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Short Communication: Fungicidal effect of flax seed oil against several fungal strains

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Abstract. *Mir RA, Ahmad MK. 2023. Short Communication: Fungicidal effect of flax seed oil against several fungal strains. Asian J Nat Prod Biochem 21: 1-5.* The plant world is provided with innate biologically active secondary metabolites, which regulate its defense mechanism. Chemical fungicides pose a great threat to the plant kingdom regarding disease resistance and phytotoxicity. Furthermore, chemical fungicides provide a short spectrum and poor control of plant pathogens. Seed oils have a great potential to act as a natural fungicide against several plant pathogens. The aim of this study was to evaluate the *in vitro* antifungal activity against eight fungal strains, *Mucor mucedo, Penicillium expansum, Trichoderma viride, Trichoderma harzianum, Phytophthora crocatum, Aspergillus niger, Phytophthora infestans,* and *Venturia inaequalis* by the poisoned food method. Antifungal activity was assessed regarding the inhibition percentage of radial growth on a solid medium (Potatoes Dextrose Agar/PDA). Flaxseed oil showed the highest (100%) antifungal activity against *M. mucedo* at a concentration of 5 µL/mL, while moderate (53% and 60%) antifungal activity was recorded for *P. expansum*, and *P. crocatum.* However, linseed oil showed negative results against *A. niger*, *T. viride,* and *T. harzianum.* Thus, the results of the present study revealed that flaxseed oil has tremendous fungicidal activity and could become an alternative to synthetic fungicides for controlling certain dreadful plant fungal diseases.

Keywords: Antifungal activity, flax seed oil, phytopathogenic fungi, secondary metabolites

INTRODUCTION

Flax has been grown by early civilizations for its tremendous health benefits and is mainly cultivated for seed and fiber production. However, plant extracts have various allopathic properties and are therefore explored for their biological use. Moreover, its seed oil is a rich source of fatty acids and secondary metabolites, which possess several biological activities. Therefore, the search for new natural products, including plant extracts, seems to be the need of the hour.

Increased fungicidal resistance of pathogenic fungi against chemical fungicides has led to an urgent need to identify alternative strategies for managing fungal pathogenicity. Agro-based industries are looking for novel natural organic compounds to be used as non-synthetic antimicrobial agents to eradicate the most notorious phytopathogens. The agriculture industry has undergone huge losses due to many fungal pathogens, namely *Mucor mucedo, Penicillium expansum, Phytophthora crocatum, Aspergillus niger, Phytophthora infestans,* and *Venturia inaequalis.* These phytopathogens are responsible for many dreadful plant diseases, such as root rots, mucor rots, citrus fruit rots, and post-harvest fungal infestations (Amiri 2019; Savary et al. 2012).

Phytopathogens have slowly developed resistance against several chemical pesticides, which led to excess chemicals with higher concentrations, proving fatal for soil and human health. Therefore, an alternative, safe, eco-friendly biopesticidal formulation is urgently needed. Much research has been done to find novel essential oils to replace chemical pesticides. Essential oils from *Artemisia annua* have been used to control the *Glyphodes pyloalis* pathogen of mulberry leaves (Oftadeh et al. 2021). Post-harvest storage is a serious problem faced by the agriculture and food industries. Chemical fungicides are used at a high concentration to reduce the losses. Essential oils can be used as an alternative to stored products and have been used against *Sitophilus granarius* (Demeter et al. 2021)

Essential oils have been continuously explored for their potential as antimicrobial agents. Several essential oils from *Allium sativum* and *Citrus sinensis* have been found effective against several phytopathogens (De Clerck 2020). Essential oils are used as spices, remedies, or for their pleasant odor; rich in terpenoids and non-terpenoid compounds, they possess various interesting allelopathic properties. Due to technological advancements, modern techniques are applied to distil volatile compounds (Baser and Buchbauer 2010; Baser and Demirci 2011). Those compounds are important in food, medicine, or cosmetics and are becoming increasingly popular as antibacterial, antioxidant, antifungal, and insecticide agents. (Mezzoug et al. 2007; M'barek et al. 2007; Rozman et al. 2007).

Natural oils are also explored for their insecticidal properties (Isman 2000). According to a recent study, seed oil has innate defence abilities (Halloin 1983; Harman 1983; Kremer et al. 1984; Ceballos et al. 1998; Ozer et al. 1999). Seed oils have been used as antimicrobial agents for rhizospheric soil (Helsper et al. 1994). Plant health is

mainly affected by early fungal infections, which affect its growth and development processes (Alvarez-Castellano et al. 2001; Skaltsa et al. 2000). Flax is an ancient Egyptian crop cultivated worldwide as an edible oil crop (Kaithwas and Majumdar 2013). The antimicrobial effect of various plant extracts was studied and documented (Paiva et al. 2010). Incorporating essential oils from *Thymus vulgaris* in milk for cheese production provides an additional defence against several exogenous detrimental pathogens of dairy products (Licon et al. 2020)

Canada is the world's biggest producer of flax, with 38%, while the annual worldwide is 3.06 million tons (Fadzir et al. 2018). The antimicrobial properties of flaxseeds were studied for their therapeutic properties (Adolphe et al. 2010). Flax seed oil has antimicrobial, anti-inflammatory, antioxidant, and wound-healing properties (Sharil et al. 2022). *Linum usitatissimum* oil is an antimicrobial agent in bovine mastitis caused by microbial infection (Kaithwas et al. 2011). In addition, the presence of polyphenols in flax seed has been considered an antimicrobial agent (Barbary et al. 2010; Pag et al. 2014). Flax seed cake was extensively studied and reported as an antimicrobial food additive (Freese et al. 1973; Zheng et al. 2005; Zuk et al. 2014). Antifungal activities of flax seeds were previously studied against many pathogenic fungal strains, including human pathogenic fungi such as *Candida albicans, Alternaria solani, Alternaria alternata, Penicillium chrysogenum,* and *Fusarium graminearum* (Guilloux et al. 2009). The *C. albicans* were highly susceptible to fixed oil than cefoperazone, with an inhibition zone of 10.33 mm and 4.33 mm, respectively (Kaithwas et al. 2011). Flax seeds also control the growth of food-borne fungal pathogens (Xu et al. 2007). Another study reported that flaxseed lignans extract demonstrated moderate (70% to 90%) antifungal activities at 2.5 to 3.0 mg/mL for both *Aspergillus flavus* and *A. niger* (Barbary et al. 2010). The antifungal nature of flax seed was reported due to the presence of α linolenic acid and linoleic acid, considered fungal growth suppressors (Abdelillah et al. 2013)

The bioactive compounds from flaxseeds were effective against *Escherichia coli, Salmonella paratyphi, Lactobacillus, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumoniae,* and *Saccharomyces cerevisiae* (Narender et al. 2016). In addition, flax seed oil extracts exhibited antimicrobial properties against the growth of *A. alternata* and *A. solani* in in-vitro conditions (Guilloux et al. 2009). Recently it was reported that genetically modified flax-type (GT) seedcake extract possesses antimicrobial activity against *S. aureus* and *E. coli* (Czemplik et al. 2012). Seedcake extract was also reported as an alternative to inhibit microbial growth of a wide range and with partial selectivity (Zuk et al. 2014). The lignans from the flaxseed interfere with the bacterial cell wall and inhibit their growth (Cowan 1999; Barbary et al. 2010).

