase in cellulase activity indicates that ST6 bacterial isolates can degrade cellulose contained in the CMC substrate to produce glucose, which is used for cell metabolism along with the length of incubation. In contrast to ST6 isolates, ST8 isolates experienced a decrease in enzyme activity on 6 days (T2) and were the lowest enzyme activity values and increased again on 9 days of incubation. The low activity is thought to be because the enzyme used is a crude extract enzyme, so it is possible that it still contains other components or other proteins that can interfere with the work of the enzyme so that the enzyme cannot work optimally and cause low enzyme activity values (Plaxton, 2019;Hardoko et al., 2019).

The highest enzyme activity in isolates ST6 and ST8 occurred at 9 days of incubation, namely 1.9153 U/ml and 1.6057 U/ml. Then, there was a decrease in cellulase enzyme activity in both isolates on the 12th day (P4). This is thought to be due to the reduction in nutrients available in the fermentation medium, so the rate of bacterial cell division is the same as cell death. According to Laila et al. (2019) and Yudiati et al., (2020), metabolite production, including enzymes, tends to decrease because the source of nutrients for cell growth has begun to decrease.

Table 5. Effect of Incubation Time on the Activity of β- glucosidase Produced by Bacterial Isolates ST 6 and ST 8 (U/ml)

Incubation Period	Isolate Type	
	ST 6	ST 8
T0	0.0279±0.0058 ^{rs}	0.0038±0.0117 ^{pq}
T1	0.0013 ±0.0069 ^{p}	0.0123±0.0073 ^{pq}
T2	0.0429±0.0 094 ^{s}	0.0402±0.0 157 ^{s}
T3	0.0417±0.0119 ^{s}	0.0211±0.0122 $^{q r}$
T4	0.0284±0.0 175 ^{rs}	0.0201±0.0 077 $^{q r}$

Note: Different superscript ^{pqrs} indicate significant differences ($p<0.05$). T0: 0 days incubation, T1: 3 days incubation, T2: 6 days incubation, T3: 9 days incubation, T4: 12 days incubation

In Table 5, the β-glucosidase activity varies with incubation time for both bacterial isolates, with the highest activity observed at 6 days (T2). Specifically, ST6 shows a β-glucosidase activity of 0.0429 U/ml, while ST8 shows 0.0402 U/ml at this incubation period, indicating that 6 days is optimal for maximizing β-glucosidase activity in both isolates. Lower activity at other time points highlights the critical role of incubation period optimization for effective enzyme production. These findings suggest that a carefully controlled incubation period can enhance enzyme output, benefiting industrial processes dependent on efficient cellulose

breakdown.The results of the variance analysis showed that the type of bacterial isolate had a significant effect on the activity of the β-glucosidase. This is thought to be because the types of bacteria found in the ST6 and ST8 bacterial isolates are different. Each type of bacteria has different abilities in degrading cellulose, the cellulase enzyme complex produced by cellulolytic bacteria varies depending on their genes and the substrates used (Ma et al., 2024).The difference in incubation time has a significant effect on the activity of the β-glucosidase, presumably because at the specified incubation time, the cellulase enzyme produced by cellulolytic bacteria has the opportunity to interact with the substrate at different times, thus producing products in different amounts. Metabolite, such as enzyme production, will increase with increasing incubation time because the cellulase enzyme has enough time to interact with the substrate and produce glucose. The small size of the CMC substrate also facilitates the interaction between the enzyme and the substrate. Yeh et al., (2010) found that small particle sizes increase the rate of cellobiose production 11.8 times compared to large particle sizes. Torrado et al. (2014); Yeh et al., (2010) Also explained that the smaller the particle size, up to the optimum limit, the surface area becomes more expansive, and the porosity becomes smaller, making it easier for reactions to occur.The interaction between the type of bacterial isolate and the incubation period also significantly affects the activity of theβ glucosidase. This shows that the difference influences the isolate used in incubation time in enzyme production. The two bacterial isolates are suspected to have different responses in degrading cellobiose into glucose at a predetermined incubation time.β-glucosidase activity showed that both isolates had very low cellulase activity on 0 day (T0) and 3 days 3 (T1) incubation. After that, it increased and reached the highest activity value on the 6th day of fermentation (T2). The increase in cellulase activity was balanced by factors such as the nature of microorganisms to the environment, nutrient content, temperature, pH, incubation time and substrate concentration (Laila et al., 2019). In addition, it was also caused by microorganisms in the sample that could degrade the substrate optimally and use cellulose as their main nutrient.The addition of incubation time to 12 days caused a decrease in the activity of the β- glucosidase. Razie et al., (2011) stated that the decrease in the activity of the β- glucosidase with the addition of incubation time after reaching maximum activity is likely caused by reduced available nutrients, accumulation of harmful substances from products in the fermentation medium and enzyme proteolysis. This is also in line with the opinion of Ikram-ul-Haq et al. (2006) who said that the decrease in enzyme activity with increasing fermentation time after reaching the highest level of activity can be caused by the depletion of micronutrients and macronutrients in the fermentation medium, in line with the ongoing fermentation process, thus suppressing the physiology of bacteria which causes inactivation of enzyme-producing cell organelles.

4. Conclusions

Based on the research that has been conducted, it can be concluded that the CMC ase activity of 2 isolates of buffalo rumen cellulolytic bacteria is influenced by the incubation period and the interaction between the type of isolate and the incubation period with the best incubation period at 9 days, while the β-glucosidase activity is influenced by the type of isolate and the incubation period as well as the interaction between the type of isolate and the incubation period with the best results at an incubation period of 6 days. Bactericellulolytic isolates from buffalo rumen can be utilized in the degradation process of feedstuffs and cellulose foods with an incubation period of 9 days. The potential use of cellulase enzymes in industries reliant on cellulose breakdown highlights their importance for advancing sustainable biotechnological applications.

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Research article

CHARACTERISTICS OF EDIBLE FILM BASED ON WATER HYACINTH (*Eichornia crassipes*) AS FOOD PACKAGING INNOVATION

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Abstract

This study explores the potential of water hyacinth (*Eichhornia crassipes*) cellulose in developing edible films as a sustainable alternative for food packaging. Edible films are biodegradable and eco-friendly materials that minimize plastic waste while maintaining food safety and quality. Water hyacinth, an invasive aquatic plant with a cellulose content of approximately 62.15%, offers a promising source for bioplastic production. This research investigates the effects of varying concentrations of hyacinth cellulose (3%, 4%, and 5%) and glycerol (1% and 1.5%) on edible films' physical and chemical properties, with carrageenan as a structural agent. Results indicate that higher cellulose concentrations increase tensile strength and reduce water absorption, while higher glycerol concentrations enhance flexibility and increase water absorption and thickness. The optimal combination of glycerol, carrageenan, and cellulose in the edible film formulation is necessary to balance flexibility, mechanical strength, water absorption, and the desired moisture content. Based on the film thickness, tensile strength, water absorption, and moisture content test, the best edible film is 5% hyacinth cellulose and 1.5% glycerol.

1. Introduction

Foodstuffs are prone to quality degradation because of chemical, environmental, microbiological, and biochemical factors. Water, light, temperature, and oxygen stimulate quality degradation. Proper packaging plays a role in slowing down this phenomenon (Wahyuni & Arifan, 2018). Packaging is an integral part of a food product and an important aspect that is needed to realize comsumer desires as product protector (Yusuf et al., 2018). It serves as containment, preservation,

information, transportation, and storage. The most familiar packaging is plastic due to its moulding convenience, high mechanical strength, low price, lightweight, and heat sealability. On the other hand, plastic packaging has disadvantages such as being hard to degrade and incineration of it will produce toxic gases. Therefore, sustainable food packaging is experiencing an increasing trend. It has a positive impact on protecting the environment and reducing waste (Ma & Li, 2024; Pascall, DeAngelo, Richards, & Arensberg, 2022; V et al., 2022). An example of sustainable food packaging is edible film, as it reduces the problem of waste disposal (Prasetyaningrum et al., 2021). Edible film typically uses sustainable, biodegradable material in the form of a solid sheet, which is used as food wrap and is consumable. Edible film materials are classified into polysaccharides, proteins, lipids, and composites (Petkoska et al., 2021). The primary function of edible film is to prevent moisture loss and suppress adverse chemical reaction rates to improve the safety and quality of fresh and processed foods (V et al., 2022).

Water hyacinth (*Eichhornia crassipes*) is an invasive aquatic plant that becomes a threat to waters because it affects water conditions, becomes a habitat for mosquitoes, and triggers sedimentation (Prasetyo et al., 2021). The invasion of water hyacinth blocks the light, inhibits photosynthesis, and absorbs nutrients from the water (Zeleke et al., 2024). Therefore, the utilization of water hyacinth is necessary to suppress its amount. The applications of water hyacinth are biogas, liquid fertilizer, fiber reinforcement for prosthetics socket, art paper, sound absorber composite, craft material, and cellulose from membrane isolation (Hadiyarto et al., 2018; Istirokhatun et al., 2015; Natari et al., 2024; Paramitha et al., 2023; Wibawa, et al., 2023; Widhata et al., 2019). The utilization of water hyacinths contributes to lake ecosystem conservation and rehabilitation (Hidayati et al., 2018).

Water hyacinth fiber consists of 62,15% cellulose content and 14,82% hemicellulose content (Arivendan et al., 2022). The composition of water hyacinth, like cellulose, hemicellulose, and lignin, makes it a suitable candidate for biodegradable film (Kusuma et al., 2024). In addition, water hyacinth is a raw material for cellulose-base polymers with a higher economic value than the other source (Istirokhatun et al., 2015). In this study, the edible film will be made using hyacinth cellulose, carrageenan, and glycerol. Water hyacinth cellulose forms a bioplastic that potentially substitutes for non-degradable plastic in food packaging use (Anantachaisilp et al., 2021). Carrageenan is a hydrocolloid and usually called as polysaccharide which is extracted from red algae (Pratama et al., 2018). It has excellent mechanical and physical properties for making transparent films (Prasetyaningrum et al., 2021). The addition of plasticizers to manufacture edible film is necessary to increase its mechanical, barrier properties, and flexibility (Putri et al., 2024). Glycerol is the plasticizer used in this study. It is widely used and effective because its small molecular weight can improve plastic properties (Fadilah et al., 2020). This study aims to explore the effect of different hyacinth cellulose and glycerol concentrations on the physical and chemical properties of the resulting edible film and determine the optimum concentration of hyacinth cellulose and glycerol to produce the best edible film.

2. Materials and methods

2.1. Materials

Water hyacinths are obtained from waters in Demak Regency, Central Java Province, Indonesia, kappa carrageenan (IndoGum, Indonesia), glycerol (Kimia Jaya Abadi, Indonesia), aquades, HCl 0.05 N, filter paper, ethanol 96%. The tools used include analytical scales (Ohaus, China), beaker glasses (pyrex), hot plate stirrer (IKA, Malaysia), magnetic stirrer (IKA, Malaysia), thermometer, oven (members), desiccator, sieve size 100 mesh, sieve size 50 mesh, plastic mold/plate, magnetic stirrer, extraction tool 1 set, Mittoya micrometer, blender, spatula, mixer. The research was carried out using water hyacinth starch. The independent variables in this study were the cellulose mass of water hyacinth (3%, 4%, 5%)

and glycerol concentration (1% and 1.5%). The dependent variables are tensile strength, thickness, moisture content, water absorption, FTIR, and shelf life.

2.2. Methods

2.2.1. Cellulose Manufacturing

Water hyacinths are washed and cleaned until the dirt disappears, dried, and then mashed until they become powder. Hyacinth powder is sifted with a size of 50 mesh. Hyacinth powder is dissolved with 500 ml of 4% NaOH and heated for 4 hours, then washed, filtered, and dried so that hyacinth fiber is free of hemicellulose. Hyacinth powder is dissolved with 500 ml of H_2O_2 1.5% and heated for 3 hours, then washed, filtered, and dried until hemicellulose-free hyacinth fibers are obtained.

2.2.2. Edible film formulation

There were variations of hyacinth cellulose in this study, namely 3%, 4%, and 5%. Then, for the glycerol variation, use 1 % and 1.5 %, the carrageenan concentration as much as 2.5 grams. Six combination treatments were repeated three times with the following levels:
Cellulose water hyacinth 3 %, glycerol 1 % (A1B1)
Cellulose water hyacinth 3 %, glycerol 1.5 % (A1B2)
Cellulose water hyacinth 4 %, glycerol 1 % (A2B1)
Cellulose water hyacinth 4 %, glycerol 1.5 % (A2B2)
Cellulose water hyacinth 5 %, glycerol 1 % (A3B1)
Sodium hyacinth 5 grams, glycerol 1.5 % (A3B2)

In each treatment, the cellulose of water hyacinth is dissolved in 100 ml of aquadest and heated for 20 minutes until the temperature on the thermometer reaches 70°C, keeping the temperature of the cellulose solution at 70°C. Then, dissolve 2.5 grams of carrageenan in different glass beakers in 100 ml of aquadest, heat it on a hotplate, and heat it until the temperature on the thermometer shows 80°C. After 20 minutes, the solution is mixed with the carrageenan solution while maintaining a temperature of 80°C. After homogeneity, pour glycerol into the cellulose-carrageenan mixture and stir until homogeneous again. Filter and pour the solution on the mold, then dry it using a dehydrator for 15 hours at a temperature of 50°C. Repeat the same steps with different treatments.

2.2.3. Moisture content meaurement

The empty aluminum cup is heated in the oven (±105)°C for 5 hours, then cooled in the desiccator for 30 minutes. The aluminum cup was weighed and recorded; 100 grams of the sample was put into the aluminum cup. The sample cup was put in the oven (±105)°C for 3 hours. The cup and sample are left in the desicator for 30 minutes. The final weight of the cup and its contents is weighed to a constant (y gram)

2.2.4. Edible film thickness

The thickness was measured using a Mitutoyo micrometer (accuracy 0.01 mm) by placing a film between the micrometer's jaws.

2.2.5. Tensile strength

The magnitude of the force used to determine the edible film of water hyacinth is measured in Kg. Furthermore, the force must be divided by the size of the surface area of the test specimen to obtain the value of the edible tensile strength of the hyacinth film in Kg/cm^2. The value obtained must first be converted into the standard unit of the tensile strength value, namely MPa, by dividing it by the number 10.2.

2.2.6 Water absorption test

Weigh the sample's initial weight (wo) and put it into a container containing the aquadest for 10 seconds. The sample is then lifted from the container, and the water on the plastic surface is removed with paper towels, after which weighing (w) is carried out. The soaking and weighing are carried out again until the sample's final weight is constant.

2.2.7 Fourier Transform Infrared (FTIR) test

This method utilized infrared spectroscopy to analyze the absorption of infrared light by different materials, providing insights into their molecular composition and structure.

2.3. Data analysis

Data analysis uses ANOVA with a confidence level of 95%. If the difference is noticeable, continue with the Duncan Multiple Range Test (DMRT) test.

3. Results and discussions

3.1. Edible film manufacture

The first step is the mixing of NaOH to separate cellulose from lignin. The second stage, or the bleaching stage, is the addition of H_2O_2 to separate the cellulose from hemicellulose. The cellulose results of water hyacinth are yellowish-white, as shown in Figure 1. Water hyacinth has a content of about 60% cellulose, 8% hemicellulose, and 17% lignin (Abdel-Fattah & Abdel-Naby, 2012). Its high cellulose content makes it a potential resource for a variety of environmentally friendly industrial applications, such as edible films. The manufacture of hyacinth edible film also requires glycerol, which functions to provide flexibility and hardness to the edible film. Glycerol is also easy to digest and safe for making edible film. Carrageenan is also important in the manufacture of edible film because the addition of carrageenan concentration will increase the tensile strength value of edible film. The manufacture of edible film using these three ingredients produces an edible film with a clear white color, as shown in Figure 2.

Figure 1. Cellulose water hyacinth

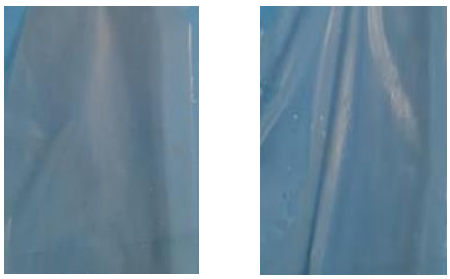

Figure 2. Edible hyacinth film

3.2. Thickness of edible film

Thickness is a very important parameter because it will affect the purpose of its use for packaging or coating products. The thickness will affect the rate of water vapor and gas transmission, thus affecting the packaged product. The higher the thickness value, the more rigid and hard the properties of the edible film produced and the safer the packaged product will be from outside influences.

Based on Figure 3, it is found that the thickness of edible film with various treatments has different thicknesses, which are around 0.08 – 0.14 mm. This result is in accordance with the thickness standard of an edible film according to the JIS (Japanese Industrial Standard), which is a maximum of 0.25 mm (Ningrum et al., 2021). The results showed that the treatment with the addition of 1.5% glycerol was able to increase the film thickness compared to the addition of 1% glycerol. The addition of glycerol tends to increase the film thickness because the glycerol molecules occupy the space between the polymer chains, causing an increase in the distance between the molecules and an increase in the thickness of the film. An increase in glycerol concentration from 0% to 0.9% led to an increase in the thickness of carrageenan and

beeswax-based edible films (Harumarani et al., 2016).

Figure 3 also shows an increase in the thickness of the edible film along with the addition of cellulose. This suggests that the addition of cellulose can increase the thickness by strengthening the polymer matrix and improving the interaction between molecules. Research shows that the addition of cellulose to starch film increases the thickness and resistance to water (Arik Kibar & Us, 2017). The addition of carrageenan, a sulfate polysaccharide, acts as a gelling agent that improves the structure and strength of the film. In addition, the concentration of raw materials affects the thickness of the edible film because it will produce a lot of dissolved solids after drying so that it forms a thick edible film (Ningrum et al., 2020).

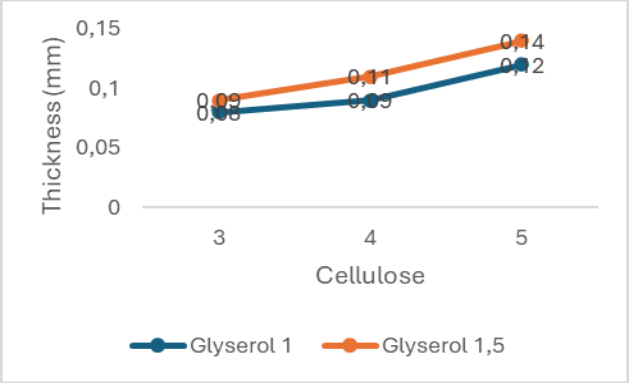

Figure 3. Thickness of edible film

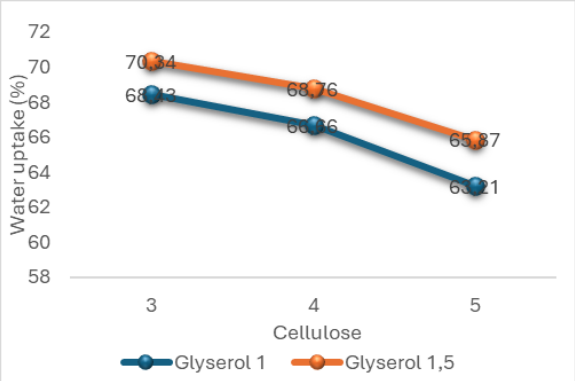

Figure 4. Water absorption

3.3. Water absorption

The water absorption test measures how much the film absorbs water. This measurement is important to determine the resistance of the edible film to moisture, which will affect the film's ability to protect against the coated material, texture stability, and shelf life of the product. Based on Figure 4, the water absorption produced by edible film in this study is around 63.21% – 70.34%. The addition of 1.5% glycerol results in a high absorption value compared to the addition of 1% glycerol. This suggests that the addition of glycerol tends to increase water absorption due to its hygroscopic properties, which allows glycerol to attract and retain water molecules from the surrounding environment. Research by (Zahra et al., 2020) shows that an increase in glycerol concentration from 1% to 2% leads to an increase in the water absorption of banana peel peptide-based edible film. The graph also shows that the higher the cellulose level, the smaller the water absorption capacity. The addition of cellulose can decrease water absorption by strengthening the polymer matrix and reducing the space between molecules that can be occupied by water. The

addition of cellulose to starch film decreases water absorption and increases water resistance (Kibar & Us, 2017). Edible film is used as a food protective layer; lower water absorption is usually preferred because a film that is resistant to water and moisture will be more effective in maintaining the integrity of the product. In this study, the edible absorption yield of water hyacinth film is still high, above 60%, so further formulation is needed to reduce water absorption.

3.4. Tensile strength

Tensile strength is one of the important mechanical properties of edible film, as it is related to the ability of edible film to protect the product it is coated with. An edible film with high tensile strength is required in use as food product packaging aimed at protecting food ingredients during handling, transportation, and marketing. The following is the tensile strength produced in this study. In Figure 5, the average tensile strength of hyacinth-based edible film can be seen between 6.62-12.31 MPa. The best edible film tensile strength is between 10-100 Mpa, while edible film with a tensile strength range of 1-10 Mpa is classified as marginal. The more cellulose composition, the higher the tensile strength value of the edible film. This is also shown in the results of this study, where the higher the cellulose level added, the higher the tensile strength will also be. The tensile strength of edible film in this study is almost the same compared to edible film from star fruit pectin, which is between 6.81-8.51 Mpa (Sulistriyono et al., 2014). The addition of higher glycerol increases the tensile strength even more. Glycerol functions as a plasticizer that increases the flexibility of edible film, improves mechanical properties, maintains the film integrity, and prevents crack formation (Putri et al., 2023). However, the addition of glycerol tends to decrease the tensile strength because it reduces the interaction between polymer molecules. Research by (Harumarani et al., 2016) showed that an increase in glycerol concentration from 0% to 0.9% led to a decrease in the tensile strength of carrageenan and beeswax-based edible films.

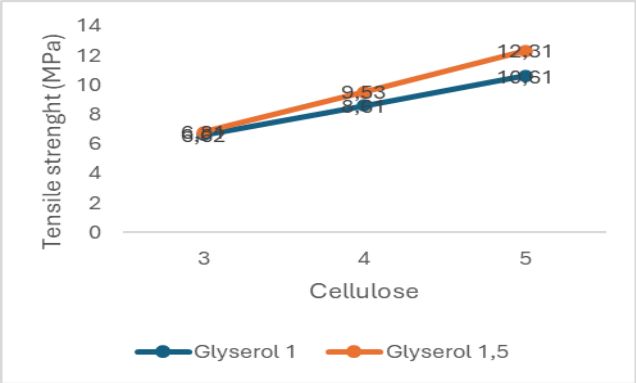

Figure 5. Tensile strength

3.5. Moisture content

The moisture content of edible film has an important role in the stability of the product. Therefore, edible film is expected to have a low moisture content so that as a primary packaging, it does not contribute water to the product, which will impact product damage and decrease shelf life. Based on Figure 6, the average moisture content of edible film is 29.21%- 35.54%. In this study, the lowest moisture content was 5 grams of cellulose with 1.5% glycerol. The concentration of cellulose and glycerol affects the moisture content of the edible film. However, in this study, the water content was still quite high. The use of hygroscopic basic materials, such as starch or cellulose, can increase moisture content due to the ability of these materials to absorb moisture from the environment. Meanwhile, in the other research, edible film carrageenan and glycerol were

obtained with a moisture content of 17.14 – 20.86% (Rusli et al., 2017; Pratama et al., 2018).

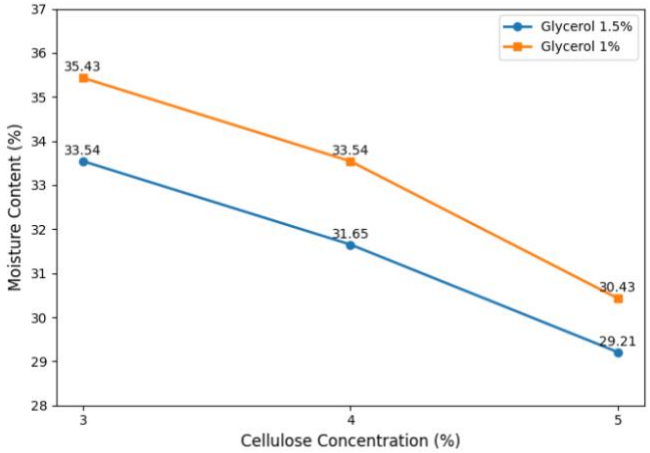

Figure 6. Moisture content

3.6.Fourier Transform Infrared Spectroscopy (FTIR)

FTIR aims to identify spectral groups and functional groups in edible films. The relationship between wavenumber and transmittance is presented in the FTIR spectra (Figure 7), allowing the identification of characteristic chemical bonds in the film matrix. Here is the FTIR spectrum of edible water hyacinth film. The black line is a 3-gram cellulose sample with 1.5% glycerol, the red line is a 4-gram cellulose sample with 1.5% glycerol, and the blue line is a 5-gram cellulose sample with 1% glycerol. In the FTIR spectral graph there a peak indicates the presence of a C-H group, which indicates the presence of a cellulose structure in the sample. However, absorption regions also indicate the presence of C-O-C groups, which are building structures of lignin compounds, indicating that the treatment given for the delignification process is less effective.

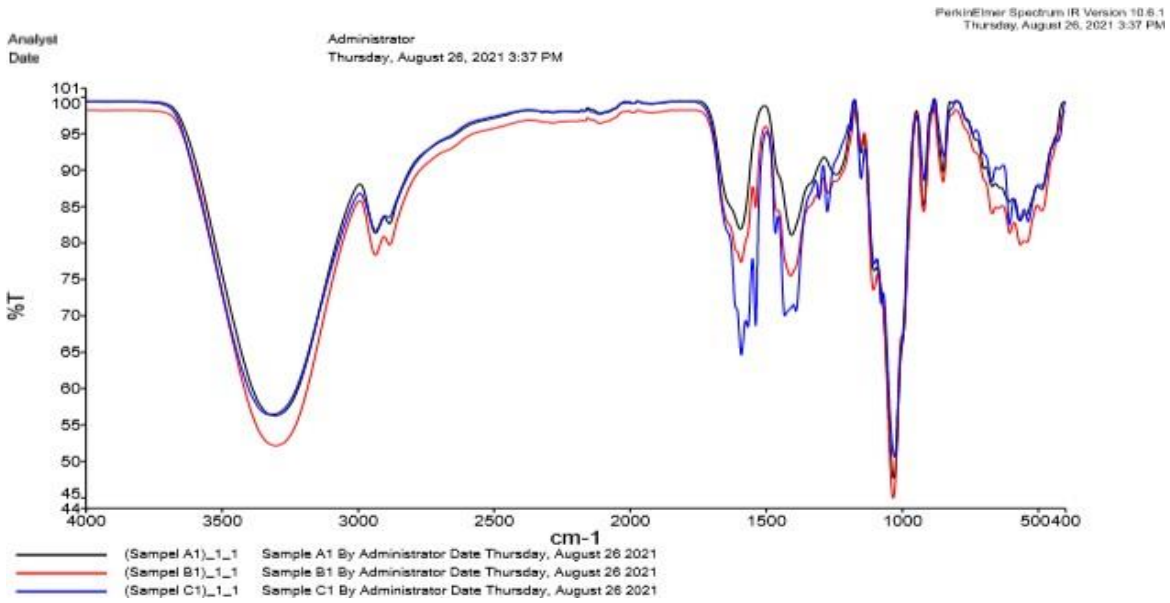

Figure 7. FTIR spectra of edible hyacinth film

4. Conclusions

The optimal combination of glycerol, carrageenan, and cellulose in the edible film formulation is necessary to achieve a balance between flexibility, mechanical strength, water absorption, and the desired moisture content. Overall, from the film thickness, tensile strength, water absorption, and moisture content test, the best edible film is with 5% hyacinth cellulose and 1.5% glycerol.

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Research article

INFLUENCE OF EXTRACTION SOLVENTS ON THE ANTIBACTERIAL PROPERTIES OF *PAEDERIA FOETIDA* LEAF EXTRACTS AGAINST *E. COLI*

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Leaves extract.

Abstract

This study aimed to solvent selection for testing the medicinal plant Paederia foetida leaf extracts for combating bacterial infection. UV–VIS spectroscopy, therefore the extraction factor (EF) analysis and FTIR spectra indicate that ethanol is more efficient solvent over the acetone. Antibacterial activity against Escherichia coli was evaluated, and a concentration-dependent inhibition was observed. The antibacterial activity of the Paederia foetida leaf extracts consist of specific phytochemicals that may disrupt bacterial membrane integrity and inhibit metabolic pathways. Therefore, our findings illustrate Paederia foetida has the potential to be a promising resource for natural antibacterial agents, where ethanol is the preferred extraction solvent.

1. Introduction

Antibiotics are widely used to treat microbial infections; however, the emergence of antibiotic resistance has become a major concern. To address this issue, several alternative treatment strategies are being explored, including medicinal plants, which have long been used in traditional medicine for combating microbial infections. *Paederia foetida*, commonly known as skunk vine, is a perennial climbing vine belonging to the Rubiaceae family. It is indigenous to tropical and temperate regions of Asia, particularly in countries such as China, India, Japan, and Indonesia, and is also found in the Mascarenes, Melanesia, Polynesia, the Hawaiian Islands, and some southeastern parts of the United States (Langeland KA et al., 2008). In India, it is predominantly found in the north-eastern states. According to the eFlora of India, *Paederia foetida* is a habitant of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and West Bengal. It plays a significant role in traditional medicine for gastrointestinal ailments as well as local cuisine. Traditionally,

the plant has been used to treat a variety of ailments, including toothaches, dysentery, sores, enterosis, enteromegaly, rhinitis, rheumatism, edema, night blindness, and various digestive disorders such as gastritis, diarrhea, and ulcers (De S et al., 1994). Several studies have reported that extracts of *Paederia foetida* possess anti-inflammatory, antidiarrheal, antioxidant, antihepatotoxic, antitussive, and gastroprotective properties. The leaves are consumed either raw or boiled to relieve stomach aches. These regional uses highlight the ethnobotanical significance of *Paederia foetida* (De S et al., 1994; Afroz S et al., 2006; Osman H et al., 2009; De S et al., 1993; Nosaovaa G et al., 2007; Chanda S et al., 2015).

Such medicinal properties are due to the bioactive phytochemicals of *Paederia foetida*. These include iridoid glycosides such as asperuloside, scandoside, and paederoside; volatile oils like linalool, geraniol, and α-terpineol; triterpenoids including ursolic acid and oleanolic acid; as well as β-sitosterol, arachidic acid, alkaloids (paederine A and B), flavonoids, and significant amounts of essential minerals (Ojha S et al., 2018).

It has been reported that the leaf extracts of *Paederia foetida*, showed inhibitory effects against pathogenic bacteria (Yunita M, 2023). However, the mode of extraction plays a crucial role in determining the antibacterial efficacy, as it can influence the quantity and availability of secondary metabolites in the plant extract (Upadhyaya S, 2013). For example, ethanolic leaf extracts showed stronger antimicrobial activity than crude extracts. These active fractions also exhibited antibiofilm properties, effectively inhibiting biofilm formation and eradicating established biofilms (Priyanto JA et al., 2022).

In the present study, we investigate the antibacterial activity of *Paederia foetida* leaf extracts, which were obtained using two different solvents, ethanol and acetone, against gram-negative bacteria.

2. Materials and methods

2.1. Materials

Spectroscopic-grade Ethanol and Acetone were purchased from Spectrochem (India). All other chemicals/reagents were from Sigma-Aldrich, USA; otherwise specified in the corresponding section of the manuscript. Dried powder of *Paederia foetida* leaves was used for the downstream studies.

2.2. Methods

2.2.1. Preparation of ethanolic and acetone leaves extract of Paederia foetida

First, the leaves (*Paederia foetida*) were dried in the subjected to a rotary evaporator for several hours. The extract samples obtained from the rotary evaporator were dried. Stock solution (100 mg/ml) in DMSO for each solvent extract was prepared and followed preparation of a 1 mg/ml sub-stock solution. The whole process is represented in Figure 1.

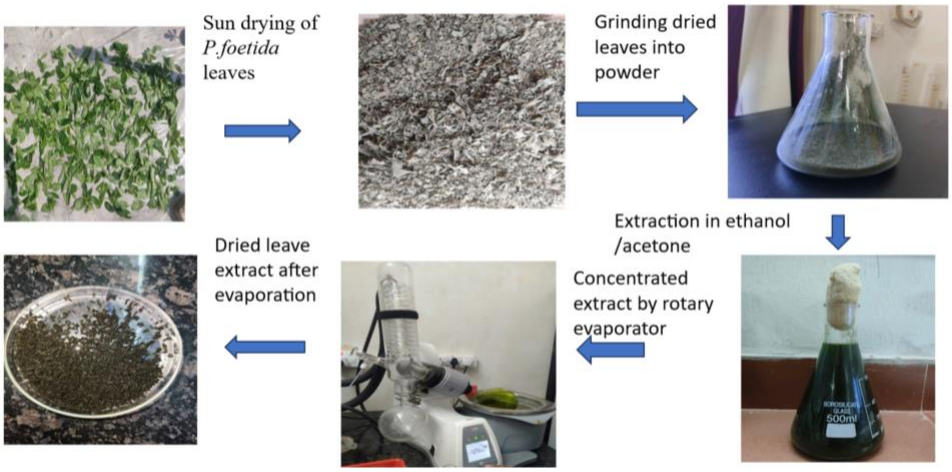

Figure 1. The complete flow for the preparation of ethanol and acetone extract from *Paederia foetida* leaves.

2.2.2. UV-Vis studies:

The steady-state absorption spectra were recorded in a UH–5300 (Hitachi, Tokyo, Japan) spectrophotometer, equipped with a pulsed Xe-lamp. In the spectrophotometer, the slit width was maintained at 2.5 nm, and the entire data was recorded within the range of 250nm-700nm. Spectroscopic-grade Ethanol and Acetone were purchased from Spectrochem (India) and used for making the experimental solutions. To achieve a homogeneous solution of the plant extracts, the mixture was stirred using a magnetic stirrer and kept undisturbed for ~5 min, and finally proceeded for the spectroscopic measurements.

2.2.3. FTIR study of both plant extracts:

The Fourier Transform Infrared spectrum (FTIR) of each extract was recorded in the IR region, from 4000 to 500 cm^{-1}, using a BRUKER FTIR spectrometer. The spectra were registered as evaporated extracts.

2.2.4.Antibacterial activity of both leaf extracts on agar plate by the well diffusion method

To study the antibacterial effect of both ethanolic and acetone leaves extract of the *Paederia foetida,* 1.5% of nutrient agar and 1.3% of nutrient broth were used for the preparation of agar plates. We used the well diffusion method for the antibacterial activity experiment. For that, 200 µl of *E. coli* culture was spread on the agar plates and incubated the plates for 30 minutes at 37°C in an incubator. Well was made by using 200 µl tips, and the agar plates were labelled as NIL, DMSO, 10, 30, 50, 70, 80, 100 µg/ml for both ethanol and acetone plates aseptically. The plant extract of six different concentrations was loaded in each well, except for the control (NIL), and kept in an incubator for 24 hours. We kept blanks for control, and we added 100µl of DMSO in one of the wells to check its effect. After 24 hours, the zone of inhibition was measured and recorded.

2.2.5. Effect of both leaf extracts on the growth of E. coli bacteria in broth media

In that connection, bacterial nutrient broth culture was prepared, and *E. coli* bacteria were treated with different concentrations of both ethanol and acetone extracts (0-100µg/ml) and kept in an incubator shaker at 37°C for overnight. The OD values of bacterial culture were measured at 600nm. Based on the OD value, we plotted the graph of OD against concentrations of plant extract.

3. Results and discussions

3.1. UV–VIS spectra

The UV–VIS spectra (700–250 nm) were recorded for *Paederia foetida* extract (in acetone [red] and ethanol [cyan] solvents), and the wavelengths of specific absorption maxima of poly-phenolic compounds (330 nm), carotenoids and terpenoids (400–450nm), and/or chlorophylls (665 nm) were identified (Fig. 2) (Dutta PP et al., 2023; Ojha S et al., 2018; Caunii A et al., 2012). To compare the yields of extraction in two solvents, the Extraction Factors (EF) of the bioactive molecules from each extract have been calculated considering the absorbance ($A_{\lambda max}$) recorded for each corresponding λ_{max} value, multiplied by the dilution factor (d) (Ojha S et al., 2018).

The formula applied was: $EF = A(\lambda_{max}) \times d$ (1)

The results, expressed as mean values of two samples of *Paederia foetida* extract in both solvents, are represented in Table 1. According to Table 1 and Figure 2, the EF in ethanol was much superior to that in acetone. Polyphenols and phenolic acid derivatives like scopoletin, quercetin, rutin, vanillin, catechin, etc (absorption in the 310-330 nm region) prefer the more polar and protic ethanol over acetone for their extraction, as also suggested from their ~10 times higher EF in EtOH than acetone. Also, the extraction of carotenoids present in the plant extracts was augmented in the polar EtOH solvent.

Table 1. The absorption maxima λmax (nm) of each plant extract from UV-Vis spectra and the mean values calculated (X ± SD) for extraction factors (EF).

Solvent used	λ_{max} (nm)	EF
Ethanol	328	37.6 ± 0.08
	410	82.8 ± 0.18
	665	29.2 ± 0.11
Acetone	342	3.6 ± 0.008
	408	8.4 ± 0.01
	665	3.05 ± 0.01

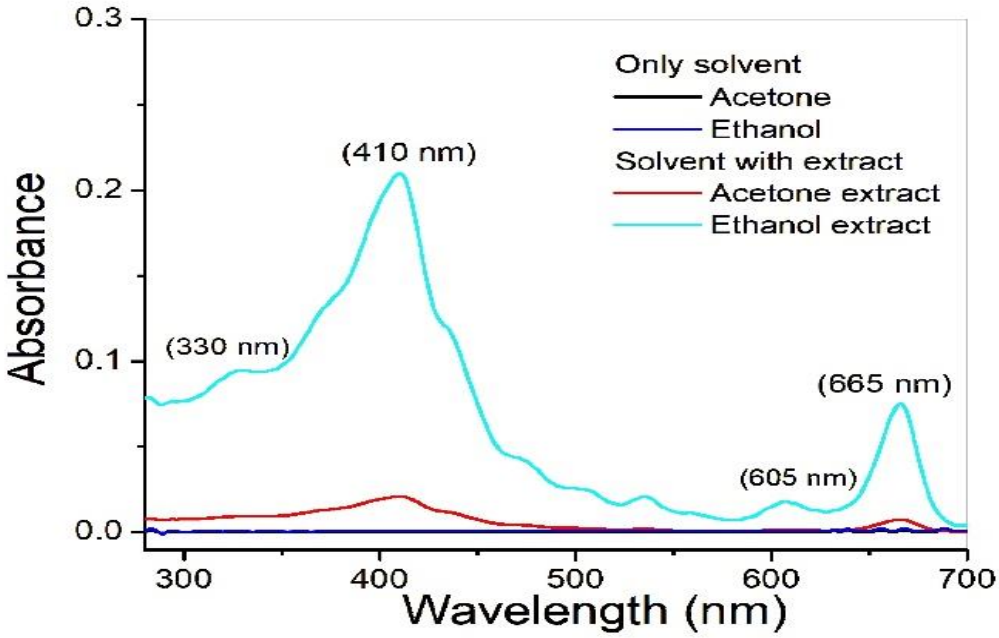

Figure 2. UV-Vis spectra of the acetone extract (Red) in acetone solvent and the ethanol extract (Cyan) in EtOH solvent. The baseline for the two pure solvents is given for reference.

3.2. FTIR studies

The FT-IR spectra (4000-500 cm-1) of the extracts in each solvent were registered, and the specific wave numbers and intensities were considered. The functional groups identification was based on the FTIR peaks attributed to stretching and bending vibrations. Five areas (marked from 1 to 5) (Fig. 3) were identified in the IR domain, and the fingerprint region was localized between 900 and 1400 cm-1.

Vibrations in the region of less than 1000 cm-1 correspond to C–H bending vibrations from carotenoids and terpenoids. Area 5 (1000-1150 cm-1) includes stretching vibrations C–O of glycosides and mono-, oligo- and carbohydrates, with signals at 1032, 1052, 1152 cm-1, while area 4 (1250-1450 cm-1) corresponds to stretching vibrations of carbonyl C-O, O-H bendings, and C-C stretchings from phenyl groups (Dutta PP et al., 2023; Kaushik N et al., 2023; Osman H et al., 2009) Area 3 is a complex one (1580-1760 cm-1) mainly including N-H bending vibrations, C=O stretchings (aldehydes and ketones, esters as well free glycerides (1730 cm-1) and the aromatic domain (Kaushik N et al., 2023; Osman H et al., 2009) Area 2 (2800-3000 cm-

1), corresponds to C-H stretching vibrations specific to CH3- and CH2- from lipids, methoxy derivatives, C-H (aldehydes). Area 1 (3350-3600 cm-1) corresponds to stretching vibrations of -OH groups from water, alcohols, phenols (3342 cm-1), carbohydrates, and peroxides. (Dutta PP et al., 2023; Kaushik N et al., 2023; Osman H et al., 2009). In the ethanolic extract, IR-peaks in the domain 1050-1100 cm-1 and 3340 cm-1 have much higher integrated IR-absorption areas, suggesting a greater specific concentration of the functional groups in the solvent, which remains in agreement with other spectroscopic determinations. Notably, for therapeutic reasons, it could be considered that evaporated ethanol extracts would provide higher concentrations of bioactive molecules from these plants.

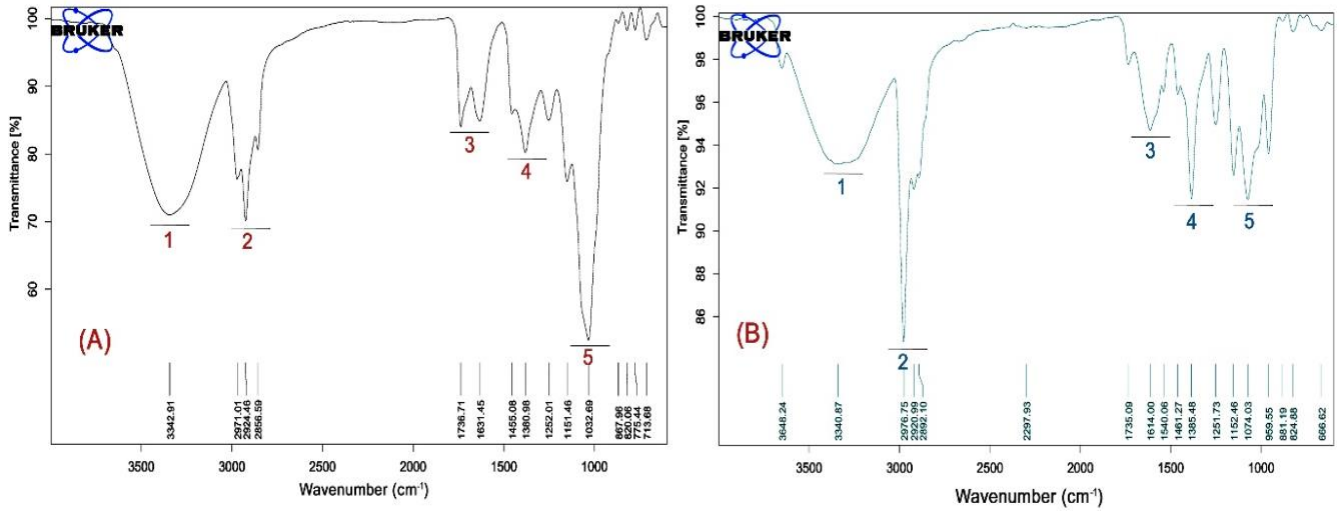

Figure 3. FTIR spectra of the P. foetida extract in (A) Ethanol and (B) Acetone solvent.

3.3. Antibacterial Activity

To address the antibacterial effects of leaves extracts of *Paederia foetida* zone of growth inhibition of *E.coli* bacteria has been observed. Figure 4 showed a significant zone of growth inhibition upon treatment of different concentrations of ethanolic leaves extract of *Paederia foetida* into wells of agar plates (10, 30, 50, 70, 80, 100 µg/ml). The diameters of the zone of inhibition were increased upon increasing the concentration of ethanolic leaf extract (12 mm, 15 mm, 17 mm, 19 mm, 21 mm, 23 mm, respectively). But in the case of untreated and DMSO treatment, no zone of inhibition was observed. The maximum zone of growth inhibition was found at a concentration of 100 µg/ml of ethanolic leaves extract is 23 nm by using the well diffusion method.

On the other hand, *E. coli* bacteria were treated with same concentrations of ethanolic extract of *Paederia foetida* in nutrient broth culture; found decrease of optical density of bacterial culture. Treatment with 10 µg/ml of ethanolic leaves extract with *E. coli* bacteria showed negligible growth inhibition, but upon increasing the concentration (30-100 µg/ml) of ethanol extract, bacterial growth gradually decreased, and at a concentration of 100 µg/ml, almost complete bacterial growth inhibition occurred (Fig. 5).

Similarly, the acetone leaves extract of *Paederia foetida* also revealed significant growth inhibition against *E. coli* bacteria by the well diffusion method (Fig. 6). The concentration of acetone leaves extract of *Paederia foetida* from 10 µg/ml to 100 µg/ml, the diameter of the zone of inhibition increases from 13 nm to 20 nm. Simultaneously, when *E.*coli bacteria were treated with a varied number of concentrations, such as 10, 30, 50, 70, 80, and 100 µg/ml of acetone leaves extract of *Paederia foetida, a* similar incident was found in ethanol extract. The bacterial growth gradually decreased, and upon treatment with

100 µg/ml of acetone extracted from *Paederia foetida* leaves drastically reduced the OD value of the bacterial culture and almost complete bacterial growth inhibition was observed (Fig. 7).

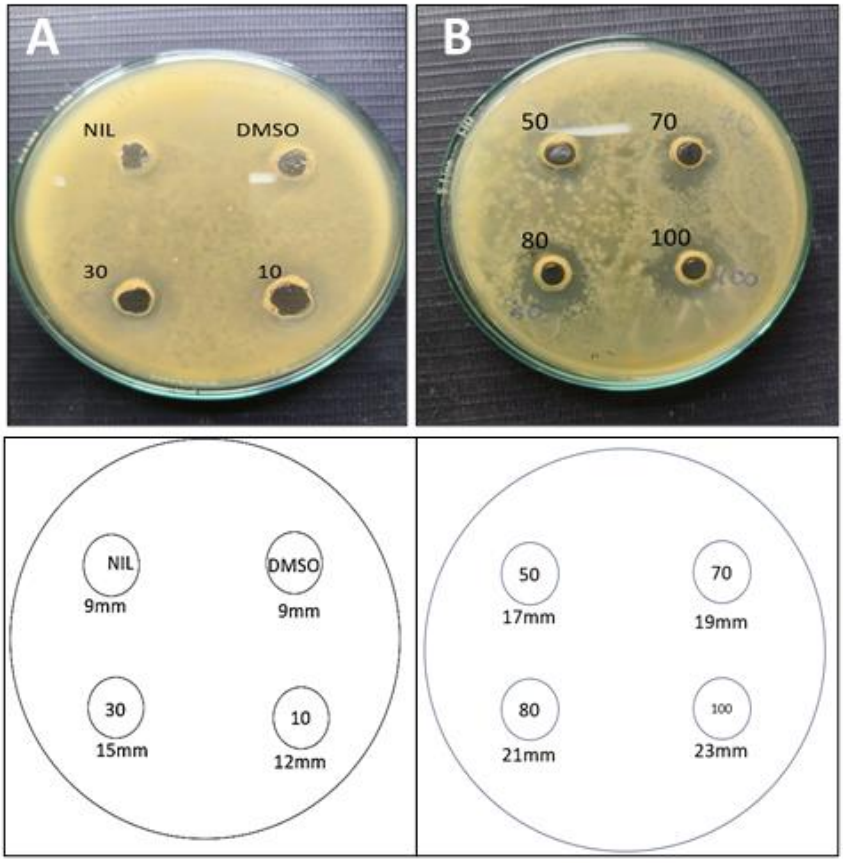

Figure 4. Zone of inhibition after treatment of ethanolic leaf extract of *Paederia foetida* (0-100 µg/ml) with *E. coli* bacteria by the well diffusion method. Corresponding downwards are represented the measurements of the diameter of zone of inhibition by the ethanolic leaf extract of *Paederia foetida* at a lower dose (0-100 µg/ml).