The present work aimed to investigate the antimicrobial effects of flax seed oil against many phytopathogens in-vitro so that an eco-friendly bio-sustainable organic formulation can be developed to eradicate the pathogenicity of tested fungi.

MATERIALS AND METHODS

Samples and materials

Commercially available flax seed oil was purchased from a local distributor in New Delhi, India. Peptone dextrose agar powder and peptone dextrose broth powder were purchased from Hi-Media, Mumbai. The antibiotic chloramphenicol was purchased from BM Scientific company Srinagar Kashmir.

Culture and maintenance of the fungal isolates

The antimicrobial activity of flax seed fixed oil was evaluated against different microbial strains. A total of eight plant fungal strains, namely *M. mucedo, P. expansum, Trichoderma viride, Trichoderma harzianum, P. crocatum, A. niger, P. infestans,* and *V. inaequalis* were tested in this study. First, the pure cultures were obtained from Globils Agri and Food Enterprises Igc Lassipora Pulwama Jammu and Kashmir, India. Next, the fungal cultures were identified based on the colony characteristics of the respective fungi. The stock culture was maintained on a Potato Dextrose Agar (PDA) medium. Next, the poison food method of antifungal activity was used for antimicrobial testing of flax seed oil.

Preparation of media

Fungal media

Thirty-nine grams of PDA Powder (high media) was dissolved in 1 L of distilled water and then sterilized in an autoclave at 121°C (15 lbs pressure) for 15 minutes.

Determination of the antifungal activity of flax seed essential oil

The antifungal assay was performed in two parts: Inhibition Concentration (IC50) and Minimum Inhibitory Concentration (MIC).

Inhibition Concentration (IC50)

The pure cultures of fungal test strains were maintained on a PDA medium for seven days. The assessment of fungitoxicity was performed by the poisoned food method (Grover and Moore1962; Adjou et al. 2012). Specific initial concentrations (1, 2, 3, 4, and 5 μL/mL) were prepared by adding the appropriate amount of flax seed essential oil containing 0.5% Tween 80 (Tween-80: 500mg/100mL). Next, 100 mL of dissolved flax oil were aseptically poured into the Petri plate, and 20 mL of melted PDA was added at 45°C. Petri plates were gently shaken to avoid entrapment of air bubbles in the oil. The medium was allowed to solidify at room temperature for about one hour. 6 mm in diameter fungal discs were cut from the periphery of seven-day-old pure cultures using a sterile cork borer and aseptically placed in the center of Petri plates. Finally, 100mg/1000mL chloramphenicol was used as an antibiotic to avoid bacterial growth. Flax oil was not mixed with the media in control plates. Three replicates were maintained for each treatment. All the plates were incubated at 28°C

for five days. The statistical data was collected after three and five days of incubation, and mean data was used to calculate the inhibition percentage. The percentage inhibition of the mycelia growth of the test fungi was calculated using the formula by (Rao and Srivastava 1994). The inhibition percentage was compared with the control plates.

Mycelial growth inhibition I (%) = C-T/C×100

Where:
I : Inhibition percentage
C : Colony diameter in control (cm)
T : Colony diameter in treatment (cm)

Data analysis

All data were presented as mean ± Standard Error (SE) of replicates and were analyzed using Data Processing Software (DPS, version 7.05) following one-way Analysis of Variance (ANOVA). Significant differences ($P < 0.05$) among treatment means after controlling for multiple comparisons were determined from a Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Antifungal activity

The present study showed that flax seed oil exhibited a strong action against the mycelia growth of eight test fungi; *M. mucedo, P. expansum, T. viride, T. harzianum, P. crocatum, A. niger, P. infestans,* and *V. inaequalis* (Figure 1). A complete mycelia growth inhibition was observed at 5µL/mL concentration. In addition, flax seed oil had a significant ($p \leq 0.05$) higher inhibitory effect on *M. mucedo* and *P. crocatum* (Figure 2, Table 1). The range of mycelia growth inhibition was between 18% to 100% at a concentration of 5 µL/mL. However, the minimum inhibitory effect was observed in *T. viride*, *T. harzianum,* and *A. niger* (Table 2).

Minimum inhibitory concentration

Inhibition of the mycelial growth of test fungi by flax seed essential oil after five days of incubation ranged from 7.33 % to 100% (Table 3). The highest and lowest inhibition of mycelial growth at a concentration of 1 µL/mL were observed in *M. mucedo* (75.47%) and *T. viride* (7.33%), respectively. The inhibition of mycelial growth in all the test fungi at an EO concentration of 1 µL/mL was significantly ($p \leq 0.05$) different.

Table 1. Mycelial growth inhibition (in percentage) by flax oil on the radial growth of different fungi

Fungus species	Growth inhibition (%)	
	Control	100 µL conc.
Mucor mucedo	0	100
Penicillium expansum	0	53
Aspergillus niger	0	20
Phytophthora infestans	0	45
Venturia inaequalis	0	40
Phytophthora crocatum	0	60
Trichoderma harzianum	0	20
Trichoderma viride	0	18

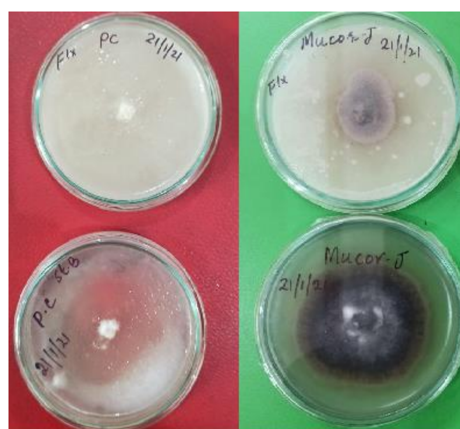

Figure 2. Effect of flax oil on *Phytophthora crocatum* and *Mucor mucedo*

Figure 1. Antifungal activity of flax seed oil against eight different fungi

Table 2. Mycelial growth inhibition (%) of eight fungal strains after three days of incubation

Essential oil concentrations (µL/mL)	Mycelial growth inhibition (%)							
	Mucor mucedo	*Penicillium expansum*	*spergillus niger*	*Phytophthora infestans*	*Venturia inaequalis*	*Phytophthora crocatum*	*Trichoderma harzianum*	*Trichoderma viride*
1	39±0.57	21.66±0.33	8.5±0.28	17.16±0.16	17.16±0.72	25.16±0.60	8.16±0.44	7.33±0.33
2	49.33±0.44	26.5±0.28	10.16±0.33	21.5±0.28	21.33±0.88	31.5±0.76	10.16±0.44	9±0.28
3	59.33±0.66	31.66±0.33	12.66±0.33	25.66±0.33	25.66±1.20	34.33±3.28	12±0.57	10.33±0.33
4	79±1.15	42.33±0.88	15.66±0.33	32.66±1.45	33.33±0.88	50±1	16±0.57	14±0.57
5	100±0	53±0.57	20.33±0.66	43±0.57	42.66±1.76	63±1.52	20.5±0.86	18±0.57

Note: Data: means ±SE statistically significant differences between treatments (P<0.05)

Table 3. Mycelial growth inhibition e (%) of eight fungal strains after five days of incuba