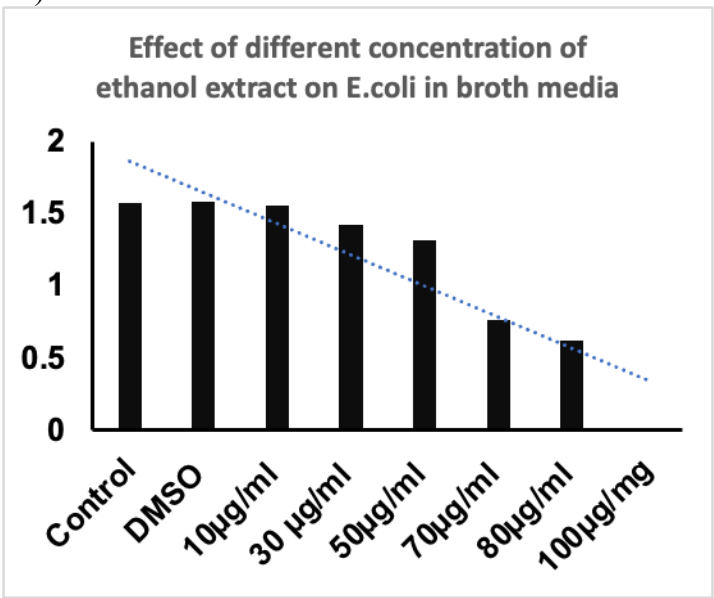

Figure 5. Optical density of bacterial culture upon treatment with different concentrations of ethanolic leaf extract of *Paederia foetida*.

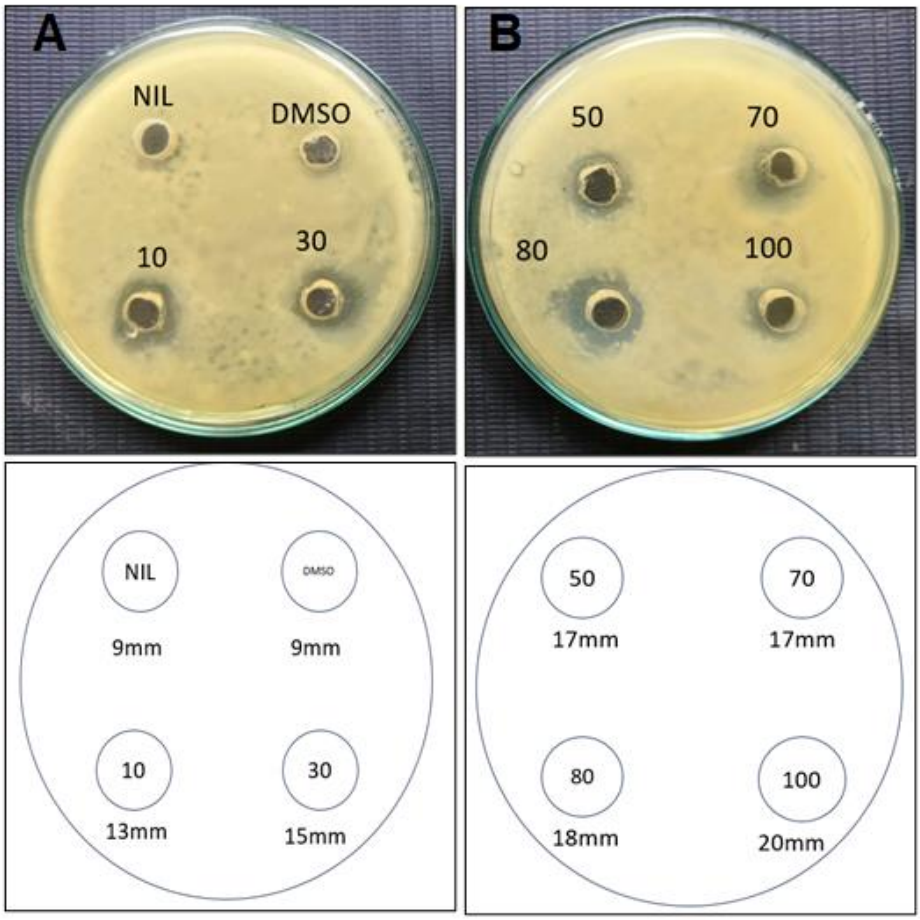

Figure 6. Zone of growth inhibition after treatment of the acetone leaf extract of *Paederia foetida* (0-100 µg/ml) with *E. coli* bacteria by the well diffusion method. Corresponding downwards are represented the measurements of the diameter of the zone of inhibition by the acetone leaf extract of *Paederia foetida* at a lower dose (0-100 µg/ml).

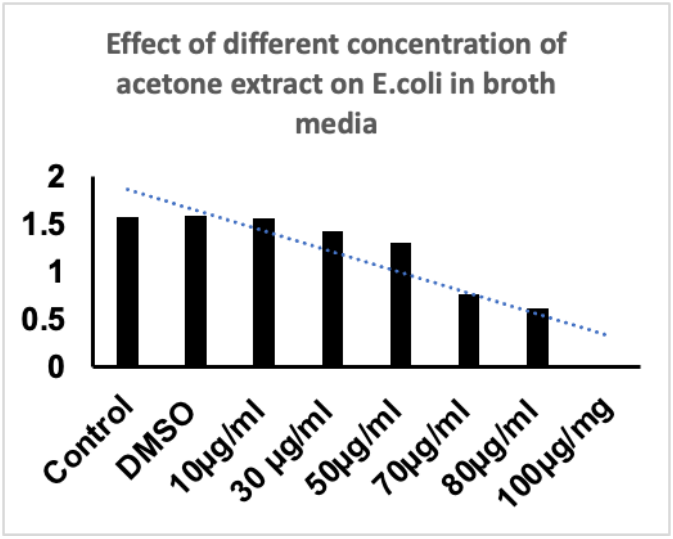

Figure 7. Optical density of bacterial culture upon treatment with different concentrations of acetone leaf extract of *Paederia foetida*.

Both ethanolic and acetone leaf extracts of *Paederia foetida* demonstrated antibacterial activity against the gram-negative bacterium *Escherichia coli* (*E. coli*). The degree of growth inhibition was comparable between the two extracts. However, ethanol can be preferred for the extraction due to its several advantages: it is cost-effective, polar, easily available, environmentally friendly, less toxic, relatively more soluble, and has a lower boiling point (Khotimah H et al., 2020). The low boiling point of ethanol is particularly beneficial during the solvent removal process, as it allows for separation at lower temperatures, thereby minimizing the risk of degrading heat-sensitive bioactive compounds (Jeyaseelan CE et al., 2012). Most importantly, ethanolic extraction of *Paederia foetida* leaves yields maximum bioactive molecules. The observed antibacterial activity of the leaf extracts is likely due to the presence of various bioactive molecules. Previous studies have shown that *Paederia foetida* contains a wide range of phytochemicals, including saponins, tannins, phenols, flavonoids, terpenoids, cardiac glycosides, alkaloids, and reducing sugars (Jeyaseelan CE et al., 2012). Our UV-VIS and FTIR analysis confirmed the presence of phenolic compounds, flavonoids, glycosides, and possibly terpenoids or carotenoids in both leaf extracts. These compounds are believed to contribute to the plant's antibacterial properties. Nevertheless, the potential mechanisms by which these bioactive compounds exert antibacterial effects vary. For example, alkaloids may inhibit protein and nucleic acid synthesis, disrupt bacterial cell membrane function, inhibit ATP synthesis, and block efflux pump activity on bacterial membranes (Yan Y et al., 2021). Flavonoids are known to interfere with bacterial energy metabolism, inhibit nucleic acid synthesis, and disrupt cytoplasmic membrane function by inhibiting ATPase and phospholipase enzyme binding, thereby compromising membrane permeability (Xie Y et al., 2014). Additionally, saponins, terpenoids, and steroids may exhibit antibacterial activity by reducing membrane permeability or by interacting with proteins in the lipopolysaccharide layer of gram-negative bacteria (Yunita M, 2023). However, the mode of action of acetone-extracted phytochemicals needs to be explored.

4. Conclusions

UV-VIS and FTIR studies confirmed the presence of polyphenolic compounds, carotenoids, and terpenoids in the leaf extract of *Paederia foetida.* IR-peaks of ethanolic extract at the domain 1050-1100 cm-1 and 3340 cm-1 indicated higher integrated IR-absorption areas and suggested a greater specific concentration of the functional groups in the solvent, which remains in agreement with other spectroscopic determinations. Furthermore, evaporated ethanol extracts would provide higher concentrations of bioactive molecules from these plants that are therapeutic in nature. And both ethanol and acetone leaves extract revealed strong growth inhibition activity against Gram-negative *E. coli* bacteria.

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Research article

PROXIMATE ANALYSIS, PHYSICAL QUALITIES AND CONSUMER PREFERENCES OF GLUTEN-FREE CHICKEN *OTAK-OTAK*

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Abstract: The development of *otak-otak* utilizing locally sourced food ingredients remains to be an area of interest. This study aimed on developing a gluten-free *otak-otak* by incorporating arrowroot flour and mocaf as alternatives for wheat and tapioca flour. The study assessed its chemical composition, physical quality, and sensory attributes to evaluate its overal quality. The study was divided into four different formulations combining arrowroot flour and mocaf flour in varying proportions; P1, P2, P3 (50:50, 70:30, and 90:10, respectively), while P0 served as the control using a 50:50 ratio of wheat and tapioca flours. A randomized design (CRD) with five replications tested for each formulation was applied in the experimental design. The proximate analyses results indicated that thefat content at P0 – P3 levels ranged from 2.70 to 4.04%; protein content from 7.12 to 8.18%; moisture content from 47.95 to 51.16%; ash content from 2.03 to 2.35%; and carbohydrates content from 33.05 to 38.67%. Additionally, cooking loss was between 14.94 and 18.61%, and water holding capacity ranged from 12.4 to 20.8%. Results from sensory evaluation indicated that consumers exhibited no significant preference for the ak-otak prepared with wheat and tapioca flour compared to those made with varying levels of arrowroot and mocaf flour. The hedonic scores varied from 2.35 to 2.87.. All parameters exhibited no significant effect (P>0.05) on *otak-otak* quality; yet, it met SNI standards, suggesting that arrowroot and mocaf flour are viable alternatives to wheat and tapioca flour.

1. Introduction

Otak-otak is prepared from Mackerel (*Scromberomorus commersonii*) specifically formed from spicy ground fish dough coupled with flour then boiled and drained (Susbandi et al., 2019). However, with a little supply and high

demand, the price of mackerel becomes expensive, so it is necessary to diversify raw materials by using chicken meat as an alternative basic ingredient for producing *otak-otak* (Putra, et al., 2015). Global chicken meat consumption is increasing due to the rising population, as it is regarded as food with high protein content (Putri et al., 2024). By 2030, poultry meat is projected to comprise 41% total meat sources (Shahbandeh, 2024). Since 1990, the global population of chickens has more than doubled, the number of chicken globally increased from 13,9 billion in 2000 to approximately 26,56 billion in 2022 (Putri et al., 2024). According to this research, chicken meat is suitable as a primary ingredient for the production of *otak-otak*.

In general, *otak-otak* can be produced using chicken meat blended with flour, coconut milk, eggs, pepper, and other spices (Srihidayati & Firdamayanti, 2021). In the following procedure, the dough is put into banana leaves, rolled, then steamed or baked. The production of *otak-otak* uses wheat flour, which contains gluten, imparting a soft and elastic texture. The production of *otak-otak* uses wheat flour, which contains the ingredient gluten, which has a soft and elastic texture. However, Dahlia (2014) stated that certain sufferers will have difficulties affected by consuming food containing gluten, like celiac disease and autism spectrum disorder (ASD). About 25% of Americans consume Gluten Free Products (GFP), which is a significantly higher than the number of Americans with celiac disease (Prada et al., 2019). Approximately 6 million individuals without celiac disease in Italy have adopted a gluten-free diet (Kamiński et al., 2020). This suggests that most of Gluten Free Products (GFP) users do not consume them for any health-related purposes. A gluten-free diet is healthier and more effective approach for weight management, contributing to the rise in GFP consumption (IMARC Group, 2023).

Currently, limited studies investigated gluten-free flour substitution in traditional Indonesian cuisines regarding both nutritional and sensory qualities. This study involved the diversification of indigenous gluten-free raw resources to manufacture *otak-otak*. Wheat flour is frequently used as an ingredient in *otak-otak*, athough it can be substituted with regional gluten-free options such as arrowroot starch flour and mocaf flour. Arrowroot starch is recognised for its high viscosity, almost double that of other flours. This characteristic facilitates the development of transparent, easily digestible products that are gentle on the digestive system (Hakim et al., 2013). The optimal texture is obtained by combining it with other flours, such as adding mocaf flour since this flour has the qualities of high viscosity, the capacity to form a gel that has dehydrating power and is easily soluble in water (Augustyn et al., 2017). Mocaf flour, derived from cassava, abundant in many regions of Indonesia, serves as a substitute for wheat flour (Wahjuningsih et al., 2024). Addition of a large proportion of mocaf flour will cause the product texture to become softer (Simanjuntak & Effendi, 2017), therefore a combination of the two types of flour is needed to produce better texture according to market desire (Yusuf et al., 2018). Given these challenges, it is imperative to conduct a comprehensive study on the effects of substituting arrowroot flour and modified cassava flour (mocaf) in the production of gluten-free *otak-otak*.

2. Materials and methods

2.1. Materials

2.1.1. Raw material preparation

A 35-day-old male broiler breast meat were obtained from chicken meat local traders in Rejowinangun Market, Magelang city. The ingredients used in the otak-otak making were wheat flour, tapioca flour, arrowroot starch flour, mocaf flour, spinach, shallots, garlic, chicken eggs, pepper, salt, sugar, water, coconut milk, flavouring, banana leaves, and cooking oil. Chemical reagents used for proximate analyses were analytical grades.

2.2. Methods

2.2.1. Production of Chicken Otak-otak

Otak-otak was produced as method conducted by Susbandi et al., (2019). Shallots and garlic were peeled, lightly dried over

medium heat for 3–5 minutes, and subsequently. Spinach leaves were washed, separated from their stems, steamed for 20 seconds, pureed using a chopper, and accurately weighed to 50 grams. Eggs were beaten and weighed to a precise quantity of 30 grams. Chicken breast was pureed with ice cubes using a chopper to preserve its texture. The pureed chicken was subsequently mixed with the prepared shallots, garlic, spinach, sugar, salt, pepper, coconut milk, and seasoning. This mixture was then processed in a chopper for approximately 4 minutes to achieve a homogeneous consistency. The resulting otak-otak mixture was transferred into a ziplock plastic bag and stored in a chiller. For the final preparation, the mixture was portioned onto banana leaves, steamed for 30 minutes, and briefly baked for 2 minutes to enhance its flavor and texture. The finished product was then analyzed to assess its chemical, physical, and sensory characteristics. The use of arrowroot starch is based on the fact that arrowroot starch can replace wheat flour in various processed foods with a percentage addition of 50%-100% (Novitasari et al., 2022). The specific formulation of each treatment can be seen in the Table 1.

Table 1. The ingredient and percentage of chicken *otak-otak*

Material	Treatment			
	PO	**P1**	**P2**	**P2**
Chicken Meat (%)	50	50	50	50
Flour (%)	15	-	-	-
Tapioca Flour (%)	15	-	-	-
Arrowrout Flour (%)	-	15	21	27
Mocaf Flour (%)	-	15	9	3
Spinach (%)	5	5	5	5
Shallot (%)	2.5	2.5	2.5	2.5
Garlic (%)	2.3	2.3	2.3	2.3
Salt (%)	0.8	0.8	0.8	0.8
Sugar (%)	0.5	0.5	0.5	0.5
Coconut Cream (%)	3	3	3	3
Egg (%)	3	3	3	3
Pepper (%)	3	3	3	3
Flavoring (%)	0.4	0.4	0.4	0.4
Water (%)	2	2	2	2

Notes: P0 = wheat flour 50 : tapioca flour 50, P1 = arrowroot flour 50 : mocaf flour 50, P2 = arrowroot flour 70 : mocaf flour 30, P3 = arrowroot flour 90 : mocaf flour 10

2.2.2. Protein Content Analysis

The methodology employed for the determination of protein content was based on Aini & Sani, (2022). The operational procedure is as follows, Initially fresh samples were dried in an oven at 105˚C for 4 hours, followed by grinding the sample; 2) The sample was weighed as much as 2 grams on a watch glass and transferred to a destruction flask; 3) Seven grams of K_2SO_4 and 5 grams of $CuSO_4$ were added; 4) Slowly added fifteen mL of concentrated H_2SO_4 and allowed it to stand for 10 minutes in the acid chamber; 5) Digestion step proceeded at 410˚C for approximately 2 hours or until the solution became clear, then allowed it to cool to room temperature and 50 mL of distilled water then was added; 6) An Erlenmeyer flask containing 25 mL of 4% H_3BO_3 solution with an indicator was prepared as a container for the distillate; 7) The ash containing the results of the digestion in the steam distillation apparatus circuit was installed ; 8) Fifty mL of sodium hydroxide-thiosulfate solution were added; 9) Distillation was carried out and The distillate was collected in the Erlenmeyer flask until the minimum volume of

150 mL (the distillate will turn yellow); 10) The distillate was titrated with standardized 0.1 N HCl until the color changes from green to neutral gray (natural gray).

$$\%protein = \frac{(V_A - V_B) \times HCl \times N\ HCl \times 14.007 \times 6.25 \times 100\%}{Wx1000} \quad (1)$$

Notes:
V_A : mL HCl for sample titration
V_B : mL HCl for blank titration
N : Normality of the standard HCl used
14.007 : Atomic weight of hydrogen
6.25 : Protein conversion factor
W : Sample weight

2.2.3. Fat Content Analysis

The method used for the analysis of crude fat content is the Soxhlet method (Aini & Sani, 2022). The procedures carried out were as follows, 1) Fresh samples were first dried using an oven at a temperature of 105˚C for 4 hours, then the sample was ground 2) The fat flask to be used was dried in an oven at a temperature of 105˚C for 30 minutes; 3) The fat flask was cooled in a desiccator for 15 minutes and weighed (W2); 4) A sample of ± 5 g was weighed (W1) and wrapped; 5) The extraction tool was assembled from a heating mantle, fat flask, **98oxhlet**, and condenser; 6) The sample was inserted into the **98oxhlet** which was then added with sufficient hexane solvent for 1½ cycles; 7) Extraction was carried out for ± 2 hours until the solvent returns through the siphon into the clear colored fat ash; 8) Distillation I used to separate the extracted fat and hexane from the extraction results from the fat flask; 9) The fat that had been separated with hexane was then heated using an oven at a temperature of 105˚C for 1 hour; 10) The fat ash was cooled in a desiccator for 15 minutes and weighed; 11) Heating using an oven was carried out again for 1 hour, if the difference in weighing the last extraction result with the previous weighing does not reach 0.0002 g.

$$\%fat = \frac{W3 - W2}{W1}\ x\ 100\% \quad (2)$$

2.2.4. Moisture Content Analysis

This moisture content analysis was based on the oven method (Aini & Sani, 2022). Porcelain crucibles were dried in a 105˚C oven for 3 hours, then placed in a desiccator for 1 hour. After that, it was weighed with an analytical balance (a). Then 2.5 grams of sample was added to the crucible (b); The crucible containing the sample was placed in a 105˚C oven for 3 hours. After that, it was placed in a desiccator for 1 hour.

$$\%\text{Dry Weight} = \frac{c - a}{b}\ x\ 100\% \quad (3)$$

$$\%Moisture\ content = 100 - \%\text{Dry Weight} \quad (4)$$

A = weight of the weighing crucible
B = sample weight
C = weight of crucible + dry sample

2.2.5. Ash Content Analysis

The ash content analysis was conducted in accordance with the method outlined by Aini & Sani, (2022). Porcelain crucibles were subjected to drying in an oven at 105˚C for 3 hours, followed by a 1-hour placement in dessicator. The crucible was subsequently weighed using an analytical balance (a). Then 2.5 grams of the prepared sample was added to the crucible (b). The crucible and sample were subjected to drying in an electric furnace at 550˚C for a duration of 4 hours. The ash sample was thereafter placed in a desiccator for 1 hour. The weight of the cup and ash was weighed (c).

$$\%Ash\ content = \frac{(\mathrm{c} - \mathrm{a})}{b} x100\% \quad (5)$$

2.2.6. Carbohydrate Content Analysis

Carbohydrate analysis was conducted according to (Novitasari et al., 2022). This method was commonly known as carbohydrate

by difference or carbohydrates through differences.

Carbohydrate analysis calculation formula:

$$\%Carbohydrate = 100\% - (fat\ content + protein\ content + ash\ content + moisture\ content) \quad (6)$$

2.2.7. Cooking Loss Analysis

Cooking loss analysis was conducted according to the (Soeparno, 2005). *Otak-otak* samples wrapped in banana leaves were weighed. Then the *otak-otak* was steamed for 30 minutes and baked for 10 minutes. The cooked *otak-otak* was weighed again The percentage was calculated using the formula:

$$\frac{weight\ before\ cooking - weight\ after\ cooking}{weight\ before\ cooking} x\ 100\% \quad (7)$$

2.2.8. Water Holding Capacity

The water holding capacity (WHC) was measured utilizing the centrifugal method as outlined by (Muchtadi & Sugiyono, 1992). The *otak-otak* were collected and subsequently chopped. and the weight of the chopped sample was measured at 5 grams. The sample was placed in a 10-mL centrifuge tube. 5 mL of distilled water was added and the mixture was centrifuged for 20 minutes at 3000 rpm. The supernatant in the tube underwent separation and its volume was subsequently measured. The calculation of the percentage of WHC is performed using the following formula:

$$\frac{Volume\ of\ water\ absorbed(ml)}{sample\ weight\ (gr)} x100\% \quad (8)$$

2.2.9. Sensory Analysis

The sensory analysis carried out was an organoleptic test using a hedonic test with 40 untrained panelists, both male and female. The panelist were Tidar University students, aged 19-23 years old. Before entering the laboratory room, panelists were given instructions by the researcher and must obey the rules during the test. Each panelist was given 4 samples with random three-digit codes and given a questionnaire to assess color, aroma, taste, texture and overall appearance. The test was carried out in one session using five-point hedonic scale (Simanjuntak & Effendi, 2017) (1: really like it, 2: like it, 3: neutral, 4: don't like it, 5: really don't like it)

2.3. Data analysis

A completely randomized design (CRD) was used and data were analyzed using analysis of variance (ANOVA). Tukey's post-hoc test was used to know the differences among treatments using SPSS Statistics 22.0 (IBM, USA).

3. Results and discussions

3.1. Proximate Analysis

The proximate analysis results presented in Table 2 indicate that no significant differences among P0 (control), P1, P2, and P3 in all measured parameters. The results of fat content analysis conducted using the Soxhlet method on gluten-free *otak-otak* showed no statistically significant difference (P >0.05). The maximum allowable fat content in otak-otak is 16%. Consequently, the gluten-free chicken *otak-otak* produced in the three treatments complies with the Indonesian national standard. The results that were not statiscally significant coulde be due to the limited range of flour percentages utilized, which may have constrained the capacity to identify meaningful differences or trends. The fat content of arrowroot flour is 0.59 % (Lewis, 2012), mocaf flour 0.83 % (Badan Standarisasi Nasional, 2013); wheat flour 1.0%; and tapioca flour 0.5% (Asmoro, 2021). The relationship between fat content and protein content is inversely proportional; an increase in protein content corresponds to a decrease in fat content, and conversely (Idrus & Rossi, 2016). The fat content in a product directly influences the resulting taste and texture, thereby impacting the sensory quality.

Table 2. Proximate analysis of chicken *otak-otak* with substitution of arrowroot flour and mocaf flour

Parameter	Fat^{ns}	Protein^{ns}	Moisture^{ns}	Ash^{ns}	Carbohydrate^{ns}
P0	4.04±0.92	8.18 ± 8.42	50.48±1.66	2.03 ± 0.22	35.26±9.58
P1	3.63±1.37	7.99 ± 7.75	47.95±5.04	2.08 ± 0.54	38.67±10.38
P2	3.91±1.59	7.93 ± 7.06	50.93±4.21	2.35 ± 0.64	33.05±7.01
P3	2.70±0.43	7.12 ± 7.71	51.16±2.74	2.34 ± 0.66	36.85±9.24

Notes:

P0 : *Otak-otak* (control) wheat flour with tapioca flour (50:50).
P1: Substitution of flour with arrowroot flour and flour mocaf (50:50).
P2: Substitution of flour with arrowroot flour and mocaf flour (70:30).
P3: Substitution of flour with arrowroot flour and flour mocaf (90:10).
ns : not significantly different

The results indicating a non-significant protein content may be due to the limited range of flour percentages utilised. The analysis of crude protein content in gluten-free chicken otak-otak, conducted using the Kjeldahl method, indicatedno significant difference (P >0.05). According to the quality requirements outlined in the Indonesian National Standard for fish otak-otak (7757 - 2013), the minimum protein content specified is 5%. Therefore, the gluten-free chicken otak-otak produced in the three treatments complies with this standard. Arrowroot flour has a protein content of approximately 3.05%. (Lewis, 2012) indicates that mocaf flour has a protein content of approximately3.42 % (Badan Standarisasi Nasional, 2013), wheat flour contains 9.0% protein, and tapioca flour contains 1.1% protein (Novitasari et al., 2022).According the regulation protein in the jurisdictations and codex, food contains at least 6,5 g primarily intact protein in a reasonable daily adult intake(Sundari et al., 2015).

The non-significant moisture content results might be attributed to the narrow range of percentages of flour used. According to Indonesia National Standard (Lewis, 2012), mocaf flour has a maximum moisture content of 13%. In addition, the moisture content of *otak-otak* is also influenced by the fiber content, while mocaf flour has a high fiber content of 6% (Faridah et al., 2014). The higher the fiber, the higher the moisture content produced (Rosita et al., 2015); (Wibawanti et al., 2021); (Pratama et al., 2018)

The results of ash content was non-significant which might be attributed to the narrow range of percentages of flour used. This result is might be affected by the processing of several types of flour used and can also caused by differences in starch types and their environment (Kang et al., 2017). The ash content results of the flour gluten-free chicken *otak-otak* samples met the requirements set by SNI (Badan Standarisasi Nasional, 2013) of a 2% as a maximum percentage. Fermentation of mocaf flour affects the ash content produced. The longer the fermentation period, the lower the ash concentration tends to be. This is due to the dissolution of mineral content in cassava as the main ingredient in making mocaf flour. The dissolution of mineral content in cassava serves as the main factor in production of mocaf flour. Fermented cassava has the potential to leas to the dissolution of certain minerals, thereby decreasing the ash content that comprises various mineral contents (Setyani & Astuti, 2017).

3.2. Physical Quality Analysis

The results of the study below pertain to the physical quality of chicken *otak-otak* when substituting arrowroot flour and mocaf flour, as presented in Table 3.

Table 3. Physical quality of chicken *otak-otak* with substitution of arrowroot flour and mocaf flour

Treatment	Parameters (%)	
	Cooking Loss ^{ns}	Water Holding Capacity ^{ns}
P0	17.06±6.85	12.4±7.26
P1	15.94±8.49	17.6±7.53
P2	18.61±6.47	16.0±5.47
P3	14.94±7.63	20.8±7.29

Notes:
P0 : *Otak-otak* (control) wheat flour with tapioca flour (50:50).
P1: Substitution of flour with arrowroot flour and flour mocaf (50:50).
P2: Substitution of flour with arrowroot flour and mocaf flour (70:30).
P3: Substitution of flour with arrowroot flour and flour mocaf (90:10).
ns : not significantly different.

Cooking loss is a condition when meat or processed products shrink during processing, such as heating. The substitution of arrowroot flour and mocaf flour in the chicken *Otak-otak* did not result in a significant impact on cooking losses. Reduced cooking losses indicate superior product quality, as there is less moss loss during the cooking process. Conversely, higher cooking loss values are associated with a deterioration in product quality (Widyawati et al., 2023). Studies on noodles have consistently shown that cooking losses greater than 12% correlate with lower-quality noodles (Kamiński et al., 2020). Several factors have been identified as contributing to cooking loss in noodles, including incomplete starch gelatinization, weakened gluten strength, and the incorporation of additional ingredients (Hajriatun et al., 2017).

Water holding capacity (WHC) is the ability of meat to absorb and retain water during mechanical treatment (stirring, tenderising, seasoning and moulding), temperature treatment, storage and transport (Zayas, 1997) The results of the analysis of variance showed that the substitution of arrowroot flour and mocaf flour did not have a significant effect (P >0.05). The non-significant results might be attributed to the narrow range of percentages of flour used. Study conducted by (Firahmi et al., 2015)explained that water holding capacity (WHC) was significantly influenced by the properties of the ingredients, particularly flour. The proportion of arrowroot flour affected water-binding ability, when pressure was applied to the nuggets, water release occured. The extent of water release is determined by the amylose content in the flour and the formation of matrices involving in water, flour, and meat proteins (Firahmi et al., 2015).

3.3. Organoleptic test

The results of the sensory evaluation, which assess the level of liking (hedonic) for chicken *otak-otak* made with arrowroot starch flour and mocaf flour, are presented in Table 4. The aspect of colour serves to capture consumer interest in a product, derived from the visual sense (Jayanti et al., 2023). The results presented in Table 4 indicate that the colour scores for P0 (control), P1, P2, and P3 did not show significant differences (P>0.05). The colouration of chicken otak-otak exhibited a predominantly white hue, with a subtle greenish tint. The colour of the Otak-otak increases in darkness with a higher proportion of arrowroot starch. Study conducted by (Herlambang et al., 2019) explained tha the bright color of the meatballs produced comes from the high proportion of mocaf flour. The addition of spinach showed a white color with a slight greenish due to its high chlorophyll content as a natural food coloring (Soenarno et al., 2023).

Table 4. Organoleptic test of chicken *otak-otak* with substitution of arrowroot flour and mocaf flour

Parameter	Treatment			
	P0	**P1**	**P2**	**P3**
Color^{ns}	2.65±0.89	2.47±0.87	2.57±0.74	2.72±0.84
Aroma^{ns}	2.40±0.92	2.37±0.95	2.55±0.81	2.87±0.99
Taste^{ns}	2.57±0.95	2.67±1.11	2.55±0.90	2.50±1.03
Texture^{ns}	2.55±0.93	2.52±0.98	2.45±0.81	2.55±0.93
Overall Appearance^{ns}	2.57±0.87	2.45±0.90	2.40±0.70	2.35±0.80

Notes:
P0 = wheat flour + tapioca flour (50:50) as control, P1 = arrowroot starch flour + mocaf flour (50:50), P2 = arrowroot starch flour + mocaf flour (70:30), and P3 = arrowroot starch flour + mocaf flour (90:10). ns= *not significantly different*.
Hedonic scale: 1= like very much, 2= like, 3= neutral, 4= don't like, 5= really don't like

Aroma is a sensory attribute that creates an impression of a product through the sense of smell (Saptadinata & Putra, 2022). The results presented in Table 4 indicate that the aroma levels of chicken otak-otak produced in P0 (control), P1, P2, and P3 did not exhibit a statistically significant difference (P>0.05). The addition of arrowroot starch and mocaf flour was suspected to have no influences on the panelists' preference of the aroma attributes of chicken *otak-otak*. Volatile compounds and water vapor can affect the aroma during the cooking process. One of the spices used in making *otak-otak*, such as shallots and garlic, releases a distinctive aroma when cooked, which can attract consumers to the product (Purwanto et al., 2015).

The flavour profile of a food product is influenced by the interplay between its shape and ingredients, as detected by the gustatory system (Herlambang et al., 2019). The data presented in Table 7 indicate that there were no significant differences in the taste of Otak-otak among the groupsP0 (control), P1, P2, and P3 (P>0.05). The perceptions held by consumers regarding products can impact the flavour profile of a food item (Augustyn et al., 2017). (Srihidayati & Firdamayanti, 2021) stated that taste can be influenced by the raw materials used, such as the type of raw materials and the addition of spices. This was also conveyed by (Augustyn et al., 2017) who stated that the taste of a product is greatly influenced by the spices added, and the amount of spices can be adjusted to suit consumer preferences

Texture also plays an important role in all types of food or influences the selling value of consumer acceptance of a product (Herlambang et al., 2019). The texture of chicken *otak-otak* in P0 (control), P1, P2, and P3 exhibited no significantl differences in consumer preference (P>0.05). The results indicated that the texture score levels for P0, P1, P2, and P3 fell within a value range of 2. The combination of arrowroot starch and mocaf flour was evaluated and assigned a value equivalent to that of the control treatment, according to the preferences of the panellists. The observed outcome can be attributed to the increased proportion of arrowroot starch, which resulted in a chewier texture. This aligns with the findings of (Soenarno et al., 2023), who reported that increasing the proportion of arrowroot starch results in a chewier texture in sausage products.

The overall appearance is the panelist's assessment before tasting the product which can be seen in general based on product's color, flavor, texture, and taste while evaluating it (Purwanto et al., 2015). Based on Table 7, the overall appearance of the chicken *otak-otak* was not significantly different (P>0.5). In general, the panelists gave a score to the overall attribute of chicken *otak-otak* with a value range of 2 (like). This showed that the panelists liked the overall appearance attributes of chicken *otak-*

otak in all proportions of arrowroot starch and mocaf flour.

4. Conclusions

In conclusion, the substitution of arrowroot flour and mocaf flour in chicken *otak-otak* results in a gluten-free that is safe for consumption. These formulations preserve the levels of fat, protein, moisture, ash, and carbohydrate levels, as well as cooking loss and water-binding capacity, in compliance with SNI standards. The study findings demonstrate that the incorporation of arrowroot starch and mocaf flour does not lead to notable variation in consumer acceptance of chicken otak-otak. This substitution represents a potential innovation for the development of gluten-free products and holds promising applications in the food industry

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Review article

FOOD-DERIVED GARLIC POLYSACCHARIDES AS EMERGING FUNCTIONAL INGREDIENTS: STRUCTURE, MICROBIOTA–IMMUNITY INTERACTIONS, AND HEALTH IMPLICATIONS

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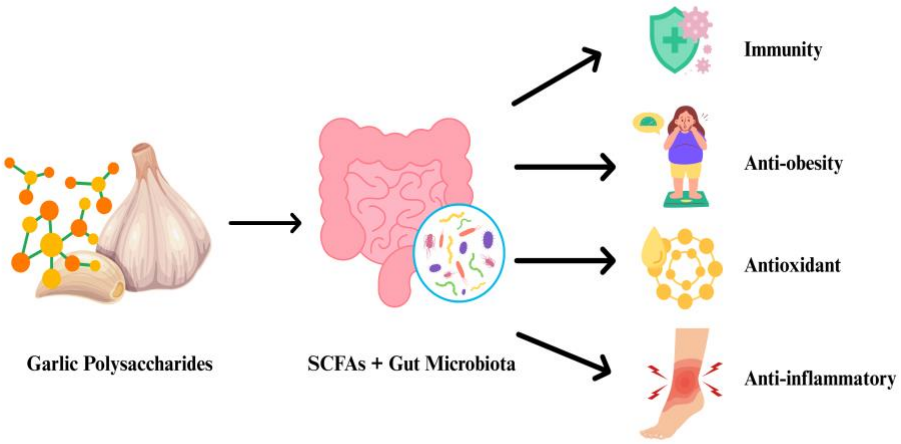
Abstract. Garlic polysaccharides (GPs) are emerging as important non-sulfur bioactives that complement the well-studied organosulfur compounds of Allium sativum. With diverse structures and molecular weights, GPs exert antioxidant, anti-inflammatory, immunomodulatory, and metabolic benefits that are increasingly linked to their role as prebiotic substrates for gut microbiota. This review consolidates recent advances in the extraction and structural characterization of GPs and examines how their physicochemical features shape fermentability, microbial enrichment, and production of metabolites such as short-chain fatty acids, bile acids, and tryptophan derivatives. These microbiota-derived signals, together with direct immune modulation by specific GP fractions, underpin improvements in mucosal barrier function, systemic immunity, and metabolic outcomes in preclinical models of obesity, diabetes, fatty liver disease, and atherosclerosis. By integrating structure–function relationships with microbiota–immunity interactions, we outline the dual role of GPs as prebiotics and immunonutrients, and compare their actions with those of established dietary polysaccharides. Current limitations include methodological variability, lack of standardized structural reporting, and scarce clinical validation. Future directions call for multi-omics approaches, personalized nutrition strategies, and well-designed human trials to translate the promising microbiota–immune mechanisms of GPs into functional food and therapeutic applications.



Graphical Abstract

1.Introduction

Garlic (*Allium sativum L.)* has a significant history as a culinary ingredient and a folk remedy, attributed with extensive health effects, including cardiometabolic protection and immune modulation, among others. Modern studies have long focused on organosulfur-based compounds, but there is growing interest in the role of carbohydrate fractions in determining the functional profile of garlic, namely polysaccharides and oligosaccharides. According to recent narrative and systematic literature, garlic-derived polysaccharides (GPs) have a wide range of bioactivities and should be given special emphasis in food and health studies (El-Saadony *et al*., 2024).

Among the non-sulfur constituents, GPs are structurally heterogeneous: most edible-bulb fractions are fructan/oligofructose-like (inulin-type) with β- (2-1)-linkages and isolated branching, though by-product streams (peels, leaves, pomace) may be pectin-rich, having galacturonic acid and rhamnogalacturonan-I/homogalacturonan domains. Since 2018, progress has mapped extraction-structure relationships (hot water/enzymatic routes; ultrafiltration; chromatographic fractionation) and has related molecular weight/branching to functional performance (Jiang *et al*., 2022; Qiu *et al*., 2024; Sunanta *et al*., 2024).

Concurrently, the gut microbiota has emerged as a central mediator of diet–health relationships. In vitro and in vivo studies show garlic saccharide fractions act as prebiotic substrates, selectively enriching beneficial taxa (e.g., *Bifidobacterium*, *Akkermansia*), enhancing short-chain fatty acid (SCFA) production, and improving barrier and inflammatory readouts; human evidence is still limited but growing. Notably, water-soluble garlic polysaccharides (WSGP) alleviate colitis and restore mucosal integrity in murine models, and aged/processed garlic saccharides can remodel microbial communities alongside favorable metabolic markers—supporting a microbiota-linked mode of action (Ettehad-Marvasti *et al*., 2022; Ha *et al*., 2024; T. Li *et al*., 2024a; Shao *et al*., 2024; Zhao *et al*., 2022a).

This review synthesizes the structural features, biological activities, and gut-microbiota interactions of garlic polysaccharides/oligosaccharides, emphasizing a structure–function–activity lens rather than a mere catalogue of studies. We integrate recent advances (2018–2025) across extraction/characterization, prebiotic and immunomodulatory outcomes, and microbiota-linked health implications, highlighting where structure (degree of polymerization, branching, and pectin vs. fructan signatures) plausibly shapes fermentability and host responses. Our approach is narrative and integrative, drawing together chemistry, microbiology, and nutrition

to clarify current evidence, limitations, and opportunities for translational research and functional food development (Holmes *et al*., 2022; Qiu *et al*., 2024).

2.Structural Features of Garlic Polysaccharides

2.1. Extraction and purification methods

Garlic polysaccharides (GPs) are most commonly obtained by hot-water extraction of fresh or dried garlic tissues, followed by removal of low-molecular impurities and concentration, with ethanol precipitation used to recover crude polysaccharide fractions. Subsequent deproteinization (Sevag or enzymatic), dialysis or ultrafiltration, and chromatographic fractionation (ion-exchange and size-exclusion) produce purified fractions of defined molecular weight ranges. Ultrasonic/microwave-assisted extraction and aqueous two-phase systems have also been applied to improve yields and reduce extraction time, while enzymatic-assisted methods enable milder conditions that better preserve native structures. Gradient ethanol precipitation and membrane separation (ultrafiltration) are widely used to obtain oligosaccharide vs. higher molecular-weight polysaccharide fractions for downstream characterization and bioactivity testing (M. Wang & Cheong, 2023; Y. Zhang *et al*., 2024).

Choice of extraction/purification method strongly affects yield, degree of polymerization (DP), and apparent bioactivity — for example, hydrolytic or harsh chemical methods can shorten chain length and increase fermentability, whereas gentle aqueous extraction preserves higher-MW fractions. Recent method comparisons and optimizations (including response-surface and design-of-experiments) have been reported to balance yield and structural integrity (Zhi *et al*., 2023).

Table 1. Extraction methods and structural features of garlic polysaccharides.

Extraction / Purification Method, Yield (if reported), and Structural Features	Analytical Techniques Used	References
Hot-water extraction (60 °C, 180 min, 1:10 w/v) followed by ethanol precipitation, deproteinization and dialysis. Yield not specified. Polysaccharide fraction rich in fructose (82.8%) and glucose (16.8%), Mw ≈3.7 kDa; inulin-type β-Fruf linkages.	HPLC for monosaccharides, SEC for Mw, FTIR, UV/CD spectra, SEM, thermal analysis.	Preparation & characterization of garlic polysaccharides (Bai *et al*., 2022)
Acidolysis of crude GPs + ultrafiltration to obtain low- and high-Mw fractions (e.g., U0.3, U6). Yield not expressed. Fractions varied in DP and fermentability; fructose and glucose were dominant.	HPAEC/HPLC, HPSEC, simulated digestion/fermentation, 16S rRNA sequencing.	Digestive properties and prebiotic activity of garlic saccharides (Zhao *et al*., 2022a)
Three-phase partitioning + gradient ethanol fractionation. Yield fraction-dependent. Produced multiple Mw populations with distinct monosaccharide ratios; separated oligosaccharides from polysaccharides.	SEC-MALLS/HPSEC, monosaccharide analysis, FTIR, GC–MS linkage analysis.	Three-phase partitioning + gradient ethanol fractionation (example of advanced fractionation used for

		raw garlic) (Yan *et al*., 2021)
Sequential acidic extraction and fractionation of garlic biomass. Reported higher yields in pectin-rich fractions. Structures dominated by galacturonic acid (>61%), with Gal and Rha; homogalacturonan and RG-I domains; Mw ≈350 kDa; degree of methylation 44–56%.	HPLC, methylation/GC–MS, FTIR, viscometry, Mw profiling.	Fractionation & characterisation of pectin-rich extracts from garlic biomass (Sunanta *et al*., 2023)
Hot-water extraction followed by purification (based on prior protocols) was used to obtain water-soluble GP fraction (WSGP). Yield not reported. Composition consistent with fructan-rich, fermentable oligosaccharides; bioactive in colitis model.	Monosaccharide analysis, LC–MS metabolites, WB/IF for bioactivity.	Water-soluble garlic polysaccharide (WSGP) improves ulcerative colitis (Shao *et al*., 2024a)
Extraction/fractionation of aged garlic produced aged garlic oligosaccharides (AGOs). Yield details not main focus. Fractions were low-DP oligosaccharides increasing SCFAs and reducing TMAO; increased Akkermansia reported.	SEC/HPSEC, monosaccharide analysis, GC/LC–MS for SCFAs, 16S/metagenomics.	Aged garlic oligosaccharides (AGOs) prepared from aged garlic (X. Wang *et al*., 2025)

2.2. Structural characteristics

Garlic polysaccharide fractions are compositionally diverse. Fructan-type fractions (common in garlic bulbs) are dominated by fructose (with terminal glucose residues in some chains), while other garlic-derived fractions—especially from peels, leaves or processing residues—can be pectin-rich, containing high proportions of galacturonic acid plus neutral sugars such as galactose, arabinose, glucose, xylose and rhamnose. Reported monosaccharide profiles in recent studies list combinations of glucose, fructose, galactose, arabinose, mannose and xylose depending on tissue source and extraction method (Chen *et al*., 2024; Qiu *et al*., 2024; Sunanta *et al*., 2024).

The predominant backbone in bulb-derived garlic saccharides is inulin-type fructan built from β-(2→1) fructofuranosyl linkages; occasional β-(2→6) branching is reported in some preparations. Pectin-type fractions contain homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) regions with side chains of arabinans/galactans. Degree of polymerization (DP) ranges from short chain fructooligosaccharides (DP < 10) to long inulins (DP up to several tens), and measured molecular weights (Mw) for garlic polysaccharide fractions span from a few kDa (oligosaccharides) to high-MW polysaccharides (>100 kDa), depending on fractionation and analytical method. Linkage analysis (methylation/GCMS) and NMR studies confirm the β-(2→1) fructan motif in many bulb fractions, and pectic linkages in peel/leaf fractions (Karimi *et al*., 2025; Qi *et al*., 2022).

Operationally, garlic oligosaccharides generally refer to low-DP, water-soluble

fructooligosaccharides (FOS) and short fructans that are highly fermentable by gut microbes; polysaccharides indicate broader, higher-Mw fractions including long-chain fructans and pectin-type polymers with different physicochemical behaviors. Fractionation (e.g., graded ethanol precipitation, ultrafiltration, or chromatographic collection) is typically used to produce and distinguish these classes for functional testing (Ito *et al*., 2011; M. Li *et al*., 2023). Fig. 1 shows the schematic structure of garlic polysaccharides.

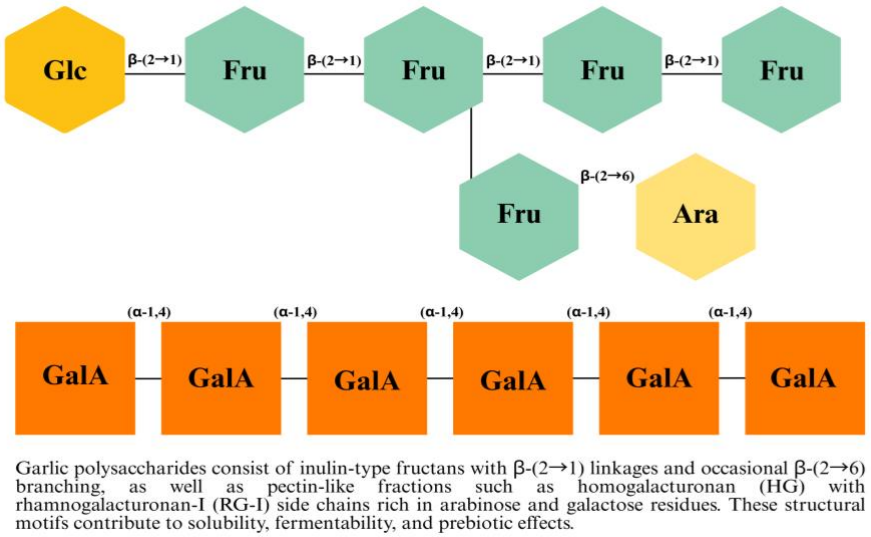


Figure 1. Schematic structure of garlic polysaccharides

2.3. Structure-Function Relationship

Water solubility is a primary determinant of microbial accessibility and fermentability. Low-DP, water-soluble oligosaccharides are rapidly fermented by saccharolytic gut bacteria resulting in quick SCFA production, whereas high-Mw, less soluble polymers may be more slowly fermented or partially resistant, stimulating different microbial consortia. Thus, solubility and molecular size together shape fermentation kinetics and selective enrichment of taxa (D. T. Wu *et al*., 2022; Xia *et al*., 2025).