Essential oil concentrations (µL/mL)	Mycelial growth inhibition (%)							
	Mucor mucedo	*Penicillium expansum*	*Aspergillus niger*	*Phytophthora infestans*	*Venturia inaequalis*	*Phytophthora crocatum*	*Trichoderma harzianum*	*Trichoderma viride*
1	75.47±2.18	39±0.57	12±0.57	25.66±0.33	20.33±0.66	31.66±0.33	12.66±0.33	7.33±0.33
2	78.62±1.66	40±1	12.66±0.33	26.5±0.28	25.16±0.60	42.33±0.88	15.66±0.33	10.33±0.33
3	84.28±1.66	44.65±2.27	15.66±0.33	31.5±0.76	33.33±0.88	54±2.30	17.16±0.72	16±0.57
4	93.71±1.66	64.33±1.85	17.16±0.72	34.33±3.28	39±0.57	56.66±1.20	18±0.57	17.16±0.72
5	100±00	50.35±0.54	19.31±0.63	40.85±0.54	40.53±1.67	59.85±1.45	19.47±0.82	17.1±0.54

Note: Data: means ±SE statistically significant differences between treatments (P<0.05)

The Minimum Inhibitory Concentrations (MICs) of flax seed oil on the test pathogens were within 1-5 µL/mL (Table 3). At a concentration of 5µL/mL, the mycelia growth of *M. mucedo* was completely inhibited. The essential oil of flax seed oil showed a fungicidal effect on three of the eight studied fungi: *P. expansum*, *P. crocatum,* and *M. mucedo*. Whereas linseed oil showed negative results against *A. niger, T. viride,* and *T. harzianum.*

The traditional use of medicinal plants to manufacture commercial drugs and their substitutes has been predominant since ancient times. These drugs help in the treatment of different forms of ailments caused by bacterial and fungal pathogens and in other important health-related activities (Cowan 1999).

The antimicrobial efficacy of flax seed oil has been carried out against many fungal pathogens, including *M. mucedo, P. expansum, P. crocatum, A. niger, P. infestans,* and *V. inaequalis.* The flax seed oil behaves differently in controlling the fungal growth *in vitro*. The *M. mucedo* and *Phytophthora* growth were completely inhibited at 100 microlitres per mL concentration. The other test fungal growth was also inhibited but not completely. The effect of antimicrobial inhibition was detected by observing growth patterns, sporulation, and mycelia growth inhibition. The present result follows Kreander et al. (2006), who reported that ethanol extract of *L. usitatissimum* showed antibacterial activity against *K. pneumonia* and *Pseudomonas aeruginosa*. The most common pathogen which causes serious problems in fruit trees is *P. crocatum*, which causes root rot in the apple, causing considerable losses to the apple industry during fruit growth.

The *M. mucedo* is a fungal plant pathogen that causes many fungal diseases, including mucor rot. Bukar et al. (2009) conducted a pathogen survey in Nigeria's sweet orange (*C. sinensis*) and found that 25% of decayed fruit were associated with Mucor spp. Mucor species has been found to cause fingernail infection among workers who squeezed cull orange fruit with bare hands (Sutherland-Campbell and Plunkett 1934). The *L. usitatissimum* extracts have antibacterial activity against three types of Gram-negative bacteria (*Shigella flexneri, Salmonella typhimurium,* and *E. coli*), and the maximum zone of inhibition was observed in *S. flexneri*. This may be considered promising natural-based antibiotics.

Recently, many studies have been conducted to explore the potential antimicrobial agents from natural resources. Plant extracts, especially essential oils, have been extensively studied for their natural phytochemicals. For example, genetically modified flax seed has many antimicrobial agents, including flavonoids (Zuk et al. 2011). Therefore, further studies should be done to know flax seed oil's exact mechanism.

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Risk assessment of heavy metal content in yam tubers locally produced in selected local government areas of Taraba State, Nigeria

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Abstract. *Olawale OF, Abah MA, Emmanuel OP, Otitoju GT, Abershi AL, Temitope DF, Andrew AE, Abdulkadir S, John A. 2023. Risk assessment of heavy metal content in yam tubers locally produced in selected local government areas of Taraba State, Nigeria. Asian J Nat Prod Biochem 21: 6-12.* Yam is one of the most cultivated tubers in northern Nigeria. The increasing yam consumption and pest attack on the crop have led to increased use of fertilizers and pesticides during the cultivation process to prevent and control pest attacks. This study aims to determine the concentration of several heavy metals (Hg, Cr, Cd, Pb, and As) in locally produced yam samples harvested from Jalingo, Takum, Mutum Biyu, Wukari and Zing Local Government Area (LGA) of Taraba state as well assessing the Total Cancer Risk and Hazard Quotient associated with consuming the analyzed yam samples. The samples were collected in duplicates from five LGAs of Taraba state (Jalingo, Takum, Mutum Biyu, Wukari, and Zing). They were washed, peeled, and dried before being analyzed for heavy metal content using the method of Nitric acid-hydrochloric acid digestion (APHA 3030 f. 2018) by Atomic Absorption Spectroscopy (AAS). The data were analyzed using ANOVA ($P<0.05$). The results showed that Hg and As values were below the permissible limit recommended by WHO, with values ranging from 0.02-0.05 mg/kg. Cadmium content was significantly high, ranging from 8.00-14.00 mg/kg. Chromium content from Zing LGA was higher than in other locations, ranging from 100.00 to 103.00 mg/kg. The risk assessment analysis revealed that chromium and arsenic had the highest and lowest hazard quotient values of 37.32 and 0.00180, respectively, for yam samples cultivated in Jalingo LGA. The highest TCR value was obtained in chromium (3.2×10^{-3}) and the lowest in the lead (1.23×10^{-8}). High levels of heavy metals present in yam samples could be due to excessive use of pesticides or fertilizers during cultivation, burning of plastics and rubbers, deposition from mountains, and other anthropogenic activities. The results of this study are expected to gain public awareness that consuming locally produced yam from LGAs contaminated with heavy metals is at risk of heavy metal toxicity, such as renal, neural, and respiratory disorders, among others.

Keywords: Bioaccumulation, cancer estimation, carcinogenesis, heavy metals, risk assessment, toxicity

INTRODUCTION

Food shortages are becoming a major problem in most Nigerian states. It could be due to increasing crop damage by pests leading to a poor crop yield (Fu et al. 2008). Therefore, to solve this problem, farmers have resorted to applying pesticides and fertilizers during cultivation to control the activities of pests and increase crop yield, respectively (Cao et al. 2010; Abah et al. 2021). Unfortunately, the use of pesticides and fertilizers on crops has been associated with the release of heavy metals such as Cadmium (Cd), Chromium (Cr), Arsenic (As), Lead (Pb), Nickel (Ni), and Stannum (Sn) (Olawale et al. 2022). Consumption of food contaminated with heavy metals seriously affects the health and economic status of the population (Asha et al. 2010; Tchounwou et al. 2012). Heavy metal contamination could originate from several sources, such as rocks, industrial wastes, and chemicals deposited into the soil (Seepersaud et al. 2018). In addition, heavy metals may enter soils from different natural and anthropogenic (human activities) sources, including industrial or domestic wastewater, application of pesticides and inorganic fertilizers, storm runoff, leaching from landfills, shipping and harbor activities, geological weathering of the earth's crust and atmospheric deposition. Rapid industrialization also resulted in the discharge of potentially toxic trace metals such as mercury, cadmium, copper, chromium, and nickel into the marine environment (Sawidis et al. 2001).