Multiple recent studies across plant polysaccharides show that lower Mw/DP fractions tend to be fermented more readily and promote greater increases in SCFAs (acetate, propionate, butyrate) and beneficial microbes (e.g., *Bifidobacterium*, *Lachnospiraceae*), whereas higher-Mw fractions produce slower but sometimes more sustained effects; these patterns have been observed for fructans and pectic-oligosaccharides alike and are reported for garlic fractions as well. Experimental work demonstrates that enzymatic hydrolysis or acidolysis to reduce DP can increase prebiotic potency (M. Li *et al*., 2023; Xia *et al*., 2025; Xiao *et al*., 2025).

Branching can influence enzyme accessibility and the spectrum of microbes able to degrade the polymer: more highly branched polysaccharides may favor specialized taxa possessing debranching enzymes, while linear β-(2→1) fructans are broadly fermented by common saccharolytic bacteria. Likewise, the presence of uronic acids (as in pectic regions) introduces charged groups that affect solubility and interactions with host mucins, and thus may modulate colon localization and immunomodulatory potential (Qi *et al*., 2022; D. T. Wu *et al*., 2022).

Structural features also correlate with reported biological effects beyond prebiotic fermentation: low-Mw garlic oligosaccharides are often linked to stronger in vitro prebiotic effects and SCFA increases that relate to metabolic endpoints (glycemic control, lipid modulation), while certain pectin-rich fractions display viscosity/emulsifying properties and antioxidant capacities that may contribute to gut barrier protection and anti-inflammatory effects.

However, direct structure–activity causal links remain understudied in human trials and require standardized fractionation and comparative head-to-head experiments (Chen *et al*., 2024; Qiu *et al*., 2024; M. Wang & Cheong, 2023).

3. Biological Activities of Garlic Polysaccharides

3.1. Antioxidant activity

Garlic polysaccharides (GPs) consistently show free-radical scavenging (e.g., DPPH, ABTS, •OH) and reducing power in vitro, with activity influenced by molecular weight and chemical modification. Carboxymethylated or metal-complexed derivatives generally enhance total antioxidant capacity compared with native GP, suggesting that electron-donating substituents and coordination with metal ions improve redox performance (Bai *et al*., 2022; Cheng *et al*., 2020). Recent in vivo work also reports improved antioxidant enzyme activities (SOD, CAT, GSH-Px) following GP supplementation, alongside mitochondrial energy benefits via AMPK/PGC-1α signaling in mice subjected to exhaustive exercise, supporting functional relevance beyond test-tube assays (T. Li *et al*., 2024b).

Table 2. Biological activities of garlic polysaccharides.

Study	Model / GP fraction & dose (as reported)	Key outcomes (bioactivity)
(Shao *et al*., 2024b)	DSS-induced colitis mice; WSGP (water-soluble garlic polysaccharide); in vivo dosing 200–400 mg/kg (oral).	Attenuated colitis: ↓histological damage, ↑tight-junction proteins (ZO-1, occludin), ↓pro-inflammatory cytokines (TNF-α, IL-6), modulated NF-κB/STAT3 signaling; increased fecal SCFAs.
(X. Wang *et al*., 2025)	ApoE$^{-/-}$ mice on HFD/HCD; Aged garlic oligosaccharides (AGOs) isolated from aged garlic; fraction doses per Methods.	Reduced atherosclerotic lesion formation, ↓TMA/TMAO, ↑fecal SCFAs (acetate/propionate/butyrate), remodeled microbiota (↑Akkermansia), improved lipid profile.
(Wu *et al*., 2024)	In vitro RAW264.7 macrophage cells & immunosuppressed mice; fructan-type garlic polysaccharide (purified fraction).	Immunostimulatory under immunosuppression: ↑NO, ↑TNF-α, ↑IL-6 in macrophages; restored immune indices in immunosuppressed mice (enhanced macrophage function).

(Liu *et al*., 2024)	HFD-induced obese mice; fermented garlic polysaccharides (BGP/OPS); dosing per study methods.	Anti-obesity & hypolipidemic effects: ↓body weight gain, ↓serum TG/TC, improved adipose inflammation; associated with altered gut microbiota and increased fecal SCFAs.
(T. Li *et al*., 2024)	Mouse fatigue model; soluble garlic polysaccharide from industrial garlic waste; dosing per Methods.	Improved exercise endurance, ↑hepatic & muscle glycogen, ↑antioxidant enzymes (SOD, GSH-Px, CAT), activated AMPK/PGC-1α signaling—showing antioxidant and metabolic benefits.
(Xie *et al*., 2022)	Animal metabolic models; garlic polysaccharide fractions (reported).	Demonstrated hypolipidemic and metabolic benefits; proposed GP as a nutraceutical for metabolic syndrome/T2D models.
(Cheng *et al*., 2020)	In vitro antioxidant assays & fraction testing (P, SP, PP, CMP) using garlic raw material.	Demonstrated significant DPPH/ABTS/•OH scavenging and reducing power for garlic polysaccharide fractions; chemical modification (e.g., carboxymethylation) enhanced antioxidant capacity.

3.2. Anti-inflammatory activity

Water-soluble garlic polysaccharides (WSGP) attenuate experimental colitis by dampening NF-κB/STAT3 signaling, reducing pro-inflammatory cytokines (e.g., TNF-α, IL-6), and enhancing mucosal barrier integrity (tight-junction proteins), with concurrent modulation of gut microbial metabolites. These findings have been reproduced in multiple DSS colitis models and updated analyses (2020–2024), highlighting a robust anti-inflammatory profile (Shao *et al*., 2020, 2024). Mechanistically, lower-MW ("small molecular") GP fractions often show stronger anti-inflammatory effects—consistent with improved solubility/fermentability—though authors call for standardized, head-to-head comparisons across fractions (M. Lu *et al*., 2023) .

3.3. Immunomodulatory activity

Beyond generic anti-inflammation, specific GP fractions activate innate immune cells. A 2024 study on a fructan-type GP demonstrated macrophage (RAW264.7) activation (↑NO, TNF-α, IL-6) and immune enhancement in immunosuppressed mice, linking activity to fructan structure (J. Wu *et al*., 2024). Broader reviews on plant polysaccharides corroborate that structural motifs and branching patterns shape macrophage polarization and downstream cytokine milieus, positioning GPs as candidate

immunonutrients; however, direct clinical validation for garlic-specific fractions remains limited (Wei *et al*., 2024).

3.4. Metabolic Health Benefits

Evidence is accumulating that GPs improve metabolic phenotypes, often in concert with microbiota remodeling. Polysaccharides from fermented garlic reduced weight gain and improved lipid profiles in HFD mice, with shifts in gut microbiota and faecal SCFAs indicating a prebiotic mechanism (Q. Liu *et al*., 2024). Purified GP improved glycemic control and hepatic glycogen metabolism in T2DM models, complementing broader evidence that dietary polysaccharides modulate glucose homeostasis (He *et al*., 2023; Xie *et al*., 2023). Aged garlic oligosaccharides mitigated atherosclerosis in ApoE$^{-/-}$ mice fed a high-fat/high-cholesterol diet, accompanying microbiota and metabolic improvements; separate studies report GP-mediated protection in MAFLD models (J. Liu *et al*., 2022; X. Wang *et al*., 2025).

4. Garlic Polysaccharides and Gut Microbiota

4.1. Prebiotic effects

Garlic polysaccharides (GPs), particularly low-molecular-weight fructan/oligosaccharide fractions obtained by controlled hydrolysis or graded fractionation, display clear **prebiotic activity** in vitro and in vivo. Several studies demonstrate that GPs (or garlic saccharide fractions) are selectively fermented by beneficial gut microbes, promoting the growth of genera commonly associated with health, such as *Bifidobacterium* and *Lactobacillus*. In vitro fermentation assays using human faecal inocula showed that hydrolysed garlic saccharides increased bifidogenic activity and produced greater levels of short-chain fatty acids (SCFAs) compared with native, high-DP fractions — consistent with the established relation between degree of polymerization and fermentability (X. Lu *et al*., 2021; Zhao *et al*., 2022).

Animal studies corroborate these findings: dietary supplementation with garlic oligosaccharide or water-soluble GP fractions increased relative abundances of *Bifidobacterium*, *Lactobacillus*, and other saccharolytic taxa, while reducing opportunistic/pathobiont groups in rodent models. These microbial shifts were accompanied by rises in faecal acetate, propionate, and butyrate — metabolites that mediate many downstream physiological effects, including epithelial energy supply, barrier integrity, and immune signalling. Such prebiotic responses appear more pronounced for low-DP/low-Mw fractions and for processed/aged garlic oligosaccharides versus unprocessed high-Mw polysaccharides (X. Wang *et al*., 2025; Zhao *et al*., 2022b). Lu *et al*. (2021) characterized garlic neutral polysaccharides and showed enhanced in vitro fermentation and bifidogenic effects after controlled hydrolysis. A series of more recent in vivo reports (2022–2024) confirm that water-soluble and aged garlic saccharides enrich beneficial taxa and increase fecal SCFAs in animal models (T. Li *et al*., 2024a; X. Lu *et al*., 2021).

Table 3. Comparative features of garlic vs. other polysaccharides.

Feature	Garlic polysaccharides (GPs)	Inulin / Fructooligosaccharides (FOS)	β-Glucans
Source & primary composition	Mainly fructan-type (inulin-like) oligo-/polysaccharides in bulbs (β-(2→1) Fruf	Fructans composed largely of β-(2→1) fructofuranosyl linkages (inulin) or shorter DP (FOS); well-	β-(1→3)/(1→6)-linked glucose polymers from yeast, fungi, oats, barley; structure

	backbone; occasional β-(2→6) branches); pectin-rich fractions (GalA, RG-I) often found in peels/leaves/waste (Jiang *et al*., 2022).	characterized commercial prebiotics (Hughes *et al*., 2022)	(branching/conformation) varies with source. Strongly associated with immunomodulatory activity (Zhong *et al*., 2023).
Typical molecular size / DP	Broad: GP range from low-DP oligosaccharides (few kDa) to high-Mw polysaccharides (tens to hundreds kDa), depending on extraction/fractionation. DP influences fermentability (Zhao *et al*., 2022a).	Commercial inulin DP typically 2–60; FOS DP <10 (short-chain), consistent and reproducible manufacturing profiles. DP correlates with fermentation kinetics (Hughes *et al*., 2022).	Mw and degree of branching highly source-dependent (oligomeric to >100 kDa); triple-helix vs single chain conformations influence receptor binding and activity (Zhong *et al*., 2023).
Primary biological actions	Prebiotic, antioxidant, immunomodulatory (macrophage activation, cytokine modulation), anti-inflammatory, metabolic benefits in animal models (anti-obesity, hepatoprotection) (Shao *et al*., 2024a).	Prebiotic effects (bifidogenic), improved laxation, enhanced mineral absorption, metabolic benefits (improved insulin sensitivity, reduced TGs) in human and animal studies (J. Li *et al*., 2025).	Immunomodulation via pattern recognition receptors (Dectin-1, CR3, TLR cross-talk); stimulates macrophages, dendritic cells and NK cells; also acts as a fermentable fiber in some sources (partial prebiotic effects) (Singh & Bhardwaj, 2023).
Prebiotic potency & taxa stimulated	Promotes *Bifidobacterium*, *Lactobacillus* and some SCFA-producers (e.g.,	Robust bifidogenic effect in humans and animals; reliably increases SCFAs (acetate/propionate/butyrate)	Variable: some β-glucans (e.g., oat β-glucan) increase SCFAs and beneficial taxa, but effects are source- and

	Lachnospiraceae); potency depends on DP and fraction (low-DP often more fermentable). Evidence primarily in vitro and rodent models; some human-relevant fermentation shown in vitro (Zhao *et al*., 2022a).	and beneficial taxa in many RCTs and reviews (Holmes *et al*., 2022).	solubility-dependent; yeast/fungal β-glucans more prominent for direct immune effects (Feng *et al*., 2025).
Mechanisms linking microbiota to immunity	Indirect: promote fermentation leading to SCFAs (GPR41/43, HDAC inhibition) and likely effects on bile acids/trp-metabolites that support Treg induction and barrier protection; Direct: certain GP fractions can stimulate innate immune cells (macrophages) in vitro. Evidence largely preclinical (Y. Zhang *et al*., 2024).	Indirectly via SCFA production, increased mucosal health, and lowered endotoxemia; substantial human mechanistic evidence for SCFA-driven effects on metabolism and some immune endpoints (Holmes *et al*., 2022).	Direct receptor-mediated immune activation (Dectin-1, others) leading to cytokine modulation, adaptive responses; Indirect microbiota-metabolite effects also reported for some dietary β-glucans. Strong mechanistic evidence from in vitro, animal studies and some clinical trials (Zhong *et al*., 2023).
Clinical evidence	Limited direct RCTs with isolated GP fractions; most evidence from in vitro and animal models,	Multiple human RCTs and meta-analyses supporting tolerance, bifidogenic response, improvements in bowel habits and some	Several human studies and trials (mostly for yeast/beta-glucan supplements and oat β-glucan for cholesterol) show immune or metabolic

	with a few human studies using whole/processed garlic or GP supplements showing microbiota/modulatory signals — calls for well-designed human trials (T. Li *et al*., 2024).	metabolic markers; inulin/FOS are clinically established prebiotics (J. Li *et al*., 2025).	benefits; more trials exist for β-glucan than for GPs, though specifics depend on source and formulation (Muroya *et al*., 2025).
Unique advantages / translational potential	Dual role: acts as a prebiotic and contains fractions with direct immunomodulatory activity — promising for metabolic diseases via flora-metabolites-immune axis; also potential to use agricultural waste (peels) as pectin sources. However, heterogeneity and lack of human RCTs limit current translation (Zhao *et al*., 2022a).	Well-characterized, consistent (commercial) source; predictable fermentation; widely used as benchmark prebiotics and included in many functional foods; regulatory acceptance for many uses (Hughes *et al*., 2022).	Strong evidence for immune activation and some metabolic endpoints (cholesterol reduction for oat β-glucan); established as immunonutrients in clinical nutrition and as functional ingredients. Source variability requires careful standardization (Singh & Bhardwaj, 2023).

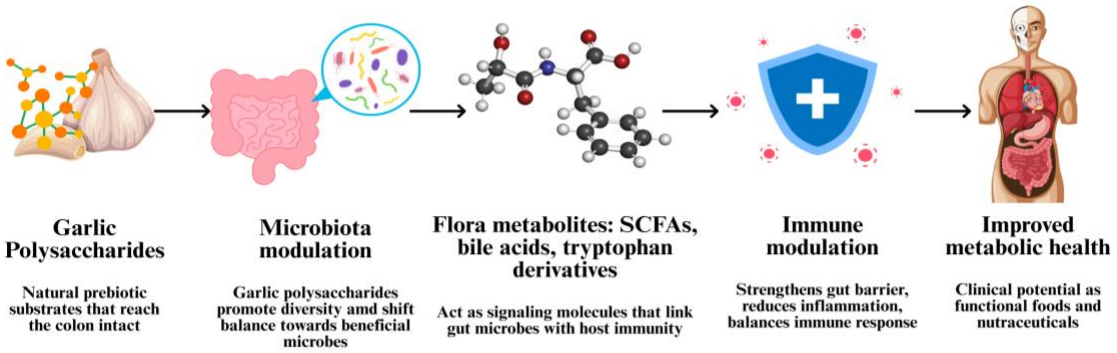


Figure 2. Mechanism of garlic polysaccharides in modulating gut microbiota and their metabolites

4.2. Impact on microbial diversity and balance

Beyond selective stimulation, GPs can reshape overall gut community structure and improve dysbiosis in disease models. In DSS-induced colitis and other inflammatory rodent models, supplementation with water-soluble garlic polysaccharides restored alpha diversity (Shannon/Chao indices) and partially reversed disease-associated shifts such as Firmicutes/Bacteroidetes imbalance. These community-level changes frequently co-occur with reduced pro-inflammatory taxa and increased mucin-degrading or SCFA-producing lineages (e.g., *Akkermansia*, Lachnospiraceae members), suggesting that GPs can promote a more resilient, functionally favorable microbiome (Shao *et al*., 2024).

In metabolic disease contexts (high-fat diet or ApoE$^{-/-}$ atherosclerosis models), aged garlic oligosaccharides and other garlic saccharide supplements have been reported to lower the Firmicutes: Bacteroidetes ratio, increase abundances of SCFA-producers, and reduce microbial signatures linked to TMA/TMAO production. These compositional shifts parallel improvements in body weight, lipid profiles, and inflammatory markers, supporting a microbiota-mediated route for garlic polysaccharide benefits in metabolic disorders (Ha *et al*., 2024; X. Wang *et al*., 2025). Most robust data come from animal and in vitro studies; human interventional data specifically with isolated garlic polysaccharide fractions remain scarce. Heterogeneity in extraction/fractionation methods, dosing regimens, and sequencing/analysis pipelines also complicates direct comparisons across studies. Nevertheless, the converging evidence supports the concept that garlic polysaccharides—especially low-DP fractions—act as meaningful prebiotic modulators that improve microbial diversity and functional outputs in models of intestinal inflammation and metabolic perturbation (X. Lu *et al*., 2021; Vinelli *et al*., 2022).

4.3. Flora Metabolites as Key Mediators

Dietary polysaccharides (including garlic-derived oligo-/polysaccharides) primarily exert systemic effects by being fermented or transformed by the gut microbiota into small bioactive molecules. The three categories most relevant to garlic polysaccharide (GP)-driven host effects are short-chain fatty acids (SCFAs), microbiota-modified bile acids, and tryptophan-derived metabolites. These metabolites act as signaling molecules coupling microbial activity to host metabolic and immune responses and therefore form the mechanistic bridge between GP intake and improvements in metabolic disease phenotypes (Hou *et al*., 2023; Zhu *et al*., 2024) .

4.3.1.Short-Chain Fatty Acids (SCFAs)

SCFAs (mainly acetate, propionate, and butyrate) are produced by saccharolytic bacteria during fermentation of fermentable carbohydrates such as low-DP fructans/oligosaccharides. They have multiple host targets: they serve as energy substrates for colonocytes, modulate intestinal barrier function, signal through G-protein coupled receptors (GPR41/FFAR3 and GPR43/FFAR2), and

inhibit histone deacetylases (HDACs) to alter gene expression in immune cells. Through these routes SCFAs promote regulatory T cell (Treg) differentiation, suppress pro-inflammatory cytokine production, and improve insulin sensitivity and lipid metabolism—mechanisms that are central to metabolic disease amelioration (Facchin *et al*., 2024; D. Zhang *et al*., 2023) .

Garlic polysaccharide studies report increased fecal SCFA production following administration of low-MW/oligosaccharide fractions or processed/aged garlic saccharides. For example, water-soluble garlic polysaccharide (WSGP) supplementation in murine colitis models increased SCFAs while improving barrier proteins and reducing inflammatory signaling. Aged garlic oligosaccharides used in high-fat/high-cholesterol models similarly raised fecal acetate/propionate/butyrate and were associated with improvements in atherosclerotic and metabolic readouts. These findings support a model in which GP-driven enrichment of SCFA-producing taxa mediates downstream immune and metabolic benefits (Shao *et al*., 2024; X. Wang *et al*., 2025).

4.3.2.Bile Acids (Microbiota-modified)

Primary bile acids synthesized by the liver are metabolically transformed by gut microbes into secondary bile acids; the composition of the bile acid pool is therefore microbiota-sensitive. Bile acids are potent signaling ligands for host receptors such as FXR (farnesoid X receptor) and TGR5 (GPBAR1), which regulate lipid and glucose metabolism, intestinal barrier function, and immune cell activity. Microbiota-driven changes in bile acid metabolism can thus influence metabolic disease risk and inflammatory status (Y. Li *et al*., 2024; Zhu *et al*., 2024).

Although direct studies of garlic polysaccharides altering bile acid pools are fewer than those for SCFAs, interventions with garlic-derived oligosaccharides and related garlic preparations have been linked to microbiota shifts that plausibly reduce pro-atherogenic metabolites (e.g., trimethylamine to Trimethylamine-N-oxide TMAO) and alter bile-acid-related signaling. Aged garlic oligosaccharide supplementation in an atherosclerosis model decreased TMAO formation and was accompanied by microbial and metabolic reprogramming, suggesting that GP intake can indirectly modulate bile-acid and other microbially-derived lipid mediators relevant to cardiovascular and hepatic health (Mao *et al*., 2024).

4.3.3.Tryptophan-derived Metabolites

Microbial metabolism of tryptophan yields indoles and related compounds (e.g., indole-3-propionic acid, indole-3-aldehyde) that act on host receptors including the aryl hydrocarbon receptor (AhR). AhR activation by these microbial indoles supports mucosal barrier integrity, induces IL-22 production (important for epithelial repair and antimicrobial peptide expression), and modulates innate and adaptive immune responses—pathways implicated in metabolic and inflammatory diseases. Another fraction of tryptophan is processed via the host kynurenine pathway, which also interfaces with immune regulation; the balance of these routes is microbiota-influenced (Hezaveh *et al*., 2022; Hou *et al*., 2023).

While direct reports linking garlic polysaccharide intake to specific tryptophan-metabolite profiles are still emerging, broader polysaccharide interventions demonstrate shifts in microbial tryptophan metabolism and enhanced AhR ligand availability. Given that garlic GPs alter microbiota composition—promoting taxa that are competent in tryptophan catabolism—the mechanistic plausibility is strong that GP consumption affects immune tone via tryptophan-derived AhR ligands. Reviews summarizing microbial-Trp–AhR crosstalk recommend targeted metabolomics in future GP studies to confirm these pathways (Dai *et al*., 2021; S. Li, 2023).

4.4. Interaction with Intestinal Immunity

Garlic polysaccharides (GPs) influence intestinal immunity both indirectly (via microbially produced metabolites) and directly (via interactions with immune cells), producing measurable effects on mucosal barrier integrity

and immune cell regulation, such as regulatory T cells (Tregs) and cytokine profiles.

4.4.1.Effects on mucosal barrier integrity

Multiple in vivo studies report that water-soluble and low-molecular-weight garlic polysaccharide fractions protect or restore mucosal barrier structure in models of intestinal injury. In DSS-induced ulcerative colitis (UC) mice, supplementation with a water-soluble garlic polysaccharide (WSGP) reduced histological damage, preserved goblet cell mucin, and increased expression of tight-junction proteins (ZO-1, occludin), thereby decreasing intestinal permeability and disease activity index. These protective effects coincided with suppression of NF-κB/STAT3 inflammatory signalling and shifts in microbiota/metabolite profiles, indicating a coordinated barrier–microbiota–immune effect (Shao *et al*., 2020, 2024a).

Similar barrier-protective outcomes have been reported in other GP interventions: studies noted increased mucin staining, reduced epithelial erosion, and recovery of villus/crypt architecture after GP or garlic oligosaccharide feeding in inflammatory and metabolic models, again often parallel to rises in SCFAs and increases in SCFA-producing taxa (e.g., Lachnospiraceae, Akkermansia). These observations support the view that GP-driven microbiota changes and SCFA increases mediate much of the mucosal protection, though direct interactions between polysaccharides and epithelial or immune cells may also contribute (T. Li *et al*., 2024; Shao *et al*., 2020). Mechanistically, SCFAs produced from GP fermentation (butyrate, propionate) provide colonocyte energy, promote tight-junction assembly, and stimulate mucus production—processes that reduce translocation of microbial components and downstream systemic inflammation. Reviews summarizing SCFA biology emphasize these epithelial actions as fundamental routes by which prebiotic fibres improve barrier integrity in inflammatory and metabolic disease contexts (Ney *et al*., 2023; Venegas *et al*., 2019).

4.4.2.Regulation of Tregs and Cytokines

GP interventions modulate immune cell phenotypes and cytokine responses in both local (intestinal) and systemic compartments. In animal models, GP or garlic oligosaccharide supplementation decreased pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and increased anti-inflammatory markers such as IL-10 and TGF-β in colon tissue and serum, aligning with improved histology and clinical scores in colitis and metabolic disease models. These cytokine shifts are consistently reported alongside microbiota/metabolite changes, highlighting the role of a microbe-metabolite-immune axis (M. Lu *et al*., 2023).

Regulatory T cells ($FoxP3^+$ Tregs) are a key cellular target of microbiota-derived metabolites. SCFAs enhance Treg differentiation and function via GPR43/GPR109A signaling and HDAC inhibition, increasing FoxP3 expression and IL-10 production—mechanisms shown in multiple preclinical studies and reviews. Although direct quantification of Treg expansion after GP administration is limited, studies that measure downstream markers (increased IL-10, reduced Th17 markers) suggest Treg-mediated immunoregulation contributes to GP benefits. Targeted studies measuring Treg frequency and function following GP treatment remain a priority (Hu *et al*., 2022; Kim, 2023). There is also evidence that certain GP fractions can directly stimulate innate immune cells: fructan-type garlic polysaccharides enhanced macrophage activation (↑NO, TNF-α, IL-6) in RAW264.7 cells and restored immune parameters in immunosuppressed mice, indicating context-dependent immunostimulatory potential that may help restore immune competence while concurrent metabolite-driven signals temper excessive inflammation in disease settings (Sun *et al*., 2025; Wu *et al*., 2024).

5.The Flora Metabolites – Immunity Axis in Metabolic Disease

Dietary garlic polysaccharides (GPs) alter gut community structure and fermentation outputs, producing metabolites that act as signalling intermediates between the microbiota

and the host immune system. These metabolite-mediated immune effects are central to how GPs can influence metabolic diseases such as obesity, type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD)/MAFLD, and atherosclerosis.

5.1.SCFAs and Immunomodulation in Obesity and Diabetes

Short-chain fatty acids (SCFAs)—mainly acetate, propionate and butyrate—are produced by microbial fermentation of fermentable carbohydrates such as low-DP oligosaccharides and fructans. SCFAs influence host metabolism and immunity through multiple mechanisms: they serve as energy substrates for colonocytes, engage G-protein coupled receptors (GPR41/FFAR3, GPR43/FFAR2, GPR109A), and inhibit histone deacetylases (HDACs), thereby shaping gene expression in immune cells and promoting regulatory T cell (Treg) differentiation and anti-inflammatory cytokine production. These pathways reduce intestinal permeability, systemic inflammation, and improve insulin sensitivity—mechanistic routes relevant to obesity and T2D prevention/amelioration (Kim, 2023; Van *et al*., 2024; D. Zhang *et al*., 2023).

Evidence linking SCFAs to metabolic improvements includes preclinical and clinical observations: increased colonic butyrate and propionate are associated with improved glucose homeostasis, reduced adipose inflammation, and enhanced Treg numbers in metabolic tissues (rodent models and translational human data). Multiple reviews synthesize how SCFA-driven immune regulation (Treg induction, suppressed pro-inflammatory cytokines) contributes to improved insulin sensitivity and reduced adipose/tissue inflammation in obesity and T2D contexts (Anachad *et al*., 2023; Cui *et al*., 2023). GP fractions, especially low-MW/processed oligosaccharides, increase fecal SCFAs and enrich SCFA-producing taxa in animal models, offering a plausible link from GP ingestion to SCFA-mediated immunometabolic benefits (Van *et al*., 2024).

5.2. Bile Acids and Liver

Bile acids (BAs), synthesized in the liver and modified by gut microbes into secondary BAs, function as signaling molecules that regulate lipid and glucose metabolism, energy expenditure, and immune responses via receptors such as FXR (farnesoid X receptor) and TGR5 (GPBAR1). FXR signaling in the intestine and liver controls bile acid synthesis (via CYP7A1), lipogenesis, and systemic metabolic pathways; TGR5 activation on immune and metabolic cells modulates energy balance and inflammation. Dysregulated bile acid composition and signaling are implicated in NAFLD/MAFLD progression and cardiometabolic disorders (Chiang & Ferrell, 2020; Fleishman & Kumar, 2024).

Microbiota shifts induced by polysaccharide interventions can reshape the bile-acid pool and FXR/TGR5 signaling: for example, microbial communities that favor deconjugation/7α-dehydroxylation change the ratio of primary to secondary BAs and thereby alter receptor activation patterns linked to hepatic lipid handling and systemic inflammation. In models of metabolic disease, interventions that favor beneficial bile-acid signaling (appropriate FXR/TGR5 balance) reduce hepatic steatosis, lower inflammatory markers, and improve lipid profiles (Chiang & Ferrell, 2020; Zhu *et al*., 2024). Specific to garlic preparations, aged garlic oligosaccharides and other GP fractions have been associated in animal studies with reduced TMA/TMAO (a microbial-derived pro-atherogenic metabolite) and microbiota changes that are consistent with favorable bile-acid modulation, suggesting a mechanistic path from GP to microbiota, bile-acid signalling and improved hepatic/cardiovascular outcomes. However, direct, targeted bile-acid profiling after isolated GP supplementation is still limited and remains an important research need (Zerem *et al*., 2025; Zhu *et al*., 2024).

5.3. Tryptophan Metabolites and Systemic Inflammation

Tryptophan (Trp) metabolism represents a critical microbiota–host interface. Microbial catabolism of Trp produces indole derivatives

(indole-3-propionic acid, indole-3-aldehyde, indoleacetic acid, etc.) that act as ligands for the aryl hydrocarbon receptor (AhR), promoting mucosal barrier integrity, IL-22 production, and antimicrobial peptide expression—actions that reduce epithelial inflammation and maintain immune homeostasis. The host kynurenine pathway, also influenced by microbiota and inflammation, generates metabolites that can be immunomodulatory (or immunosuppressive) and are linked to metabolic and neuroinflammatory outcomes (Miyamoto *et al*., 2024; G. Wang *et al*., 2024).

Microbial production of AhR ligands from Trp helps restrain excessive inflammation and supports epithelial repair—mechanisms relevant in obesity and metabolic endotoxemia where barrier dysfunction drives systemic inflammation. Emerging studies show that dietary fibers/polysaccharides that reshape the microbiota can increase beneficial indoles and thus indirectly promote AhR-mediated anti-inflammatory effects. Direct evidence connecting garlic polysaccharide consumption to specific Trp-metabolite shifts is still emerging, but the pathway is mechanistically plausible and supported by analogous polysaccharide interventions (Miao *et al*., 2025; G. Wang *et al*., 2024).

5.4. Integration Model

Ingestion of GP leads to selective fermentation by saccharolytic microbes (particularly when GP fractions are low-DP/soluble), leading to increased production of SCFAs and shifts in microbial taxa (Van *et al*., 2024). SCFAs promote Treg differentiation and anti-inflammatory cytokine production (e.g., IL-10), indole derivatives activate AhR supporting barrier repair and mucosal IL-22 production, and favorable shifts in microbiota reduce pro-atherogenic metabolites and alter bile-acid pools to engage FXR/TGR5 signaling that improves lipid/glucose handling. These combined immune and metabolic receptor pathways reduce tissue inflammation and metabolic dysfunction (Chiang & Ferrell, 2020; Kim, 2023).

Reduced adipose and hepatic inflammation, improved insulin sensitivity, lower atherogenic metabolite burden (TMAO), and enhanced gut barrier integrity converge to ameliorate obesity, T2D, NAFLD/MAFLD, and associated cardiovascular risk. Animal studies with GP/aged garlic oligosaccharides show concordant microbiota/metabolite/phenotype improvements consistent with this model; translational human data remain limited (Anachad *et al*., 2023; Zerem *et al*., 2025).

6.Comparison with Other Dietary Polysaccharides

Dietary polysaccharides commonly studied for prebiotic and immunomodulatory effects include inulin, fructooligosaccharides (FOS), and β-glucans. Garlic polysaccharides (GPs) share important functional similarities with these established fibers but also display unique features—particularly a dual influence on gut microbiota composition and host immunity—that merit attention.

6.1.Similarities in Prebiotic Role

Like inulin and FOS, many garlic polysaccharide fractions (especially low-DP/oligosaccharide fractions and processed/aged saccharides) are resistant to upper-GI digestion and are fermented in the colon, producing short-chain fatty acids (SCFAs) and stimulating saccharolytic taxa such as *Bifidobacterium* and *Lactobacillus*. In vitro and in vivo studies indicate that hydrolysed garlic saccharides and low-Mw GP fractions exert bifidogenic and SCFA-generating effects broadly comparable to other fructan-type prebiotics, although potency varies with DP and extraction method. Reviews of inulin/FOS consistently report strong bifidogenic activity, and garlic saccharides often fall within this functional class due to their inulin-type fructan backbone (X. Lu *et al*., 2021; Teferra, 2021). Clinical and animal literature for established prebiotics (inulin, FOS) documents benefits on gut ecology, bowel function, and metabolic markers; garlic polysaccharide studies reproduce several of these endpoints in animal and in vitro models, supporting the classification

of GPs as functional prebiotic polysaccharides when appropriately fractionated (Dou *et al*., 2022; Zhao *et al*., 2022b).

6.2. Differences and Unique Features of Garlic Polysaccharides

While inulin and many commercial FOS are relatively well-characterized linear fructans (β-(2→1) fructofuranosyl linkages) with predictable DP ranges, garlic polysaccharide extracts display greater heterogeneity. Depending on source material and processing, garlic yields both inulin-type fructans (bulb) and pectin-rich fractions (peel, leaf, pomace) containing galacturonic acid, RG-I/HG domains, and neutral sugar side chains. This structural diversity confers varied solubility, fermentability, and physicochemical properties not seen in single-source prebiotics like purified inulin (Xie *et al*., 2024; Zhao *et al*., 2022b).

6.2.1.Dual Microbiota and Immunity Impact

β-glucans are celebrated for their direct immunomodulatory activity (via Dectin-1 and other pattern recognition receptors) and also have prebiotic effects depending on source and solubility; inulin/FOS are primarily recognized for microbiota/SCFA-mediated immune benefits. Garlic polysaccharides occupy an intermediate/dual niche: many GP fractions behave as prebiotics (promoting SCFA production and beneficial taxa), and specific GP fractions show direct immunomodulatory actions (macrophage activation, cytokine modulation) in vitro and in vivo. This dual action—microbiota-mediated metabolite signaling plus direct innate immune engagement—distinguishes garlic polysaccharides from purely fermentable fibers and aligns them partially with immunoactive polysaccharides such as β-glucans (Singh & Bhardwaj, 2023; Wu *et al*., 2024).

6.2.2.Processed/Aged Garlic Products with Unique Metabolic Effects

Aged or processed garlic oligosaccharides have been reported to reduce pro-atherogenic metabolites (e.g., TMA/TMAO) and remodel microbiota in ways that directly impact cardiovascular and hepatic outcomes—effects that are not always observed with generic inulin or FOS supplementation. These specific metabolic endpoints (TMAO reduction, bile-acid-related signalling shifts) have been demonstrated in recent animal models using garlic oligosaccharides and provide mechanistic rationale for garlic's application in cardiometabolic contexts (T. Li *et al*., 2024; X. Lu *et al*., 2021).

7.Challenges and Future Perspectives

7.1.Current Limitations

Most studies on garlic polysaccharides (GPs) remain preclinical: in vitro fermentations and rodent models dominate the literature, with relatively few randomized controlled trials (RCTs) using well-characterized GP fractions in humans. Several recent reviews highlight this translational gap and call for human intervention studies that integrate microbiome and immune endpoints to validate preclinical findings (El-Saadony *et al*., 2024; Jiang *et al*., 2022). There is substantial methodological heterogeneity in how GPs are extracted, fractionated, and characterized—ranging from traditional hot-water + ethanol precipitation to enzymatic, ultrasonic/microwave-assisted, and membrane/column fractionation approaches. This diversity leads to wide differences in degree of polymerization (DP), molecular weight, branching, and composition across studies, making direct comparisons difficult and complicating reproducibility and meta-analysis. Several recent reviews and methodological papers emphasize the need for standardized extraction and reporting guidelines (Irianto *et al*., 2025; Jiménez-Amezcua *et al*., 2025). Dose-response relationships for specific GP fractions are poorly defined: animal studies use a broad range of doses, and human data—when present—often use whole garlic preparations rather than isolated, characterized polysaccharide fractions. Bioavailability of polysaccharide-derived metabolites (e.g., systemically measured SCFAs, indoles, bile-acid species) depends on fermentation kinetics and intestinal transit, but targeted pharmacokinetic data for GP fractions are scarce. Safety profiles for isolated GP fractions appear favorable in preclinical work, yet systematic toxicology and allergenicity assessments, as

well as well-controlled human safety/dose-finding trials, are lacking. Reviews of garlic bioactives also note unresolved issues around standardizing "active" dose equivalents across preparations (Lawson & Hunsaker, 2018; Shao *et al*., 2024a; Sunanta *et al*., 2023).

7.2. Future Directions

To move from associative to causal inference, future GP research must combine shotgun metagenomics (taxonomic + functional capacity) with targeted metabolomics (SCFAs, bile acids, tryptophan metabolites, TMA/TMAO) and glycomics (detailed GP structure—DP, linkage, branching). Multi-omics integration will enable mapping of which GP structures drive specific microbial metabolic pathways and immune outcomes. Recent methodological reviews highlight the power of combined metagenomics–metabolomics platforms for nutritional intervention studies and recommend standardized pipelines for reproducibility (Aya *et al*., 2025; Yang *et al*., 2025). Interindividual variability in baseline microbiota composition strongly influences response to dietary fibers and prebiotics. Personalized or stratified approaches—using baseline microbiome signatures to predict who will respond to a given GP fraction—could substantially improve efficacy of interventions for metabolic disease. Reviews and pilot studies in personalized diets and microbiome stratification provide frameworks that should be applied to GP trials (e.g., enterotype-informed supplementation, responder/non-responder analyses) (Hernández-Calderón *et al*., 2022; Song & Shin, 2022). For translational impact, GPs must be developed into standardized ingredients (with defined DP/Mw profiles) and formulated into food matrices or nutraceuticals with demonstrated stability, palatability, and shelf life. Process innovations (green extraction, membrane fractionation, microencapsulation) can improve yield and bioavailability while keeping structural integrity. Regulatory, scalability, and cost considerations will also be central to bringing GP-based products to market. Recent reviews on extraction innovation and food-component characterization outline practical steps for industrial translation (Irianto *et al*., 2025). Well-designed human RCTs should: (1) use chemically characterized GP fractions (with DP, Mw, and linkage data), (2) include multi-omics endpoints (microbiome, metabolome, immunophenotyping), (3) incorporate dose-finding and safety arms, and (4) stratify participants by baseline microbiome or metabolic phenotype to identify responders. Such studies will be essential to demonstrate causality and to support health claims or therapeutic uses in metabolic disease. Method papers and reviews stress the importance of these integrated designs for dietary-microbiome interventions (Chinta *et al*., 2025; Yang *et al*., 2025). Finally, creating community standards for reporting extraction/purification methods, structural characterization, and bioactivity assays (analogous to CONSORT for clinical trials) will improve comparability. Collaborative consortia that share standardized reference GP materials and harmonized protocols will accelerate progress and reduce duplication. Reviews and editorials advocating for standardization across microbiome–nutrition research provide blueprints for such efforts (Falsafi *et al*., 2025; Yang *et al*., 2025).

8.Conclusion

Garlic polysaccharides (GPs) represent an emerging class of non-sulfur bioactives with dual roles as prebiotics and immunonutrients. Their structural heterogeneity spanning fructan- and pectin-type fractions shapes fermentability, microbial selectivity, and downstream production of key metabolites such as short-chain fatty acids, bile acids, and tryptophan derivatives. Through these microbiota–immune interactions, GPs exert antioxidant, anti-inflammatory, immunomodulatory, and metabolic benefits in preclinical models of obesity, diabetes, fatty liver disease, and atherosclerosis. Compared with established dietary polysaccharides, GPs show distinctive potential by coupling microbiota-driven metabolite signalling with direct immunological effects. However, translation remains limited by methodological variability, insufficient structural standardization, and a lack of well-

controlled human clinical trials. Future work should focus on multi-omics integration, dose–response characterization, and personalized nutrition strategies to confirm efficacy and enable the development of GP-based functional foods or therapeutics.

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Authors' contributions

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Data availability

No new data were created or analyzed in this study. Data sharing does not apply to this article.

Declarations

Ethics approval and consent to participate

The authors declare that they have no human and/ or animal studies in this manuscript.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research article

PHYSICAL PROPERTIES IMPROVEMENT OF GADUNG (*Dioscorea hispida* Dennst.) STARCH OXIDIZED BY HYDROGEN PEROXIDE

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Abstract

Oxidation is chemical modification which alters and improves the properties of starch. This study investigated the effect of oxidation using hydrogen peroxide on Gadung starch towards its physical characteristics. The different concentrations of oxidant (0; 2; 4; 6 and 8%) were studied. The effect of oxidation were evaluated based on swelling power, solubility, freeze thaw stability, viscosity, and whiteness of the oxidized starch. A significant difference was observed on the value of swelling power, solubility, freeze-thaw stability, viscosity and whiteness of Gadung starch. H_2O_2 at 4% concentration showed the best swelling power with 263% increase than that of native starch. The highest solubility was shown by 8% H_2O_2 concentration which was 55.66 g/100g. The syneresis of 8% H_2O_2 was also the lowest, 11.67% which showed the highest freeze thaw stability. The viscosity has decreased to 94.3% compared to native starch at 8% H_2O_2. The whiteness of oxidized starch is increased along with increasing concentration of hydrogen peroxide that given.

1. Introduction

Gadung (*Dioscorea hispida* Dennst) tuber is an easy-growing vine in tropical climates such as in Indonesia, which also wildly grows in the forest. Gadung has very good nutrition with a starch content of 38.80%, with amylose and amylopectin portions of 12.42% and 87.58%, respectively (Santoso et al. 2015). Therefore, Gadung has been considered one of the staple foods in Indonesia. Despite the potential of Gadung being very high, the tuber is underutilized and have not been commercially important in the food industry (Kumoro et al. 2014). Part of the reason is due to its cyanide content, which is toxic; thus, it needs a special step of preparation to reduce it to an acceptable/safe level (Kumoro et al. 2016). The other reason is its derived product's poor characteristics, which limits the potential application. For instance, Gadung starch, as the

main derivative, has a very low solubility of about 5.6 g /100 g (Subroto et al. 2024).

Native starch has many disadvantages, such as lack of heat resistance, too high viscosity, and a high tendency of retrogradation and syneresis (Hazarika and Sit 2016). Hence many researchers are motivated to modify native starch's properties in order to enhance its utilization. Modification of starch can be done either by physically, chemically and enzymatically. Among starch chemical modifications, oxidation is a popular technique. The technique includes reaction that occurs between starch and oxidizing agent under controlled temperature, pH, and time. Several benefits have been reported from starch oxidation, such as lower viscosity, high thermal stability, brighter color, and good binding ability (Moreno et al. 2017). Hydroxyl groups in the starch hydrocarbon chain, primarily at C-2, C-3, and C-6 positions, are converted to carbonyl and/or carboxyl groups by the oxidation process, thus inducing physical and chemical characteristics change (Ojogbo, Ogunsona, and Mekonnen 2020).

Conventional oxidation reactions usually use inorganic substances such as hypochlorite as oxidizing agents. However, with environmental concerns as a priority and also potential residue that may cause food safety concerns, hydrogen peroxide has gained much interest from researchers. Hydrogen peroxide is characterized by its low cost and easily decomposed; hence, it is safe for the water environment and leaves no harmful residues in food products (Kumoro et al., 2015). Oxidized starch prepared by hydrogen peroxide has already been investigated in many starchy material such as in wheat starch (Sun et al. 2017a), maize starch and sweet potato starch (Zhang et al. 2012). However, there has been no research on the effect of oxidation using hydrogen peroxide on the characteristic of Gadung starch. Therefore, this study aimed to improve Gadung starch's properties by the use of oxidation method and to evaluate the effect of oxidation using hydrogen peroxide on its swelling power, solubility, viscosity, freeze-thaw stability and whiteness.

2. Materials and methods

2.1. Starch Isolation

Gadung starch was isolated according to (Shofiyah et al. 2020) method with some modifications. Gadung tubers (obtained from farmer groups in Podorejo Mijen, Semarang, Indonesia) were cleaned, peeled, cut and soaked in 10% salt solution for 48 hours in order to remove the cyanides. Gadung was crushed with a blender and added with water at a ratio of 1:3. Gadung slurry was then filtered to produce a starch suspension. Starch suspension was precipitated for 24 hours. The precipitate was then separated and dried by oven at 45 ºC for 24 hours. The dried starch was passed through 100 mesh sieve.

2.2. Oxidation

The oxidation of Gadung starch was carried out based on (Sumardiono et al., 2021). Appropriate amount of $CuSO_4.5H_2O$ (MERCK, Germany) was added to 42% (w/w) starch suspension to achieve concentration of 0.04 g of Cu_2+/100 g starch. The mixture was stirred at 250 rpm (IKA RW 20 overhead stirrer, Germany) and heated for 15 min at 40 °C. Subsequently, 35% (w/w) solution of H_2O_2 (MERCK, Germany) was added dropwise in order to achieve the final H_2O_2 concentration of 0; 2; 4; 6 and 8% of dry starch (v/w). The reaction was continued for 45 min. The slurry was then washed with aquadest and filtered. After drying at 45 °C for 24 hour, the resulted starch was passed through 100 mesh sieve. The treatments were carried out in four repetitions.

2.3. Swelling Power and Solubility

Swelling power and solubility were evaluated according to (Kusumayanti, Handayani, and Santosa 2015) method with some modifications. Starch was dispersed in aquadest at the concentration of 0.5 g/100 g. The starch dispersion was heated to 95 °C and kept for 30 min under constant stirring. The resulted starch paste was immediately cooled in an iced water bath until it reached ambient temperature. A 10 g of suspension was prepared in a centrifuge tube and then centrifuged at 2300 rpm for 30 min (Hettich Zentrifugen, Germany). An

aliquot of decanted supernatant was then dried in an oven at 110 °C for 4 h. Swelling power and solubility were calculated using below equations where W is the total weight of the suspension in the tube, W1 is the weight of dry tube, W2 is the weight of the tube after the supernatant was decanted and W3 is weight of the dried supernatant.

$$\text{Swelling power (g/g)} = \frac{W_2 - W_1}{W \text{ x } 0.5/100} \quad (1)$$

$$\text{Solubility (g/100g)} = \frac{W_3 \text{ x } 100}{W \text{ x } 0.5/100} \quad (2)$$

2.4. Swelling Power and Solubility

The measurement of freeze-thaw stability was based on the method described by (Siswo Sumardiono et al. 2022). Starch suspension at the concentration of 5 g/100 g was heated at 95 °C while constantly stirred for 30 min and then brought to room temperature in an iced water bath. A 10 g of resulting starch paste was put in a centrifuge tube and frozen at -14 °C for 24 hours. Frozen starch was then thawed at 30 °C for 1.5 hours followed by centrifugation at 2300 rpm for 30 min. The supernatant was decanted and weighed. Freeze-thaw stability is shown as % syneresis which calculated as follow:

$$\% \text{ Syneresis} = \frac{\text{Supernatant (g) x } 100\%}{\text{Total weight of suspension in the tube (g)}} \quad (3)$$

2.5. Viscosity

The viscosity was determined as the Final Viscosity according to the method described by (Siswo Sumardiono et al. 2021) with some modifications. Starch in aquadest suspension (15 g/100 g) was prepared and heated at a temperature of 95 °C for 15 min. The viscosity was measured by viscometer (Rion VT-06, Japan) after the suspension was cooled to 50°C.

2.6. Whiteness

Whiteness was determined by digital colorimeter using CIE L*a*b colour scale. Whiteness was expressed as L (lightness) value which value ranges from 0 (black) to 100 (white).

2.7. Statistical Analysis

Data were statistically analyzed using Analysis of Variance (ANOVA) with 5% level of significance. Duncan Multiple Range Test (DMRT) was applied to any significant difference.

3. Results and discussions

3.1. Swelling Power

As can be seen in Fig 1, the swelling power of native and oxidized Gadung starch was significantly different ($P<0.05$). The swelling power of native starch (0%) showed increasing value by adding H_2O_2 at concentrations of 2% and 4%, followed by a decrease of 6% and 8%. Native Gadung starch had the lowest swelling power with 4.09 g/g, while the highest swelling power was found at an H_2O_2 concentration of 4%, which was 14.85 g/g, representing an increase of about 263%.

A similar scenario was reported by (Wang et al., 2003), where the swelling power of corn starch increased by 37.3% as the oxidant concentration was increased from 0.5% to 1% but further decreased at concentrations of 1.5% and 2% with a reduction of thepercentage of 25%. On the contrary, (Halal et al., 2015) found a decreasing swelling power in barley starch with increasing oxidant concentration. Research (Sun et al. 2017b) has also reported that the swelling power of wheat starch, which is oxidized by 12% hydrogen peroxide, was decreased from the native one with a reduction rate of about 6.8%. Comparatively, the current study showed a much higher increase in swelling power at the optimum concentration than other similar studies.

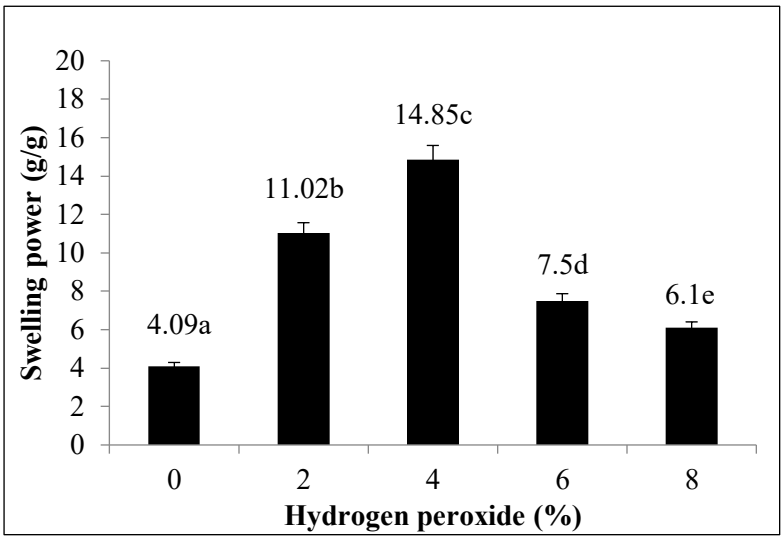

Figure 1. Swelling power of native and oxidized gadung starches.

Swelling power has been used to demonstrate differences among various types of starches because it indicates the starch's water-holding capacity (Pramana et al. 2024). In the current study, swelling power has reached the peak at the H_2O_2 concentration of 4% and then decreased. The increase can be explained by the fact that at the lower oxidant concentration, the oxidation reaction mainly affects the amylose fraction and results in the amylopectin: amylose ratio being higher. It is in agreement with (Vanier et al., 2017), who argued that amylose readily reacts with an oxidizing agent, possibly due to its linear structure, which makes it more susceptible to oxidative degradation. Therefore, less oxidizing agent was available for the oxidation of the amylopectin. Amylose depolymerization causes a decrease in the degree of crystallinity, and water molecules present in the system can be readily accessed by amylopectin molecules, thus causing an increase in starch swelling power (Cahyono et al., 2023).

The following trend of swelling power decrease at concentrations 6% and 8% was speculated to be caused by the amount of hydroxyl radicals in the system being too high. It resulted in the oxidation of hydroxyl groups from both amylose and amylopectin into carboxyl groups and cleaving the polymer into a shorter chain (Sumardiono et al. 2024) . The depolymerization of amylopectin molecules causes the starch to lose its ability to absorb water, hence decreasing its swelling power (Kumoro et al. 2020).

3.2. Solubility

Fig 2 shows that the oxidation treatments gave a significant ($P<0.05$) effect on the solubility of Gadung starch. The solubility of the Gadung starch increased with the increasing oxidant concentration, where the highest solubility was at an 8% concentration of H_2O_2 at 55.66 g/100g. The solubility enhancement from the native starch reached 351%. This value is higher when compared with an even higher oxidation level (12% H_2O_2) in wheat starch (Sun et al., 2017), which had a solubility increase of about 158%. A comparable result was reported by (Sun et al. 2017a), where a 391% solubility increase was found in corn starch oxidized by 12% H_2O_2 concentration.

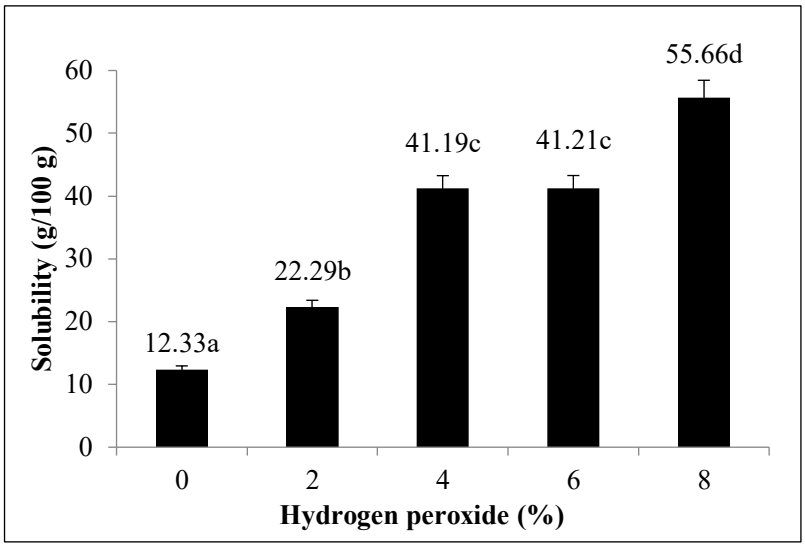

Figure 2. Solubility of native and oxidized Gadung starch.

Solubility indicates the percentage of starch molecules released after the swelling of starch granules (Rahma et al. 2017). The positive correlation between solubility and oxidant concentration has also been reported by (Halal et al. 2015) in barley starch, whose solubility increased substantially as the amount of oxidant (NaOCl) increased. The increased solubility is caused by the depolymerization of starch granules and the weakening of the starch structure by the oxidation process. According to (Fonseca et al. 2015), the formation of carboxyl groups lead to porous structures on starch granules and hence it could absorb water but could not hold it. The degradation of starch molecules increases the mobility of the starch molecules and makes the molecules interact easier with water, most probably due to the increased polarity (Sumardiono et al., 2017). High-soluble starch can be applied in the encapsulation of food products and also as a food additive to products that require high solubility (Sunarti, Pasaribu, and Winarti 2020).

3.3. Freeze-thaw Stability

Starch's freeze-thaw stability can be reflected in its syneresis, which is defined as the portion of water that separates out after freezing and thawing (Kumoro et al. 2014). This stability is particularly relevant to the potential application of modified starch in frozen foods, e.g., sausages, and meatballs. Therefore, in the current study, freeze-thaw stability is expressed as the magnitude (in percent) of syneresis. Lower % syneresis represents higher freeze-thaw stability.

As shown in Fig 3, the H_2O_2 oxidation had a significant effect ($P<0.05$) on the starch syneresis value. The percentage syneresis of oxidized starch decreased with increasing numbers of oxidants that were given. The highest syneresis was shown by native starch in the amount of 47.83%, while the lowest syneresis was shown in oxidant concentration of 8% with 11.67%, showing an approximate 75% reduction. This reduction percentage is higher than cross-linked oxidized corn starch at 12% oxidant concentration, which decreased syneresis from its native starch by about 55.2% (Arunyanart and Charoenrein 2008). Similarly, it is also higher than syneresis reduction in wheat starch oxidation (12% oxidant level), about 26.3% of its native starch (Sun et al. 2017a). The higher syneresis reduction at a lower oxidant level shows the effectiveness of H_2O_2 oxidation treatment in improving the Gadung starch's freeze-thaw stabili

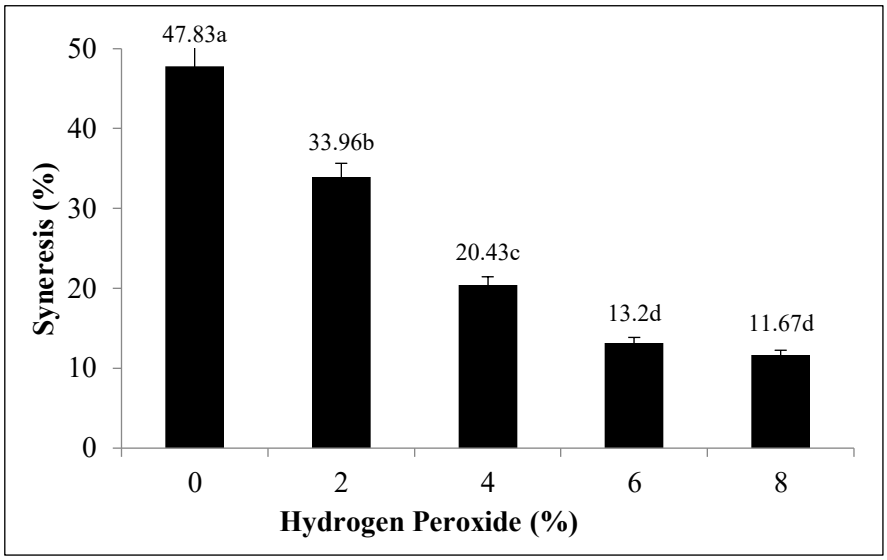

Figure 3. Freeze-thaw stability of native and oxidized Gadung starch.

The increasing freeze thaw stability can be attributed to the formation of carboxyl groups in oxidation reaction. Higher oxidant concentration results in more carboxyl groups and thus produces higher freeze thaw stability (Sumardiono et al., 2021). Carboxyl and carbonyl functional groups reduce the syneresis of starch because the carboxyl group has a strong hydrophilicity that prevents the release of water and improves water-holding capacity (Wardhani et al. 2023).

3.4. Viscosity

The viscosity, expressed as final viscosity, was measured at the starch paste's temperature of 50°C after its complete gelatinization at 95 °C heating condition. The simulated setup can illustrate the possible application of the oxidized starch, for instance, as an added substance in instant soup or beverage powder. Typical preparation of such products may include dilution with hot water, to induce gelatinization, prior to consumption which normally done at milder temperature.

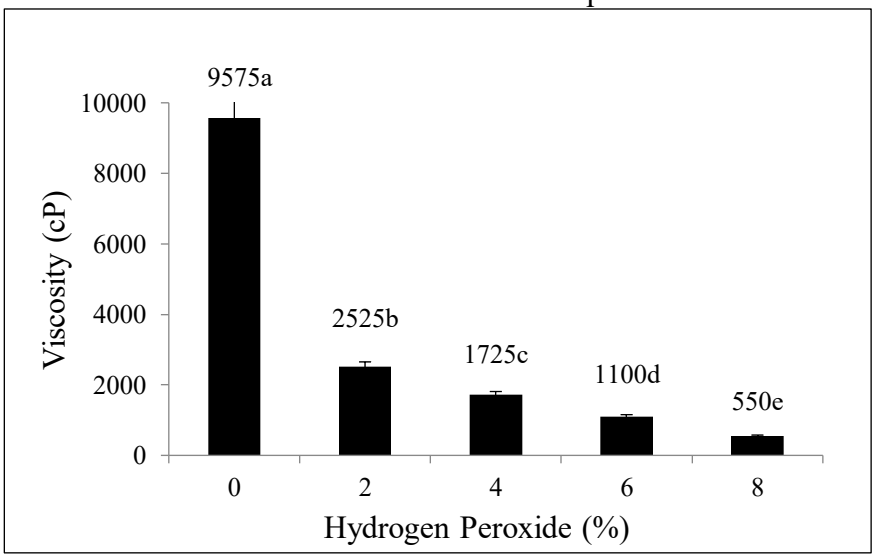

Figure 4. Viscosity of native and oxidized Gadung starch.

As can be seen in Fig 4, H_2O_2 oxidation treatment gave significant effect ($p<0.05$) to Gadung starch final viscosity. Starch paste viscosity decreased with increasing oxidant level. The highest starch paste viscosity was shown by native starch that was 9575 cP, while the lowest viscosity was resulted by oxidized starch with the highest oxidant concentration which was 550 cP, hence showing a reduction percentage of 94.3%. This reduction value is greater than that of oxidized wheat starch, which has decreased by about 89.2% from its native starch (Sun et al. 2017a). It is also higher than that reported by (Sandhu et al., 2008) where the final viscosity of oxidized corn starch has lowered about 32% from its native starch.

Viscosity is influenced by the ability of starch to be hydrated and its water-binding capacity (Pratama et al. 2018). Oxidation may cause disintegration of starch glycosidic bond resulting in loss of water-binding capacity, so that the viscosity of starch decreases (Sugiharto 2023). Furthermore, (Vanier et al. 2017) argued that the presence of carboxyl groups when compared with the hydroxyl groups would weaken the structure of the starch granules and contribute to lower the viscosity of starch.

3.6. Whiteness

The L value could be used as a parameter for characterizing starch color, and it is a direct measurement of its whiteness (Pramono et al. 2021). A higher L value (closer to 100) indicates a whiter material. Fig 5 shows that the whiteness of oxidized Gadung starch increased significantly ($p<0.05$) as the oxidant concentration increased. The highest L value was 83.89, shown by the highest oxidant level (8%). It represents an increase of about 4.71% from the native Gadung starch. The result is in accordance with (Vanier et al. 2017) study which reported an increase of 1.2% whiteness in oxidized potato starch if compared with its native starch.

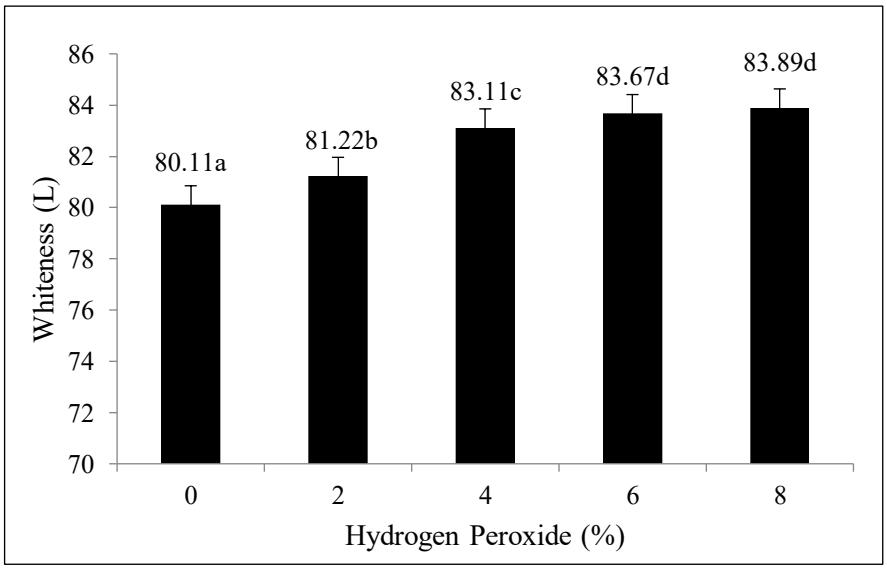

Figure 5. Whiteness of native and oxidized Gadung starch.

Oxidizing agents, which are generally used in starch modification, such as hydrogen peroxide or sodium hypochlorite, could function as bleaching agents. A bleaching agent enhances the white colour by oxidizing the pigments except white. Research by (Argüello-García et al., 2014) stated that sodium hypochlorite increases the whiteness value of starch by oxidizing impurities such as carotene, xanthophyll, and other related pigments. Therefore, oxidizing/bleaching agent is useful to improve the starch color, which normally undergoes a browning reaction during the extraction and drying process (Al-Baarri,

Legowo, and Widayat 2018).

4. Conclusions

It can be concluded that the difference oxidant concentration that given has a significant effect on the value of swelling power, solubility, freeze-thaw stability, viscosity and whiteness of Gadung starch. Giving an oxidant concentrations above 4% reduce the value of Gadung starch swelling power. Solubility, freeze-thaw stability and whiteness increased when the H_2O_2 concentration was increase. The higher concentration of oxidant that given resulted lower Gadung oxidized starch paste viscosity.

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Research article

COMPARATIVE STUDIES ON PHYSICOCHEMICAL AND NUTRITIONAL VALUES OF ORGANICALLY AND CONVENTIONALLY GROWN LUFFA ACUTANGULA L. ROXB STORED IN DIFFERENT HOUSEHOLD PACKAGING AND STORAGE TEMPERATURES

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Abstract

The organically grown fresh produce has 20% lesser yield and of premium range (10–40% more) than conventionally grown foods. *Luffa acutangula* L. Roxb (*Luffa*) is a widely consumed vegetable and commonly found in Indian dietaries. The present study, using the principles of modified atmosphere packaging, the changes in physicochemical and nutritional values of organic and conventional *Luffa* during storage in different packaging at ambient and low temperatures for 2 storage durations were experimented. There were significant differences in moisture, dry weight, total dietary fibre and protein content of both organic and conventional *Luffa* in all packaging. The study showed that during storage in PP container, LDPE zipper bags and cling film wrap at ambient(25◦C) and low (4◦) temperatures can produce a modified atmosphere which can extend the shelf life of *Luffa*.

1.Introduction

The popularity of organic foods is increasing globally as organic foods are perceived to have more nutritional values, have little or no additional contaminants and promotes sustainable agriculture (Das et al.2020; Greene et al. 2009).

Organically grown fresh produce has become quite demanding to the producers and consumers owing to their nutritional benefits as compared to conventionally grown crops (Funk and Kennedy, 2016; Durham and Mizik 2021).

The organically grown foods have 20% lower yield with 10–40% higher premium range as compared to conventionally grown foods (Durham and Mizik 2021; Winter and Davis 2006; Gutierrez et al. 1999). Therefore, it is of utmost importance to store at the domestic level this lower yielded and premium range organically grown fresh produce.

Luffa acutangula L. Roxb (*Luffa*) belongs to genus *Luffa* of Cucurbitaceae family and is commonly known ridge gourd and consumed as a popular other vegetable among Asian, African, and Arabic countries. Ridge gourd is considered as a very common vegetable in Indian dietaries (Jyothi et al., 2010). Ridge gourd is a commonly known medicinal plant as a potent blood purifier, has laxative actions, a hepatoprotective, anti-diabetic, body weight lowering, immunity booster, has antimicrobial activities and anti-inflammatory effect, and promote gut and skin health (Barik et al., 2018). Moreover, organically grown ridge gourd composed of greater amount of minerals, has more total sugars and reducing sugars, and more ascorbic acid than conventional ridge gourd(Barik et al., 2018).

Modified atmosphere packaging (MAP) of food commodity refers to the technique of sealing actively respiring produce (i.e., the fresh produce like fruits and vegetables) in polymeric films so that the O_2 and CO_2 levels are modified within the package headspace (Mangaraj et al. 2009). MAP of vegetable in combination with cold storage is considered as the best process to extend the shelf-life period (Kargwal et al. 2020; Sandhya 2010) and maintain sensory and microbiological quality of fresh-cut produce (Fang & Wakisaka 2021; Kader et al.1989; Philips1996).

The MAP has found to be effective for long-term storage of fruits like apple, orange, kiwi, pears, potato, and cabbage and for storage of strawberries, cherries, bananas, guava, tomato etc. for short term storage (Kargwal et al., 2020).

Polypropylene (PP) and Low-density polyethylene (LDPE) films are polymeric films generally used for MAP (Alimardani et al.2015). According to a study by Wang and Qi (1997) on other gourd family vegetable-Cucumis when stored within perforated or sealed 31.75-µm low-density polyethylene (LDPE) packets had showed less severe chilling injury than unpackaged fruit stored at 5 °C with 90–95% relative humidity (RH).

Therefore, in the present study, we have been conducted to find out the effect of domestic packaging (PP container, LDPE zipper bags, and cling film)and storage temperature controls(25^0C and 4^0C) on the changes in physicochemical and nutritional values between organic and conventional *Luffa* stored in at different temperatures.

2.Methods and Materials

2.1.Sample collection and sample preparation

The ridge gourd (*Luffa acutangula* L. Roxb) of Swarna Manjari cultivar were collected from certified organic farms and conventional farms carefully from two localities (Baruipur and Mathurapur of south 24 Parganas, West Bengal, India), and their average results were interpreted.

The organic *Luffa* was cultivated during the January to April (summer) or June to July (rainy) months in sandy loam concentrated in organic matter. Different organic fertilizers, such as vermicompost (1000 kg per acre), Ghana Jeevamrutham, and bone meal (50–75 kg per acre) rich in phosphorous, calcium and nitrogen compost are commonly added before cultivation to supply the nutrients, essential for plant growth, such as nitrogen, phosphorus, and potassium. In addition, provision for good drainage and pH range 6.5–7.5 at warm temperature (above 16°C) were also maintained. Organic plant fertilizer containing seaweed extract, humic acid, and fulvic acid was applied for growth promoter.

For plant protection, neem cakes (150–200 kg/ acre), Trichoderma viride biofungicide (1.5% w.p.), neem oil (1–2 ml/litre sprinkled 1–2 times per week), Bacillus thuringiensis (Bt) power or liquid inoculums (spray), Curcumin (spray) and pheromone trap (one trap per 25 square feet area) were used.

During intercrop time and for crop rotation, cultivation of dhaincha (*Sesbania spp*) was also advocated for enhancing the soil fertility.

The *Luffa* was conventionally grown using conventional cultivation techniques including inorganic manure, such as ammonium nitrate, calcium phosphate, muriate of potash added during soil preparation with N:P: K−10:26:26 and urea was at the middle of cultivation for

growth of plant. For plant protection, various pesticides and conventional fungicide were applied.

Three types of domestic packaging made of polymeric film/bag/box such as polypropylene or PP (of 0.23 mm thickness) container, low-density polyethylene or LDPE (of 2.5 mm thickness) zipper bag and polyethylene cling film (of 0.06 mm thickness) were used as domestic packaging for the study.

The samples were treated immediately after harvesting with chlorinated (100 ppm, 20 °C) (Safe Practices for Food Processes, 1998) to remove the free heat and to disinfect the vegetables. The samples were kept without packaging, PP containers, in LDPE zipper and wrapped in cling film (Jacobsson et al. 2004; Thompson 2010) and ambient temperature (25 °C) and low (4 °C) at a relative humidity of 70% and 90%, respectively, for 3 days and 7 days of storage durations. Then, the physicochemical and nutritional values were measured.

2.2.Physicochemical Parameters

2.2.1.Moisture Content and Dry matter

The moisture content was determined by the procedure outlined by Raghuramulu et al (2003). Moisture content was estimated by keeping 15 g of each vegetable samples in hot air oven at 105°C until a constant weight. Moisture content was determined as:

$$\text{Moisture content (g \%)} = \frac{W1-W2}{W1} \times 100 \quad (1)$$

where, W1=Initial weight of meat sample, g;
W2=Final weight of meat sample, g
Dry Matter= (100- moisture content) g

2.2.2.pH and Titratable Acidity

The titratable acidity was measured according to the AOAC (2000) methods. The pH of the vegetable slurry was measured by electrode pH meter (Labman, LMPH-15). The vegetable slurry (obtained from blended and filtered vegetable sample) was centrifuged (REMI Bench top Centifuge, R4C) at 3000 rpm at RT for 15 minutes and the supernatant clear fluid was taken for estimation. 6g of supernatant was diluted with 50ml of double distilled water and the mixture was titrated (after adding 0.3ml of 1% phenolphthalein) with 0.1 (N) NaOH to the end point of pH 8.1 to 8.2 with permanent pink colour. Titratable acidity was expressed in gram per 100gram to the predominant organic acids as follows:

1ml of 0.1 (N) of NaOH ≈ Malic acid- 0.067

2.2.3.Titratable acidity(g/%)

=[ml of 0.1(N) NaOH used] X [concentration of 0.1(N) NaOH] x [milliequivalent factor] X 100 Gram of sample (2)

2.2.4.Tissue Respiration Rate

The tissue respiration rate was measured as per method described by Guo et al(2008). 1000 g of vegetable sample was placed in a closed glass container, separated with 20 ml 0.1 M sodium hydroxide on the bottom. After 1 hour, the sodium hydroxide was mixed with 2 ml saturated $BaCl_2$ to precipitate the carbon as carbonates. The mixture was then titrated (using two drops of 1% phenolphthalein) by 0.2 M hydrochloride acid until pink colour disappeared, based on which, the carbon dioxide was calculated by a conversion table (Zhang et al., 2003).

2.3.Nutritional Parameters

2.3.1.Carbohydrate content

The total carbohydrate content was estimated by the Anthrone method (Dubois et al., 1956). In this method, carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone and measured at 630 nm.

2.3.2.Total Protein

The estimation of nitrogen was done by Kjeldahl method (Raghuramulu et al., 2003) which was based on the principle that organic nitrogen when digested with sulphuric acid in the presence of a catalyst (selenium oxide,

mercury, or copper sulphate) was converted into ammonium sulphate. Ammonia liberated by making the solution alkaline was distilled into a known volume of a standard acid, which was then back- titrated. The protein content was obtained by multiplying the nitrogen value with 6.25.

2.3.3.Total Dietary Fibre

The total dietary fibre content was determined according to the AOAC (1986) methods. The principle of the method is that the defatted food samples are gelatinized, and protein and starch are removed by enzymatic digestion. The residue was filtered and washed and then measured gravimetrically. The total dietary fibre content calculated as:

$$B = \text{Blank (mg)} = \text{Weight Residue} - P_B - A_B \quad (3)$$

Where weight residue = Average of residue weights (mg) for duplicate blank determinations; and P_B and A_B = weights (mg) of protein and ash, respectively, determined in first and second residues.

$$\text{The TDF (g\%)} = [(\text{weight residue} - P - A - B)/ \text{weight test portion}] \times 100 \quad (4)$$

Where weight residue = average of weights (mg) for duplicate blank determination; and P and A = weights (mg) and ash (mg) respectively, in first and second test portion residues; weight portion= average of 2 test portion weights (mg) taken.

2.4.Statistical analysis

The study was a completely randomized design (CRD), 2 farming varieties of *Luffa* (organic and conventional) collected from 2 locations stored in 3 types of packaging (plus sample stored without any packaging) at 2 storage temperatures for 2 storage durations. Fresh *Luffa* of both organic and conventional varieties were also experimented. The total number of samples for the study was 68. All the parameters were estimated in three replicas.

The statistical methods used in the study include percentage analysis and one way analysis of variance.

Statistical analysis was performed with standard technique of multivariate analysis using SPSS 20 software.

3.Results and Discussion

In this study, results were interpretated from both organic and conventional *Luffa* before and after storage in different domestic packaging at 25°C and 4°C with RH of 90% and 70% respectively both for 3 days and 7 days.

The physical–chemical properties of the *Luffa* were influenced by the types of domestic packaging, temperature, and storage duration. When stored in domestic packaging, it creates a altered environment within it causing changes in physicochemical and nutritional parameters. The following abbreviations were used for farming type and packaging types in the study: ORGN = organic Cucumis, CONV = conventional Cucumis, WP = without Packaging, CONT = PP container, ZIPPER = LDPE zipper bags and CF = cling film wrap, thereafter, in this study results and discussion.

3.1.Impact on Moisture Content and Dry matter(g%)

Table 1 and Fig 1 showed the comparison between organic and conventional *Luffa* in moisture content. From the Multivariate ANOVA, it was observed that there were no significant differences in moisture content among different types of farming (organic and conventional) packaged in different packaging systems (PP container, LDPE zipper bags, and Cling film wrap) and without any packaging and stored at different temperatures (25◦C and 4◦C) for 3 days and 7 days durations of storage. A similar study was conducted by Jahan et al (2020) on other gourd family vegetable i.e., conventional variety of cucumber in plastic wrap at ambient temperature and 5°C, showed that moisture content decreased with the extension in storage duration.

Table 1. Comparison between Organic and Conventional *Luffa* in Moisture Content (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY FRESH MEAN ± SE	3 DAYS WP MEAN ± SE	3 DAYS CONT MEAN ± SE	3 DAYS ZIPPER MEAN ± SE	3 DAYS CF MEAN ± SE	7 DAYS WP MEAN ± SE	7 DAYS CONT MEAN ± SE	7 DAYS ZIPPER MEAN ± SE	7 DAYS CF MEAN ± SE
ORGN	25ºC	95.73±0.04	94.08±0.01	96.44±0.13	95.66±0.49	96.46±0.05	92.76±0.13	96.49±0.09	92.17±0.03	95.34±0.08
	4 ºC		95.05±0.04	93.91±0.01	94.24±0.02	95.92±0.62	93.92±0.02	95.32±0.05	92.12±0.16	95.31±0.03
CONV	25ºC	95.39±0.01	95.03± 0.01	96.49±0.26	96.66±0.02	96.87±0.04	95.93±0.02	97.08±0.03	97.59±0.05	97.92±0.03
	4º C		94.75±0.002	94.75±0.01	95.08±0.001	95.27±0.04	94.90±0.03	95.14±0.08	95.85±0.02	96.37±0.12

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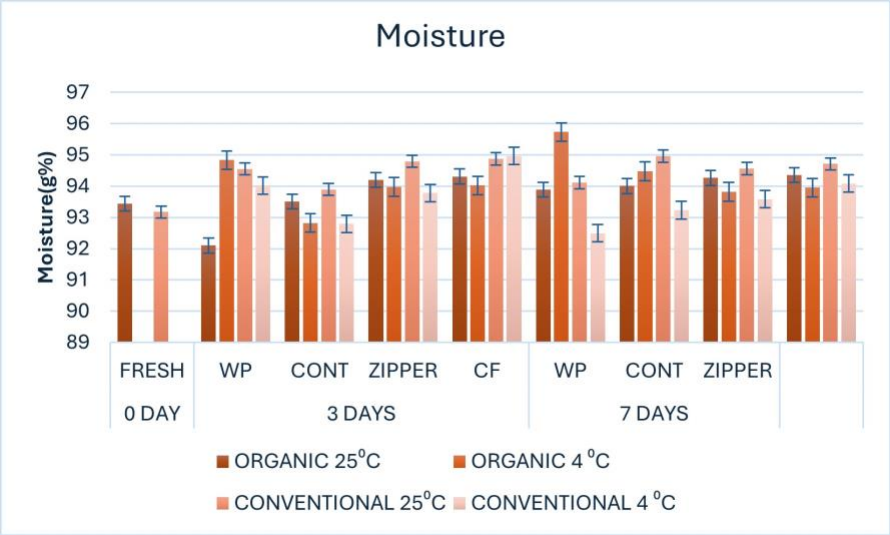

Figure 1. Comparison between Organic and Conventional *Luffa* in Moisture Content (g%)

Table 2 and Figure 2 showed the comparison between organic and conventional *Luffa* in dry weight. From the Multivariate ANOVA, it was observed that that dry weight of *Luffa* of both organic and conventional varieties, stored in different packaging systems (without packaging, PP container, LDPE zipper bags, and Cling film wrap) had a significant effect. A study by Valverde-Miranda et al (2021) concluded that dry matter may influence the shelf life of *Cucumis* i.e., the higher the dry weight at fresh condition, the longer the shelf life will be.

Table 2. Comparison between Organic and Conventional *Luffa* in Dry Weight (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY FRESH MEAN ± SE	3 DAYS WP MEAN ± SE	3 DAYS CONT MEAN ± SE	3 DAYS ZIPPER MEAN ± SE	3 DAYS CF MEAN ± SE	7 DAYS WP MEAN± SE	7 DAYS CONT MEAN ± SE	7 DAYS ZIPPER MEAN ± SE	7 DAYS CF MEAN ± SE
ORGN	25ºC	6.56±0.18	7.87±0.07	6.49±0.02	5.80±0.01	5.5±0.30	6.11±0.06	6.65±0.04	5.77±0.04	5.65±0.03
	4 ºC		5.26±0.17	7.18±0.03	6.04±0.10	5.98±0.04	4.27±0.02	5.49±0.01	6.11±0.01	6.05± 0.04
CONV	25ºC	6.83±0.20	5.5±0.02	6.45±0.57	5.78±0.50	5.13±0.06	5.89±0.18	5.04±0.02	5.46±0.01	5.33± 0.17
	4º C		5.74±0.43	7.20±0.011	6.23±0.02	5.04±0.03	6.77±0.02	7.51±0.08	6.46±0.05	5.92±0.05

 # Values bearing same or no superscript between column do not differ significantly.

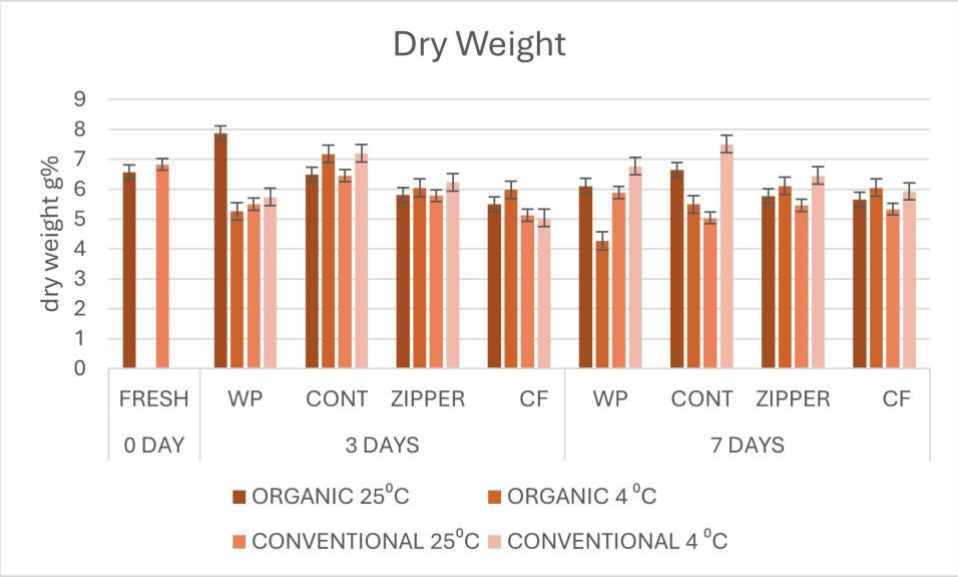

Figure 2. Comparison between Organic and Conventional *Luffa* in Dry weight(g%)

3.2.Impact on pH and Titratable Acidity

Table 3, Table 4 and Figure 3, Figure 4 showed the comparison in pH and Titratable acidity of organic and conventional *Luffa of* stored in different packaging (PP container, LDPE zipper bags, and Cling film wrap) and without any packaging at different temperatures (25ºC and 4ºC) for different days (3 days and 7 days).

The multivariate ANOVA represented that the pH and Titratable acidity of *Luffa* for different types of farming (organic and conventional) stored in different packaging (without packaging, PP container, LDPE zipper bags, and Cling film wrap) at different temperatures (25ºC and 4ºC) for different days (3 days and 7 days) had a significant effect.

It was observed that the TA (g%) was reduced during storage of both organic and conventional *Luffa*. The result obtained was correlated with results found in pH value. The results obtained was in agreement with the study findings of Patil et al(2010) who had conducted the similar study with bottle gourd stored in polyethylene bag and Corrugated Fiber Board (CFB) box packing.

Table 3. Comparison between Organic and Conventional *Luffa* in pH (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY	3 DAYS				7 DAYS			
		FRESH MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE
ORGN	25ºC	6.63±0.09	6.25±0.03	6.43±0.01	6.46±0.01	6.53±0.05	6.44±0.07	6.52±0.09	6.42±0.007	6.51±0.006
	4 ºC		6.17±0.01	6.36±0.01	6.33±0.09	6.26±0.07	6.37±0.03	6.46±0.07	6.36±0.007	6.40±0.02
CONV	25ºC	6.59±0.02	6.08±0.03	6.22±0.07	6.22±0.07	6.30±0.02	6.36±0.01	6.49±0.07	6.50±0.003	6.62±0.01
	4º C		5.95±0.03	6.16±0.07	6.13±0.01	6.24±0.02	6.26±0.03	6.43±0.05	6.46±0.01	6.52±0.01

 # Values bearing same or no superscript between column do not differ significantly.

Table 4. Comparison between Organic and Conventional *Luffa* in Titratable Acidity (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY FRESH MEAN ± SE	3 DAYS WP MEAN ± SE	3 DAYS CONT MEAN ± SE	3 DAYS ZIPPER MEAN ± SE	3 DAYS CF MEAN ± SE	7 DAYS WP MEAN ± SE	7 DAYS CONT MEAN ± SE	7 DAYS ZIPPER MEAN ± SE	7 DAYS CF MEAN ± SE
ORGN	25ºC	0.69±0.05	0.17±0.01	0.11±0.01	0.09±0.01	0.09±0.003	0.22±0.02	0.17±0.02	0.16±0.01	0.15±0.02
	4 ºC		0.17±0.01	0.11±0.001	0.09±0.02	0.08±0.01	0.22±0.01	0.17±0.02	0.16±0.01	0.15±0.01
CONV	25ºC	0.89±0.004	0.08±0.02	0.06±0.02	0.07±0.01	0.06±0.003	0.47±0.01	0.17±0.01	0.90±0.06	0.77±0.02
	4º C		0.07±0.01	0.05±0.01	0.07±0.02	0.05±0.003	0.45±0.09	0.17±0.01	0.89±0.02	0.79±0.01

 # Values bearing same or no superscript between column do not differ significantly.

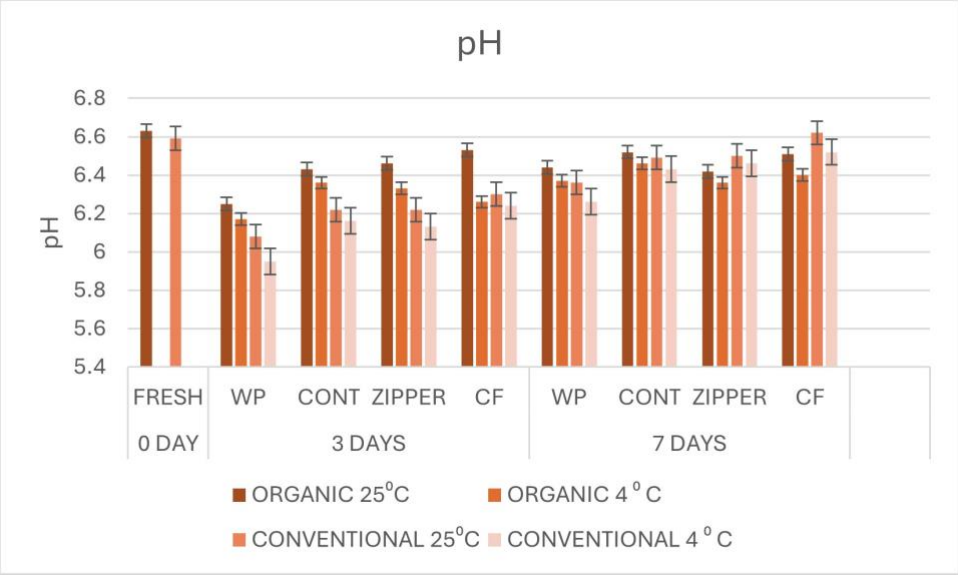

Figure 3. Comparison between Organic and Conventional *Luffa* in pH

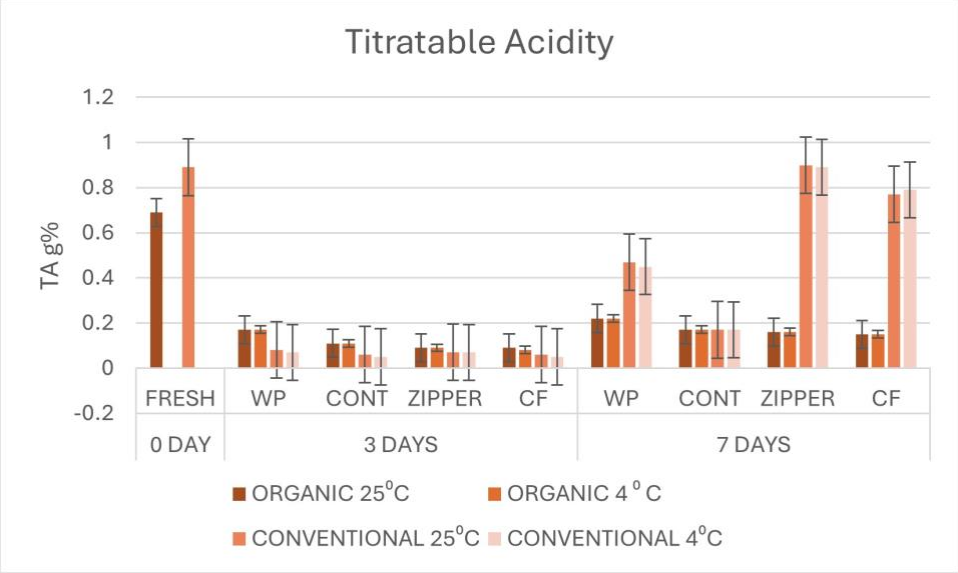

Figure 4. Comparison between Organic and Conventional *Luffa* in Titratable Acidity(g%)

3.3.Impact of Storage Conditions on Tissue Respiration Rate

The comparison of tissue respiration rate (TRR) in both conventional and organic *Luffa in* different storage conditions *was* presented in Table 5 and Figure 5. It was observed that the TRR of cling film wrapped *Luffa* was low both for organic and conventional *Luffa,* whereas for PP container the TRR was the highest. Cling film creates an optimum modified atmosphere with optimal membrane gas permeability thus controlling the postharvest TRR (Exama et al, 1993).

As postharvest losses are caused by continuous respiration and transpiration, So reduction in TRR rates can be the strategy to retain quality of vegetable during storage (Kaur, Kumar, Devgan, and Kumar, 2019). In a recent study by Das et al (2022), it was observed that TRR decreased during storage in different packaging, thus enhancing shelf life of other gourd family vegetable cucumber. Such studies on *Luffa* were not found in previous literature.

It was observed from multivariate ANOVA that the tissue respiration rate of both organic and conventional *Luffa*, stored in different packaging (PP container, LDPE zipper bags, and Cling film wrap) and without packaging, at different temperatures (25ºC and 4ºC) for different days (3 days and 7 days) had a significant effect.

Table 5. Comparison between Organic and Conventional *Luffa* in Tissue Respiration Rate (mg of CO2/ kg)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY	3 DAYS				7 DAYS			
		FRESH MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE
ORGN	25ºC	13.78±0.05	6.05±0.01	8.9±0.01	4.37±0.01	6.65±0.03	5.68±0.02	7.46±0.02	5.06±0.01	0.15±0.02
	4 ºC		4.76±0.01	3.1±0.01	2.04±0.02	3.04±0.01	3.04±0.01	3.84±0.01	3.93±0.01	0.15±0.01
CONV	25ºC	11.23±0.04	5.77±0.02	8.81±0.02	3.54±0.01	6.82±0.03	6.82±0.01	7.55±0.01	5.18±0.06	0.77±0.02
	4º C		4.81±0.01	4.22±0.01	3.03±0.02	4.11±0.03	4.11±0.09	4.29±0.09	3.2±0.02	0.79±0.01

 # Values bearing same or no superscript between column do not differ significantly.

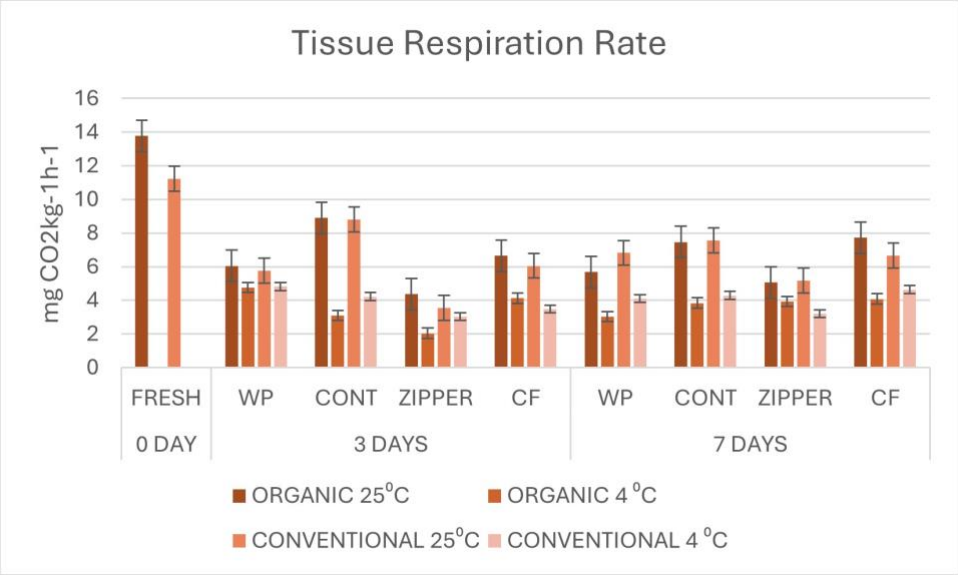

Figure 5. Comparison between Organic and Conventional *Luffa* in Plant Tissue Respiration Rate (mg CO2kg-1.h-1)

3.4.Impact of Storage Conditions on Carbohydrate Content

Table 6 and Figure 6 represented the comparison between organic and conventional *Luffa* in carbohydrate content at different storage conditions. During storage due to increased microbial growth induced carbohydrate breakdown, the carbohydrate content decreased (Swain, Anandharaj, Ray, and Parveen Rani, 2014).

In the present study, substantial losses in carbohydrate content was found may be due to respiratory activity in extended storage at relatively high temperature as described in an earlier study by Kramer(1977). Similar studies by Das et al (2022) on other gourd family cucumbers showed similar results.