Tubers such as yam, potatoes, cassava, and others are good food sources in Africa and other countries worldwide. Production of tubers provides employment, food supply, and food security (Yerima et al. 2020). Several African countries have long used yam as a food source and traditional medicine. Yam is composed of starch with small amounts of sugars, cellulose, proteins, lipids, and minerals and plays an essential role in ensuring food security and livelihood (Flora et al. 2016). Tubers like yam could be marketed as fresh or processed products such as powdered, pounded, or direct boiling (Lanre-Iyanda and Adekunle

2012). Furthermore, plants that grow in contaminated soil absorb heavy metals and other pollutants and accumulate them in their tubers since they cannot metabolize them completely (Valko et al. 2005). The ability of yam to absorb heavy metals is affected by several factors such as soil pH, metal solubility, conductivity, the soil type, amount of the metal (s) in the soil or air, its nature, and the form of chemicals used on the farm such as the type of the fertilizer (Ellen et al. 1990; Rani et al. 2014). Different plants have different capacities and abilities to absorb heavy metals depending on their metabolism, leading to uneven plant distributions of the metals. Higher consumption of contaminated foods leads to various illnesses due to toxicity (Tchounwou et al. 2004). Exposure to these heavy metals, such as cadmium (Cd), lead (Pb), zinc (Zn), arsenic (As), and copper (Cu), is hazardous to human health (Benavides et al. 2005). Cadmium and copper are harmful to the environment, and their accumulation in farmlands used for yam cultivation could result in the contaminated product (De Mattias Sartori et al. 2004). These heavy metals have a long half-life, non-biodegradable, depleted nutrients, and soluble in water. Lead poisoning could cause physiological and morphological changes in microalgae. In addition, several heavy metals also cause toxicity-related mutagenesis and carcinogenesis (Tchounwou et al. 2004).

Long-term heavy metals exposure to plants accumulates, while animals consume many. Heavy metal accumulation in animals or crops is difficult to decontaminate or excrete even by disposing of metal-contaminated agricultural soils for safer food production (Hudson 2021). Heavy metals estimation and analysis could be used to analyze environmental pollution and information on the risk of contaminated agricultural products (Sirot et al. 2009). Due to the harsh economic situation, the number of farmers who cultivate yam has been increasing recently in Taraba state. Using fertilizers to boost soil fertility has become a primary source of soil contamination; hence, this research aims to determine the heavy metal content in yam samples locally produced in some selected Local Government Areas of Taraba state.

MATERIALS AND METHODS

Study area

This research was conducted in Taraba State, Nigeria (Figure 1), at a Longitude of 7.9994° N and latitude of 10.7740° E from December 2021 to March 2022. The geographical coordinates of the five Local Government Areas in this study were: Wukari (7.9303° N; 9.8125° E), Takum (7.2577° N, 9.9745° E), Jalingo (8.8929° N, 11.3771°E), Zing (8.9952°N, 11.7467°E), and Mutum Biyu (8.6450°N, 10.7718°E).

Sample collection

Two tubers of white yam were collected from each location of five Local Government Areas of Taraba state that commonly produced yam (Jalingo, Takum, Mutum Biyu, Wukari, and Zing) were treated as individual treatment. Samples were sent to the laboratory in January 2022 and stored at room temperature before analysis.

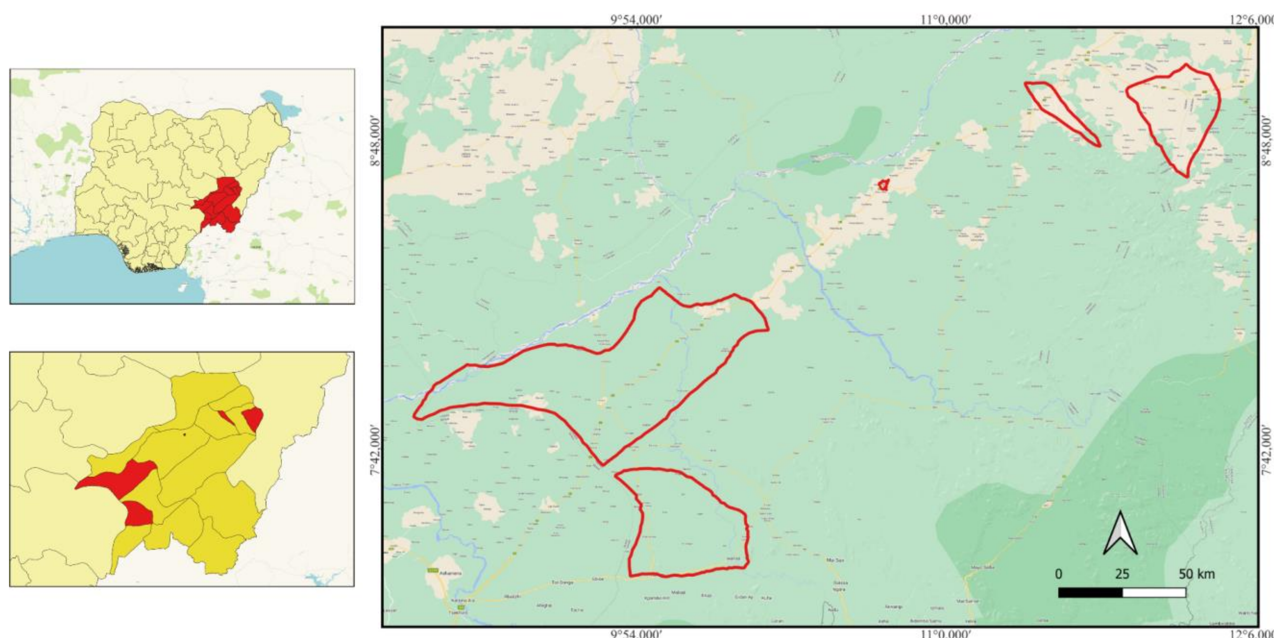

Figure 1. Yam-producing area in Local Government Areas of Taraba State, Nigeria indicated by red color

Sample collection

Two tubers of white yam were collected from each location of five Local Government Areas of Taraba state that commonly produce yam (Jalingo, Takum, Mutum Biyu, Wukari, and Zing) and were treated as individual treatment. Samples were sent to the laboratory in January 2022 and stored at room temperature before analysis.

Sample preparation

The samples were thoroughly washed with distilled water to remove surface dirt, dust, and other deposits which may cause contamination and then peeled. The samples were rewashed thoroughly with distilled water to remove contamination. The cleaned samples were cut into smaller pieces to speed up the drying process. The samples were air-dried for three days and ground using a pestle and mortar. They were again washed and allowed to dry. They were placed into well-labeled envelopes for further analysis. The samples were labeled in the order shown below:

Takum farms 1 and 2 as TK1 and TK2, respectively
Mutum Biyu farm 1 and 2 as MB1 and MB2, respectively
Zing farms 1 and 2 as ZG1 and ZG2, respectively
Jalingo farms 1 and 2 as JL1 and JL2, respectively
Wukari farms 1 and 2 as WK1 and WK2, respectively

Sample digestion

Sample digestion was performed using the method by Okoli et al. (2022). Five (5) mL of a well-mixed acid appropriate for the heavy metals of interest was transferred to a flask. In a fume cupboard, 3 mL of concentrated HNO_3 was added to the flask, covered with a ribbed watch glass, and then placed on a heating mantle, and the mixture was cautiously evaporated to less than 5 mL. The mixture was cooled, and the flask walls were rinsed. The glass was washed with metal-free water, and then 5 mL of concentrated HNO_3 was added and covered with a no-ribbed watch glass. The flask was put into the heating mantle. The heating continued until digestion was complete. Additional heating for 15 mins was done to dissolve any precipitate. The mixture was cooled down, and the flask walls were washed with distilled water. Insoluble materials were filtered, and the filtrate was transferred to a 100 mL volumetric flask and then mixed thoroughly.