Table 6. Comparison between Organic and Conventional *Luffa* in Carbohydrate (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY	3 DAYS				7 DAYS			
		FRESH MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE	WP MEAN ± SE	CONT MEAN ± SE	ZIPPER MEAN ± SE	CF MEAN ± SE
ORGN	25ºC	1.68±0.13	3.26±0.01	1.49±0.01	1.35±0.01	2.04±0.31	2.74±0.01	1.70±0.01	1.56±0.003	2.79±0.03
	4 ºC		3.91±0.01	1.60±0.002	1.44±0.01	2.09±0.04	2.96±0.013	1.93±0.01	1.71±0.002	2.8±0.04
CONV	25ºC	1.75±0.01	2.82±0.01	1.07±0.01	1.46±0.01	2.03±0.015	2.63±0.04	1.72±0.01	1.79±0.007	2.04±.0.03
	4º C		3.89±0.02	1.75±0.01	1.89±0.07	2.04±0.015	2.78±0.03	2.09±0.04	2.10±0.01	2.04±0.03

 # Values bearing same or no superscript between column do not differ significantly.

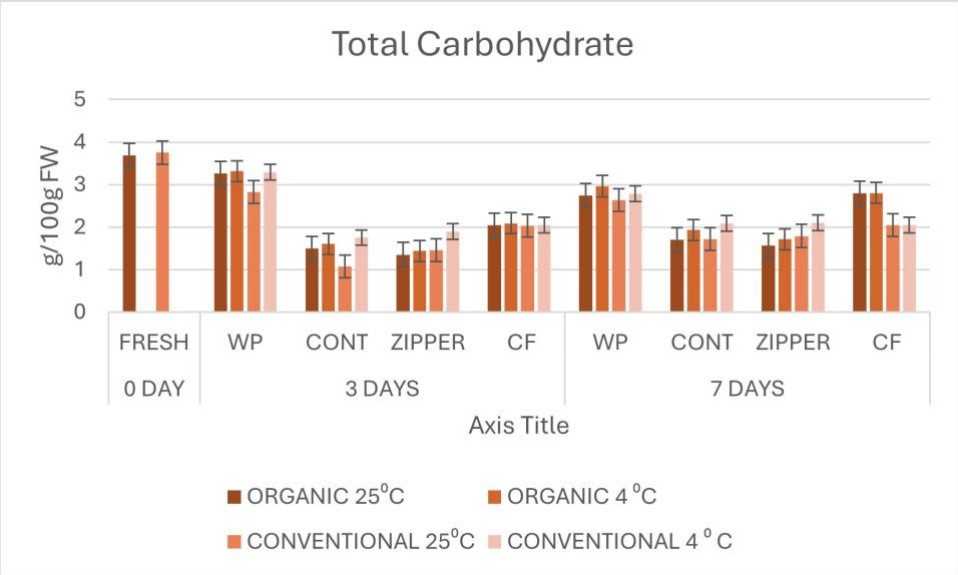

Figure 6. Comparison between Organic and Conventional *Luffa* in Carbohydrate Content(g%)

3.5.Impact of Storage Conditions on Protein Content

Table 7 and Figure 7 represented the comparison between organic and conventional Luffa in carbohydrate content at different storage conditions.

From the multivariate ANOVA, it was found that the total protein content of organic and conventional *Luffa*, stored in different packaging (PP container, Polyethylene zipper bags, and Cling film wrap) and without packaging at different temperatures (25ºC and 4ºC) for different days (3 days and 7 days) had a significant effect. A recent similar study by Das et al (2022) on other gourd family cucumber showed similar results.

Table 7. Comparison between Organic and Conventional *Luffa* in Protein (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY FRESH MEAN ± SE	3 DAYS WP MEAN ± SE	3 DAYS CONT MEAN ± SE	3 DAYS ZIPPER MEAN ± SE	3 DAYS CF MEAN ± SE	7 DAYS WP MEAN ± SE	7 DAYS CONT MEAN ± SE	7 DAYS ZIPPER MEAN ± SE	7 DAYS CF MEAN ± SE
ORGN	25ºC	1.43±0.03	1.42±0.003	1.32±0.01	1.35±0.001	1.36±0.01	0.92±0.003	0.89±0.003	0.91±0.01	0.98±0.01
	4 ºC		1.49±0.002	1.54±0.001	1.39±0.004	1.47±0.01	1.11±0.003	0.95±0.002	0.82±0.001	1.47±0.006
CONV	25ºC	1.48±0.07	1.55±0.003	1.48±0.003	1.40±0.01	1.48±0.01	1.53±0.002	1.38±0.001	1.47±0.003	1.48±0.01
	4º C		1.47±0.02	1.47±0.003	1.54±0.001	1.01±0.02	1.93±0.003	1.51±0.01	1.27±0.01	1.39±0.01

 # Values bearing same or no superscript between column do not differ significantly.

Table 8. Comparison between Organic and Conventional *Luffa* in Total Dietary Fibre (g%)

TYPE OF VEG.	TEMP. OF STORAGE	0 DAY FRESH MEAN ± SE	3 DAYS WP MEAN ± SE	3 DAYS CONT MEAN ± SE	3 DAYS ZIPPER MEAN ± SE	3 DAYS CF MEAN ± SE	7 DAYS WP MEAN ± SE	7 DAYS CONT MEAN ± SE	7 DAYS ZIPPER MEAN ± SE	7 DAYS CF MEAN ± SE
ORGN	25ºC	3.66±0.02	3.51±0.003	3.36±0.003	3.22±0.003	2.97 ±0.01	2.92±0.003	2.90±0.01	2.91±0.003	3.02±0.001
	4 ºC		2.96±0.003	3.64±0.003	3.32±0.003	3.55±0.01	3.13±0.01	2.84±0.003	2.70±0.003	2.95±0.001
CONV	25ºC	3.47±0.03	3.25±0.003	3.16±0.003	3.08±0.003	3.11±0.003	3.15±0.01	3.17±0.001	3.22±0.001	2.98±0.01
	4º C		3.23±0.004	2.99±0.001	3.21±0.003	3.16±0.01	3.88±0.007	2.90±0.006	3.17±0.001	3.22±0.003

 # Values bearing same or no superscript between column do not differ significantly.

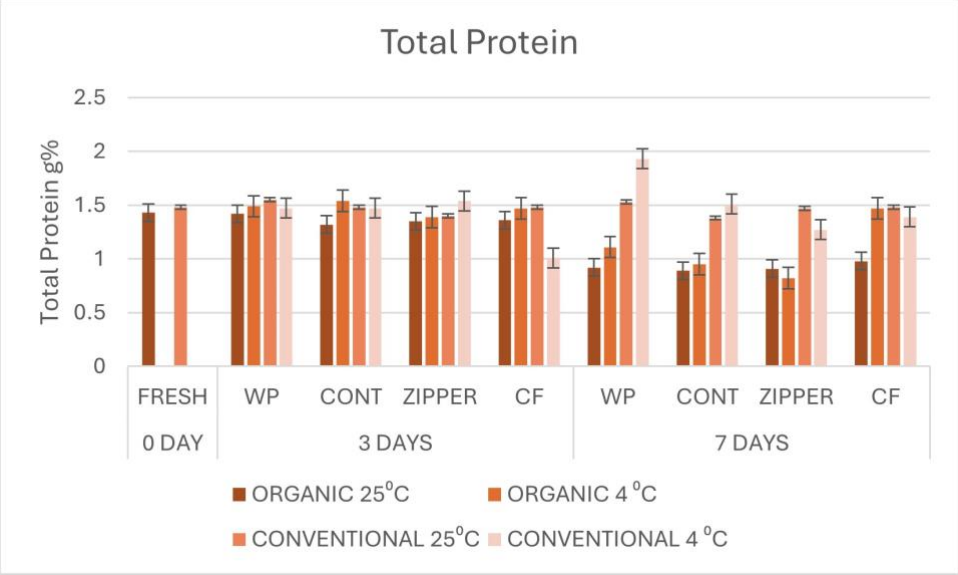

Figure 7. Comparison between Organic and Conventional *Luffa* in Total Protein Content(g%)

3.6.Impact of Storage Conditions on Total Dietary Fibre Content

Table 8 and Figure 8 represented the comparison between organic and conventional *Luffa* in total dietary fibre (TDF). From the multivariate ANOVA, it was observed that the total dietary fibre of organic and conventional *Luffa*, stored in different packaging (PP container, Polyethylene zipper bags, and Cling film wrap), and without packaging, at different temperatures (25ºC and 4ºC) for different days (3 days and 7 days) had a significant effect. In a recent study on other gourd family vegetables-cucumber showed similar results for TDF content (Das et al, 2022).

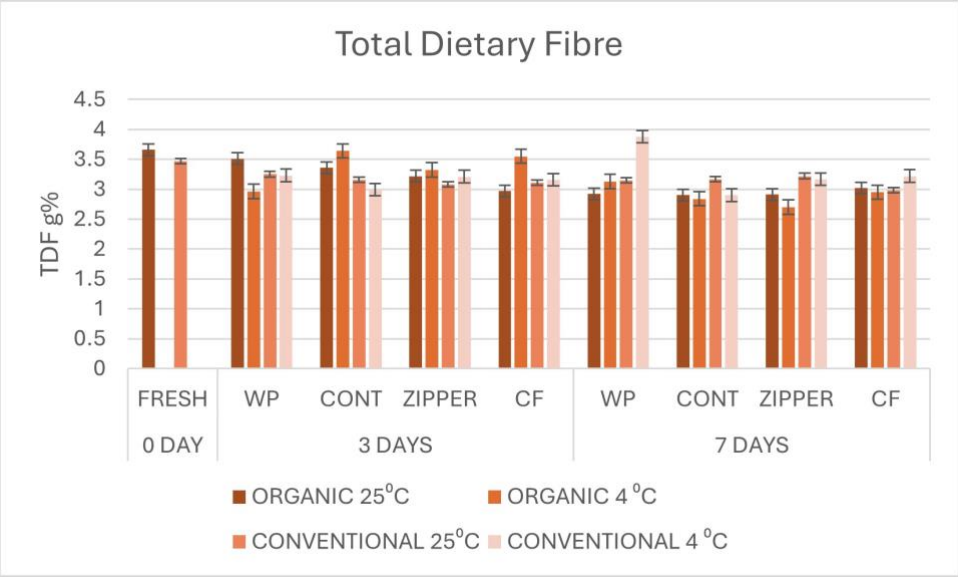

Figure 8. Comparison between Organic and Conventional *Luffa* in Total Dietary Fibre (g%)

4.Conclusions

From the study it can be concluded that there were no significant differences in physicochemical parameters-moisture, dry weight content, the pH and Titratable acidity, and tissue respiration rate, and nutritional parameters such as total protein and total dietary fibre, among different types of farming (organic and conventional) packaged in different packaging systems (PP container, LDPE zipper bags, and Cling film wrap) and without any packaging and stored at different temperatures (25◦C and 4◦C) for 3 days and 7 days durations of storage. Storage in cling film wrap decreased the tissue respiration rate, thereby increasing shelf life of both organic and conventional *Luffa.* The carbohydrate content was decreased due to microbial induced carbohydrate breakdown and hence during storage in domestic packaging carbohydrate breakdown can be prevented. Therefore, packaging in domestic packaging and temperature management can be considered as a devising strategy to enhance shelf life of both organic and conventional *Luffa.*

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Declaration of Conflict of interest

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Research article

APPLICATION OF MICROCRYSTALLINE CELLULOSE EXTRACTED FROM OIL PALM EMPTY FRUIT BUNCHES (EFB) AS A THICKENING AGENT IN ARTIFICIAL MEAT

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ABSTRACT

Empty fruit bunches (EFB), a byproduct of palm oil processing, contain approximately 40% cellulose, presenting a valuable resource for sustainable applications. This study explores the extraction of microcrystalline cellulose (MCC) from EFB and evaluates its effects on the characteristics of artificial meat. MCC was incorporated at varying concentrations (1%, 2%, and 3%) into artificial meat formulations using different red bean-to-soybean flour ratios (1:3, 3:1, and 1:1). Fourier-transform infrared (FTIR) spectroscopy confirmed the successful extraction of MCC, as evidenced by the presence of OH, CH, C=C, C-O, and C-O-C functional groups. The addition of soybean flour increased moisture content (50.26%–53.55%), ash (1.63%–1.66%), and protein (1.55%–1.63%), while red bean flour contributed to higher fat (1.59%–2.22%) and carbohydrate levels (44.01%–46.3%). The 1:1 flour ratio significantly enhanced crude fiber content (2.19%–3.8%). Although MCC addition had minimal impact on the overall chemical composition, it notably improved the fiber content of artificial meat, suggesting its potential as a functional ingredient for texture enhancement and dietary fiber enrichment.

1. Introduction

Agriculture in Indonesia produces a variety of commodities, one of which is oil palm, a key agricultural product. Palm oil is a major source of Crude Palm Oil (CPO) and Palm Kernel Oil (PKO) (Arifandy et al., 2021). Crude palm oil was obtained by diverse steps, one of the most important steps was the grazing process that during this process temperature has a major impact in generating high crude (Swastawati et al., 2019; Wibawanti et al., 2021). During the processing of oil palm, empty fruit bunches

(EFB) are generated as a byproduct. This waste material is renewable, abundant, and has the potential to be used as a source of cellulose. It is commonly repurposed as fertilizer or utilized as a substrate for growing fungi and plants. For every ton of fresh oil palm fruit bunches processed, approximately 230 kg of empty palm fruit bunches are produced (Kamal, 2018).

The palm oil processing process produces not only solid waste in the form of EFB but also palm kernel shells and fibers. The cellulose content in the solid waste is relatively high, at approximately 40%, while other components include hemicellulose (24%), lignin (21%), and ash (15%) (Ngatirah, 2017). Cellulose is characterized by its fibrous structure, white color, and insolubility in water and organic solvents. Cellulose molecules are straight chains consisting of about 5,000 glucose units, forming fibrils bonded by hydrogen interactions between hydroxyl groups (Nosya, 2016). Glucose molecules with a chain length of 250 units form microcrystalline cellulose (MCC) molecules (Al-Baarri et al., 2018; Nawar et al., 2010). Microcrystalline cellulose is commonly used in drug manufacturing as a binder. Beyond the pharmaceutical industry, MCC is also widely utilized in the food industry as a food additive. Its applications include use as an anti-caking agent, thickener, bulking agent, emulsifier, filler, and texturizer to improve food texture (Sundarraj & Ranganathan, 2018)

To date, studies on the utilization of microcrystalline cellulose from oil palm empty fruit bunches (EFB) have been limited to compost production and capsule shells. No research has explored the use of MCC in artificial meat products. This research seeks to utilize palm oil waste as a raw material for producing MCC to enhance the characteristics of artificial meat. The study aims to identify MCC derived from EFB and determine the effects of different MCC concentrations on the characteristics of artificial meat.

2. Materials and methods

2.1. Materials

EFB were processed using general laboratory equipment such as a sieve, scales, and glassware for sorting, weighing, and grinding. Specialized equipment, including a furnace for heating, a Soxhlet apparatus for extraction, and a Kjeldahl apparatus for protein analysis, were used during the cellulose extraction process. The structural properties of the resulting microcrystalline cellulose were analyzed using an FT-IR spectrophotometer, and the texture of the artificial meat was assessed with a texture analyzer Brookfield Ametek. Chemical treatments employed NaOH for delignification, H_2O_2 for bleaching, and HCl for neutralization, with standard filtration using filter paper.

2.2. Methods

The initial stages of the research included sorting the empty fruit bunches, followed by washing and drying. This was followed by the preparation of oil palm empty fruit bunch flour, cellulose extraction, and MCC extraction.

Cellulose extraction began with the delignification process of oil palm empty fruit bunch flour using a 12% (w/v) NaOH solution for 3 hours at 90°C. Afterward, the solution was cooled and filtered to separate the cellulose from the NaOH and other components of the flour. The next step involved bleaching with a 3% H2O2 solution (6 mL) and 2 mL of buffer solution. The mixture was then heated on a hot plate for 1 hour at 80°C. The cellulose was subsequently washed with distilled water until the pH was neutral, resulting in high-purity α-cellulose (Dewanti, 2018).

The MCC preparation process involved hydrolyzing 10 g of cellulose flour using 2 N HCl in a glass beaker, which was then heated on a hot plate for 1 hour. The precipitate was washed with distilled water until the pH of the solution was neutral. Next, the precipitate was dried in a cabinet dryer for 24 hours at 60°C, after which it was ground (Ohwoavworhua et al., 2009). Additionally, red bean and soybean flour were prepared. The final stage of this research involved the preparation of artificial meat by adding microcrystalline cellulose (MCC) from EFB and analyzing its chemical characteristics.

The research factors included the ratio of red bean and soybean flour, which were as follows:

F1 (1:3), F2 (3:1), and F3 (1:1). The MCC concentration levels were 1%, 2%, and 3%, along with the respective ratios of red bean and soybean flour: FT1 (1:3), FT2 (3:1), and FT3 (1:1).

3. Results and discussions

3.1. Identification of MCC using FTIR

Fourier transform infrared (FTIR) spectroscopy is a technique that identifies chemical bonds in a molecule by creating a spectrum of the molecule's infrared absorption. Figure 1 presents absorption bands characteristic of microcrystalline cellulose prepared from sugarcane bagasse and EFB as analyzed by means of FTIR.

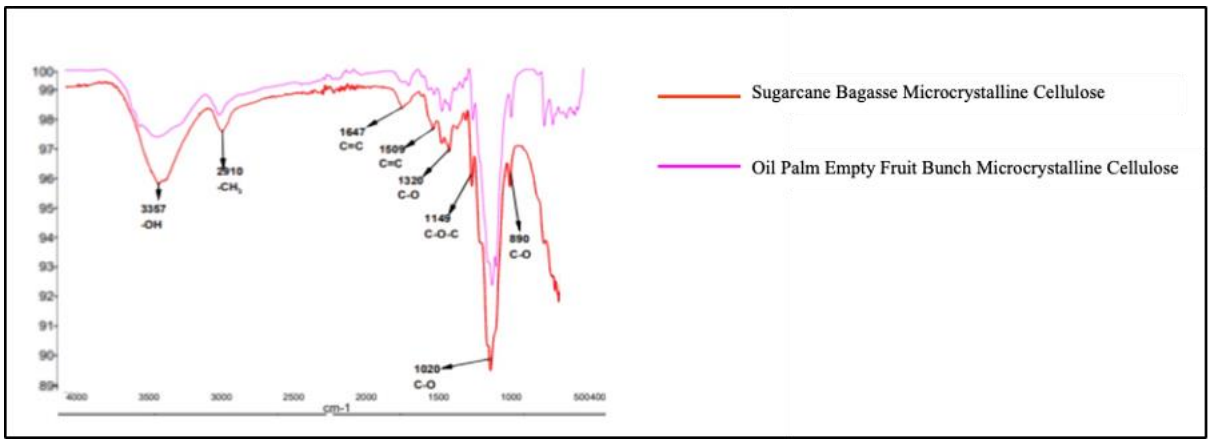

Figure 1. FTIR Analysis of Microcrystalline Cellulose Prepared from Sugarcane Bagasse and Oil Palm Empty Fruit Bunch (EFB)

At the frequency region of 3400–3200 cm^{-1}, the presence of O-H bonds from α-cellulose was observed, with the intensity of the wave showing variations and broadening. The absorption band at 2900–2901 cm^{-1} indicated the presence of C-H bonds, further confirming α-cellulose. The C=C stretching vibration group, characteristic of the lignin skeleton, appeared in the range of 1500–2000 cm^{-1}.

Additionally, the absorption at 1320 cm^{-1} indicated the presence of C=O groups, while the wavelength range of 1000–1150 cm^{-1} represented the C-O ester group. The C-H alkene group was identified at a wavelength of 700–850 cm^{-1}, signifying an increase in crystalline regions within the MCC. These results confirm the structural integrity and purity of the MCC.

3.2. Moisture content

The damage occurring in a product is a key parameter in determining its quality and shelf life. Such damage is generally caused by a high moisture content in the product, which results from internal metabolism, biological activity, or the presence of microbes that can degrade the product (Daud et al., 2019). The moisture content of the artificial meat samples in Figure 2 shows a value of 50.26%-67.34%. According to the Indonesian National Standard (SNI) (National Standardization Agency of Indonesia, 2014), the maximum moisture content in meatballs is 70%. The highest water content analysis results were produced in the chicken control, which amounted to 67.34%. As for the lowest result, artificial meat is 50.26% with the treatment ratio of red bean flour and soybean flour is 3:1 and the addition of microcrystalline cellulose as much as 1%. Thus, the water content of artificial meat results has met the SNI requirements.

Figure 2 shows that the formulation of red bean flour: soybean (1:1) produces the highest moisture content value compared to other formulations. The addition of 2% microcrystalline cellulose produced the highest moisture content compared to 1% and 3% concentrations. Cellulose, with its hydrophilic properties, can bind water molecules to its hydroxyl (OH) groups, a key component in cell

wall formation. The polymer structure of cellulose, with its weak hydrogen bonds, allows water to easily penetrate (Fatriasari et al., 2019). It is reported that the moisture content of MCC from bagasse is 4.96%, while corn cob MCC exhibited a moisture content of 6.9%(Kunusa, 2017; Nawangsari, 2019).

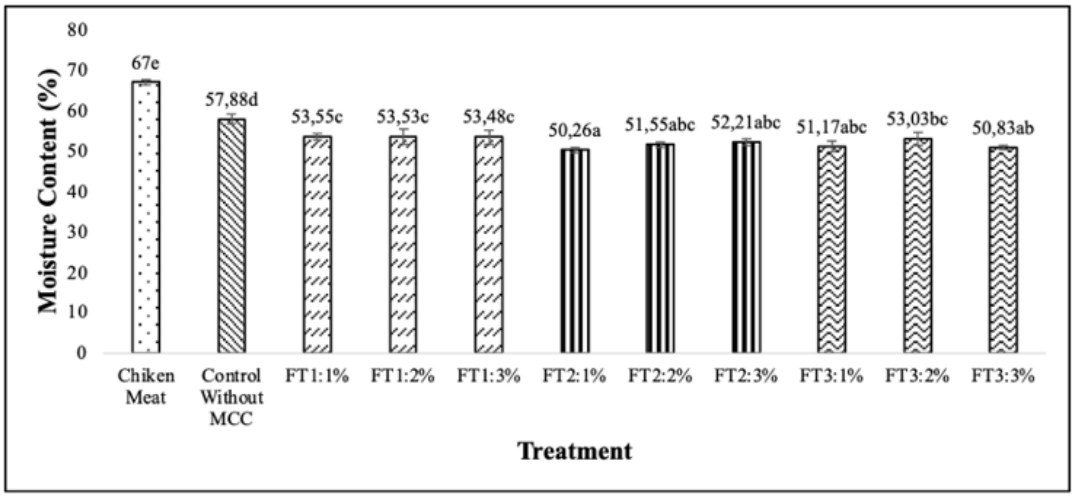

Figure 2. Moisture content of artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

The addition of red bean flour reduced water content in chicken sausage as the proportion of flour increased (Yuliatun et al., 2023), a decrease in moisture content was observed in crackers with increased peanut flour (Asfi et al., 2017), and higher red bean flour content in cork fish nugget products resulted in lower moisture content(Almayda et al., 2024). Conversely, adding soybean tempeh flour to artificial jerky increased its moisture content (Sidup et al., 2022), while higher soybean pulp flour content in cookies led to greater water retention (Wijaya et al., 2023). The ability of food ingredients to bind water is influenced by their protein content, as proteins contain hydrophilic groups that help retain water. As heat treatment causes protein molecules to form a network, this matrix can act as a water binder, preventing water molecules from escaping (Astawan et al., 2016).

3.3. Ash content

Ash content refers to the material remaining after a product has been completely burned. It refers to the proportion of inorganic mineral residue remaining after the complete combustion of organic material (Qadaryati et al., 2023). Food products typically consist of 96% organic substances and water, with the remaining portion comprising inorganic substances, or ash content (Hutomo et al., 2015). Determining ash content in food ingredients is essential for assessing product quality. Higher ash content often indicates a decline in food quality (Tahar et al., 2017). Calcium, phosphorus, and iron are mineral components that remain after ignition. These minerals are essential inorganic compounds that the human body requires for various physiological functions (Khuluqiah et al., 2109).

Figure 3 shows that the ash content values ranged from 0.67% to 6.43%. The highest value, 6.43%, was observed in the chicken control, while the lowest ash content, 0.67%, was found in the formulation with a 1:1 red bean to soybean flour ratio and 1% microcrystalline cellulose. According to the Indonesian National Standard (SNI) (National Standardization Agency of Indonesia, 2014), the maximum allowable ash content in meatballs is 3%. Therefore, the ash content of all samples, except the chicken control, meets the SNI quality requirements.

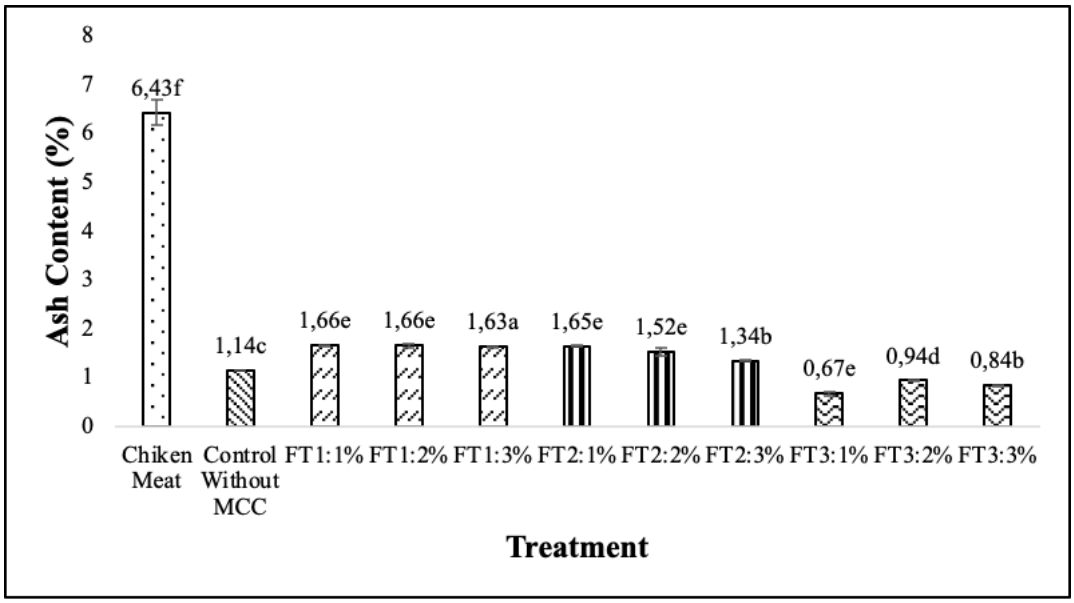

Figure 3. Ash content analysis of artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

The ability of food ingredients to bind water is influenced by their protein content, as proteins contain hydrophilic groups that help retain water. As heat treatment causes protein molecules to form a network, this matrix can act as a water binder, preventing water molecules from escaping (Astawan et al., 2016). The mineral content in soybeans includes calcium (300.36 mg/100 g), magnesium (258.24 mg/100 g), iron (16.4 mg/100 g), sodium (3.0 mg/100 g), zinc (2.7 mg/100 g), and phosphorus (695.20 mg/100 g) (Etiosa et al., 2018). The ash content in soybean flour has been reported as 2.89% (Astuti et al., 2014) and 3.88% (Fanzurna & Taufik, 2020). Increasing the amount of soybean flour in nugget products leads to a higher ash content (Parinduri & Rusmarilin, 2016) and adding soybean flour also increases the ash content (Swamilaksita et al., 2021).

Red bean flour has been reported to contain 2.75% ash (Astuti et al., 2014), though some studies have indicated values as low as 0.67% (Awalin et al., 2023). In the context of meatball production, the ash content of red bean flour was found to be 2.74% (Khuluqiah et al., 2109). Furthermore, the inclusion of higher amounts of red bean flour in porridge has been shown to decrease its ash content (Munte et al., 2019), Also observed in cookie formulations, where the addition of red bean flour resulted in a reduction of ash content (Binalopa et al., 2023).

3.4. Protein content

Protein plays a crucial role in replacing damaged tissue and maintaining existing tissue (Virgiansyah, 2018). Soybean flour contains higher protein levels compared to other beans, with 40-50% protein content, approximately 34.9 grams per 100 grams (Harleni & Nidia, 2017). In contrast, red bean flour contains 1.95 grams of protein per 100 grams (Wiranata et al., 2017). Figure 4 presents the results of the protein analysis in artificial meat. Figure 4 shows that the protein content of artificial meat with the addition of MCC from oil palm empty fruit bunches ranged from 0.83% to 3.32%. According to the quality standards set by SNI 3818:2014 (National Standardization Agency of Indonesia, 2014), the minimum protein content for meatballs is 8.0%, meaning that this artificial meat does not meet the SNI requirements.

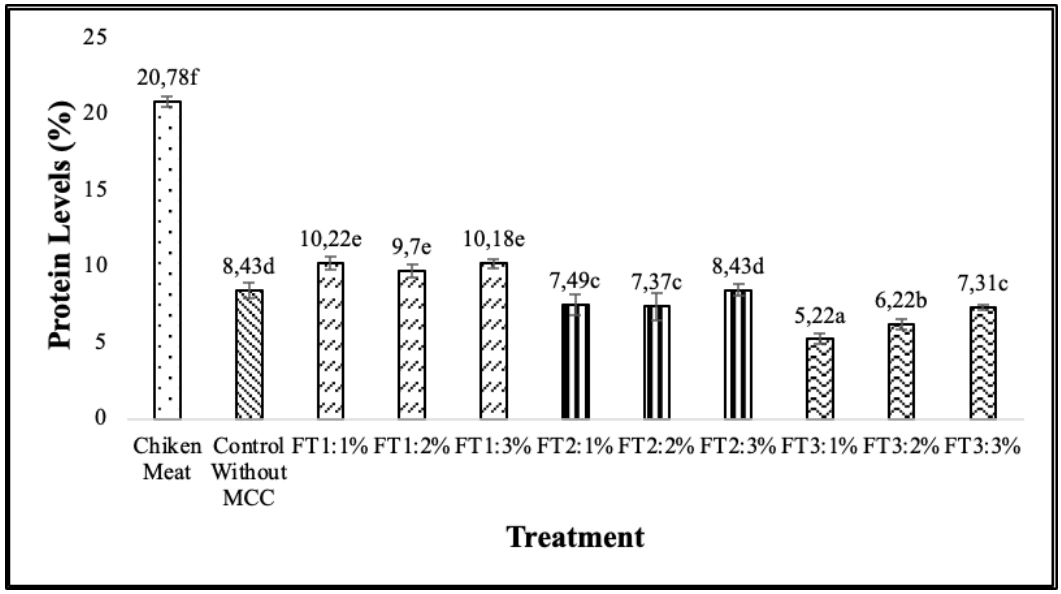

Figure 4. Protein content analysis of artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

Research has shown that the protein content in beef meatballs increases with the addition of soybean flour (Hermana et al., 2023). This is consistent with previous studies that reported an increase in protein levels in tapioca dumplings with higher soybean flour content (Aninditia et al., 2023). Similarly, it was found that adding soybean flour to buffalo meat nuggets increased their protein content (Suryaningsih et al., 2015). Conversely, other research observed a decrease in protein content in chicken sausage products as the amount of red bean flour increased (Pinardi et al., 2020). The protein content in red bean flour has been reported as follows: 21.61% (Siahaan et al., 2021), 15.3% (Audu & Aremu, 2011), 41.64% (Fanzurna & Taufik, 2020), 35.9% (Taufik, 2018), and 28.25% (Jariyah et al., 2017).

3.5. Fat content

Fats are a class of lipids, which are organic compounds insoluble in water but soluble in nonpolar solvents such as diethyl ether, benzene, chloroform, and hexane. Fats, specifically triglycerides, are solid at room temperature (Mulyani & Sujarwanta, 2018). Figure 5 shows that the fat content in artificial meat with the addition of microcrystalline cellulose from oil palm empty fruit bunches ranged from 1.01% to 5.29%. According to the quality standards outlined in SNI 3818:2014 (National Standardization Agency of Indonesia, 2014), the maximum allowable fat content in artificial meat is 10%. Therefore, the fat content of the samples meets the SNI requirements.

The fat content in soybean flour is reported to be 28.44% [25], while another study indicates it as 26.22% (Indrawan et al., 2018). This is consistent with research that found increasing the amount of soybean flour results in higher fat content (Hariadi et al., 2017). In contrast, red bean flour contributes to lower fat content in artificial meat. Red bean flour has a fat content of 9.83% (Pangastuti et al., 2013), while another study reports it as 0.48% (Tamrin & Pujilestari, 2016). Similarly, red bean flour contains 0.47% fat (Chrestella, 2020). Therefore, higher amounts of soybean flour can increase fat content in the product.

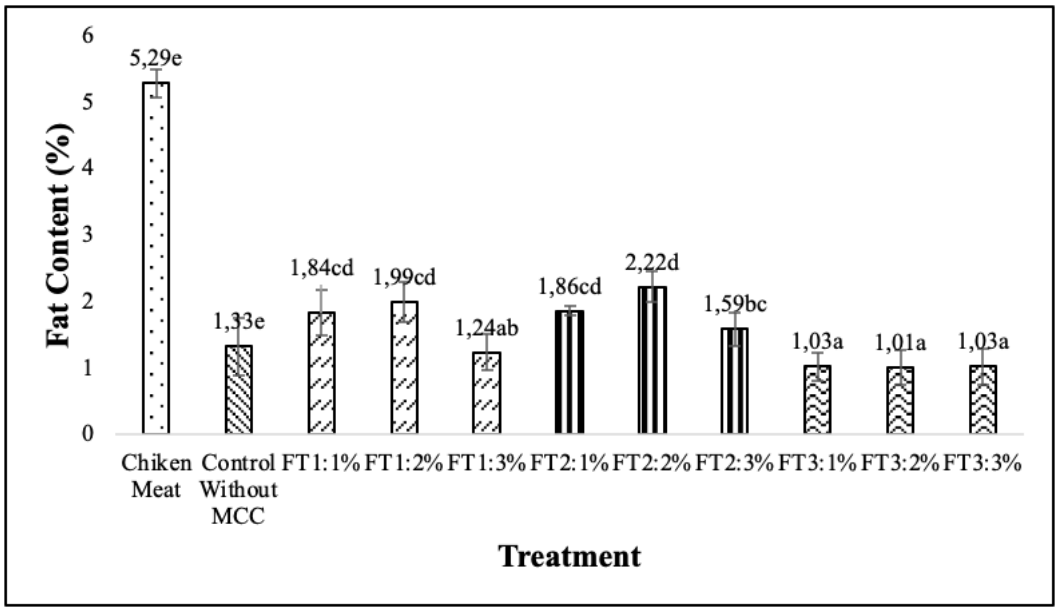

Figure 5. Fat content analysis of artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

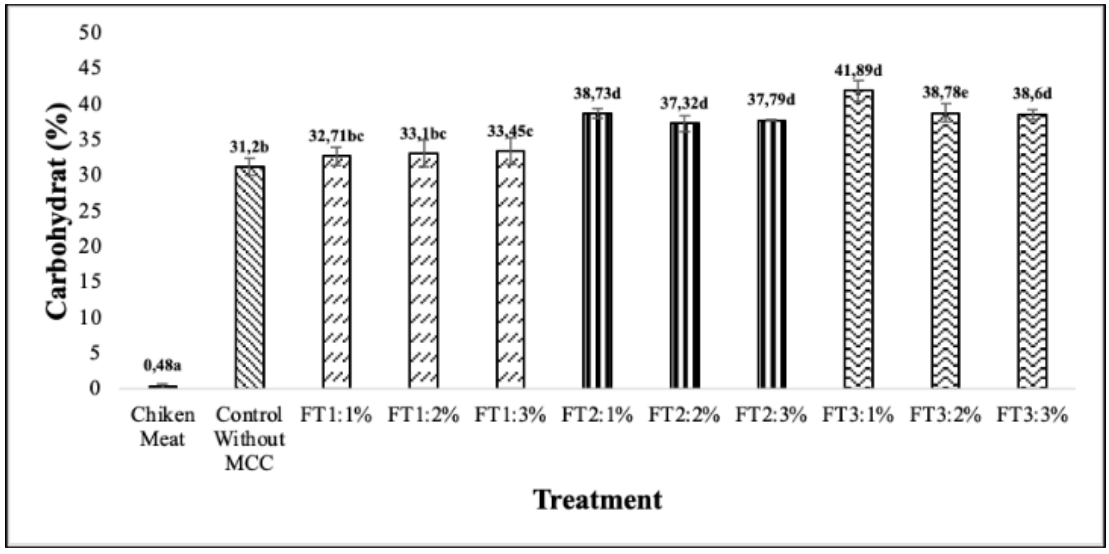

Figure 6. Carbohydrate content analysis of artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

The addition of soybean flour to gourami fish meatballs was found to increase the fat content (Tina et al., 2018). Similarly, an increase in fat content was observed in nugget products with higher amounts of soybean flour (Simanjuntak & Pato, 2021). In brownies, replacing wheat flour with soybean flour also results in higher fat content (Kunusa, 2017). In contrast, artificial meat with added soybean flour had lower fat content compared to that with red bean flour. This is because soaking soybeans reduces fat content. The soaking process activates lipase enzymes, which break down fats, resulting in free fatty acids that are easily dissolved in water during soaking (Pangastuti et al., 2013).

3.6. Carbohydrate content

Carbohydrates are essential nutrients for the body, primarily serving as energy sources. They are a group of organic compounds with diverse molecular structures (Siregar, 2014). Figure 6 shows that the carbohydrate content of artificial meat with the addition of microcrystalline cellulose from oil palm empty fruit bunches ranged from 17.61% to 46.30%. The lowest carbohydrate content, 17.61%, was found in the chicken meat control, while the highest value, 46.30%, was observed in the formulation with a 1:1 red bean to soybean flour ratio and 1% microcrystalline cellulose. Mentari et al. (Mentari et al., 2016) reported that artificial meatballs produced a carbohydrate content of 21.63%.

Increasing the substitution of soybean flour has been shown to decrease the carbohydrate content in snack bar products (Qoriah et al., 2021). Similarly, Suriany et al. (2020) found that adding more soybean flour to noodles led to a reduction in carbohydrate content. Conversely, the addition of red bean flour in food bars increased the carbohydrate content as the level of substitution rose (Utama & Anjani, 2016). Carbohydrates in food ingredients are often reduced due to the higher levels of water, ash, protein, and fat present (Ratnawati et al., 2019). The carbohydrate content in red bean flour is reported to be 12.83% (Prasetyo et al., 2014). In contrast, the carbohydrate content of soybean flour has been reported as 32.24% (Ekafitri & Isworo, 2014) and 34% (Sukaryono et al., 2017). The reduction in carbohydrate content in soybeans is attributed to the degradation of carbohydrate molecules into simpler sugars. Additionally, soaking and boiling soybeans can further decrease the carbohydrate content due to the loss of oligosaccharides (Kusuma Putri et al., 2021).

3.7. Crude fiber content

Crude fiber is a component of food that cannot be hydrolyzed by chemicals. It is determined using sulfuric acid (H_2SO_4 1.25%) and sodium hydroxide (NaOH 1.25%) (Hardiyanti & Nisah, 2021). The crude fiber content in a food ingredient depends on the fiber content of the ingredients used in the formulation (Widnyani et al., 2021). Crude fiber is a carbohydrate component that is separated from nitrogen-free extracts, primarily starch, through a simple chemical analysis. It consists of cellulose, hemicellulose, and lignin (Londok & Pandelaki, 2023).

Figure 7 presents the crude fiber content in artificial meat formulations. The analysis revealed that the crude fiber values varied across different formulations of red bean and soybean flour. Notably, when red bean and soybean flour were used in equal proportions, a higher crude fiber content was observed compared to other formulations. Furthermore, increasing the concentration of microcrystalline cellulose (MCC) in the formulations resulted in a significant increase in the crude fiber content.

It was reported that increasing the substitution of red bean flour raises the crude fiber content (Rahmawati, 2018). However, in snack bar products, the addition of red bean flour led to a decrease in fiber content as the proportion of red bean flour was reduced (Siregar et al., 2017).

Red bean flour is a rich source of fiber and increasing its amount in artificial rice made from ganyong tubers also increased crude fiber content (Salsabila et al., 2020). It was reported that red beans contain 5.77% crude fiber (Li et al., 2002), while adding red bean flour to arrowroot flour cookies increases crude fiber content. Approximately one-fifth of food fiber consists of crude fiber (Istiqomah & Rustanti, 2015) The insoluble fiber content in soybean seeds ranges from 3.08% to 5.49%(Ratnaningsih et al., 2018; Wibawanti et al., 2021). It was stated that adding soybean flour to chicken nuggets increased crude fiber values (Mawati et al., 2017). In tempeh products, a higher proportion of soybeans results in greater crude fiber content compared to tempeh made with a smaller number of soybeans (Nasrulloh et al., 2021).

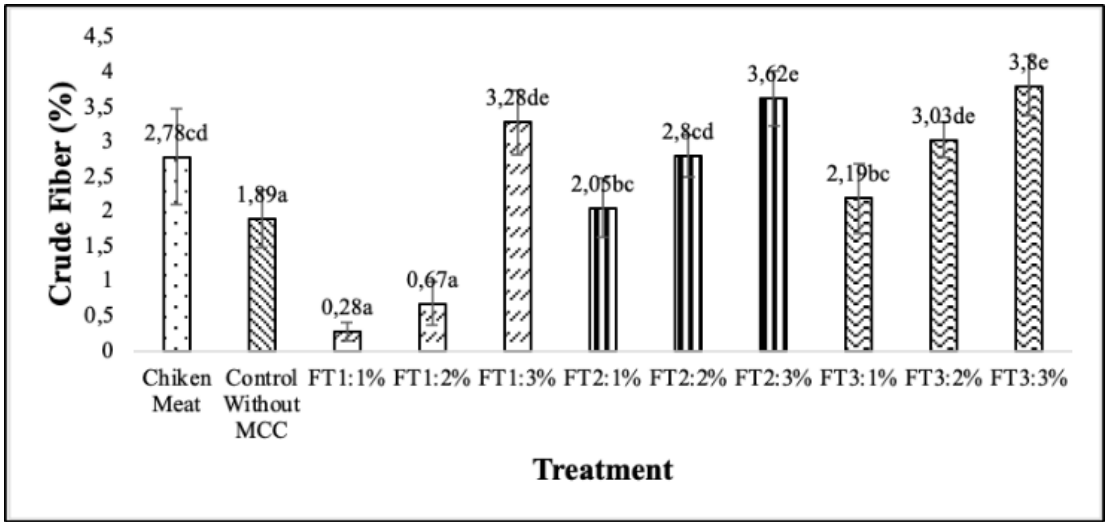

Figure 7. Crude fiber content in artificial meat with varying ratios of red bean and soybean flours and MCC concentrations. Data labels are shown as means, whereas standard deviations are shown as error bars. Different superscript letters within the same category parameter indicate statistically significant differences ($p < 0.05$).

4. Conclusions

The addition of microcrystalline cellulose to artificial meat increases crude fiber content but does not significantly affect chemical characteristics such as moisture content, ash content, protein content, fat content, or carbohydrate content. In contrast, the incorporation of soybean flour and red bean flour does impact the chemical characteristics of artificial meat.

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EFFECTS OF DIFFERENT LACTASES AND RATIOS ON THE PREPARATION OF LOW-LACTOSE PREBIOTIC LIQUID GOAT MILK

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ABSTRACT

Consumption of dairy products provides consumers with nutrients and is beneficial to human health, but lactose intolerance prevents some consumers from consuming dairy products. Using lactase to hydrolyze lactose and generate galactooligosaccharides (GOS) can not only avoid lactose intolerance but also increase the prebiotics of dairy products and improve the functionality of the product. This study investigated the effects of 14 lactases and their ratios on the generation of GOS and the hydrolysis of lactose using goat milk. It was found that there were significant differences in the effects of lactase on the synthesis of GOS and lactose hydrolysis. The rate of lactose hydrolysis (LHR) and GOS concentration are 4.70%-80.62%, and 0.82 g/L-12.60 g/L respectively. The best effect is achieved when lactase E10 and E5 are in a ratio of 4:1, The GOS concentration was 14.587±0.20 g/L and the LHR was 91.880±0.01%, respectively.

1. Introduction

When it comes to dairy products, goat milk is the most like breast milk. with the most complete nutrition and the most easily absorbed by the human body. The fat particles of goat milk are one-third of the volume of milk, which is more conducive to human absorption, and long-term moderate drinking of goat milk will not cause weight gain. In addition, the vitamins and trace elements in goat milk are significantly higher than in cow milk. However, a large proportion of people suffer from "lactose intolerance" and cannot effectively break down lactose in the body, they cannot drink goat milk. In Africa and South America, more than half the population is lactose intolerant, and the impact is more pronounced in some Asian countries, where lactose intolerance affects almost 100% of the population. (Garcia et al., 2011).

At present, there are four main methods to reduce lactose content in dairy products, namely, physical removal, chemical acid hydrolysis, genetic engineering technology, and enzymatic hydrolysis. The physical removal method and chemical acid hydrolysis method use membrane technology and high-temperature hydrolysis of strong acid and alkali respectively, which are lower in cost, but will lose nutrients in milk, so these two methods are used less. Transgenic technology has developed a new idea for the research of low-lactose dairy products. By connecting the cDNA encoding the precursor of intestinal lactase to the egg cell, the mammals that produce low-lactose liquid milk can be cultured without affecting the protein level in the milk. At present, this method is still in the process of exploration. (Liang, 2013). Enzymatic hydrolysis is to decompose lactose into glucose and galactose by adding exogenous lactase. This method can prevent the loss of other nutrients in the milk, not only that, due to the increase of glucose content in the milk to increase the sweetness, glucose, and galactose are more easily absorbed by the human body, so the enzymatic hydrolysis method is currently the main processing technology for processing low-lactose dairy products.

The products of lactose hydrolysis are glucose and galactose on the one hand, glucose is an energy source that can be absorbed directly by the body, so more easily converted into energy for the human body (Wang, 2009); Galactose, is the other hand, it is an essential monosaccharide for the body's brain and mucous membranes (Sun et al., 2004). Moreover, glucose and galactose can be combined into GOS by transglycosidation using lactase (Huang et al., 2020). GOS can reduce the secretion of inflammatory factors and relieve inflammation.

GOS is the most common prebiotic. The use of prebiotics can control the balance of intestinal flora and produce short-chain fatty acids that facilitate intestinal transport (MDSL S A et al., 2014). Butyric acid, propionic acid, and short-chain fatty acids have anti-cancer properties, these three compounds are the primary byproducts of the GOS process in the body (Cummings et al., 1981).

With the vigorous development of the dairy industry in recent years, many hydrolyzed lactose products have been developed at home and abroad, such as yogurt, buttermilk, cheese, milk, fermented milk, and so on (Jelen et al., 2003). Some of the lactose-rich whey produced in the production of cheese is discarded, causing environmental problems (Pesta et al., 2007). Some studies have shown that GOS can be produced by enzymolysis of whey by lactase, which can turn waste into treasure (Nedim et al., 2002). So now more and more research are to synthesize GOS by using milk or by-product whey and whey permeate. However, there are relatively few studies on the synthesis of GOS from goat milk products.

At present, most of the experiments at home and abroad use a single lactase to decompose lactose to synthesize GOS. The LHR varies greatly under different experimental conditions, but the change in GOS production is not much. So, in this study, different complex lactase enzymes were selected to explore their combined effects on lactose hydrolysis and GOS production in goat milk (Wang et al., 2006; Zhang et al., 2008)

In our previous study, the effects of 14 commercially available lactose enzymes on the preparation of enzymatic hydrolyzed goat milk were studied and the optimal enzyme ratio was improved (Xu et al., 2021). In this study, we used the same enzyme preparation to investigate the effects of its type and ratio on the LHR in goat milk and the production of GOS, providing a guide for the preparation of prebiotic goat milk.

2. Materials and methods

2.1.Experimental raw materials and handling The raw material used in the experiment is full-fat sheep milk powder from the laboratory. Then the raw materials are treated and rehydrated at the ratio of 1:8, after sterilization, a certain amount of lactase is added for enzymatic hydrolysis, and then the enzyme is removed and cooled, and finally, the

Enzymatic hydrolyzed goat milk required for the experiment is obtained.

2.2.Lactase

14 commercially available lactose enzymes from previous experiments were selected for this study (Xu et al., 2021).