AAS sample analysis

Heavy metal concentrations in the digested samples were determined using a Shimadzu Atomic Absorption Spectrophotometer (AAS-model 6650F with the appropriate lamps installed. Heavy metal concentration was quantified from calibration curves using the individual metal standards. The heavy metals analyzed were: Lead (Pb), Chromium (Cr), Mercury (Hg), Arsenic (As), and Cadmium (Cd).

Hazard quotient estimation

The Hazard quotient model assessed the risk of consuming yam-contaminated heavy metals in five Local Government Areas. The hazard quotient is the ratio of the potential exposure to a substance and the level of no adverse effects. The Estimated Daily Intake (EDI) was calculated using the equation described by Mai (2006).

$$\text{Hazard Quotient} = \frac{Estimated\ Daily\ Intake\ (EDI)}{Acceptable\ Daily\ Intake\ (ADI)}$$

A hazard quotient less than or equal to one indicates negligible hazard, while a hazard quotient greater than one indicates hazard possibility.

$$EDI = \frac{\text{(concentration of heavy metal as mg/ kg) x (daily intake of food in kg/person)}}{\text{Adult body weight (60 kg)}}$$

Target cancer risk analysis

The target cancer risk formula by Juan et al. (2010) was used to calculate the possibility of consumers having cancer after consuming yams contaminated with heavy metals in the five Local Government Areas.

$$TR = \frac{EFr \times EDtot \times YI \times MCS \times CPSo \times 10\text{-}3}{BWa \times ATc}$$

Where:

EFr : Exposure frequency (350 days/years)
EDtot : Exposure duration, total (30 years)
YI : Yam ingestion, gram per day (1 g) × 1000 mg/kg
MCS : Metal concentration
BWa : Adult (65 kg)
CPSo : Carcinogenic potency slope, oral (1 mg/kg/day)
ATc : Averaging time, carcinogens (25,550 days)

*Reference doses for the following heavy metals as recommended by WHO/FAO are as follows: Lead 0.004 (mg/kg, Arseni; 0.01 (mg/kg), Chromium 0.003 (mg/kg), Cadmium 0.001 and Mercury 0.001 (mg/kg).

Data analysis

The variations in the level of heavy metals in the yam samples were analyzed using Analysis of Variance (ANOVA). Statistical test was performed at $p<0.05$ level of significance with the result reported as Mean ± SD.

RESULTS AND DISCUSSION

Heavy metal concentration and risk assessment of yam

Table 1 shows the heavy metal concentration in yam samples cultivated in Jalingo LGA, and the order of these heavy metal concentrations is presented as follows: Cr>Cd>Pb>Hg>As. The risk assessment analysis revealed that chromium and arsenic had the highest and lowest hazard quotient values of 37.32 and 0.00180, respectively. The highest TCR value was obtained in chromium (3.2×10^{-3}) and the lowest in the lead (1.23×10^{-8}).

The level of heavy metal concentration in yam samples cultivated in Takum LGA shows in Table 1. The order of these heavy metal concentrations is presented as follows: Cr>Cd>Pb>Hg>As. The risk assessment analysis revealed

that chromium and arsenic had the highest and the lowest hazard quotient of 51.064 and 0.00180, respectively. In addition, the highest TCR value was obtained in chromium (4.4×10^{-3}) and the lowest in the lead (5.1×10^{-9}).

The level of heavy metal concentration in yam samples cultivated in Mutum Biyu LGA shows in Table 1. The order of these heavy metal concentrations is as follows: Cr>Cd>Pb>Hg>As. The risk assessment analysis revealed that chromium and arsenic had the highest and the lowest hazard quotient of 40.645 and 0.00180, respectively. The highest TCR value was obtained in chromium (3.5×10^{-3}) and the lowest in the lead (1.17×10^{-8}).

The level of heavy metal concentration in yam samples cultivated in Wukari LGA LGA shows in Table 1. The order of these heavy metal concentrations is as follows: Cr>Cd>Pb>Hg>As. The risk assessment analysis revealed that chromium and arsenic had the highest and the lowest hazard quotient of 56.0328 and 0.00300, respectively. The highest TCR value was obtained in chromium (4.83×10^{-3}) and the lowest in the lead (6.4×10^{-9}).

Table 1 also shows the level of heavy metal concentration in yam samples cultivated in Zing LGA. The order of these heavy metal concentrations is as follows: Cr>Cd>Pb>Hg=As. The risk assessment analysis revealed that chromium and arsenic had the highest and the lowest hazard quotient of 62.4430 and 0.00370, respectively. In addition, the highest TCR value was obtained in chromium (5.38×10^{-3}) and the lowest in the lead (9.5×10^{-9}).

Table 1. Heavy metal concentration and risk assessment of yam samples cultivated in Taraba State, Nigeria

Parameter	Concentration (ppm)	WHO limit (mg/kg)	Risk assessment parameters			Target cancer risk
			EDI	ADI	HQ	TCR
Jalingo						
Hg	0.02 ± 0.00	0.10	0.000030	0.001	0.03700	-
As	0.01 ± 0.00	0.22	0.000018	0.010	0.00180	1.88×10^{-8}
Pb	1.15 ± 0.03	0.30	0.00210	0.004	0.53000	1.23×10^{-8}
Cd	9.87 ± 0.06	0.20	0.01810	0.001	18.0950	7.9×10^{-5}
Cr	61.08 ± 0.12	0.05	0.11198	0.003	37.3200	3.2×10^{-3}
Takum						
Hg	0.03 ± 0.02	0.10	0.000055	0.001	0.05500	-
As	0.01 ± 0.00	0.22	0.000018	0.010	0.00180	1.88×10^{-8}
Pb	0.48 ± 0.04	0.30	0.000880	0.004	0.22000	5.1×10^{-9}
Cd	11.69 ± 0.05	0.20	0.021430	0.001	21.43170	2.4×10^{-5}
Cr	83.56 ± 0.62	0.05	0.153190	0.003	51.06400	4.4×10^{-3}
Mutum Biyu						
Hg	0.02 ± 0.00	0.10	0.000037	0.001	0.03700	-
As	0.01 ± 0.00	0.22	0.000018	0.010	0.00180	1.88×10^{-8}
Pb	1.10 ± 0.04	0.30	0.002017	0.004	0.50400	1.17×10^{-8}
Cd	10.37 ± 0.14	0.20	0.019000	0.001	19.01170	8.19×10^{-5}
Cr	66.51 ± 0.00	0.05	0.121940	0.003	40.64500	3.5×10^{-3}
Wukari						
Hg	0.04 ± 0.00	0.10	0.000073	0.001	0.07330	-
As	0.02 ± 0.00	0.22	0.000037	0.010	0.00300	1.88×10^{-8}
Pb	0.60 ± 0.02	0.30	0.001100	0.004	0.27500	6.4×10^{-9}
Cd	12.45 ± 0.12	0.20	0.022830	0.001	22.8250	9.8×10^{-5}
Cr	91.69 ± 0.07	0.05	0.168100	0.003	56.0328	4.83×10^{-3}
Zing						
Hg	0.02 ± 0.00	0.10	0.000037	0.001	0.03700	-
As	0.02 ± 0.00	0.22	0.000037	0.010	0.00370	1.88×10^{-8}
Pb	0.89 ± 0.05	0.30	0.001630	0.004	0.40791	9.5×10^{-9}
Cd	13.80 ± 0.11	0.20	0.025300	0.001	25.3000	1.09×10^{-4}
Cr	102.18 ± 0.26	0.05	0.187330	0.003	62.4430	5.38×10^{-3}