2.3.Screening of different types of lactases

Considering the cost of the experiment, after the milk was sterilized 65 ℃ for 20 minutes,14 lactase enzymes were added to goat milk at 0.1% each, enzymolysis at 40℃for 3h, and then enzymolysis at 100℃ for about ten minutes. After cooling, the content of glucose, galactose, and lactose in enzymatically hydrolyzed goat milk could be determined, then the LHR and the galactose oligosaccharide could be calculated. It was selected as a lactase that hydrolyzed lactose readily and contained a high level of GOS.

2.4.Matching Optimization of enzymatic hydrolyzed goat milk

Based on the results of lactase screening, the influence of complex enzymes on the content of GOS and the LHR was studied, and the ratio of complex enzymes was determined by mixing experiment design.

2.4.1.Measurement of galactose and lactose content

The galactose and lactose content after the addition of lactase to goat milk is usually based on a lactose/galactose test kit. It is based on the principle that in a weak acid environment (pH of about 6.6), Glucose and D-galactose are produced when lactase hydrolyzes lactose. Then, in a weak base environment (pH of about 8.6), D-galactose reacts with galactose dehydrogenase (Gal-DH) and coenzyme I to give galacturonic acid and coenzyme II. Therefore, the amount of coenzyme II corresponds to the amount of lactose and galactose to a certain extent, and its absorbance can be determined by a spectrophotometer at 340 nm.

2.4.2.Measurement of glucose content

While glucose in goat milk by biosensor analyzer. The measurement process is as follows: firstly, a water filter membrane of 0.2 μm is used to filter the samples which are the pure goat milk and goat milk added with lactase, and then a glucose standard solution of 1 g/L is used to calibrate the biosensor. Finally, Glucose concentration was calculated by adding 25 g/L of the filtered filtrate to the biosensor. The samples were verified by repeated experiments.

2.4.3.Measurement of LHR and galactose oligosaccharide content

The LHR can be computed based on the change in lactose quantity before and after the comparison of lactase enzymolysis through the determination methods of lactose, galactose, and glucose mentioned above. In line with the conservation of mass concept, the quantity of glucose, galactose, and galactose oligosaccharides generated is equal to the amount of lactose ingested, in other words, the quantity of galactose oligosaccharide is comparable to the amount of lactose consumption subtract the amount of growth of galactose and glucose. The LHR and galactose oligosaccharide were calculated according to previous experiments (Xu et al., 2021).

2.5.Color Measurement

The color difference of goat milk is usually determined using CIE color analysis (International Commission on Illumination) which utilizes the spectrophotometer Minolta CM-5. Before analyzing the sample, a whiteboard must be used to zero and calibrate the sample. During the measuring process, the average value is determined three times. The distinction in color between the experiment and the blank group is denoted by ΔE. ΔE can be calculated according to previous experiments (Xu et al., 2021).

2.6.Organoleptic evaluation of liquid goat milk

According to Jing and Liu's (2020) method, the color, tissue state, odor, and taste of the

enzymatically hydrolyzed goat milk were organoleptically evaluated.

3. Results and discussions

3.1.Analysis of GOS and LHR from lactose hydrolysis by different lactase

As shown in Figure 1, the lactase type has a noteworthy impact on the GOS content and LHR in goat milk, and the outcomes differ according to the enzyme. In the figure, the LHR ranged from 4.696% to 80.617%, and GOS content ranged from 0.823 g/L-12.599 g/L. Among them, 5 LHRs were higher than 50%, which were E11 (54.834%), E13 (56.630%), E14 (69.635%), E5 (71.755%), E10 (80.617%). The LHR of the remaining 9 lactase enzymes were all lower than 50%, and the lowest one was E2 (only 4.696%). There were 4 kinds of galactooligosaccharides with content higher than 6 g/L, namely E11 (6.146 g/L), E9 (8.356 g/L), E10 (10.421 g/L), and E14 (12.599 g/L). The other ten enzymes had GOS contents less than 6 g/L, and the lowest was E2 (0.823 g/L). The GOS content and LHR are not always positively correlated, as can be seen from the comparison. For example, the GOS concentration of E10 is only 10.421 g/L, despite the LHR being 80.61%, suggesting a low glycoside transfer efficiency and a high enzymatic hydrolysis efficiency. On the contrary, even though E14's LHR is just 69.635%, enzymatically hydrolyzed goat milk has a 12.599 g/L GOS concentration, suggesting a poor level of enzymatic hydrolysis efficiency, Transglycoside efficiency is high.

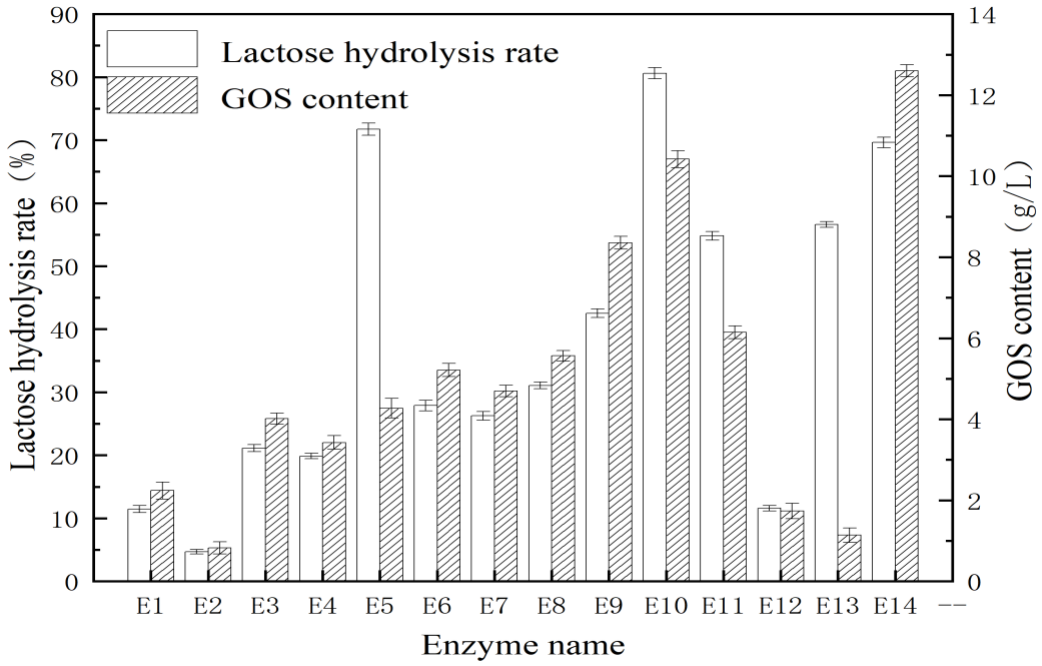

Figure 1. The impact of various lactase types on the LHR and the amount of GOS in goat milk

As can be seen from Table 1, contrasted with the goat milk's control sample color value (L=93.39±0.02, a=-2.10±0.02, b=7.1±0.03), the variation in color of the enzymatically hydrolyzed goat milk had significant changes: the milk color became darker, and most of the milk was reddish and yellow. Among them, the E8 enzymolysis milk's color became dark and yellowish green, and the E12 enzymolysis milk's color became dark and bluish green, indicating that different types of lactases possess varying impacts on the color value of goat milk. The amount that the color number is off-center from low to high is L* (92.96~90.07), a*(-1.19~-2.43), b* (8.00~5.18). The appearance of color difference is due to the

different types of lactases leading to changes in the color parameters of goat milk, and then leading to changes in the color of goat milk. The overall color difference (ΔE), can be computed, as indicated by formula 3. At ΔE =1, the minimum color variation that is discernible to the human eye is present., which is five times the threshold for visual identification. Identify it as 1NBS. The difference between the two colors is more noticeable the larger the ΔE; on the other hand, the difference is less noticeable. If it is in the range of 0 to 0.5 NBS, it means that there is little to no color variation between the two colors; If it is between 0.5 and 1.5 NBS, it indicates a small difference. If it is between 1.5-3NBS, the color distinction between the two colors can be clearly distinguished. When the color difference is greater than 3.0NBS, The most noticeable distinction between the two colors (Chen et al., 2013).

Table 1. The impact of various lactases regarding the hue of goat milk that has been hydrolyzed enzymatically.

Enzyme	L*	a*	b*	ΔE
E1	92.77±0.01	-1.80±0.01	7.3±0.03	0.72
E2	90.23±0.18	-2.43±0.01	7.44±0.10	3.19
E3	91.66±0.02	-1.19±0.01	7.98±0.03	2.15
E4	90.31±0.04	-2.47±0.01	8.00±0.03	3.23
E5	92.65±0.02	-1.71±0.07	7.37±0.034	0.87
E6	92.38±0.01	-1.53±0.01	7.65±0.02	1.29
E7	92.87±0.01	-1.83±0.01	6.99±0.01	0.59
E8	91.48±0.80	-2.12±0.13	7.54±0.02	1.96
E9	90.07±0.01	-2.25±0.02	6.73±0.39	3.34
E10	92.96±0.01	-1.98±0.01	7.20±0.01	0.46
E11	92.60±0.01	-2.10±0.01	7.51±0.04	0.89
E12	91.26±0.07	-2.38±0.01	5.18±6.38	2.88
E13	92.48±0.11	-2.12±0.03	6.77±0.16	0.97
E14	92.87±0.01	-1.97±0.01	6.85±0.14	0.59

Figure 2 shows that the color variation of goat milk is significantly influenced by the kinds of lactase. The variation in milk color caused by lactase enzymolysis ranges from 0.46NBS to 3.34NBS, among which E12 (2.88NBS), E3 (2.15NBS), and E8 (1.96NBS) have entire color disparity between 1.5NBS and 3.0NBS, respectively. The total chromatic difference of E9 (3.34NBS), E4 (3.23NBS), and E2 (3.19NBS) exceeded 3.0NBS, respectively, and the remaining eight lactases had chromatic differences of less than 1.5NBS.

From Figure 3, it is evident that the organoleptic assessment of goat milk is significantly influenced by the kind of lactase, and the sensory scores of enzymolysis goat milk range from 38.5-69.5, in which the organoleptic evaluation score with the greatest value is E10 (69.5±2.12), while the lowest score is E2 (38.5±3.54).

Among them, E2, E4, E8, E9, and E12 enzymatic hydrolyzed goat milk tastes bitter, this could be connected to the way that proteins in liquid goat milk are hydrolyzed by enzymes. (Wang et al., 2020). Pure goat milk is less sweet than goat milk made with additional lactase enzymes, which should be due to the increased sweetness of lactose after hydrolysis to produce glucose.

In summary, the enzymolysis effect of lactase on goat milk was summarized, the suitable lactases are E10, E5, E14, and E13 in order, these enzymatic hydrolysis of goat milk had higher GOS content and LHR, had less impact on organoleptic evaluation and color.

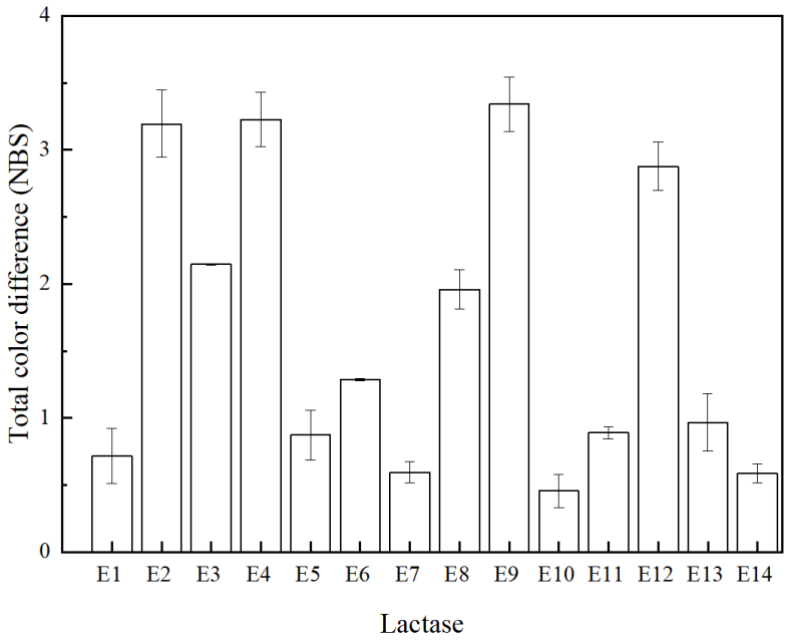

Figure 2. The impact of various lactase types on color ΔE of goat milk

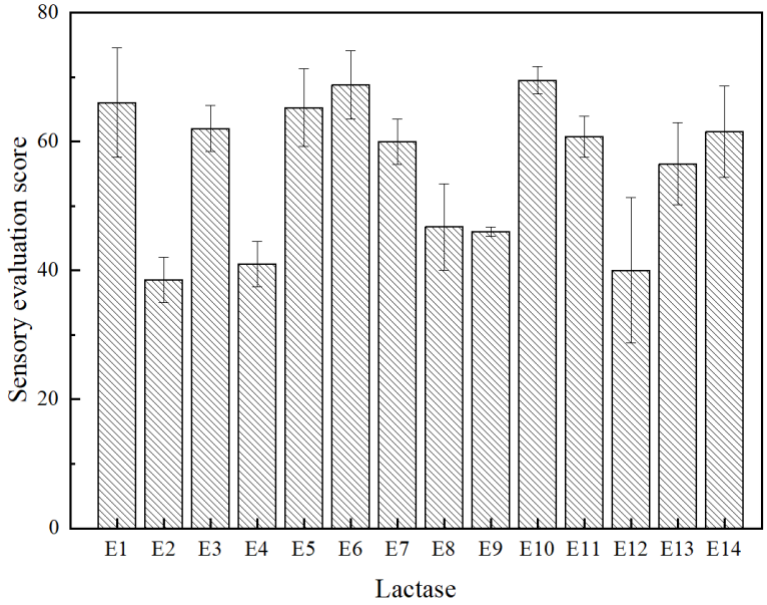

Figure 3. The organoleptic evaluation of goat milk after enzymolysis by different lactase types

3.2.Effects of different lactase combinations on GOS production from hydrolyzed lactose

Based on the test findings mentioned above, lactase E10, E5, E14, and E13 with good enzymolysis ability were selected, and the additive quantity of mixed lactase was established at 0.06%. The test findings are shown in Table 2.

As can be seen from Table 2, different lactase combinations have a noteworthy impact on the GOS content and LHR in goat milk. The GOS content varies from 12.237g /L-14.667 g/L and the LHR of goat milk varies from 69.583%-85.000%. The LHR from low to high were E10+E14 (69.583%), E10 (74.738%), E10+E5+E13+E14 (78.750%), E10+E13 (81.250%), and E10+E5 (85.000%). Production of GOS from low to high were E10 (12.237g/L), E10+E14(12.298 g/L), E10+E5+E11+E14(12.697 g/L), E10+E5 (14.476 g/L), and E10+E13(14.667 g/L). It showed that the E10+E5 had greater hydrolysis and glycoside transfer efficiency than the others, while the hydrolysis efficiency of E10+E13 was lower than that of E10+E5, and its GOS content was similar to that of E10+E5. Therefore, E10 and E5 were selected as test compound enzyme preparations.

Table 2 The impact of combining lactases on degree of hydrolysis and GOS in goat milk

Enzymes	LHR（%）	GOS（g/L）
E100.06%）	74.738±0.001	12.237±0.020
E100.03%）+E5（0.03%）	85.000±0.007	14.476±0.014
E100.03%）+E13（0.03%）	81.250±0.001	14.667±0.010
E100.03%）+E140.03%）	69.583±0.002	12.298±0.013
E100.03%）+E5（0.01%）+ E13（0.01%）+ E14（0.01%）	78.750±0.001	12.697±0.018

Different compound experimental designs were used to optimize the lactase ratio

Table 3. The setup and outcomes of the goat milk hydrolysis experiment using lactases E5 and E10

NO	A(E10)	B(E5)	LHR(%)	GOS(g/L)
1	0.667	0.333	88.817	13.911
2	1	0	87.603	13.610
3	0.5	0.5	84.521	13.115
4	0.5	0.5	84.177	12.982
5	0.25	0.75	73.831	11.168
6	1	0	87.982	13.723
7	0.75	0.25	92.166	14.509
8	0	1	44.352	4.751
9	0.333	0.667	84.751	13.177
10	0	1	39.982	3.625

A mixture test was created using DesignExpertV8.0.6 to determine the ideal complex lactase ratio. The design and outcomes of the mixed experiment are displayed in Table 3 to Table 5.

Based on the test findings shown in Table 3, the quadratic regression equations of

complex lactase (E10 and E5), LHR (Y1), and GOS content (Y2) were obtained by Design-Expert8.0.6. The quadratic regression equation is (1) and (2):

$$Y_1=86.81A+43.71B+87.61AB \quad (1)$$

$$Y_2=13.41A+4.57B+18.68AB \quad (2)$$

Table 4 demonstrates the significance of the regression equation and the no-significance of the missing fitting component, which suggests that the regression equation in place is well-fitted. The P in the table for the linear combination of influencing factors and AB is less than 0.0001, meaning that the influence on the rate of lactose hydrolysis of enzymolized goat milk is greater when E10 and E5 are raised in a linear proportion. The quadratic regression equation determination coefficient, or R2, is 96.82%, making it possible to use the model to forecast the rate at which complex lactase (E10+E5) Enzymatic hydrolyzed goat milk.

Table 5 demonstrates the significance of the regression equation and the no-significance of the missing fitting component, which suggests that the regression equation in place is well-fitted. The P in the table for the linear combination of influencing factors and AB is less than 0.01, meaning that the influence on the rate of lactose hydrolysis of enzymolized goat milk is greater when E10 and E5 are raised in a linear proportion. The quadratic regression equation determination coefficient, or R2, is 96.12%, making it possible to use the model to forecast the GOS content of goat milk hydrolyzed by complex lactase (E10+E5).

Finally, according to the examination of the regression equation, E10:E5=0.8:0.2 is the ideal complex lactase ratio. The GOS was 14.633 g/L, and the LHR was 92.208% under these circumstances. The confirmed GOS content and LHR was 14.587±0.20 g/L and 91.880±0.01%, and they were in close agreement with the estimates. The ideal lactase E10+E5 ratio found by the composite design is achievable, according to the data.

Dairy products are an important source of nutrients for the human body, providing more than 10 essential nutrients for maintaining blood health, nervous, immune systems, and promoting the growth and repair of various parts of the body. People with “lactose intolerance” are unable to efficiently break down lactose, which is the main sugar in dairy products, thus affecting the dairy industry. Therefore, reducing the amount of lactose in dairy products by hydrolyzing lactose with combined lactose enzyme.

Table 4 The LHR of goat milk by （E5+E10）was analyzed using variance

source	Degree of freedom	sum of square	Mean square	F	P	Significance
model	2	3114.53	1557.27	106.55	＜0.0001	***
Linear mixing	1	2193.5	2193.5	150.08	＜0.0001	***
AB	1	921.03	921.03	63.02	＜0.0001	***
error	7	102.31	14.62			
Lack of Fit	4	92.63	23.16	7.18		
Pure error	3	9.68	3.23			
sum	9	3216.84				

Table 5 The GOS content of goat milk by complex lactases （E5+E10）was analyzed using variance

source	Degree of freedom	sum of square	Mean square	F	P	Significance
model	2	134.11	67.06	86.68	＜0.0001	***
Linear mixing	1	92.25	92.25	119.25	＜0.0001	***
AB	1	41.86	41.86	54.12	0.0002	**
error	7	5.42	0.77			
Lack of Fit	4	4.77	1.19	5.51	0.0963	
Pure terror	3	0.65	0.22			
sum	9	139.53				

Lactase can also use glucose and galactose to synthesize GOS through glycoside transfer, which can reduce the secretion of inflammatory factors and relieve inflammation and can be digested and utilized by colon microorganisms. It is an important nutrient and immune regulatory substance and is intimately linked to the early growth and development of newborns and young children. The primary determinant of GOS content, according to Ruiz-Matutte et al (2012), was lactase's hydrolysis rate of lactose breakdown. When lactose has hydrolyzed 75–90% of it, the amount of GOS is gradually increased, and when the LHR exceeds 99%, the content of GOS begins to gradually decrease. The GOS content was the highest when the LHR 99.3%, and the lowest when the LHR was 99.9%.

At present, there are few studies on the decomposition of lactose in goat milk at home and abroad. Most studies are on the decomposition of lactose in milk by lactase, and the lactase used is single enzyme: It has been found that when the enzyme amount is 2500 U/L, the hydrolysis temperature is 40°C, and the hydrolysis temperature is 2h, the hydrolysis effect of lactose in milk is the best, and the hydrolysis rate is 74.50% (Wang et al., 2013); When the lactase dosage is 0.16%, the temperature is 45.3°C, and the pH4.95, the predicted hydrolysis rate of lactose in milk powder is 76.87% (Rong et al., 2019). These studies are based on single enzyme that changes the amount of addition, temperature, and other conditions. Therefore, this study first studied the effects of 14 lactase enzymes and their ratios on the production of LHR and GOS in goat milk, and screened out the more suitable enzymes: E10, E5, E14 and E13. Then, the compound lactase combination was applied to goat milk, and the compound experimental design was adopted to optimize the ratio and obtain the optimal ratio (E5: E10=1: 4), the GOS content was 14.587±0.20g/L, LHR was 91.880±0.01%, and the transglycosylation and hydrolysis rates were higher than those of single enzymes.

4. Conclusions

Using goat milk as raw material, the effects of 14 lactose enzymes and their ratio on LHR and GOS production were studied. A considerable difference was seen between the impact of various lactose enzymes on the GOS and LHR, with GOS ranging from 0.82 g/L to 12.60 g/L and LHR ranging from 4.70% to 80.62%. The selected E10, E5, E14, and E13 were more suitable than other enzymes. The best effect is achieved when lactase E10 and E5 are in a ratio of 4:1, the GOS content was 14.587±0.20 g/L and the LHR was 91.880±0.01%, respectively.

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Research Article

ANTI-OBESITY PROPERTIES OF KIDNEY BEAN (PHASEOLUS VULGARIS) HUSK PEPTIDES IN DIET-INDUCED OBESE RATS: A FUNCTIONAL AND METABOLIC STUDY

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Abstract

Obesity is a global problem that is spreading at an incredible rate. Bioactive peptides of plant origin, e.g., extracts of *Phaseolus vulgaris* beans, are currently considered as one of the possible ways to treat this disease. In this work, we shed a light on the peptides from *P. vulgaris* husks, evaluating their anti-obesity properties for the first time. By acetic acid treatment of the kidney bean husk extract, we obtained hydrolysis-derived peptides and fed them to rats with diet-induced obesity. During the experiment, we measured rats' weight, and compared the weight of main organs right after euthanasia. Biochemical blood parameters were measured using specialized biochemical analyzers. Serum and brain serotonin levels were determined spectrofluorometrically. It was determined that group which consumed kidney bean peptides had improved visceral, brown and subcutaneous adipose tissue weights. In addition, this group showed improvements in total protein, total and indirect bilirubin, creatinine, aspartate aminotransferase, alpha-amylase, glucose, alanine aminotransferase, gamma-glutamyl transferase levels, and had improved serotonin levels. We believe that the anti-obesity properties of our peptides are directly related to their hypoglycemic activties. It may also be related to the already discovered antioxidant activity, or anti-inflammatory properties that our peptides may have.

1. Introduction

Obesity and overweight are serious health complications that are rapidly spreading around the world. In 2022, 43% of people 18 y.o. or above were overweight (body mass index (BMI) >25 kg/m2), and 16% were obese (BMI >30 kg/m2), according to the World Health Organization (WHO). The situation for children is more serious: over 390 million children between the ages of 5 and 19 were overweight, with 160 million of them being obese (Obesity and overweight, 2025). The World Obesity

Federation estimates that there will be 1.53 billion obese adults in 2035. Furthermore, the rate at which obesity is spreading is independent of a nation's level of prosperity and is a natural occurrence in high-, middle-, and low-income nations (World Obesity Atlas 2024: No area of the world is unaffected by the consequences of obesity, 2024).

Obesity can be characterized as a complex disease, which can affect practically every organ system in the body. Moreover, the level of impairment of a particular body organ may vary across the population, since the complexity of this disease revolves around individual trait of each organism. The most common obesity-related health complications are chronic kidney disease, acute kidney injury, heart diseases, difficult breathing, loss of mobility, increased blood pressure, development of type 2 diabetes mellitus, fatty liver disease and many-many other (Anderson and Shashaty, 2021). As of 2022, there were 7 officially approved drugs for treatment of obesity: phentermine, phentermine-topiramate, naltrexone-bupropion, orlistat, liraglutide, semaglutide and tirzepatide, however, these medications have many negative adverse effects and limitations, for example their prescription is not recommended for pregnant or lactating women (Gudzune and Kushner, 2024). There is a popular opinion, that glucagon-like peptide receptor agonists (GLP-1 RA), such as abovementioned liraglutide, semaglutide and tirzepatide, is a very promising type of anti-obesity drugs, research of which may be the key to the ideal anti-obesity medication development (Bailey *et al*., 2023). In addition to that, anti-obesity and anti-diabetes activities were found in food-derived peptides, which may act as inhibitors or agonists of various receptors in human body. Plant-derived bioactive peptides, for example, are very promising candidates in this aspect, because plant material is cheap, widely available, and, in contrast to animal-derived peptides, it doesn't rise any ethical or religious concerns (Suryaningtyas and Je, 2023).

Among a wide variety of plants, kidney bean (*Phaseolus vulgaris*) is known from folk medicine for its antiglycemic, antidiabetic and anti-inflammatory activities (Helmstädter, 2010). Moreover, modern studies on mice and rats prove the presence of such effects in *P. vulgaris* beans and bean husk aqueous extracts (Bhide *et al*., 2022; Kyznetsova *et al*., 2015; Yurchenko *et al*., 2021). Recently, Shchypanskyi *et al.* have discovered prominent anti-oxidant activities of common bean husk-derived peptides in in vitro studies (Shchypanskyi *et al*., 2025). Our study was aimed to develop this topic further, investigating the effect of peptides from bean husk extracts on the obesity progression in rats, emphasizing the effect on body weight, possible morphometric changes of organs, alterations of main biochemical parameters in serum and shifts in serotonin levels.

2. Materials and methods

2.1. Bean Husk Extract Preparation

With a few minor modifications, *P. vulgaris* bean husk extracts were produced following the technique outlined by Kyznetsova *et al*. (Kyznetsova *et al*., 2015) The husks of kidney beans that we utilized came from a local field in Kyiv region, Ukraine. One litre of boiling distilled water and around 132 grams of dried, powdered bean husks were mixed, and this mixture was then placed in a covered container for twenty minutes. After that, we left it to cool overnight. After cooling and filtering, the mixture was centrifuged for 10 minutes at 1,000 g. A laboratory freeze-drier LyoQuest (Telstar, Barcelona, Spain) was used to lyophilize the resultant supernatant, producing around 8 grams of freeze-dried material.

2.2. Peptide Extraction and Purification

In our study we aimed to obtain non-native peptides by acidic hydrolysis of proteins derived from an extract. To obtain these peptides, 8 grams of lyophilized bean husk extract were mixed with 250 mL of 1 M acetic acid (Product code 502000, CARLO ERBA Reagents SAS, Val de Reuil, France). The mixture was hydrolyzed for 1 hour under constant agitation. Following this, the solution was heated to 100 °C, boiled for 1 hour, and then cooled to room temperature. The resulting suspension was

centrifuged at 2,800 g for 45 minutes. After that, the collected supernatant was dialyzed in order to remove impurities of non-protein nature, and then the purified fraction was lyophilized.

2.3. Animal Housing and Ethical Compliance

For the experiment we have chosen 30 non-linear albino male rats. Throughout the study, all animals were housed in the accredited vivarium of Taras Shevchenko National University of Kyiv. All procedures involving animals were conducted in compliance with ethical guidelines and regulations for the use of laboratory animals, as outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Additionally, the procedures adhered to Ukrainian legislation, specifically the law enacted on February 21, 2006 (No. 3447-IV), titled "On the Protection of Animals from Cruel Treatment."

The animals were maintained under the following conditions: temperature 20–24 °C, humidity 30–70%, natural light/dark cycle. Initially, the rats were randomly divided into three groups of 10 animals each. Each group was placed in separate cages, with five animals per cage, and provided with free access to water and standard rodent chow in pellet form.

2.4. Obesity Induction in Rats

After three days of acclimatization and initial weighing of the animals, we initiated the process of obesity induction using a high-calorie diet. The high-calorie chow was prepared by mixing the following ingredients in 2 liters of hot water to soften them: standard rodent chow pellets (60%), chicken eggs (10%), lard (10%), crushed peanuts (5%), white sugar (9%), dry milk powder (5%), and sunflower oil (1%) (Yurchenko *et al*., 2021). From this mixture, solid spheres approximately 4 cm in diameter were formed. After drying, these spheres were used as high-calorie food for the experimental groups.

The obesity modelling experiment lasted 12 weeks and was divided into two stages. In the first stage, all rats were weighed, randomly divided into three groups of 10 animals each, and housed in separate cages with five animals per cage. The control group was provided with filtered water and standard rodent chow pellets *ad libitum*. The other two groups were given filtered water and the custom high-calorie food described above, also *ad libitum*. Throughout the experiment, the rats' weights were measured once every two weeks. The second stage began at the 6th week. One of the experimental groups receiving the high-calorie diet were additionally provided with aqueous solutions of *P. vulgaris* peptides (200 mg/kg of body mass) in place of drinking water, *ad libitum*, yet the feeding regimen for the remaining animal groups remained unchanged. According to the weight of animals, weekly rate of body mass gain compared to the initial weight was calculated using the formula:

$$\text{Body mass gain (\%)} = 100 * (\text{Weight}_{\text{week n}} - \text{Weight}_{\text{week 0}}) / \text{Weight}_{\text{week 0}} \quad (1)$$

where $\text{Weight}_{\text{week n}}$ – animals' weight on corresponding number of week (n), $\text{Weight}_{\text{week 0}}$ - animals' initial weight at the beginning of experiment (week 0)

2.5. Euthanasia and Sample Collection

Euthanasia of the experimental animals was performed on 12th week after the start of the experiment. To collect an adequate volume of blood samples and avoid the potential influence of anesthetics on biochemical parameters in blood and tissues, the decapitation method of euthanasia was employed. Immediately after decapitation, blood samples (approximately 10 mL per animal) were collected for serum preparation. Subcutaneous fat, visceral fat, brown fat, spleen, and heart were isolated and weighed.

For the preparation of brain homogenates, 1 g of brain samples were mixed with 50 mM Tris-HCl buffer (pH 7.4) (Product code C4706, Sigma-Aldrich, Saint Louis, USA) containing 140 mM NaCl (Product code A57006, ThermoFisher Scientific, Waltham, USA) in a mass ratio of 1:9 (organ to buffer). The tissues were homogenized at +4 °C using hand-held homogenizers (Product code 11799, Reichelt

Chemietechnik GmbH+Co., Heideilberg, Germany). The resulting homogenate was centrifuged at 600 g for 15 minutes, and the supernatant was centrifuged again at 10,000 g for another 15 minutes. Rat serum samples were prepared by centrifuging freshly drawn blood at +4 °C and 1,300 g for 20 minutes. The obtained homogenates and serum samples were aliquoted and stored frozen at −20 °C.

2.6. Biochemical analysis

Determination of the main biochemical parameters, namely total protein (TP), glucose, total cholesterol (TC), direct, indirect and total bilirubin, urea, creatinine, alanine aminotransferase (ALT) aspartate aminotransferase (AST), α-amylase, alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) in rat serum were performed in a private laboratory using cobas c311 analyzer (Roche Diagnostics, Basel, Switzerland).

2.7. Serotonin content analysis

Serum and brain samples were thawed at 37 °C, after which 0.4 M perchloric acid (Product code 1005191001, Merck, Darmstadt, Germany) was added in a 1:5 ratio. The resulting mixture was incubated at 4 °C for 60 min. After that, the solution was centrifuged for 5 min at 800 g in a cooled rotor. After centrifugation, the supernatant was removed and the pH was adjusted to 5-6 with 2 N KOH (Product code P4494, Sigma-Aldrich, Saint Louis, USA). The samples were centrifuged again for 5 min at 800 g in a cooled rotor. The resulting supernatant was applied to a pre-equilibrated with 0.01 M Na-phosphate buffer solution (pH 6.2) (Product code 76847, Sigma-Aldrich, Saint Louis, USA) column with CM-Sepharose (Product code 17127703, Cytiva Life Sciences, Marlborough, USA). The elution was carried out at room temperature with buffer solution (0.03 M Na-phosphate buffer solution, pH 6.2) (Product code 76847, Sigma-Aldrich, Saint Louis, USA) which eluted serotonin. To 1 ml of the eluted fraction with serotonin was added 0.3 ml of 11.6 M HCl (Product code 528525 CARLO ERBA Reagents SAS, Val de Reuil, France). The measurements were performed using a spectrofluorophotometer RF-6000 (Shimadzu Europa GmbH, Duisburg, Germany) at an excitation wavelength of 295 nm and an absorption wavelength of 550 nm against a blank containing distilled water instead of the sample (Yurchenko *et al*., 2021).

2.8. Statistical analysis

Statistical analysis of experimental data was achieved using Statistics Kingdom software Statistics Kingdom, 2025). In order to access the normality of distribution of obtained data, the Kolmogorov-Smirnov Test of Normality was conducted. Significance of differences between the groups was assessed via one-way analysis of variances (ANOVA) with a Tukey's post hoc test at $p<0.05$. Data in figures and table is presented as mean ± standard error of mean (M ± m).

3. Results and discussions

Rapid and constant weight gain is a direct major indicator of overweight and obesity development. In this study, we examined weight gain rates in rats that consumed standard chow (Control), rats with diet-induced obesity (DIO) that ate high-calorie custom-made chow, and DIO group that also consumed aqueous solutions of peptides (DIO+P). As shown in Figure 1a, all groups exhibited continuous weight gain throughout the experiment, but at different rates. Between weeks 2 and 12, the control group gained weight at an average rate of 11.2 ± 0.7 % of initial body weight every two weeks, with a maximum increase of 16.2 ± 1.0 % at week 6 and a minimum increase of 8.6 ± 0.4 % at week 12. By the end of the experiment, the final body weight in this group was 81.4 ± 7.3 % higher than the baseline.

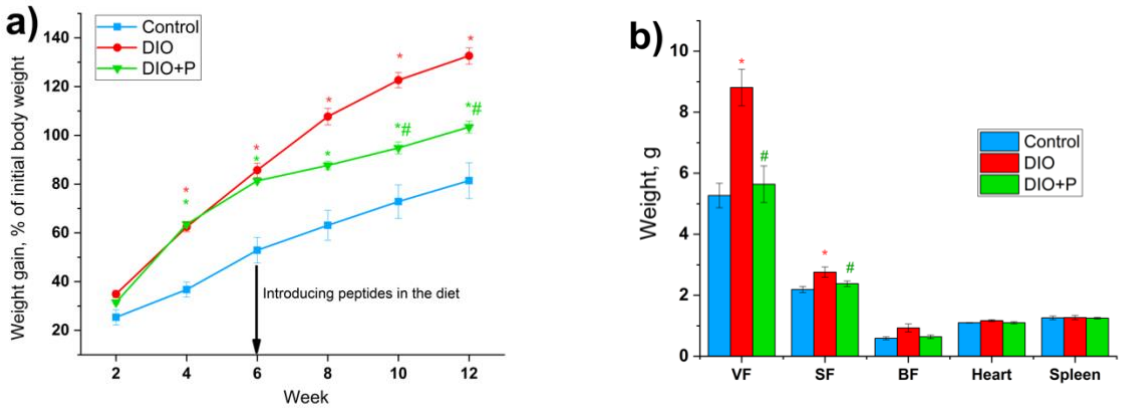

Figure 1. Weight gain rates (a) and final weights of internal organs (b). VF – visceral fat, SF – subcutaneous fat, BF- brown fat. * - p< 0.05 (compared to control group), # - p< 0.05 (compared to DIO group), n=10

In the DIO group, weight gain averaged 19.5 ± 1.0 % every two weeks, with a peak increase of 27.6 ± 0.5% at week 4 and a minimum gain of 10.0 ± 0.3 % at week 12. The final weight in this group was 132.6 ± 3.4 % above baseline. In contrast, the DIO+P group had a lower average weight gain of 14.4 ± 0.7 %, with a peak increase of 32.0 ± 0.3 % at week 4 and a minimum of 6.3 ± 1.0 % at week 8. By the conclusion of the study, the final body weight in the DIO+P group was 103.4 ± 2.5 % of the baseline values.

These findings suggest that peptide supplementation reduced the rate of weight gain, resulting in much lower total weight gain compared to DIO group. These results are supported by previous papers, that demonstrate the weight-reducing effects of *P. vulgaris* dry extracts in both animal models (Carai *et al*., 2011; Fantini *et al*., 2009) and human subjects (S. Wang *et al*., 2020). Moreover, our results prove the potential of kidney bean husk-derived peptides to be considered as bioactive anti-obesity compounds along with whole kidney bean extracts, yet the main advantage of our peptides is their cost-effectiveness, because bean husks aren't as valuable as whole beans.

Changes in the weight of internal organs during obesity progression serve as one of major indicators of its development, with adipose tissue mass being most informative. In this study, visceral fat weight in the control group was approximately 67% lower than in the DIO group (5.3 ± 0.4 g and. 8.8 ± 0.6 g, respectively) (Fig. 1b). In contrast, visceral fat weight in peptide-supplemented group was comparable to that of the control group, being 5.6 ± 0.6 g. A similar trend was observed for subcutaneous fat. In the control group, subcutaneous fat weight was 2.2 ± 0.1 g which is approximately 26% lower than in the DIO group (2.8 ± 0.2 g). In the DIO+P group subcutaneous fat weight was approximately 2.4 ± 0.1 g, respectively, which is close to the control values. Brown adipose tissue weight in the control group averaged 0.6 ± 0.1 g, which was 58% lower than in the DIO group (0.9 ± 0.1 g), whereas in the DIO+P group (0.6 ± 0.1 g), it did not differ from the control group. These findings are similar to previous researches, in which the fat-reducing effects of *P. vulgaris* extracts were proved. In a study by Wang *et al.* (S. Wang *et al*., 2020), individuals who consumed *P. vulgaris* extract for 35 days had an 8% less adipose tissue mass compared to control values. Similarly, Neil *et al.* (Neil *et al*., 2019) reported that consumption of *P. vulgaris* beans led to a 12% reduction in visceral fat and a 28% reduction in subcutaneous fat in obese C57BL/6 mice. Organ weights such as the heart and spleen are also related to obesity progression, because obesity induces structural cardiac changes, namely left ventricular enlargement (Stencel *et al*., 2023), and hypertrophy of spleen, due to its role in low-density lipoprotein clearance (He *et al*., 2022).

However, we did not observe statistically significant differences in heart or spleen weights under our experimental conditions.

As shown in Table 1, the biochemical analysis of serum revealed the most pronounced difference in ALP activity between the control and DIO groups, with the latter exhibiting a 321% increase. In the DIO+P group, ALP activity was 231% higher than in the control group, suggesting a positive effect consumption of peptides in regulation of ALP activity. ALP is one of the key liver enzymes, alterations in levels of which serve as biomarkers for liver dysfunction. Recent studies have reported an association between elevated ALP activity and obesity (Jalili *et al*., 2022). Although the precise mechanism is still unclear, it is possible that obesity may lead to excessive release of this enzyme in adipose tissue (Khan *et al*., 2015). The effect of *P. vulgaris* consumption on ALP levels was also demonstrated in a study by Forster (M. Forster, 2012), in which a diet with kidney beans significantly reduced ALP levels in obese dogs.

Table 1. Rat serum biochemical parameters

	Control	DIO	DIO+P
ALP, U/L	126.8 ± 22.5	(407.6 ± 17.3)*	(293.0 ± 9.8)*#
TC, mmol/L	2.0 ± 0.1	(3.5 ± 0.2)*	(3.0 ± 0.1)*#
Glucose, mmol/L	4.3 ± 0.1	(6.5 ± 0.2)*	(4.4 ± 0.1)#
GGT, U/L	23.0 ± 1.4	(11.8 ± 0.7)*	(19.0 ± 0.6)*#
Total protein, g/L	76.2 ± 2.0	(88.8 ± 1.2)*	(79.6 ± 1.1)#
Total bilirubin, μmol/L	10.3 ± 0.2	(8.8 ± 0.3)*	(10.2 ± 0.1)#
Direct bilirubin, μmol/L	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
Indirect bilirubin, μmol/L	9.1 ± 0.2	(7.4 ± 0.3)*	(8.5 ± 0.1)#
Creatinine, μmol/L	50.6 ± 0.9	(59.2 ± 0.9)*	(53.4 ± 1.5)#
Urea, mmol/L	6.3 ± 0.1	5.9 ± 0.3	6.3 ± 0.2
ALT, nmol/L	79.6 ± 6.5	(69.1 ± 2.6)*	(78.8 ± 1.6)#
AST, nmol/L	260.8 ± 4.9	(218.8 ± 12.8)*	(253.4 ± 2.2)#
α-amylase, U/L	636.0 ± 14.6	(758.4 ± 27.0)*	(688.6 ± 3.9)#

* - $p < 0.05$ (compared to control group), # - $p < 0.05$ (compared to DIO group), n=5

Visible differences between the control and DIO groups were also observed in serum TC and glucose concentrations, as well as GGT activity. TC is an important indicator of lipid metabolism and it is closely associated with obesity and metabolic syndrome. In the DIO group, total cholesterol levels were 77% higher than in the control group and in the DIO+P group this increase was 54% (Table 1). These findings can be supported by previous research by Nchanji and Ageyo (Nchanji & Ageyo, 2021), who reported a 19% reduction in total cholesterol levels in volunteers consuming *P. vulgaris* extract. However, clinical trials conducted by Singh *et al.* (Singh *et al*., 2024) on the commercial kidney bean extract Phaseolean® did not report significant alterations in cholesterol levels.

As shown in Table 1, glucose levels in the DIO group were 50% higher than in the control group, indicating a direct correlation between blood glucose concentration and obesity progression in our study. At the same time in the DIO+P group glucose concentration was just 3% higher – almost at the same level as in non-obese control rats. High blood glucose concentration is a major marker of carbohydrate metabolism disorders, including type 2 diabetes, which is closely related to obesity. *P. vulgaris* is widely recognized as a source of α-amylase inhibitors, which contribute not only to their hypoglycemic effects but also to their anti-obesity properties in general (Peddio *et al*., 2022). In a recent study, *P. vulgaris* extract demonstrated dose-dependent glucose-lowering effects, but, unlike our findings, glucose levels in extract-fed rats

did not return to control values (Almuaigel *et al*., 2017).

In our study, GGT activity in the DIO group was 48% lower than in the control group (Table 1). In the DIO+P group, GGT activity was reduced by 17%, compared to control values. GGT is one of the most obesity-associated liver enzymes. Elevated levels of this enzyme are often observed in overweight individuals (Jalili *et al*., 2022). A decrease in GGT activity in obese rats may be a result of dysfunctional antioxidant system, as this enzyme plays a critical role in the glutathione regeneration cycle (Bai *et al*., 2022). Similar findings were reported in a study, where four-month consumption of *P. vulgaris* extract by individuals with type 2 diabetes led to an increase in their GGT levels (Feng *et al*., 2022). However, the mechanism responsible for the reduction in GGT activity observed in obese rats still remains unclear.

We observed less pronounced changes in TP, total and indirect bilirubin, creatinine, ALT, AST, and α-amylase levels. In the DIO group, total protein levels were 16% higher than in the control group. At the same time, in the DIO+P group this increase was only 4%(Table 1). The elevation in TP levels observed in obese rats may be result of increased levels of circulating acute-phase proteins, inflammatory cytokines, and tissue-derived proteolytic products, because these molecules are usually associated with obesity-induced inflammation. The ability of *P. vulgaris* extracts to lower total protein levels was demonstrated in a rat model of induced diabetes (Khatija & Marikkar, 2022). Our findings support this evidence by confirming a similar effect in our obesity model.

Serum total bilirubin levels in the DIO group were nearly 14% lower than in the control group, while DIO+P group didn't show statistically significant differences from control values, with reductions of only 1% (Table 1). Similarly, concentration of indirect bilirubin was 18% lower in the DIO group compared to controls, while in the DIO+P group, this reduction was approximately 5% (Table 1). However, we did not observe any significant changes in direct bilirubin levels among our experimental groups. These findings are supported by previous research by Siddiq *et al*. (Siddiq *et al*., 2018), who reported no effect of kidney bean extract on total and direct bilirubin levels in healthy rabbits. Similarly, Patel *et al.* (Patel *et al*., 2024) found that anti-obesity pellets containing kidney beans had no significant effect on total bilirubin levels in Sprague Dawley rats.

Obesity is usually associated with impaired kidney function, which can be assessed through various biomarkers, including serum levels of creatinine and urea, both of which are known as indicators of renal dysregulation (Abdelfattah *et al*., 2024). As shown in Table 1, creatinine concentrations in the DIO group were 17% higher compared to the control group. In the DIO+P group, creatinine level was similar to the control group, showing 6% increase. In contrast, urea levels of DIO and DIO+P groups had no significant difference, compared to the control group (Table 1). These findings are consistent with the results of Wang *et al*. (S. Wang *et al*., 2020), who observed no significant changes in creatinine or urea levels after bean extract consumption by obese human subjects. Nonetheless, Abdelfattah *et al*. (Abdelfattah *et al*., 2024) reported elevated creatinine and urea levels in obese rats, but supplementation with a *P. vulgaris* extract-containing mixture reduced these markers to baseline levels.

ALT and AST are commonly used biomarkers of liver function, and their blood concentrations are closely associated with obesity progression. There is a direct correlation between elevated ALT and AST levels and the severity of obesity, mostly due to obesity-related liver dysfunction (Jalili *et al*., 2022). Although most studies report increased ALT and AST levels in obesity, our findings showed lower concentrations of these enzymes in obese rats compared to controls. As shown in Table 1, ALT levels in the DIO group were 13% lower than in the control group. Meanwhile, the DIO+P group exhibited reduction of 1%, compared to the control. Similarly, AST levels in the DIO group were 16% lower than in the control group, but in the DIO+P group AST concentration was reduced only by 3%, with no significant difference from control values (Table 1). The reduction in liver enzyme concentrations could

be related to metabolic adaptations in the liver without its' significant damage. The possible explanation of this phenomenon is a downregulation in the synthesis of these enzymes or alterations in their release into circulation, which may occur in the early stages of metabolic dysfunction without significant liver injury (Zheng *et al*., 2023).

In our study, α-amylase activity in the DIO group was increased by 19% compared to control values, while in the DIO+P group this indicator was increased by 8% (Table 1). Alpha-amylase is a key enzyme involved in carbohydrate metabolism. Recent studies indicate that obesity leads to increased circulating α-amylase activity, mainly due to overexpression of the Amy2 gene, which regulates pancreatic amylase production (Azzout-Marniche *et al*., 2019). Our results do not differ from previous studies that showed that *P. vulgaris* extract can significantly reduce α-amylase activity in the blood - by about 28% in an obesity model - suggesting a potential regulatory effect of bean compounds on amylase activity (Micheli *et al*., 2019). This inhibitory effect is likely due to biologically active peptides with α-amylase-inhibitory effects. As we mentioned above, P. *vulgaris* is a rich source of α-amylase inhibitors, such as phasolamine, which can reduce postprandial glucose levels (Peddio *et al*., 2022).

Summarizing the results of the biochemical analysis DIO+P group had a significant effect on most of the assessed parameters. The mechanisms of peptide effects on these biochemical markers may be complex. As we have already mentioned, proteins with α-amylase-inhibitory properties from *P. vulgaris* are able to regulate postprandial glucose levels by reducing carbohydrate digestion and absorption (Almuaigel *et al*., 2017; Peddio *et al*., 2022). Such regulation of glucose homeostasis may, in turn, reduce metabolic stress on key organs, including the liver and kidneys, contributing to improved lipid metabolism, enzymatic function, and systemic inflammation. In addition, the bioactive peptides present in legumes have been shown to have antioxidant and anti-inflammatory effects, which may also contribute to the results obtained (Matemu *et al*., 2021; Shchypanskyi *et al*., 2025). Although the exact molecular pathways underlying these effects remain unclear, it is possible that the bioactive peptides exert their effects through a combination of direct enzyme inhibition, modulation of inflammatory signalling, and enhancement of antioxidant defence systems.

Serotonin, or 5-hydroxytryptamine (5-HT) is very important neurotransmitter, which regulate many processes in our bodies, but in context of obesity its' key role lies in energy balance and appetite regulation (van Galen *et al*., 2021). Up to 10 % of total body serotonin is synthetized in the brain, mostly in raphe nuclei. However, these quantities are enough to regulate food intake and control appetite – there is an inverse correlation between brain serotonin levels and food consumption (Conde *et al*., 2023). In contrast, nearly 90 % of total serotonin, also known as peripheral serotonin is synthetized by enterochromaffin cells in the gut, and its effect on the energy regulation is the opposite to central (brain) serotonin. There are reports, suggesting that high peripheral serotonin levels in blood are related to gain weight, probably by inducing gluconeogenesis in liver, and inducing adipogenesis in adipocytes (Namkung *et al*., 2015). In our study, the brain serotonin levels in the DIO group were significantly reduced, measuring 105.6 ± 3.2 ng/mg of protein, which is approximately 2.5 times lower than in the control group (259.6 ± 12.9 ng/mg of protein) (Fig. 2A). In contrast, DIO+P group exhibited serotonin levels of 334.7 ± 13.4 ng/mg of protein, which is nearly 1.3 times greater compared to the control group. These results support the generally supported opinion about the positive impact of brain serotonin levels on appetite and weight gain reduction. In one of previous studies, serotonin levels in obese rats' brains were decreased by 1.42 times, compared to the control, but rats that consumed *P. vulgaris* beans had the same values as control rats (Yurchenko *et al*., 2021).

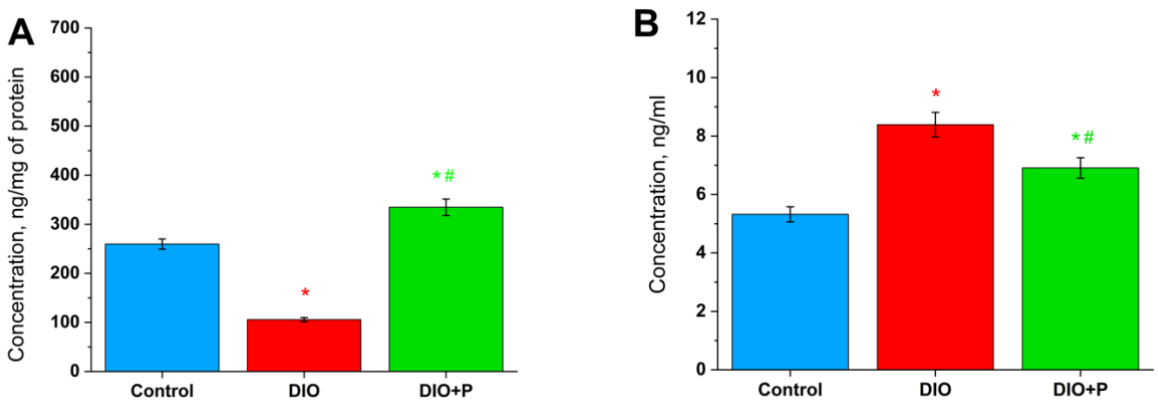

Figure 2. Concentrations of serotonin in rats' brain (a) and sera (b). * - p< 0.05 (compared to control group), # - p< 0.05 (compared to DIO group), n=10

In the DIO group serum serotonin was 8.4 ± 0.4 ng/ml, which is a 1.6-fold increase relative to the control group (5.3 ± 0.2 ng/ml). Meanwhile, the DIO+P group showed a relatively slight increase, with serotonin levels reaching 6.9 ± 0.2 ng/ml, approximately 1.3 times higher than in the control group (Figure 2B). Our results are in line with previous studies and common thought about obesity stimulating effect of increased peripheral serotonin levels (Nonogaki, 2022). However, in one of the recent studies there was a 1.8 time decrease in obese rats' serum serotonin levels, compared to the control, which differs from results of our and other studies (Yurchenko *et al*., 2021).

Our findings indicate that obesity is associated with a significant reduction of brain serotonin and simultaneous increasing of peripheral serotonin concentrations, and aligns with results of other studies on this topic (Namkung *et al*., 2015; Young *et al*., 2018). Serotonin levels greatly depend on the body inflammation status, microbiome composition, stress and many other factors (Cîmpeanu *et al*., 2025). The potential mechanism behind these effects remains unclear, yet there are a few possible explanations. For example, Buey et. al. (Buey *et al*., 2023) highlighted the possible mechanisms of milk bioactive peptide influence on serotonergic system, among which the ability of peptides to regulate tryptophan (which is 5-HT precursor) intake by gut cells, as well as to influence the activity of key 5-HT synthesis enzymes. Moreover, these peptides can possibly form complexes with 5-HT, regulating its potential, and influence gut microbiota, role of which in obesity development shouldn't be underestimated. Another potential mechanism may lie in the upregulation of 5-HT receptor synthesis by bioactive peptides, and facilitation of 5-HT binding to its receptor in their presence (J. Wang *et al*., 2022). Soy-derived peptide deprestatin was found to influence serotonin release and subsequent 5-HT_{1A} receptor activation, most likely by transmitting signal from intestinal tract to brain via vagus nerve (Mizushige, 2021). However, in order to describe precise mechanism of *P. vulgaris* peptides' effects further researches need to be conducted in the future.

4. Conclusions

In this study, we analyzed the effect of novel peptide group obtained from *P. vulgaris* husks on the organometric and biochemical parameters of rats with induced obesity. Summarizing the results of our work, we can say that these peptides have anti-obesity properties to a certain extent: we found a decrease in the rate of weight gain, and weight of adipose tissue, alignment of most biochemical parameters to control values and improved levels of serotonin in serum and brain. The exact mechanisms of the peptides' effect on all studied parameters are not yet known, but in our opinion, the decrease in weight gain and improved biochemical

parameters may be mainly a result of hypoglycemic effect inherent to *P. vulgaris* extracts. In addition, antioxidant, and potential anti-inflammatory properties of our peptides may be related to this as well.

The results of our work may also indicate that peptides from bean husks, having an advantage from an economic point of view, exhibit the same activities as bean extracts at almost the same level. These data can be taken into account in the development of new drugs based on bioactive peptides, since the cheapness of bean husks makes them an attractive type of raw material.

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Bio-Ethical certificate

MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE, THE NATIONAL UNIVERSITY NAMED AFTER TARAS SHEVCHENKO, 01033, Kyiv, Volodymyrska St., 60, **Protocol No. 7** of November 21, 2024, Committee on Bioethics of Scientific Research, **date 21. 11.2024.** *The Committee on Bioethics in Scientific Research at Taras Shevchenko National University of Kyiv (hereinafter referred to as the Committee) at its meeting (Protocol No. 7 of November 21, 2024) reviewed the materials of the dissertation research "The effect of exogenous peptides of various origins on the development of experimental obesity in rats" by graduate student Serhii Andriiovych Shchypanskyi. According to the materials submitted to the Committee for consideration, the research will be conducted in 2025, with Oleksii Mykolaiovych Savchuk as the scientific supervisor. The materials contain all the documents necessary for making a decision: an abstract, an application for permission to use laboratory animals with a detailed description of the work protocol. The research will be carried out taking into account existing bioethical and scientific standards for conducting dissertation research involving laboratory animals.*

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Research article

SENSORIAL EVALUATION OF DIFFERENT TYPES OF LEAF-WRAPPED SILVER POMFRET: A STUDY OF GUJARAT'S TRIBAL COMMUNITIES

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Pomfret.

Abstract

This study examined how plant leaf wraps affect steamed fish's sensory qualities, customer preferences, and acceptance. Leaf-wrapped samples were rated on appearance, texture, saltiness, juiciness, and flavour using a hedonic scale. Leaf wrapping considerably affected sensory qualities, with *Musa paradisiaca, Curcuma longa*, and *Tectona grandis* being the most preferred materials because they improved fish look, moisture retention, and flavour. *M. paradisiaca* preserved juiciness, while *C. longa* and *T. grandis* added colour and aroma. *P. betel* was least liked due of its bitter phenolic taste. Flavour, saltiness, and juiciness determined consumer preference, according to MCA. This study shows that plant leaves can be used as biodegradable food wrappers with sensory and environmental benefits. The findings on consumer perceptions of leaf-wrapped fish may affect food sector marketing and product development. Future research should examine fish-leaf constituent, biochemical interactions and their antibacterial effects on food preservation and safety.

1. Introduction

Fish are good natural food sources for humans, and because of their excellent taste and high digestibility, they are preferred as a perfect diet (Abelti, 2016). Fishes are highly significant and nutritious food sources around the world and are among the best sources of proteins, vitamins, and minerals (Ruxton, 2011). Fish constitute a low-cost form of animal protein that is consumed worldwide (Fawole et al., 2007). It is an important part of many people's diets and typically contributes to a healthy lifestyle. A study conducted by Idris et al., (2010) concluded that fish protein currently takes precedence over other animal proteins and that its composition compares favourably to that of other amino acid supplements. Fish contains several nutrients, such as vitamin D, selenium, and iodine. It is high in protein and low in saturated fats (Ruxton, 2011). Fish are major sources of thiamine and riboflavin, as well as minerals, phosphatides, sterols, enzymes, hormones,

hydrocarbons, and pigments (Larsen et al., 2007; Usydus et al., 2009).

Fish are a vital dietary source of n-3 long-chain polyunsaturated fatty acids (LCPUFAs), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are essential for human health. These fatty acids play critical roles in brain development, cardiovascular health, and reducing inflammation (Swanson et al., 2012). However, the ability of the human body to convert basic n-3 fatty acids into their more biologically active long-chain forms is limited, especially in certain populations(Mahaffey et al., 2011). This makes fish an indispensable source of these nutrients, particularly in low-income regions where diets are often dominated by starchy foods and lack diversity. Fish are also rich in high-quality protein and contain omega-3 and omega-6 polyunsaturated fatty acids, which help lower blood cholesterol levels and support normal bodily functions(Mei et al., 2019). Globally, fish ranks as the third most consumed food by weight, following rice and vegetables (Hels et al., 2003; Minkin et al., 1997), underscoring its importance in human nutrition.

In the contemporary, time-constrained environment, there is an increasing demand for convenience products that are ready-to-eat and ready-to-cook. This trend has also influenced seafood, with traditional preparation methods such as roasting, steaming, frying, and smoking continuing to be widely used. These techniques have historically been employed across various cultures and societies, fulfilling both culinary and preservation functions (Laudan, 2013; Pauli, 1999). The selection of processing method markedly affects the nutritional, chemical, and physical characteristics of seafood, thereby influencing its overall quality and health benefits.

Traditional methods such as steaming, smoking, drying, and salting have long been used to extend the shelf life of perishable foods, especially seafood (Hattula et al., 2001; Varlet et al., 2006). Smoking, one of the oldest preservation techniques, dates back thousands of years and is valued for its ability to preserve fish while enhancing flavour and texture (Djinovic et al., 2008). Steaming, another traditional method, is widely used in artisanal fish processing and is particularly effective at retaining heat-sensitive nutrients such as omega-3 fatty acids and vitamins (Özden & Erkan, 2008; Wang et al., 2012). In addition, compared with boiling, steaming removes extra fat and reduces cyanotoxins (bacterial toxins) in fish by up to 26% within two minutes (Castro-González et al., 2015; Liam et al., 2014; Stancheva et al., 2014).

1.1. Organoleptic analysis

Sensory evaluation, also known as organoleptic assessment, is an important tool for determining the quality of food products. The five human senses—sight, smell, taste, touch, and hearing—are used to measure, investigate, and interpret the qualities of food (Olafsdottir et al., 1997). This method not only provides information about the sensory properties of food but also aids in understanding consumer preferences and acceptability. Studies have demonstrated that traditional techniques, such as preparing and serving fish with leaf wraps, enhance their sensory qualities, such as appearance, flavour, and taste. Furthermore, many plant leaves have strong antibacterial and antifungal activities that protect against environmental and foodborne infections (Sahu & Padhy, 2013). Polyphenols in these leaves can leach into food during cooking, enhancing their function as natural antioxidants (Somayaji & Hegde, 2016). The historical practice of consuming food on leaf plates, as noted by Acharya Charaka, is regarded as a holistic approach that links the senses with the mind, highlighting its cultural and socioreligious importance (Kora, 2019). Sensory evaluation frequently uses the hedonic scale, a quantitative approach for assessing consumer acceptance and preference. This scale generally spans from "like extremely" to "dislike extremely," enabling researchers to quantify subjective responses to food products (Lawless & Hayman, 1997). Food research commonly employs quantitative methods such as descriptive analysis and

discriminative testing alongside hedonic scaling. For descriptive analysis, trained panels list and rate certain sensory characteristics. On the other hand, discriminative testing checks for detectable differences between samples (Stone et al., 2004).

1.2. Hedonic scale for sensory analysis

Hedonic scales are frequently employed in the sensory evaluation of food products to assess consumer preferences and perceptions for features, such as flavour, texture, or appearance. These scales often span from "extremely like" to "extremely dislike," enabling respondents to evaluate their degree of enjoyment or satisfaction with the product (Girardot et al., 1952; Peryam & Pilgrim, 1957). The data derived from hedonic scales assist researchers and food manufacturers in comprehending consumer preferences, pinpointing product enhancements, and ensuring that items align with the requirements of the target audience. Hedonic testing may utilize numerous scale types, such as 9-point, 5-point, or descriptive scales, contingent upon the needed analytical depth. This form of sensory evaluation is crucial for informing product development and marketing tactics.

This study seeks to investigate the effects of traditional practices, particularly the use of leaf wraps, while employing quantitative methodologies to evaluate their impact on food quality and safety. The aim of this research is to integrate organoleptic evaluation with sophisticated data analysis techniques to reconcile traditional knowledge with modern food science, providing insights into sustainable and culturally pertinent food preservation and preparation strategies.

2. Materials and methods

2.1. Study area

Daman, a union territory in India, is situated along the Arabian Sea at the mouth of the Daman Ganga River, near the southern border of Gujarat. It is geographically divided into two parts—Nani Daman (Little Daman) and Moti Daman (Big Daman)—by the Daman Ganga River, which holds cultural and economic significance for the region. Daman is known for its scenic coastline, Portuguese colonial architecture, and vibrant culture. The union territory's economy is driven by industries such as fishing, textiles (particularly ready-made garments), and plastics, which are among its most profitable sectors. Agriculture also plays a role, with major crops including paddy, ragi, bajra, jowar, groundnut, pulses, and beans. Daman experiences a tropical monsoon climate characterized by warm temperatures and distinct wet and dry seasons. The annual mean temperature ranges from 20°C to 38°C, with the hottest months being April and May. The region receives an average annual rainfall of 1,500 mm to 2,000 mm, most of which occurs during the monsoon season from June to September. The coastal location moderates extreme temperatures, making the climate relatively pleasant throughout the year.

2.2. Collection and preparation of fish samples

Silver pomfret (*Pampus argenteus* (Euphrasen, 1788)) was selected for the present study. The whole fish were purchased from the local market of Daman and Vapi. The fish were cleaned and sliced into small uniform pieces weighing 20–25 grams each for the steaming process. Leaves of twelve different plant species for wrapping were collected from nearby areas, agricultural fields, and forests or purchased from the vegetable market (Table-1). Leaves were washed with water to remove any dirt or residue. The prepared fish pieces were then wrapped with collected plant leaves and steamed in a pressure cooker for 20–30 minutes. Each sample is then coded unbiasedly for sensory analysis and presented to the panellists. One sample was used as a control (without leaf wrapping) to compare the data.

Table 1. List of plant species used for wrapping the fish samples

Sr. no.	Scientific Name	Common Name	Family
1	*Annona squamosa* L.	Custard apple	Annonaceae
2	*Artocarpus heterophyllus* Lam.	Jackfruit	Moraceae
3	*Brassica oleracea* var. *capitata* L	Cabbage	Brassicaceae
4	*Butea monosperma* Lam.	Flame of forest	Fabaceae
5	*Colocasia esculenta* L.	Colocasia	Araceae
6	*Curcuma longa* L.	Turmeric	Zingiberaceae
7	*Mangifera indica* L.	Mango	Anacardiaceae
8	*Musa paradisiaca* L.	Banana	Musaceae
9	*Piper betle* L.	Betel	Piperaceae
10	*Psidium guajava* L.	Guava	Myrtaceae
11	*Syzygium samarangense* (Blume) Merr. & L.M.Perry	Wax apple	Myrtaceae
12	*Tectona grandis* L.f.	Teak	Lamiaceae
13	*Terminalia catappa* L.	Indian Almond	Combretaceae

2.3. Sensory testing

Sixty informants from the local Koli community, aged 25–50 years, were selected randomly for the study. Participants were selected if they consumed the fish regularly and did not have any allergy to the fish. Two different tests (sensory and ranking) were carried out after the leaf-wrapped fishes were steamed. The prepared leaf-wrapped fish samples were then presented for testing to all the panellists in random order to avoid bias. Panellists were provided with clean warm water to clean the pallet after each sample was tested. A 9-point hedonic scale was used to record responses from the panellists and for further analysis. The organoleptic attributes of appearance, juiciness, saltiness, rancidity, flavour, and general acceptability of the fish samples were recorded (Table-2). The panellists were also asked to rank the fish samples in ascending order of preference from the most preferred to the least preferred for the ranking preference test (Cleaver, 2018).

2.4. Statistical analysis

Multiple correspondence analysis (MCA) was performed to interpret the data and identify which sensory parameters are more important and play a major role in the overall preferences of the dish (Alhuzali et al., 2022; Greenacre, 1992). All the statistical analyses were performed in R (R Core team, 2022) statistical software via RStudio (Posit team, 2023).

3. Results and Discussion

The sensory assessment in this study was conducted to understand the preferences of different leaf-wrapped fishes. Data from 13 distinct leaf wraps were collected, and one sample was used as a control where no leaf was wrapped. Wrapping the plant leaves significantly improved the aroma and increased the preference.

These samples of plant leaves usually received the highest sensory scores in the hedonic preference test, and the panellists expressed a pleasant flavour and aroma of that particular plant species.

The wrapping of plant leaves to fish led to an evident change in the flavour of the fish, which was defined as herbal or spicy by the panellists after they were cooked. However, the flavours released in the other three groups, i.e., leaves of *M. paradisiaca, C. esculenta, A. heterophyllus*, and *C. longa,* were among the most common in the ranking test. Among the freshly steamed samples, the panellist did not prefer the flavour of the control sample because of its pungent flavour.

The leaf wraps that were liked by most of the participants in terms of appearance were *C. longa* (95%), followed by *T. grandis* (91%) and

M. paradisiaca (88%). The only wrap that had a different frequency distribution than the others was the *P. betel* leaf-wrapped sample, which received the lowest rating (high score under the category average and bad appearance). For the control and other leaf-wrapped fish samples, the frequency distribution was distributed the best, best and average (Figure 1).

Juiciness is the second parameter that was considered in the study, in which three options were given, of which one respondent had to give their feedback as per their own experience after testing the wrapped fish.

The participants reported that almost all the leaf-wrapped fish samples and the control samples were soft and juicy, with a frequency of more than 90% under the soft parameter (Figure 2). The fish samples wrapped in *C. esculenta* (91%) and *T. grandis* (68%) received the highest rating under the hard texture. Approximately 12% of the participants experienced hard textures for *T. catappa* and *M. paradisiaca* as well.

The third parameter was the saltiness of the leaf-wrapped fish samples. Almost all the participants said that they found most of the fish samples salty. Only the samples that were not found to be salty were wrapped in the leaves of *T. catappa* (32%), *B. oleracea var. capitata* (28%), the control (22%), and *P. guajava* (18%).

In terms of the responses to the specific flavour profile, the spicy flavour had the highest frequency, followed by the tasteless, sweet, and sour flavours. *B. monosperma* (58%) had the highest frequency of spicy flavours, followed by *C. longa* (50%), *T. grandis* (45%), and *C. esculenta* (45%). The control fish samples presented the highest frequency (68%) in the taste-less category, followed by *B. oleracea var. capitata* (48%), *T. catappa* (28%), and *P. guajava* (23%). The participants experienced a more bitter taste in the *P. betel* (47%) wrapped fish sample than in the *P. guajava* (25%) leaf-wrapped sample. *M. indica* (59%) and *T. catappa* (53%) leaf-wrapped fish samples presented the highest frequency values under sour taste. For the sweet taste profile, *M. paradisiaca* (48%) presented the highest frequency value, followed by *S. samarangense* (38%) and *A. squamosa* (30%) (Figure 3).

The overall preference and liking of the different leaf-wrapped fish samples were calculated on the basis of the above mentioned parameters (Table 3). A dominant and pleasant aroma was also observed for the samples wrapped with *M. paradisiaca*, *C. esculenta*, *M. indica*, and *C. longa*.

Considering the overall frequency of the classes created, more than 50% of the participants preferred to use leaf-wrapped fish at least once, whereas approximately 39% of them liked and would accept the preparation of a dish. The fish samples wrapped with *M. paradisiaca*, *C. esculenta*, *A. heterophyllus*, *M. indica*, and *T. grandis* were accepted and preferred over the other fish samples. For the remaining species, the results showed that the participants preferred it occasionally (Figure 4).

Only the *P. betel*-wrapped fish sample had the highest frequency in the not liked category, and the participants did not prefer it again. This sample had the least intense prepared fish flavour and odor and the smallest change in colour and texture from those of the fresh fish, which echoed the lowest hedonic ratings of all the mentioned attributes (Figure 4).

Table 2. Various parameters and informant response

Sr. No.	Scientific Name	Appearance				Juiciness		Saltiness			Flavour profile					Specific taste of leaf wrap
		Bad	Average	Good	Best	Hard	Normal	Soft	No	Yes	Tasteless	Bitter	Sour	Sweet	Spicy	
1	Control	0	17	19	24	0	1	59	13	47	38	11	0	6	5	0
2	*Annona squamosa* L.	0	9	22	29	2	0	58	5	55	10	8	1	18	8	15
3	*Artocarpus heterophyllus* Lam.	0	2	15	43	0	0	60	3	57	9	1	9	13	23	5
4	*Brassica oleracea* var. *capitata* L	0	2	20	38	3	1	56	17	43	29	11	4	9	7	0
5	*Butea monosperma* Lam.	0	1	15	44	4	0	56	0	60	15	0	0	1	35	9
6	*Colocasia esculenta* L.	0	2	5	53	55	0	5	1	59	11	3	3	16	27	0
7	*Curcuma longa* L.	0	1	2	57	5	0	55	0	60	8	10	0	1	30	11
8	*Mangifera indica* L.	0	3	14	43	5	0	55	0	60	0	2	31	4	9	14
9	*Musa paradisiaca* L.	0	2	5	53	7	0	53	5	55	6	10	5	29	9	1
10	*Piper betle* L.	10	23	15	12	2	0	58	0	60	3	28	0	11	11	7
11	*Psidium guajava* L.	0	5	21	34	1	0	59	11	49	14	15	0	15	9	7
12	*Syzygium samarangense* (Blume) Merr. & L.M.Perry	0	3	36	21	3	0	57	0	60	2	1	16	23	15	3
13	*Tectona grandis* L.f.	0	2	3	55	41	0	19	0	60	8	2	17	0	27	6
14	*Terminalia catappa* L.	0	7	32	21	7	0	53	19	41	17	5	32	5	1	0

Table 3. Overall ratings and preferences of different leaf-wrapped fish samples.

Sr. No.	Scientific Name	Rating classes		
		Least preferred	Preferred once	Most preferred
1	Control	6	46	8
2	*Annona squamosa* L.	6	39	15
3	*Artocarpus heterophyllus* Lam.	1	41	18
4	*Brassica oleracea* var. *capitata* L	0	45	15
5	*Butea monosperma* Lam.	1	36	23
6	*Colocasia esculenta* L.	1	19	40
7	*Curcuma longa* L.	0	12	48
8	*Mangifera indica* L.	1	27	32
9	*Musa paradisiaca* L.	0	17	43
10	*Piper betle* L.	27	28	5
11	*Psidium guajava* L.	2	44	14
12	*Syzygium samarangense* (Blume) Merr. & L.M.Perry	3	40	17
13	*Tectona grandis* L.f.	0	11	49
14	*Terminalia catappa* L.	5	51	4

Table 4. Different parameters and corresponding values for MCA dimension 1 and dimnesion 2

Name of variable	MCA Dimensions	
	Dim. 1	Dim.2
Appearance	0.37	0.46
Juicy	0.15	0.047
Salty	0.56	0.16
Flavour	0.69	0.72

MCA resulted in 11 dimensions, of which the first two were used together to explain 100% of the variation present within the data . Considering the values of the percentage of variance, 16.05% and 12.65% of the variation were explained by Dimension 1 and Dimension 2, respectively, and can depict the 28.70% variation present in the data and hence be considered for understanding the associations among the variables. Sensorial variables of dimensions 1 and 2 represent the eigenvalues only on the basis of this, it was observed that there were no clear differentiating values allocated to each of the obtained dimensions (Table - 4). The maximum value was 0.69 (flavour), followed by 0.56 (salt) for the first dimension and 0.72 (flavour) for the second dimension, followed by 0.46 (appearance). Appearance also contributed to the first dimension (value of 0.37). The most discriminant variables for dimension 1 hierarchically were flavour, salty, appearance, and juicy; for dimension 2, the most discriminant variables were flavour, appearance, salty and juicy. Flavour was a variable that had a strong association with both dimensions, whereas the other variables had a moderate association with either of the other dimensions, as in the case of salt or appearance (Figure 6).

Multiple correspondence analysis (MCA) further demonstrated that flavour, saltiness, appearance, and juiciness were the most discriminant variables affecting consumer

preference. The biplot visualization revealed clear distinctions between the least preferred samples and the preferred samples, with some overlap between the preferred and most preferred categories. This suggests that while certain leaf wraps were distinctly favoured, individual taste preferences played a role in overall acceptability.

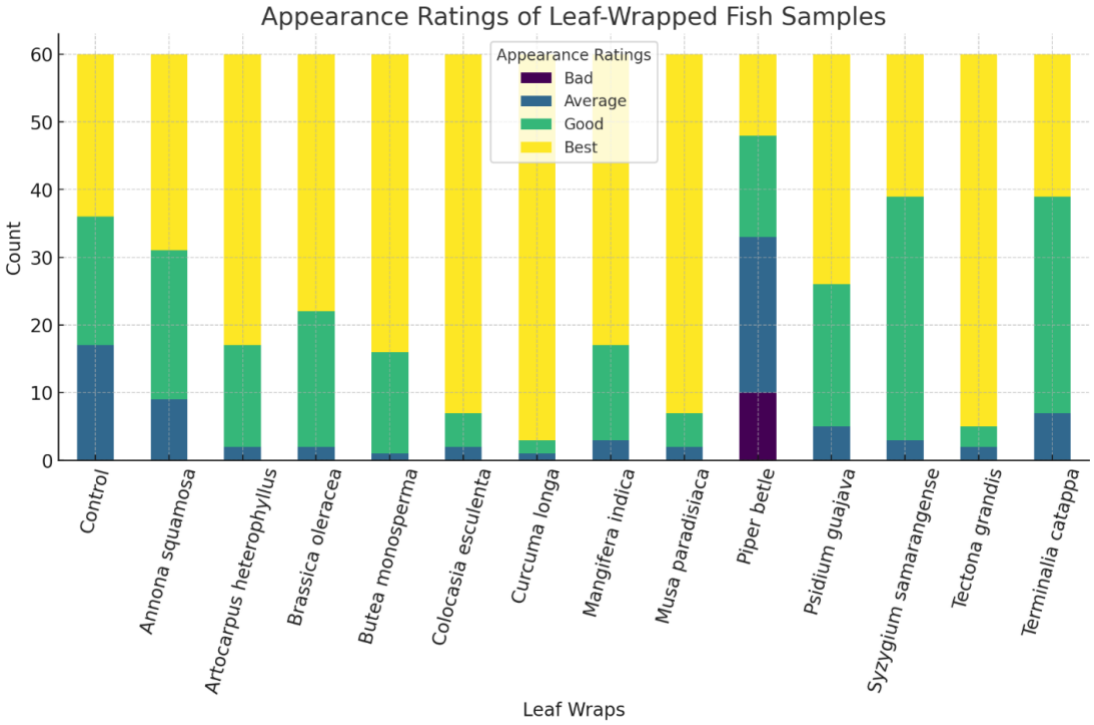

Figure 1. Appearance Ratings of leaf-wrapped Fish Samples

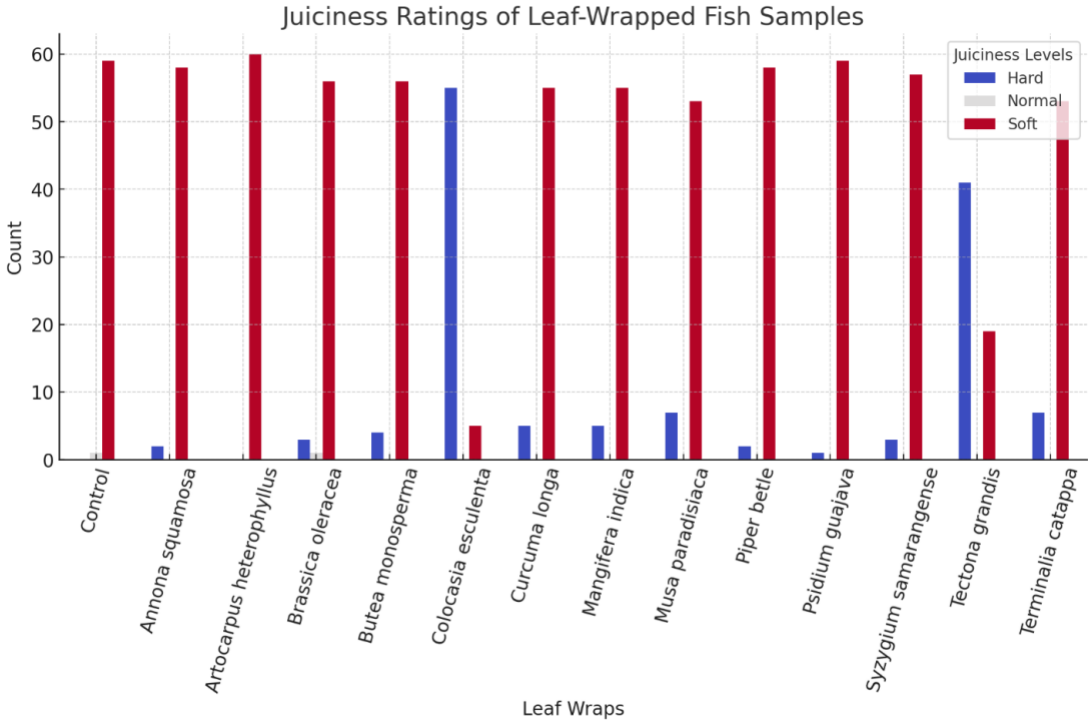

Figure 2. Juiciness Ratings of Leaf-Wrapped Fish Samples

An ellipse of the 95% confidence interval (CI) is drawn corresponding to each group. The data points of the least preferred group are very clearly distinguishable from those of the other two groups, and all the data points fall under the 95% CI ellipse. On the other hand, the data points of the most preferred and preferred groups have an overlapping area, and as a result, an ellipse of 95% for both groups overlaps each other. This most preferred group is small, and approximately 90% of the data points also fall in the ellipse of preferred points once. However, some of these two data points do not fall under the 95% CI ellipse because some uncommon combination of feedback corresponds to each parameter (Figure 7).

The study revealed that leaf wrapping generally enhanced the sensory appeal of fish, with participants showing a strong preference for certain leaf-wrapped samples over the control. The samples wrapped in *C. longa, T. grandis,* or *M. paradisiaca* received the highest ratings for appearance. Previous studies have shown that turmeric leaves contain bioactive compounds such as curcuminoids, which contribute to their vibrant colour and distinct aroma, making them suitable for culinary applications (Sahoo et al., 2021). Similarly, *T. grandis* and *M. paradisiaca* leaves are known for their phytochemical composition, which may increase the visual and textural appeal of food (Gbadamosi & Emi, 2017).

Juiciness was another important factor evaluated in this study. Most samples were rated as soft and juicy, except for those wrapped in *C. esculenta* and *T. grandis*, which had the highest percentage of responses, indicating a harder texture. This could be attributed to differences in moisture retention and heat penetration through the leaf layers. According to (Quan et al., 2023), *C. esculenta* leaves contain oxalates and other structural compounds that may influence the texture of wrapped food items.

The perception of saltiness varied across different leaf-wrapped fish samples. The samples wrapped in *T. catappa, B. oleracea var. capitata*, and *P. guajava* were found to be less salty, while the control sample was also categorized as having relatively mild saltiness. These findings suggest that certain leaves may influence the diffusion of salt into fish during cooking, possibly due to their chemical composition and permeability. Leaves, such as those from *P. betel*, are associated with a bitter taste, which likely contributes to their low preference scores. *P. betel* contains phenolic compounds such as eugenol and hydroxychavicol, which are known for their strong astringent and bitter flavours (Gunathilake et al., 2018).

Flavour profile analysis revealed that *B. monosperma*, *C. longa*, and *T. grandis* were associated with a spicy taste, whereas *M. indica* and *T. catappa* imparted a sour note to the fish (Figure 3). The use of mango leaves in traditional food preparation has been linked to the presence of terpenoids and flavonoids, which can contribute to a tangy or citrus-like flavour (Kumar et al., 2021). On the other hand, *M. paradisiaca* imparts a noticeable sweetness, likely due to the presence of natural sugars in banana leaves (Gbadamosi & Emi, 2017).

The overall preference ratings indicate that the fish samples wrapped in *M. paradisiaca*, *C. esculenta*, *A. heterophyllus*, *M. indica*, and *T. grandis* were the most liked. These findings align with previous research showing that banana leaves are commonly used in traditional cooking because of their ability to enhance food aroma while maintaining moisture content (Gbadamosi & Emi, 2017). In contrast, *P. betel* received the lowest preference rating, likely due to its bitter taste and strong medicinal aroma, which were less appealing to participants.

Among the all leaves tested, *M. paradisiaca*, *C. longa*, and *T. grandis* were the most preferred, primarily because of their ability to enhance appearance, maintain juiciness, and contribute favourable flavour (Figure 5). The high ratings for *M. paradisiaca* align with its traditional culinary usage, as its natural phytochemicals help retain moisture and impart mild sweetness. Similarly, *C. longa* and *T. grandis* presented vibrant colouration and aromatic qualities, which was supported by

their bioactive compounds. Conversely, *P. betel* leaves were the least preferred owing to their strong bitter taste, which was attributed to the presence of phenolic compounds such as eugenol and hydroxychavicol (Gunathilake et al., 2018).

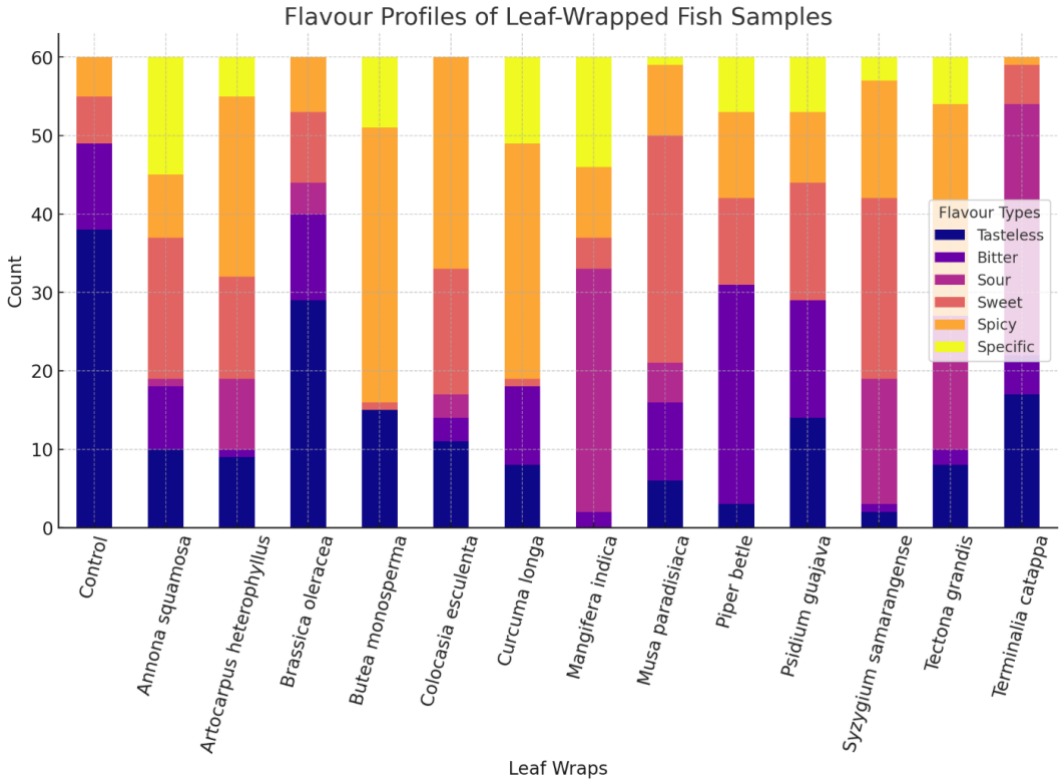

Figure 3. Flavour Profiles of Leaf-Wrapped Fish Samples

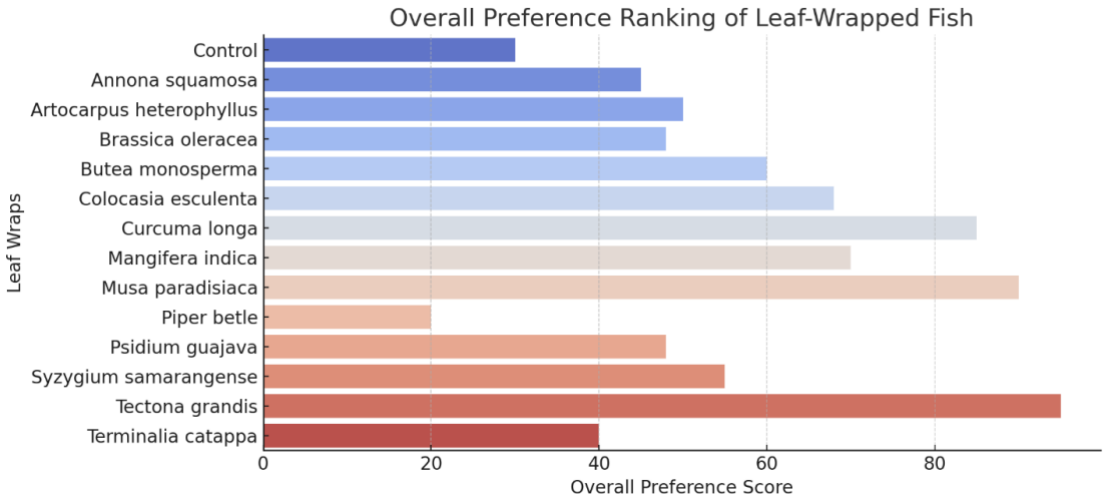

Figure 4. Overall preference Ranking of Leaf-wrapped Fish

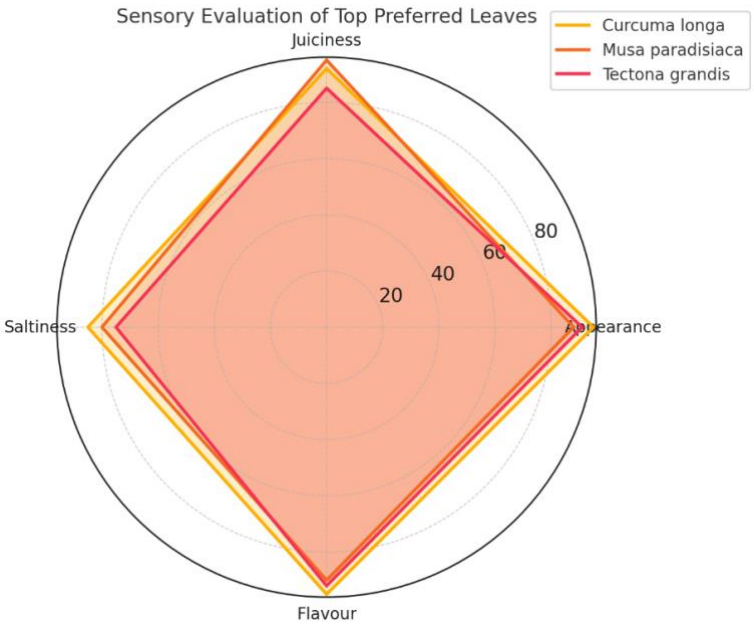

Figure 5. Sensory Parameters of Top Preferred Leaves

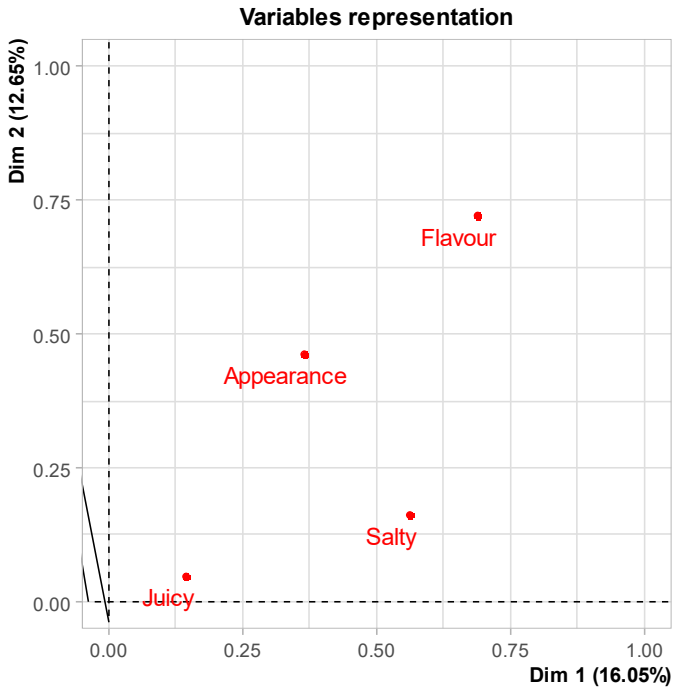

Figure 6. Scatter plot of dimension 1 and dimension 2

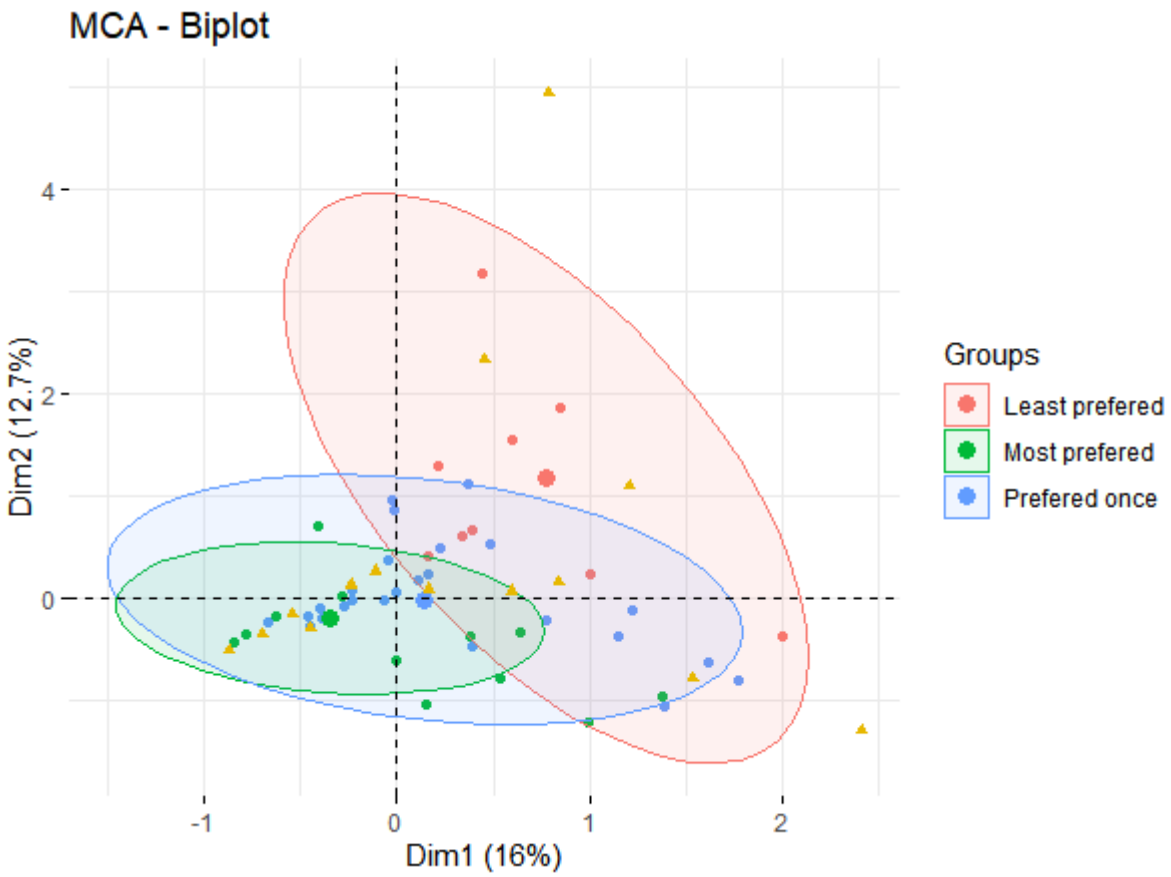

Figure 7. MCA biplot depicting the distribution of preferences among informants

4. Conclusions

The present study evaluated the sensory attributes and consumer preferences of fish samples wrapped in different plant leaves, highlighting their influence on their appearance, juiciness, saltiness, and flavour. The results demonstrated that leaf wrapping significantly altered the sensory properties of the fish, with some leaves imparting desirable flavours and aromas, whereas others were less preferred.

The study highlighted the impact of phytochemical interactions between leaves and fish during cooking, which affect salt diffusion and textural modifications. *C. esculenta* and *T. grandis* resulted in firmer textures, possibly because their structural components affect moisture retention. The variations in salt perception among different leaf-wrapped samples suggest differences in permeability and mineral composition, influencing the sensory balance of the dish. Multiple correspondence analysis (MCA) revealed clear clustering patterns, indicating that individual sensory attributes play a significant role in overall acceptability.

The results of this study hold potential implications for the food industry, particularly in promoting natural and sustainable food packaging alternatives. Traditional leaf-wrapping techniques provide not only sensory benefits but also eco-friendly and biodegradable food preparation methods. Moreover, the concept of leaf-wrapped steamed fish presents an opportunity for value addition in the food industry by improving economic and nutritional benefits. The changing consumer mindset toward natural and traditional culinary methods suggests the potential for entrepreneurial ventures that emphasize health and nutrition. Future research should further investigate the biochemical mechanisms underlying flavour transfer and texture modification in leaf-wrapped foods,

potentially integrating nutritional and antimicrobial properties to expand their functional benefits in modern gastronomy. Additionally, the findings from this study could aid in the development of effective marketing strategies to promote traditional fish cuisine in contemporary food markets.