Note: USEPA (2014). *Results are expressed in mean ± standard deviation of duplicate samples. EDI: Estimated Daily Intake, ADI: Acceptable Daily Intake, HQ: Hazard Quotient, TCR: Target Cancer Risk

Discussion

This study determined heavy metal concentration in yam samples harvested from five selected Local Government Areas (Jalingo, Takum, Mutum Biyu, Wukari, and Zing). Table 1 shows the heavy metal concentration, risk assessment, and cancer risk estimation of heavy metals in yam samples harvested from the Jalingo Local Government Area. The order of heavy metal concentration from highest to lowest is as follows: Cr>Cd>Pb>Hg>As. Chromium had the highest concentration (61.08 ± 0.12), while arsenic had the lowest concentration (0.01 ± 0.00). Chromium concentration in the yam sample from Jalingo exceeded the permissible limit recommended by WHO (0.05 mg/kg). The previous study by Oyatayo et al. (2015) showed the Cr concentration in water samples from Gashaka Gumti Park was 0.08 mg/kg. The high chromium concentration in this study could result from improper waste disposals such as leather, textile, and other industrial chemicals around the yam cultivation area. Consumption of yam originating from the Jalingo local Government Area may result in chromium toxicity. Chromium in the cells is reduced by hydrogen peroxide (H_2O_2), Glutathione (GSH) reductase, and ascorbic acid to produce reactive intermediates. Any of these states of chromium can attack DNA, proteins, and membrane lipids by forming reactive oxygen species such as O_2^-, H_2O_2, ^-OH, and OH_2, which may cause lung cancer and respiratory tract irritation (Zhema et al. 2022).

Lead exposure could result from exposure to chemicals such as paints, lead batteries, smelters, and oxides used in producing paint, pigments, and Pb sheets as the primary external source of lead in the food chain (Oancea et al. 2005). Lead concentration in yam samples harvested from Jalingo exceeded the recommended WHO value of 1.0. Stankovic et al. (2014) showed lead concentration in some medicinal plants grown in Jalingo low-lands ranged from 0.00 to 0.06 mg/kg. The differences with the results of this study may be due to the differences in metal bioaccumulation by different plant species and their ability to detoxify them. Lead can inhibit or mimic the actions of calcium and interact with proteins (Okoli et al. 2022). Lead binds to sulfhydryl and amide groups of enzymes, altering their configuration and diminishing their activities that can cause anemia, weakness, and kidney or brain damage (Wang and Sun 2013). Mercury and arsenic concentration were lower than 0.05 mg/kg; this concentration was below WHO permissible limit. However, they can bioaccumulate due to the overuse of chemical pesticides, fertilizers, and other sources of mercury and arsenic, thus inducing toxicity such as renal and neurological disorders. Arsenic can also bioaccumulate, interfering with the activities of mitochondrial enzymes and the uncoupling of oxidative phosphorylation (Okoli et al. 2022).

The risk assessment and cancer risk estimation calculated for samples harvested from Jalingo revealed that chromium had the highest hazard quotient (37.3), above 1, with a TCR value of 3.2×10^{-3}. These values indicated an increased cancer risk from consuming yam tubers contaminated with Cr. It also reveals that a significant amount of chromium was released from various sources into the environment.

The concentrations of different heavy metals, risk assessment, and cancer risk estimation in yam samples harvested from Takum LGA, Taraba State, are presented in Table 1. Chromium had the highest concentration (50.0 mg/kg) of other heavy metals estimated in the yam samples, which exceeded the WHO recommended value. The HQ and TCR values of chromium were 51.10 and 4.4×10^{-3}, respectively, indicating a high cancer risk. The high chromium concentration in the yam samples could be due to activities like the incineration of municipal waste, such as plastics and batteries, by the inhabitants of the LGA.

The concentration of cadmium, HQ, and TCR values were 11.69 ± 0.05 mg/kg, 21.43, and 2.4×10^{-5}, respectively. A previous study by Adachu et al. (2015) showed that lead and cadmium concentrations were within the range of 0.00-0.03 mg/kg in herbal decoction and beverages locally produced in Wukari. The cadmium concentration differed from the findings of Maxwell et al. (2015), that Cd concentration in dust particles along Jalingo road was 0.002 mg/kg. Cadmium causes toxicity, such as cell proliferation, lung damage, and apoptosis. These activities interact with the DNA repair mechanism, generate the reaction of oxygen species (ROS), and induce apoptosis (Wang and Sun 2013).

The level of mercury and arsenic in yam samples harvested from Takum LGA were significantly less than 0.05 mg/kg. It suggests that mercury and arsenic pollution is less in this location. The low concentrations of Hg and As reduce the risk of mercury and arsenic toxicity, such as reactions with protein sulfhydryl groups to inactivate dihydrolipoyl dehydrogenase and thiolase enzymes. It produces inhibited pyruvate oxidation and beta-oxidation of fatty acids (De Mattias Sartori et al. 2004). Ezeonu et al. (2022) reported that lead and mercury concentration in dust particles along federal high roads in Taraba state was 0.00015 mg/kg. Lead interferes with intracellular calcium cycling, altering the ability of organelle to release molecules from the stores, such as endoplasmic reticulum and mitochondria (Sirot et al. 2009).

Yam samples harvested from Mutum Biyu LGA of Taraba state had mercury and arsenic concentrations lower than the permissible values of WHO (0.10 mg/kg). Lower concentrations of Hg and As could be due to reduced burning of fossils and petroleum pollution, which are the major sources of mercury in plants. A study by Aremu et al. (2017) showed mercury and arsenic concentrations in groundwater in Wukari were low (0.0002 mg/kg and 0.003 mg/kg, respectively). The health implication of arsenic bioaccumulation includes lung and skin cancers due to free radicals attacking DNA and proteins (Hudson 2021).

Yam samples harvested from Wukari LGA had a high chromium concentration (91.69 mg/kg), followed by cadmium (12.45 mg/kg), significantly above the WHO permissible levels of 0.05 mg/kg and 0.20 mg/kg, respectively. The HQ values of chromium and cadmium were 56.0 and 22.8, respectively, while the TCR values were 4.83×10^{-3} and 9.8×10^{-5}, respectively, indicating a high

cancer risk to predisposed consumers of contaminated samples. Yerima et al. (2020) study on soil samples around the Mechanic Village, LGA Wukari, showed a high cadmium concentration (1.33 mg/kg). Increased oil pollution from petroleum and other gases in garages, lubricants, and dumping of spare parts, metals, and plastic scraps has contributed to a high concentration of these metals in the harvested yam samples (Zhema et al. 2022). Cadmium is a toxic metal that causes chromosomal aberrations, sister chromatid exchange, DNA strand breaks, and DNA- protein crosslinks in cell lines (Sirot et al. 2009). As a result, cadmium causes mutations and chromosomal deletions leading to lung damage and gastrointestinal and neurological disorders. In addition, contaminated chromium in foods can cause an attack on DNA, proteins, and membrane lipids, disrupting cellular integrity and functions (Sirot et al. 2009).

The result of heavy metal concentration, risk assessment, and TCR in yam samples harvested from Zing LGA is presented in Table 1. Yam samples from Zing LGA contained high concentrations of chromium and cadmium with HQ of 62.4 and 25.3, respectively, and TCR of 5.38×10^{-3} and $1.09\times10^{-}4$, respectively. These results indicate a high risk of cancer to predisposed consumers. The high concentration of Cr and Cd in yam samples from Zing LGA could be due to weathering rocks and waterfalls from the mountains in Zing LGA, as a source of cadmium and chromium to the food chain (Hudson 2021)., This result differed from the report of Achadu et al. (2015) that heavy metal concentrations in soil samples from Wukari LGA. High Cr consumption has adverse health effects, such as kidney damage (renal tubular damage). Cr (VI) can pass through cell membranes and be reduced intracellularly to reactive intermediates that can react with cellular molecules leading to upper abdominal pain, edema, pulmonary congestion, nose irritation, and skin irritations (Yerima et al. 2020).

In conclusion, analyzing heavy metal content in food samples commonly consumed in a particular location is important in ensuring human health. Elevated levels of heavy metals in foodstuffs pose a high risk to health and a potential cause of cancer due to their bioaccumulation. Cadmium and Chromium concentration in these sampling sites has exceeded the WHO permissible limit with a hazard quotient. It reveals a high risk of toxicity and carcinogenic due to increased environmental pollution. Sources of environmental pollution could be waste disposals, incinerations, and burning of tires, plastics, rubbers, and scrapes from mechanic sites to reduce the contamination of agricultural lands used to produce yam. Daily consumption of foods with high levels of heavy metals will accumulate, resulting in different toxic effects such as cancers, renal failures, neurological disturbances, respiratory tract infections, and even high intoxication deaths. The continuous consumption of foods contaminated with mercury and arsenic at low concentrations results in their accumulation in the body since heavy metals have a long half-life, are soluble in water, and pass through the cell membrane.

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Comparative evaluation of cassava composite flours and bread

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Abstract. *Abu MS. 2023. Comparative evaluation of cassava composite flours and bread. Asian J Nat Prod Biochem 21: 13-17.* Wheat imports into Nigeria have a high monetary value of NGN 635 billion annually. The recent data from the National Bureau of Statistics (NBS) trade report shows that Nigeria has spent NGN 258.3 billion on wheat imports in the first three months of 2021, despite the government continuing to encourage local production, such as composite flour. Composite flour combines several flours from roots, tubers, cereals, and legumes with or without adding wheat flour. This study evaluated the functional properties of composite flour produced from potato, cassava, and soybean and the effect of fortification on microbial growth on the produced bread. The flour variations were prepared at 50: 50 (%w/w), then the proximate composition, mineral contents, and functional properties were analyzed. The composite flour comprises WCF = 400 g wheat flour + 400 g cassava flour. CPF = 400 g of cassava + 400 g of sweet potatoes. CSF = 400 g of cassava + 400 g of soybean. The dry ingredients for making dough were 800 g of flours/composite flours, 100 g of butter, 100 g of sugar, and 5 g of yeast thoroughly mixed with warm water to obtain the dough. Exactly 110 g of the dough was placed in the baking pan and kept at room temperature for 20 minutes to ripe. The ripe dough was baked in the oven for 15 minutes. The bread was allowed to cool for further analysis, including the sensory evaluation and the microbial load of the finished products. Bread production uses CP, CS, WC flours, whole wheat, and cassava flours. The Swelling Capacity (SWC) showed that the Wheat Flour (WF) (11.00 ± 1%) was significantly ($p<0.05$) higher than Cassava Flour (CF) (6.33 ± 0.58%) and Cassava-Soybean Flour (CSF) (6.67 ± 0.58%). On the other hand, Water Absorption Capacity (WAC) revealed that the Wheat Flour (WF) (1.74 ± 0.24 g H_2O/g flour) was significantly ($p<0.05$) lower than CF (2.10 ± 0.08 g H2O/g flour) and Cassava-Potato Flour (CPF) (2.16 ± 0.07 g H2O/g flour). The Oil Absorption Capacity (OAC) showed that wheat flour (WF) (1.80 ± 0.15 g oil/g flour) was significantly ($p<0.05$) lower than CF (2.41 ± 0.07 g H2O/g oil), CPF (2.21 ± 0.81 g oil/g flour) and CSF (2.15 ± 0.10 g oil/g flour). The microbial density was higher on the Wheat-Flour Bread, WFB, followed by cassava flour bread, CFB. There was a lower microbial growth in cassava-potato flour bread, CPFB. The functional properties and the sensory evaluation of the composite flour bread indicated substantial feasibility of using legume/tuber composite flours in bread production.

Keywords: Cassava flour, composite four, functional properties, proximate, sensory evaluation

INTRODUCTION

Functional properties are intrinsic physico-chemical of food proteins that interact with other food constituents directly or indirectly, affecting food processing applications, quality, and general acceptability (Berchie et al. 2010). Food proteins are expected to possess vital functional characteristics such as water and oil absorption capacities, nitrogen solubility, bulk density, and emulsifying and foaming properties. It determines the acceptability of novel proteins as food supplements and components for formulating new food items (Kiin-Kabari et al. 2015).

Cassava (*Manihot esculenta* Crantz) is an important root and tuber crop, especially in Africa. In areas where cassava is a main staple, people have processed it into storable products such as tapioca, starch, dough, and gari (Dixon 2002). It significantly alleviates the African food crisis because of its efficient production, year-round availability, and tolerance to extreme stress conditions (Hahn 1987). Cassava has some attractive characteristics, especially for smallholder farmers in Ghana (Bokanga 1992).

Cassava provides the primary source of dietary calories for about 500 million people, many of them in Africa (Yeoh et al. 1998). Of all the tropical root crops, cassava is the most widely distributed and cultivated in different parts of Africa (Onwueme and Sinha 1991). It is crucial in those areas where the food supply is constantly threatened by environmental constraints, such as drought and pest outbreaks, because of its ability to grow under conditions considered suboptimal for most food crops (El-Sharkawy 2004; Berhanu and Feyissa 2020.). It can be harvested 6 to 24 months after planting and left in the ground as a food reserve for household security during famine, drought, and war.

The increasing cassava demand due to fast-expanding feed and starch markets as well as other cassava-based industries across the globe and rising prices of close substitutes such as rice and maize are rapidly re-ordering the dynamics of the cassava market in the tropics [Africa, Asia, and Latin America] (Ikuemonisan and Akinbola 2019). Cassava was known for its wide ecological adaptability and could perform relatively well, although other crops may not be able to produce reasonable yield (Otekunrin and Sawicka 2019). Because of these characteristics, cassava can be a reliable food security for

farming households in the tropics (Ikuemonisan and Akinbola 2019) and a livelihood for millions of farmers and traders worldwide (FAO 2018). In Nigeria, cassava is an integral part of the household diet. The emerging market dynamics may disrupt the availability of foodstuffs to consumers, which is of concern to policymakers and researchers (FAO 2018).