5.References

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Research article

EFFECTS OF FRUIT PARTS AND POST-FLOWERING TIME ON THE CHARACTERISTICS AND BIOACTIVITIES OF JACKFRUIT (*ARTOCARPUS HETEROPHYLLUS* LAM.) IN CAN THO CITY AND THE MEKONG REGION

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Abstract

The study qualitatively and quantitatively analyzed bioactive compounds (alkaloids, flavonoids, phenolics & tannins, proteins, amino acids, carbohydrates, saponins, polyphenols, carotenoids) and antioxidant activities (DPPH, ABTS, TAC) in jackfruit pulp, fibers, and seeds collected from six regions of the Mekong Delta, including Thoi Lai, Can Tho (TL-CT); Phong Dien, Can Tho (PD-CT); Co Do, Can Tho (CD-CT); Cai Lay, Tien Giang (CL-TG); Chau Thanh, Hau Giang (CT-HG); and Chau Thanh, Ben Tre (CT-BT), at three maturity stages (100, 110, and 120 days). The results indicated that harvest time played a more significant role than locality. The pulp was richest in phenolics, tannins, and flavonoids at 100–110 days, the fibers contained higher levels of alkaloids and flavonoids at 120 days, while the seeds were consistently rich in proteins, amino acids, and carbohydrates at all stages. The external appearance and physical properties of jackfruit (shape, size, weight, component ratios, color, aroma, taste) increased and changed markedly with ripening, reaching optimal values at 120 days, with the highest pulp ratio, attractive color, and pronounced sweetness. The contents of bioactive compounds and antioxidant capacities generally declined with increasing maturity, with total polyphenols ranging from 0.07 to 0.32 mg GAE/gDW and flavonoids from 0.03 to 0.44 mg QE/gDW. In contrast, carotenoid content increased, ranging from 0.11 to 0.63 mg/mL. Notably, jackfruit seeds exhibited superior total antioxidant capacity (TAC) (123.67 mg AA/gDW) compared to pulp and fibers. Antioxidant activity decreased with

advancing maturity, as reflected by IC_{50} values of DPPH (99.32–367.33 μg/mL) and ABTS (46.21–287.07 μg/mL), indicating that seeds and pulp demonstrated stronger antioxidant potential than rag.

1. Introduction

Jackfruit (*Artocarpus heterophyllus*), belonging to the family Moraceae and genus *Artocarpus*, is a widely cultivated fruit plant in Southeast Asia and Brazil (Sreeja Devi et al., 2021; Swami et al., 2012). Numerous cultivars of jackfruit exist, each differing considerably in fruit characteristics and properties. The fruit size varies greatly, ranging from small types weighing only 300–400 g to large types reaching several tens of kilograms. The tree is a woody perennial, typically 8–15 m in height, and begins to bear fruit after three years of age. The fruit is a syncarp, oval in shape, measuring about 30–60 cm in length and 20–30 cm in diameter. Jackfruit usually sets fruit in mid-spring and ripens by late summer (July–August). It is not only a nutritious fruit but also a plant with various medicinal applications. Several well-known cultivars include *mit mat*, *mit dai*, *mit thai*, and *mit nghe*. Jackfruit also represents an important income source for small farms through trade and serves as a nutrient-rich feed for livestock (Laishram & Ghosh, 2018; Ranasinghe et al., 2019a; Van et al., 2023a)

Jackfruit is a fleshy, sweet, and aromatic fruit. Except for the spiny rind and fibrous core, most parts of the fruit are edible. The pulp contains high sugar content and provides considerable energy (Barbosa et al., 2019). The edible bulbs are bright yellow, thick, dry, crispy-sweet, and fragrant, with small seeds and little fiber. Seeds are dark brown to brown, ellipsoid in shape, about 2–3 cm long and 1–1.5 cm in diameter, and surrounded by a thin white sheath. They are starchy and hard, with a storage capacity of about one month under low-temperature conditions. Medicinally, seeds have been reported to tonify qi, improve digestion, relieve hunger, and reduce cough, among other benefits (Palamthodi et al., 2021; Van et al., 2023b). Studies on the proportion of fruit components, including pulp, seeds, peel, and core, indicated that the inedible portion (peel and core) accounted for 59.20% of the total fruit weight. In Indonesia, the edible portion (pulp) was reported to account for 30–35%, while the peel and seeds contributed 55–62% and 8–15%, respectively (Saxena et al., 2011; Thanh et al., 2020).

Jackfruit contains a wide range of bioactive compounds, including carotenoids that act as antioxidants (Baliga et al., 2011). The antioxidant activity of jackfruit pulp extracts has been correlated with total phenolic and flavonoid contents (Jagtap et al., 2010). Both fresh pulp and seeds demonstrate antioxidant capacity comparable to ascorbic acid, with phenolic contents equivalent to 27.7 and 0.9 mg gallic acid, respectively, contributing approximately 70% of the total antioxidant activity (Soong & Barlow, 2004). Jackfruit is also a rich source of essential minerals, particularly magnesium, which plays a vital role in calcium absorption, bone strengthening, and the prevention of bone-related disorders such as osteoporosis.

The Mekong Delta is the largest fruit-producing region in Vietnam, with a cultivated area of approximately 390,000 ha, accounting for more than 33% of the country's fruit-growing area, and an annual production of about 4 million tons (Department of Agriculture and Rural Development of Can Tho City). Within this, jackfruit cultivation occupies around 30,600 ha (Department of Agriculture and Rural Development of Can Tho City). Different parts of the jackfruit have been investigated at various maturity stages and across different areas to determine the most suitable cultivation areas for achieving optimal chemical composition and bioactivity. These findings provide practical insights for farmers to expand jackfruit production, taking advantage of favorable soil, water, and alluvial conditions to improve productivity and quality.

2. Materials and methods

2.1. Materials

Thai jackfruits were collected and separated into individual components (pulp, fibers, and seeds), which were subsequently analyzed and evaluated. The jackfruits were cultivated in Dinh Mon Commune, Thoi Lai District, Can Tho City; Truong Long Commune, Phong Dien District, Can Tho City; Thoi Hung Commune, Co Do District, Can Tho City; My Thanh Nam Commune, Cai Lay District, Tien Giang Province; Dong Phuoc A Commune, Chau Thanh District, Hau Giang Province; and Tan Phu Commune, Chau Thanh District, Ben Tre Province.

2.2. Analysis methods

2.2.1. Qualitative methods

The qualitative screening of phytochemical constituents in jackfruit pulp was carried out using standard chemical tests. The presence of alkaloids, flavonoids, phenolics, tannins, proteins, organic acids, amino acids, saponins, and carbohydrates was confirmed by characteristic color changes or precipitate formation with specific reagents.

2.2.2. Determination of total carotenoid content (TCC)

One gram of the sample was homogenized with 10 mL of an acetone:water mixture (4:1) for 2 minutes until uniform. To determine the effect of ultrasonic treatment on extraction yield, the samples were sonicated for 3 minutes (5 cycles, 30 s pulse, 10 s pause) under the same conditions. The samples were placed in an ice-water bath to prevent overheating. The homogenized samples were centrifuged at 5000 rpm for 10 minutes at 20 °C. The absorbance spectra of each compound were measured and recorded at 663.6 nm for chlorophyll a, 646.6 nm for chlorophyll b, and 470.0 nm for total carotenoids.

2.2.3. Determination of total polyphenol content (TPC)

The total polyphenol content was determined using the Folin–Ciocalteu method as described by Nhi et al. (2020) . Diluted extracts (0.1 mL) were mixed with 0.5 mL of 10% Folin–Ciocalteu reagent, vortexed, and allowed to stand for 5 min. Then, 0.4 mL of 7.5% Na_2CO_3 was added, and the mixture was incubated at room temperature in the dark for 1 h. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer, and gallic acid was used as the calibration standard. Results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g DM).

2.2.4. Determination of total flavonoid content (TFC)

The total flavonoid content was determined using the aluminum chloride colorimetric ($AlCl_3$) method as described by Nguyen et al. (2020) with minor modifications. Diluted extracts (0.5 mL) were mixed with 4.3 mL ethanol, 0.1 mL of 10% $AlCl_3$, and 0.1 mL of 1 M CH_3COOK. The mixtures were incubated for 30 min at room temperature. Absorbance was measured at 510 nm using a UV–Vis spectrophotometer. TFC values were calculated from a quercetin calibration curve and expressed as mg quercetin equivalents per g dry matter (mg QE/g DM).

2.2.5. Determination of total acidity capacity (TAC)

The phosphomolybdenum method was performed following the modified procedure of Van et al. (2024).The reagent solution was prepared by mixing 0.6 M concentrated sulfuric acid (95–97%) with 4 mM ammonium molybdate (98%) and 28 mM sodium dihydrogen phosphate. A volume of 3 mL of reagent was transferred into test tubes, followed by the addition of 0.3 mL extract at different concentrations (100–500 µg/mL). For the negative control, 0.3 mL methanol was added instead of the extract. All tubes were incubated at 95 °C for 90 min, cooled to room temperature, and absorbance was measured at 695 nm.

2.2.6. Investigation of free radical scavenging activity by DPPH· method

The DPPH• free radical scavenging assay was performed. One gram of jackfruit sample was homogenized with 50 mL ethanol, diluted, and 0.5 mL of the extract was mixed with 1.5 mL of DPPH• solution (OD517 nm = 1.1 ± 0.02). Ethanol (99.5%) was used as the blank.

The mixtures were incubated in the dark for 30 minutes, and absorbance was measured at 517 nm using a UV–Vis spectrophotometer. IC_{50} values were determined from inhibition curves, and vitamin C (ascorbic acid) was used as the standard.

2.2.7. Investigation of free radical scavenging activity by ABTS·+ method

The ABTS•+ radical cation solution was prepared by mixing 10 mL of 7.4 mM ABTS•+ with 10 mL of 2.6 mM $K_2S_2O_8$ and incubating in the dark for 24 h, then diluted to an absorbance of 1.1 ± 0.02 at 734 nm. Diluted samples (0.5 mL) were transferred into test tubes and mixed with 1.5 mL of the adjusted ABTS•+ solution, while ethanol (99.5%) was used as the blank. The mixtures were incubated in the dark for 30 min, and absorbance was measured at 734 nm using a UV–Vis spectrophotometer. For IC_{50} determination, six test tubes were prepared, including one blank (only reagent) and five tubes containing different extract volumes (100–500 µL) with corresponding ethanol dilutions, each mixed with 1500 µL of ABTS•+ solution. The mixtures were incubated in the dark for 30 min and absorbance was measured at 734 nm. Vitamin C (ascorbic acid) was used as the reference standard.

2.3. Data analysis

Results were analyzed by one-way analysis of variance (ANOVA) method and significant differences among means from triplicate analyses at ($p<0.05$) were determined by Fisher's least significant difference (LSD) procedure using the Statgraphics software (Centurion XV).

3. Results and discussions

3.1. Qualitative analysis of bioactive phytochemicals in major parts of jackfruit at different post-flowering times in the Mekong delta region

Figure 1 presented the images of jackfruit parts cultivated in six areas of the Mekong Delta. Table 1 showed the qualitative analysis of bioactive compounds in jackfruit parts (aril, fiber, seed) collected from six areas of the Mekong Delta: Thoi Lai, Can Tho (TL-CT); Phong Dien, Can Tho (PD-CT); Co Do, Can Tho (CD-CT); Cai Lay, Tien Giang (CL-TG); Chau Thanh, Hau Giang (CT-HG); and Chau Thanh, Ben Tre (CT-BT). Jackfruit samples were collected at three technical maturity stages (100, 110, and 120 days after flowering) to qualitatively determine the presence of compounds such as alkaloids, flavonoids, phenolics & tannins, proteins, amino acids, carbohydrates, and saponins. The degree of presence was indicated by the symbols +, ++, +++ (with +++ representing the highest level). The study focused on evaluating the accumulation trends of these compounds across different areas and maturity stages to draw conclusions regarding the potential applications of jackfruit in the food sector. The results showed that the presence of bioactive compounds was relatively uniform among the surveyed areas, regardless of specific soil and climatic conditions. In the aril, phenolics, tannins, and flavonoids were strongly present in all six areas when jackfruits reached 100–110 days of maturity. This demonstrated that the harvest stage played a more critical role than locality in the accumulation of these compounds. Phenolics and tannins are known for their strong antioxidant capacity; therefore, jackfruit arils from any locality could be harvested at 100–110 days to optimize nutritional value and their potential use in antioxidant-rich functional foods. The fiber, which is often considered a by-product, also exhibited a significant presence of alkaloids and flavonoids in all areas, especially at 120 days of maturity. The stability of these compounds in the fiber among areas highlighted the potential for utilizing jackfruit fiber as a valuable bioresource, thereby contributing to reducing waste in the agricultural value chain.

The seeds were also remarkable for their high levels of proteins, amino acids, and carbohydrates across all six areas and maturity stages. This part of the fruit exhibited superior nutritional value and was less affected by

environmental factors or harvest timing. Another compound, saponin, was detected in all jackfruit parts across all areas, with the highest levels observed at 110–120 days of maturity. The stable presence of saponins across regions indicated that jackfruit from the Mekong Delta could serve as an important natural resource for the development of health-promoting products.

Figure 1. Photograph of jackfruit parts cultivated in the mekong delta region. A: Thoi Lai, Can Tho (TL-CT), B: Phong Dien, Can Tho (PD-CT), C: Co Do, Can Tho (CD-CT), D: Cai Lay, Tien Giang (CL-TG), E: Chau Thanh, Hau Giang (CT-HG), F: Chau Thanh, Ben Tre (CT-BT).

Table 1. Qualitative analysis of compounds in different parts of jackfruit across regions and technical maturity stages

Parts	Area	TL-CT			PD-CT			CD-CT			CT-HG			CL-TG			CT-BT		
	Time (day)	100	110	120	100	110	120	100	110	120	100	110	120	100	110	120	100	110	120
	Compound																		
Pulp	Alkaloid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Flavonoid	+++	+++	++	+++	+++	+ +	+++	+++	+ +	+++	+++	+ +	+++	+++	+ +	+++	+++	+ +
	Phenolic và tanin	+ +	++	+	+ +	++	+	+ +	++	+	+ +	++	+	+ +	++	+	+ +	++	+
	Protein	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Amino acid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Organic acid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Carbohydrate	++	+	+	++	+	+	++	+	+	++	+	+	++	+	+	++	+	+
	Saponin	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++
Rag	Alkaloid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Flavonoid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Phenolic & Tanin	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+
	Protein	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++
	Amino acid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Organic acid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Carbohydrate	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+
	Saponin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Seed	Alkaloid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Flavonoid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Phenolic & Tanin	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+	++	+ +	+
	Protein	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++
	Amino acid	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	Organic acid	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++
	Carbohydrate	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+	+ +	+	+
	Saponin	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++

3.2. Characteristics and physical properties in major parts of jackfruit at different post-flowering times in the Mekong delta region

Table 2 presented the external characteristics and physical properties of jackfruit parts from different areas and technical maturity stages. In terms of morphology, at all three maturity stages (100, 110, and 120 days after flowering), jackfruits from all areas exhibited an elongated shape with a slightly swollen middle portion. Both fruit length and width showed an increasing trend with maturity across all areas, which was consistent with the natural growth process of the crop. At TL-CT, the average length increased from 35.33 cm at 100 days to 36.65 cm at 120 days, while the width increased from 24.53 cm to 25.51 cm. This trend was also observed in other areas, with a slight increase as maturity advanced. These results indicated that the accumulation of dry matter and water in the fruit peaked at 120 days, leading to an increase in fruit size. However, slight differences in fruit size were observed among areas. At 120 days, fruit length ranged from 35.16 cm (CT-BT) to 37.46 cm (PD-CT), suggesting that growth conditions, soil type, and climate were relatively similar across regions. This supported the establishment of common quality standards for jackfruit production among areas, thereby maintaining product uniformity in the market.

Fruit weight also showed an increasing trend with maturity. Specifically, at TL-CT, fruit weight increased from 8.02 kg (100 days) to 8.54 kg (120 days). A similar trend was observed across all other areas, with fruit weight ranging from 8.02 to 8.89 kg. The increase in fruit weight with advancing maturity resulted from higher accumulation of water and dry matter, which led to maximum size and weight at 120 days.

The proportions of internal components, including aril, fiber, and seed ratios, changed markedly with maturity. The aril ratio tended to increase with maturity. At TL-CT, the aril ratio increased from 26.54% (100 days) to 30.53% (120 days), while the fiber ratio decreased from 16.05% (100 days) to 12.53% (120 days). The increase in aril ratio and decrease in fiber ratio indicated that the fruit had reached full ripeness, with the edible portion occupying a larger proportion, thereby enhancing economic value and product quality. This also reflected nutrient accumulation in the aril during ripening, which reduced fiber content and increased nutritional composition. However, aril and other component ratios showed no substantial variation among areas. Across all areas, aril ratios ranged from 26.40% to 30.70%, while fiber ratios ranged from 12.65% to 17.70%, indicating only minor differences among regions. The seed ratio exhibited a slight decrease with maturity. At 100 days, seed ratios ranged from 14.70% (CT-HG) to 14.90% (CD-CT), but declined to approximately 13.20% (PD-CT) to 13.40% (CL-TG) at 120 days. This could be explained by the disproportionate development between the aril and seed, as the aril accumulated dry matter and gained weight more rapidly than the seed during ripening. The peel ratio ranged from 16.90% (CT-HG, 110 days) to 26.40% (PD-CT, 120 days), with an overall increasing trend as maturity advanced. The ratio of other parts (including peduncle, core, and non-edible tissues) ranged from 17.25% to 25.50% and generally decreased with ripening across most areas. A similar decline was recorded elsewhere, suggesting that as the fruit ripened, nutrients were translocated from these parts to the edible portion.

Sensory attributes such as color, aroma, and taste also changed markedly with maturity. At 100 days, fruits displayed a dark green external color, which shifted to light green or slightly yellow at 120 days. Internal color also varied from pale yellow to deep yellow, reflecting increased concentrations of carotenoid pigments.

Table 2. Characteristics and physical properties of jackfruit parts across different areas and maturity stages

Area	**TL-CT**			**PD-CT**			**CD-CT**		
Time (Day) / **Characteristic**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
Fruit length (cm)	35.33 ± 2.58[a]	36.33 ± 4.53[a]	36.66 ± 3.51[a]	34.53 ± 2.34[a]	35.74 ± 2.36[a]	36.27 ± 3.72[a]	35.33 ± 2.58[a]	36.33 ± 4.53[a]	36.94 ± 3.51[a]
Fruit width (cm)	24.53 ± 2.53[a]	24.58 ± 2.65[a]	25.51 ± 3.74[a]	23.37 ± 3.26[a]	24.73 ± 2.46[a]	25.15 ± 2.64[a]	23.53 ± 2.53[a]	23.58 ± 2.65[a]	24.85 ± 3.74[a]
Fruit weight (Kg)	8.02 ± 1.58[a]	8.32 ± 1.21[a]	8.54 ± 2.68[a]	8.36 ± 1.63[a]	8.52 ± 2.63[a]	8.84 ± 2.68[a]	8.12 ± 2.35[a]	8.59 ± 2.73[a]	8.89 ± 3.35[a]
Pulp ratio (%)	26.54 ± 2.27[a]	27.69 ± 1.43[a]	30.53 ± 1.42[b]	26.48 ± 2.31[a]	27.80 ± 1.50[a]	30.55 ± 1.40[b]	26.60 ± 2.25[a]	27.90 ± 1.55[a]	30.70 ± 1.45[b]
Rag ratio (%)	16.05 ± 1.07[a]	17.69 ± 2.86[a]	12.53 ± 2.31[b]	16.12 ± 1.15[a]	17.65 ± 2.05[a]	12.60 ± 2.20[b]	16.00 ± 1.20[a]	17.70 ± 2.10[a]	12.50 ± 2.15[b]
Seed ratio (%)	14.81 ± 1.04[a]	14.62 ± 1.46[a]	13.25 ± 1.42[a]	14.80 ± 1.05[a]	14.60 ± 1.20[a]	13.20 ± 1.35[a]	14.90 ± 1.10[a]	14.65 ± 1.25[a]	13.30 ± 1.30[a]
Rind ratio (%)	17.28 ± 2.15[a]	16.92 ± 1.53[a]	25.43 ± 1.52[b]	17.30 ± 2.00[a]	17.15 ± 1.80[a]	26.40 ± 1.75[b]	17.40 ± 2.05[a]	17.00 ± 1.75[a]	23.35 ± 1.80[b]
Other parts (%)	25.22 ± 1.43[a]	23.08 ± 2.31[a]	18.26 ± 3.21[b]	25.30 ± 1.85[a]	22.80 ± 1.90[a]	17.25 ± 2.05[b]	25.10 ± 1.80[a]	22.75 ± 2.05[b]	20.15 ± 2.10[b]
External color	Light green	Dark green	Dark green with black spots and streaks	Light green	Dark green	Dark green with black spots and streaks	Light green	Dark green	Dark green with black spots and streaks
Internal color	Pale yellow, almost white	Light yellow	Yellow	Pale yellow, almost white	Light yellow	Yellow	Pale yellow, almost white	Light yellow	Yellow
Aroma	No aroma	Slight aroma	Strong aroma	No aroma	Slight aroma	Strong aroma	No aroma	Slight aroma	Strong aroma
Taste	No taste	Slightly sweet taste	Distinctly sweet taste	No taste	Slightly sweet taste	Distinctly sweet taste	No taste	Slightly sweet taste	Distinctly sweet taste

Area	CT-HG			CL-TG			CT-BT		
Time (Day) / **Characteristic**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
Fruit length (cm)	34.63 ± 1.56[a]	35.83 ± 2.26[a]	35.66 ± 3.51[a]	34.73 ± 2.84[a]	35.82 ± 2.57[a]	37.46 ± 2.46[a]	34.38 ± 3.63[a]	35.74 ± 3.27[a]	35.16 ± 2.63[a]
Fruit width (cm)	24.36 ± 1.61[a]	25.16 ± 1.58[a]	25.93 ± 3.74[a]	23.47 ± 1.57[a]	24.74 ± 3.37[a]	24.93 ± 2.75[a]	24.73 ± 2.74[a]	23.64 ± 1.75[a]	25.25 ± 3.27[a]
Fruit weight (Kg)	8.37 ± 2.47[a]	8.64 ± 3.28[a]	8.73 ± 3.63[a]	8.27 ± 2.64[a]	8.64 ± 3.47[a]	8.93 ± 3.74[a]	8.05 ± 1.58[a]	8.32 ± 1.21[a]	8.84 ± 3.73[a]
Pulp ratio (%)	26.40 ± 2.35[a]	27.75 ± 1.60[a]	30.50 ± 1.50[b]	26.50 ± 2.30[a]	27.85 ± 1.55[a]	30.60 ± 1.55[b]	26.55 ± 2.28[a]	27.87 ± 1.53[a]	30.65 ± 1.52[b]
Rag ratio (%)	16.20 ± 1.25[a]	17.60 ± 2.00[a]	12.65 ± 2.25[b]	16.05 ± 1.30[a]	17.68 ± 2.15[a]	12.55 ± 2.20[b]	16.08 ± 1.22[a]	17.67 ± 2.12[a]	12.57 ± 2.18[b]
Seed ratio (%)	14.70 ± 1.15[a]	14.55 ± 1.30[a]	13.35 ± 1.40[a]	14.85 ± 1.10[a]	14.62 ± 1.35[a]	13.40 ± 1.45[a]	14.82 ± 1.12[a]	14.61 ± 1.33[a]	13.37 ± 1.42[a]
Rind ratio (%)	17.20 ± 2.10[a]	16.90 ± 1.80[a]	22.55 ± 1.70[b]	17.35 ± 2.00[a]	16.92 ± 1.85[a]	24.45 ± 1.80[b]	17.32 ± 2.08[a]	16.91 ± 1.83[a]	25.47 ± 1.82[b]
Other parts (%)	25.50 ± 1.75[a]	23.20 ± 1.95[ab]	21.25 ± 2.00[b]	25.25 ± 1.90[a]	22.93 ± 2.00[b]	19.00 ± 2.15[c]	25.23 ± 1.88[a]	22.94 ± 2.05[b]	17.94 ± 2.13[c]
External color	Light green	Dark green	Dark green with black spots and streaks	Light green	Dark green	Dark green with black spots and streaks	Light green	Dark green	Dark green with black spots and streaks
Internal color	Pale yellow, almost white	Light yellow	Yellow	Pale yellow, almost white	Light yellow	Yellow	Pale yellow, almost white	Light yellow	Yellow
Aroma	No aroma	Slight aroma	Strong aroma	No aroma	Slight aroma	Strong aroma	No aroma	Slight aroma	Strong aroma
Taste	No taste	Slightly sweet taste	Distinctly sweet taste	No taste	Slightly sweet taste	Distinctly sweet taste	No taste	Slightly sweet taste	Distinctly sweet taste

a, b, c: Values represent statistically significant differences ($p < 0.05$)

This enhancement in color improved fruit attractiveness, thereby increasing market value and consumer appeal. In terms of aroma and taste, fruits at 100 days exhibited only faint or no aroma. By 120 days, the aroma became more distinct and pleasant, while sweetness was more pronounced. At all areas, fruits at 120 days consistently exhibited a strong characteristic aroma and sweetness, indicating completion of the natural ripening process and improved flavor quality. Thus, 120 days represented the optimal harvest stage for jackfruit intended for fresh consumption.

Overall, analysis of the parameters indicated that 120 days after flowering was the ideal harvest stage for jackfruit in the fresh market. At this stage, fruits exhibited the largest size and weight, the highest aril ratio, attractive color, strong aroma, and pronounced sweetness, thereby enhancing commercial value and consumer satisfaction. In contrast, earlier maturity stages (100 and 110 days) could be more suitable for processed products such as dried jackfruit or green jackfruit, meeting diverse market demands. The similarity in basic parameters across areas further demonstrated that jackfruit from different regions achieved nearly uniform quality, facilitating standardization and the development of branded products. In summary, fruit size, weight, internal component ratios, color, aroma, and taste varied significantly with maturity but remained consistent across areas. The 120-day maturity stage was the most optimal for harvesting and fresh consumption, due to superior sensory attributes and quality. The uniformity of jackfruit quality among areas provided a foundation for production standardization, enhanced competitiveness, and improved export potential of Vietnamese jackfruit.

3.3. Chemical composition and antioxidant activity in major parts of jackfruit at different post-flowering times in the Mekong delta region

Polyphenols were the major groups of compounds in the chemical composition of plants, flowers, and ripened fruits. These compounds exhibited strong biological activities and exerted positive effects on human health, such as antioxidant activity, prevention of the formation of singlet oxygen radicals, control of cancer cell proliferation, and mitigation of human diseases (Van et al., 2023a; Le et al., 2019). Table 3 presented an overview of polyphenol contents in different parts of jackfruit (flesh, fiber, and seeds) and their variations according to cultivation regions and ripening stages. In general, polyphenol content tended to decrease as the ripening stage advanced. The highest polyphenol content was recorded in jackfruit seeds cultivated in CL-TG at 100 days of maturity with a value of 0.32 ± 0.02 mg GAE/g dry weight, while the lowest value was observed in jackfruit fiber cultivated in CD-CT at 120 days with 0.07 ± 0.02 mg GAE/g dry weight. Polyphenol contents did not differ significantly among regions for the same fruit part. Table 3 showed that the polyphenol content in jackfruit seeds was considerably higher than that in flesh and fiber. This trend was consistent with the study of Jagtap et al. (2010), in which the highest TPC was reported in seeds (27.7 mg GAE/g), but it contrasted with the findings of Shrikanta et al. (2015), who reported polyphenol contents of 1.27 mg GAE/g in flesh and 1.00 mg GAE/g in seeds. The present measurements showed similarity with the polyphenol content of 0.21 mg GAE/g in ripe jackfruit flesh using methanol extract and 0.46 mg GAE/g using ethanol extract (Jagtap et al., 2010). However, these results were lower than those reported by Shrikanta et al. (2015), who obtained polyphenol levels ranging from 1.00 to 1.27 mg GAE/g for flesh and seeds.

Flavonoids were secondary phenolic metabolites mainly distributed in plants. They exhibited a wide range of biological activities in plants, animals, and even microorganisms (Khalid et al., 2019). Table 3 indicated that flavonoid contents tended to decrease as ripening progressed. The highest flavonoid content was recorded in jackfruit fiber cultivated in TL-CT at 100 days of maturity (0.44 ± 0.02 mg QE/g dry weight), whereas the lowest value was observed in seeds from CT-

BT and CD-CT at 120 days (0.03 ± 0.01 mg QE/g dry weight). Overall, flavonoid contents in jackfruit fiber were higher than those in flesh and much higher than in seeds. CT-HG and TL-CT exhibited superior flavonoid contents compared to other regions. Previous studies suggested that environmental temperatures ranging from 30 °C to 40 °C could suppress flavonoid biosynthesis, while low-light conditions could also inhibit flavonoid accumulation (Shi et al., 2022). These findings were consistent with Jagtap et al. (2010), who reported 0.24 mg RE/g (rutin equivalent) in ethanol extract. Flavonoid contents in jackfruit flesh ranged from 13.12 mg QE/100 g to 109.44 mg QE/100 g in ethyl acetate and methanol extracts, respectively (Shafiq et al., 2017). Flavonoids possessed multiple biochemical and antioxidant activities with beneficial effects against diseases such as cancer, Alzheimer's disease, and atherosclerosis (Panche et al., 2016). Similar to TPC, as the fruit ripened, increasing enzymatic activity hydrolyzed substantial amounts of flavonoids, leading to decreased TFC over time. Ranasinghe & Marapana (2019b) also reported that the seed coat and outer endosperm contained high flavonoid contents. At 100 days, the seed coat was thick, but it gradually thinned at 110 and 120 days, explaining the decline in flavonoid content in seeds.

Carotenoids were plant pigments functioning as antioxidants, hormone precursors, and natural colorants. They were found in most plant organs and tissues and determined the characteristic color of fruits. Table 3 presented carotenoid contents in jackfruit (flesh, fiber, seeds) across regions and ripening stages (100, 110, 120 days). In general, carotenoid contents increased with ripening. Jackfruit flesh contained higher carotenoid levels (0.27–0.63 mg/mL) compared to fiber (0.19–0.46 mg/mL) and seeds (0.11–0.39 mg/mL). The highest value was 0.63 ± 0.01 mg/mL in flesh at 120 days in PD-CT, while the lowest was 0.11 ± 0.01 mg/mL in seeds at 100 days in TL-CT. Differences across regions could be attributed to environmental factors such as climate, soil, cultivation practices, temperature, and light exposure. Direct exposure to sunlight and higher temperatures enhanced carotenoid biosynthesis, increasing carotenoid contents (de Azevedo & Rodriguez-Amaya, 2005). The present results agreed with previous reports by Jagadeesh et al. (2007), who reported 0.592 µg/g, and Nansereko et al. (2022), who recorded 60.47 µg/100 g.

DPPH radical was widely used to assess the antioxidant capacity of compounds. Table 3 showed IC_{50} values for DPPH radical scavenging activity in different jackfruit parts at 100, 110, and 120 days. DPPH IC_{50} values increased with ripening. The lowest IC_{50} was observed in CT-HG at 100 days (99.32 ± 2.42 µg/mL), while the highest was in CD-CT at 120 days (367.33 ± 4.72 µg/mL). Antioxidant activities in flesh and seeds were comparable and considerably higher than in fiber. These differences were likely due to variations in polyphenol, flavonoid, and carotenoid contents, which influenced antioxidant capacities (Rosa et al., 2009).

The antioxidant potential of jackfruit varied with maturity because maturity influenced enzymatic activities and nutrient levels. As seeds matured, their antioxidant levels increased, protecting them from environmental stressors such as UV radiation and pollutants. Mature seeds also contained higher essential fatty acid levels, which further contributed to antioxidant activity. However, Baliga et al. (2011) reported different values for DPPH scavenging capacity in jackfruit seed extracts, with dichloromethane–methanol (1:1) extract showing IC_{50} = 0.6433 ± 0.0029 mg/mL and acetone extract IC_{50} = 0.7867 ± 0.0104 mg/mL. Although DPPH and ABTS assays were based on radical scavenging, ABTS was not inherently a free radical and required oxidation by a strong oxidant such as $K_2S_2O_8$. Upon oxidation, ABTS lost one electron and generated the ABTS radical.

Table 3. Chemical composition and antioxidant activity of jackfruit parts across different areas and maturity stages

Parts	**Pulp**								
Area	**TL-CT**			**PD-CT**			**CD-CT**		
Time (day) / **Compound**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
TPC (mg GAE/g DW)	0.25 ± 0.04a	0.19 ± 0.06b	0.14 ± 0.02c	0.24 ± 0.02a	0.16 ± 0.05b	0.14 ± 0.04b	0.20 ± 0.02a	0.14 ± 0.02b	0.12 ± 0.02b
TFC (mg QE/ g DW)	0.31 ± 0.01a	0.29 ± 0.05a	0.19 ± 0.03b	0.25 ± 0.00a	0.24 ± 0.05a	0.15 ± 0.01b	0.23 ± 0.02a	0.18 ± 0.05b	0.10 ± 0.00c
TCC (mg/mL)	0.30 ± 0.00a	0.33 ± 0.01a	0.53 ± 0.01b	0.29 ± 0.02a	0.41 ± 0.00b	0.63 ± 0.01c	0.31 ± 0.00a	0.44 ± 0.01b	0.61 ± 0.01c
IC50 DPPH (µg/mL)	108.59 ± 2.75a	114.08 ± 5.30b	165.16 ± 1.39c	116.52 ± 3.16a	124.23 ± 4.66b	170.21 ± 8.22c	128.29 ± 2.37a	135.10 ± 4.61b	202.34 ± 4.40c
IC50 ABTS (µg/mL)	56.60 ± 1.33a	71.40 ± 7.08b	77.64 ± 4.23c	58.61 ± 3.27a	75.36 ± 0.33b	78.55 ± 0.56b	58.65 ± 1.48a	77.32 ± 1.18b	81.54 ± 3.04c
TAC (mg AA/g DW)	113.52 ± 2.33a	44.02 ± 2.17b	35.90 ± 7.33c	93.13 ± 7.38a	36.71 ± 2.04b	34.65 ± 7.37b	87.21 ± 4.94a	32.80 ± 1.59b	29.15 ± 0.69b
Parts	**Pulp**								
Area	**CT-HG**			**CL-TG**			**CT-BT**		
Time (day) / **Compound**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
TPC (mg GAE/g DW)	0.22 ± 0.01a	0.16 ± 0.04b	0.13 ± 0.01b	0.21 ± 0.04a	0.14 ± 0.02b	0.12 ± 0.03b	0.18 ± 0.01a	0.15 ± 0.03b	0.13 ± 0.01b
TFC (mg QE/ g DW)	0.29 ± 0.02a	0.25 ±0.04a	0.16 ± 0.01b	0.26 ± 0.01a	0.22 ± 0.03a	0.13 ± 0.01b	0.22 ± 0.03a	0.17 ± 0.02b	0.13 ± 0.01c
TCC (mg/mL)	0.28 ± 0.01a	0.37 ±0.02b	0.51 ± 0.04c	0.27 ± 0.01a	0.37 ± 0.02b	0.57 ± 0.05c	0.29 ± 0.02a	0.41 ± 0.03b	0.55 ± 0.02c
IC50 DPPH (µg/mL)	99.32 ± 2.42a	108.24 ± 4.24b	154.22 ± 2.47c	112.37 ± 2.41a	136.12 ± 2.21b	164.32 ± 3.33c	114.38 ± 1.45a	147.21 ± 3.13a	195.43 ± 4.12b
IC50 ABTS (µg/mL)	46.21 ± 2.21a	65.27 ±3.32b	76.83 ± 3.12c	49.23 ± 4.43a	68.26 ± 1.27b	80.37 ± 1.34c	54.32 ± 3.41a	71.18 ± 2.25b	77.35 ± 2.43c
TAC (mg AA/g DW)	112.34 ± 2.53a	53.35 ±1.53b	37.43 ± 4.43c	102.43 ± 3.52a	41.25 ± 4.43b	38.34 ± 3.53c	93.43 ± 2.45a	39.34 ± 4.42b	35.43 ± 3.25c

Parts	**Rag**								
Area	**TL-CT**			**PD-CT**			**CD-CT**		
Time (day) / **Compound**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
TPC (mg GAE/g DW)	0.23 ± 0.02a	0.19 ± 0.05b	0.11 ± 0.01c	0.21 ± 0.03a	0.14 ± 0.01b	0.08±0.00c	0.18 ± 0.02a	0.14 ± 0.00b	0.07 ± 0.02c
TFC (mg QE/ g DW)	0.44 ± 0.02a	0.24 ± 0.06b	0.16 ± 0.02c	0.32 ± 0.09a	0.21 ± 0.09b	0.16±0.00c	0.27 ± 0.01a	0.18 ± 0.05b	0.15 ± 0.04c
TCC (mg/mL)	0.19 ± 0.00a	0.21 ± 0.00a	0.32 ± 0.02b	0.20 ± 0.00a	0.21 ± 0.00a	0.33±0.01b	0.24 ± 0.00a	0.24 ± 0.00a	0.44 ± 0.00b
IC50 DPPH (µg/mL)	264.32 ± 2.14a	293.18 ± 5.13b	334.84 ± 3.74c	294.79 ± 4.42a	304.60 ± 3.11b	366.62±3.08 c	311.03 ± 3.37a	315.86 ± 1.72b	367.33 ± 4.72c
IC50 ABTS (µg/mL)	89.13 ± 3.26a	105.24 ± 3.22b	284.32 ± 1.32c	97.55 ± 6.78a	108.82 ± 3.66b	264.97±1.41 c	122.91 ± 2.88a	130.06 ± 2.15b	287.07 ± 3.78c
TAC (mg AA/g DW)	108.31 ± 2.43a	59.32 ± 2.93b	41.39 ± 3.83c	93.13 ± 7.38a	36.71 ± 2.04b	34.65±7.37c	87.21 ± 4.94a	32.80 ± 1.59b	29.15 ± 0.69c
Parts	**Rag**								
Area	**CT-HG**			**CL-TG**			**CT-BT**		
Time (day) / **Compound**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
TPC (mg GAE/g DW)	0.21 ± 0.01a	0.18 ± 0.02b	0.10±0.02c	0.23 ± 0.01a	0.17 ± 0.02b	0.09 ± 0.00c	0.20 ± 0.01a	0.16 ± 0.01b	0.08 ± 0.01c
TFC (mg QE/ g DW)	0.42 ± 0.03a	0.27 ± 0.01b	0.19 ± 0.01c	0.36 ± 0.02a	0.26 ± 0.03b	0.14 ± 0.01c	0.29 ± 0.02a	0.19 ± 0.03b	0.14 ± 0.02c
TCC (mg/mL)	0.21 ± 0.02a	0.29 ± 0.01b	0.34 ± 0.03c	0.23 ± 0.01a	0.24 ± 0.02a	0.38 ± 0.01b	0.22 ± 0.01a	0.31 ± 0.02b	0.46 ± 0.02c
IC50 DPPH (µg/mL)	273.24 ± 6.23a	303.32 ± 4.29b	352.21 ± 5.31c	283.12 ± 3.31a	301.23 ± 2.24b	346.35 ± 2.15c	317.36 ± 4.39a	319.26 ± 5.18a	352.21 ± 2.31b
IC50 ABTS (µg/mL)	91.41 ± 7.23a	109.13 ± 5.23b	264.14 ± 7.26c	96.24 ± 21.15a	104.35 ± 4.14b	271.97 ± 12.41c	114.35 ± 4.31a	135.43 ± 5.19b	276.13 ± 6.13c
TAC (mg AA/g DW)	113.52 ± 10.33a	44.02 ± 2.17b	35.90 ± 7.33c	89.34 ± 3.24a	48.43 ± 1.27b	39.49 ± 1.95c	81.34 ± 2.35a	39.42 ± 2.78b	21.87 ± 2.21c
Parts	**Seed**								
Area	**TL-CT**			**PD-CT**			**CD-CT**		
Time (day)	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**

Compound									
TPC (mg GAE/g DW)	0.28 ± 0.04a	0.24 ± 0.02b	0.14 ± 0.03c	0.27 ± 0.05a	0.23 ± 0.00b	0.14 ± 0.04c	0.25 ± 0.04a	0.22 ± 0.00b	0.13 ± 0.02c
TFC (mg QE/ g DW)	0.06 ± 0.02a	0.05 ± 0.00a	0.04 ± 0.00a	0.05 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.01a	0.04 ± 0.01a	0.03 ± 0.01a	0.03 ± 0.01a
TCC (mg/mL)	0.11 ± 0.01a	0.14 ± 0.01a	0.30 ± 0.01b	0.11 ± 0.02a	0.15 ± 0.01a	0.33 ± 0.03b	0.12 ± 0.02a	0.16 ± 0.01a	0.39 ± 0.03b
IC50 DPPH (µg/mL)	108.59 ± 9.75a	114.08 ± 5.30b	165.16 ± 1.39c	116.52 ± 3.16a	124.23 ± 4.66b	170.21 ± 8.22c	128.29 ± 2.37a	135.10 ± 4.61b	202.34 ± 17.40c
IC50 ABTS (µg/mL)	56.60 ± 6.33a	71.40 ± 7.08b	77.64 ± 4.23c	58.61 ± 11.27a	75.36 ± 0.33b	78.55 ± 0.56b	58.65 ± 8.48a	77.32 ± 1.18b	81.54 ± 3.04c
TAC (mg AA/g DW)	123.67 ± 3.70a	88.11 ± 5.82b	56.03 ± 2.82c	115.40 ± 4.47a	80.09 ± 2.18b	48.43 ± 2.62c	110.80 ± 3.33a	62.56 ± 3.78b	42.87 ± 5.83c
Parts	**Seed**								
Area	**CT-HG**			**CL-TG**			**CT-BT**		
Time (day) / **Compound**	**100**	**110**	**120**	**100**	**110**	**120**	**100**	**110**	**120**
TPC (mg GAE/g DW)	0.31 ± 0.02a	0.26 ± 0.03b	0.12 ± 0.01c	0.32 ± 0.02a	0.26 ± 0.01b	0.13 ± 0.02c	0.26 ± 0.02a	0.21 ± 0.01b	0.15 ± 0.01c
TFC (mg QE/ g DW)	0.09 ± 0.01a	0.06 ± 0.00b	0.05 ± 0.00b	0.11 ± 0.01a	0.07 ± 0.00b	0.06 ± 0.01b	0.08 ± 0.01a	0.05 ± 0.00b	0.03 ± 0.01b
TCC (mg/mL)	0.13 ± 0.01a	0.18 ± 0.02b	0.33 ± 0.01c	0.12 ± 0.01a	0.17 ± 0.01b	0.36 ± 0.02c	0.14 ± 0.02a	0.21 ± 0.01b	0.36 ± 0.01c
IC50 DPPH (µg/mL)	111.32 ± 2.35a	119.01 ± 3.21b	167.32 ± 3.41c	114.32 ± 2.12a	132.53 ± 4.66b	168.37 ± 3.53c	115.31 ± 2.37a	127.13 ± 3.24b	197.43 ± 4.13c
IC50 ABTS (µg/mL)	54.43 ± 3.25a	75.31 ± 4.15b	79.32 ± 2.86c	59.18 ± 3.38a	78.74 ± 1.36b	82.84 ± 1.46c	54.54 ± 2.43a	75.64 ± 3.21b	83.26 ± 2.15c
TAC (mg AA/g DW)	114.32 ± 9.21a	79.34 ± 5.22b	59.24 ± 3.29c	119.53 ± 4.14a	85.23 ± 4.35b	44.24 ± 3.21c	118.35 ± 6.12a	68.19 ± 2.61b	41.32 ± 6.14c

a, b, c: giá trị thể hiện khác biệt có ý nghĩa thống kê ($p<0.05$)

Table 3 indicated that the highest ABTS IC_{50} was observed in fiber (287.07 ± 3.78 µg/mL) in CD-CT, while the lowest was in flesh (46.21 ± 2.21 µg/mL) in CT-HG. Overall, ABTS IC_{50} values increased with ripening, indicating a decline in antioxidant capacity across jackfruit parts. Regional differences were also observed, similar to those found for DPPH. Values reported by Cregger et al. (2014) differed, with an IC_{50} of 7.62 ± 0.13 mg/mL, but were consistent with studies showing ABTS scavenging activity in jackfruit seed extracts with acetone and dichloromethane–methanol (1:1), yielding IC_{50} = 0.0491 ± 0.0005 mg/mL and IC_{50} = 0.0556 ± 0.0002 mg/mL, respectively.

Table 3 also illustrated the influence of ripening stages (100, 110, 120 days) and cultivation regions on total antioxidant capacity (TAC) in jackfruit. TAC values tended to decrease with ripening and varied significantly among fruit parts. The highest TAC was observed in seeds at 100 days in TL-CT (123.67 ± 3.70 mg AA/g dry weight), while the lowest was recorded at 120 days in CT-BT (21.87 ± 2.21 mg AA/g dry weight). TAC values exhibited trends consistent with IC_{50} values from DPPH and ABTS assays, with seeds and flesh showing higher antioxidant capacity than fiber. Three methods (ABTS, DPPH, TAC) were employed to evaluate antioxidant activity in jackfruit. Among them, ABTS and DPPH were more reliable compared to TAC. IC_{50} values were critical indicators of antioxidant capacity in extracts for ABTS and DPPH methods. TAC, however, did not yield IC_{50} values because the standard compound, ascorbic acid, generated H_2O_2 during oxidation, which reduced overall antioxidant potential. ABTS yielded lower IC_{50} values than DPPH, possibly because ABTS radicals were measured at 734 nm (far from the visible region), whereas DPPH radicals were measured at 517 nm (closer to the visible region), which could lead to optical interference and differences between the two methods.

From the above results, it was observed that the differences among the areas were negligible, with a consistent trend of similar bioactive compound profiles. This could be explained by the natural conditions of the Mekong Delta, where climatic, soil, and cultivation factors did not differ significantly. Such uniformity not only facilitated the consistent exploitation of the biological value of jackfruit across regions but also created favorable conditions for developing sustainable harvesting and utilization strategies, particularly by focusing on the 120-day maturity stage to achieve optimal efficiency.

4. Conclusions

The study revealed relatively uniform presence of bioactive compounds across different areas. The pulp was richest in phenolics, tannins, and flavonoids at 100–110 days of maturity. The fibers contained significant levels of alkaloids and flavonoids, particularly at 120 days. The seeds were notable for their protein, amino acid, and carbohydrate contents at all maturity stages. Saponin levels remained stable across all parts and locations, with the highest values recorded at 110–120 days. Polyphenol content tended to decrease with maturity, flavonoid content peaked in fibers (TL-CT, 100 days), while carotenoids increased with maturity. DPPH radical scavenging activity increased with maturity, whereas TAC decreased. The assessment of raw material characteristics (shape, size, weight, component ratios, color, odor, taste) in pulp, fiber, and seeds showed maturity-dependent variations, with the 120-day stage being generally optimal for fresh consumption. The study provided a comprehensive database of physicochemical properties, chemical composition, and antioxidant activities of jackfruit pulp, fiber, and seeds, serving as a basis for proposing appropriate preservation and processing methods. Furthermore, it identified cultivation regions that offered higher nutritional value, thereby suggesting potential applications in various fields.

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