Wheat (*Triticum aestivum* L) is the world's most extensively grown cereal crop, covering about 237 million hectares annually, accounting for 420 million tonnes (Olabanji and Omeje 2007). It is an annual grass growing between ½ to 1 ¼ meters in height, with a long stalk that terminates in a tightly formed cluster of plump kernels enclosed by a beard of bristly spikes (Smith 2010). It is a global commodity, with about 150 MT (metric tonnes) traded annually (World Agricultural Outlook Board 2014). The increased global demand for wheat was due to increasing consumption, industrialization, and westernization. In particular, the gluten protein fraction's unique properties allow wheat processing to produce bread, other baked foods, noodles and pasta, and a range of functional ingredients (Shewry and Hey 2015). These products may be more convenient to produce or consume than traditional foods and as part of a "Western lifestyle."

Notably, the contribution of wheat to total energy in terms of kilo calories increased significantly in Nigeria (from less than 1% to 6.64%), India (11.85% to 20.41%), and China (12.20% to 17.83%) (Shewry and Hey 2015). However, the percentage contributions of all cereals declined in these three countries. Hence, in these three countries, increased wheat consumption occurred at the expense of other cereals, particularly minor cereals (millets and sorghum) (Shewry and Hey 2015). Increased wheat production has been accompanied by decreased imports in the UK, China, and India, but wheat imports have risen dramatically in African countries, Turkey and Mexico, despite increased production.

Nwanekezi (2013) states that wheat imports into Nigeria reach NGN.635 billion annually. However, the recent data from the National Bureau of Statistics (NBS) trade report shows that Nigeria has spent NGN.258.3 billion on wheat import during the first three months of 2021, despite the government's continuous to encourage local production. Despite being a major market for wheat products, Nigeria only produces 400,000 million tons annually out of a total demand of 3.6 million metric tons, according to data from the Federal Ministry of Agriculture. In recent years, the Boko Haram insurgency has obstructed efforts to increase local wheat production. Wheat farmers in Borno, the country's major producing state, had abandoned their farmlands and fled to other areas or took residency at the Internally Displaced Person's (IDP) camp (Business Day 2021).

Nigeria and several developing countries have encouraged the initiation of programs to evaluate the feasibility of alternative locally available flour as a substitute for wheat flour. Composite flours may be considered as blends of wheat and other flours or fully non-wheat combinations of flours for producing leavened bread, unleavened baked products, pasta, porridges, and snack. On the other hand, composite flour could be defined as a mixture of flours from starch-rich tubers (e.g., cassava, yam, sweet potato) and protein-rich flours (e.g., soy, peanut) and cereals (e.g., maize, rice, millet, buckwheat), plus or minus wheat flour (Suresh et al. 2015). Therefore, this study was designed to fortify cassava flour with local non-wheat flour to produce bread/other confectioneries products and enhance the nutritional content of such products in Nigeria.

MATERIALS AND METHODS

Materials

Refined wheat (*Triticum aestivum* L.) flour, Full-fat soy (*Glycine max* (L.) Merr.) flour, potato (*Solanum tuberosum* L.) flour, and cassava (*M. esculenta*) flour, Sugar, Yeast, and Butter were obtained from the local market.

Methods

Composite flour preparation and bread production

The composite flours were produced in the ratio of 1: 1. WCF = 400 g of wheat flour + 400 g of cassava flour. CPF = 400 g of cassava + 400 g of sweet potatoes. CSF = 400 g of cassava + 400 g of soybean. And then, 800 g of flours/composite flours, 100 g of butter, 100 g of sugar, and 5 g of yeast were thoroughly mixed with warm water to obtain the dough. Exactly 110 g of the dough was placed inside the baking pan and kept at room temperature for 20 minutes to ripe. The ripe dough was taken to the oven and baked for 15 minutes. The bread was allowed to cool for further analysis.

Swelling capacity

The swelling capacity was determined by the method described by Okaka and Potter (1977). A 100 mL graduated cylinder was filled with the ripe dough to the 10 mL mark, and the distilled water was added to give a total volume of 50 mL. The top of the graduated cylinder was tightly covered and mixed by inverting the cylinder. The suspension was inverted again after 2 minutes and left to stand for a further 8 min. The volume occupied by the sample was taken after 8 minutes.

Water Absorption Capacity (WAC) and Oil Absorption Capacity (OAC)

WAC and OAC were determined according to the slightly modified method described by Niba et al. (2001). Next, 2 grams of each sample were put into a centrifuge tube, and 50 mL of water was added. The mixture was shaken for about 5 minutes at room temperature. The mixture was kept in a water bath for about 15 minutes and centrifuged at 5,000 rpm for 15 minutes. The supernatant was decanted and discarded, the adhering drops of water/oil were removed, and the resultant sediment was reweighed. WAC and OAC were calculated as the weight of the residue (M2) divided by the initial weight of the sample (M1) (g/g).

$$\text{WAC/OAC} = \frac{M2}{M1}\, g/g$$

Determination of bread weight or baking loss

Weight loss was determined by weighing the ripe dough of each sample before baking (M1) and the bread sample after sufficient cooling (M2). Weight loss was expressed as a percentage.

$$\text{Weight Loss} = \frac{M1-M2}{M1} x100$$

Determination of microbial density

Accurately 1 g of the sample was weighed into a test tube containing 9 mL of distilled water (Ekici et al. 2019). The test tubes were arranged in ascending order from 1 to 10. The first test tube containing the sample was shaken vigorously till homogenous. Next, 1 mL of the mixture from the first tube was transferred to the second test tube and shaken. The same was done to the last test tube. All the test tubes were labeled from 1^1 to $1^{10,}$ respectively.

$$\text{Colony forming unit (C.F.U)} = \frac{No\ of\ count}{100} \times unit\ factor$$

RESULTS AND DISCUSSION

Functional properties of flours/composite flours

Table 1 showed that the wheat flour (WF) had a swelling capacity (11.00 ± $1^{\%}$) significantly ($p<0.05$) higher than the cassava flour (CF) (6.33 ± $0.58^{\%}$) and cassava-soybean flour (CSF, (6.67 ± $0.58^{\%}$).

The water holding/water absorption capacity (WHC/WAC) of the wheat flour, (WF), (1.74 ± 0.24 g H_2O/g flour) is significantly ($p<0.05$) lower than the cassava flour (CF) (2.10 ± 0.08 g H_2O/g flour) and the cassava-potato flour (CPF) =2.16 ± 0.07 (g H_2O/g flour).

The oil absorption capacity (OAC) showed that the wheat flour (WF) (1.80 ± 0.15 g oil/g flour) was significantly ($p<0.05$) lower than the cassava flour (CF) (2.41 ± 0.07 g H_2O/g oil), cassava-potato flour (CPF) (2.21 ± 0.81 g oil/g flour) and cassava-soybean flour, CSF (2.15 ± 0.10 g oil/g flour).

Bacterial growth of raw materials of flour and finished product after cooling

The population density of bacteria on raw material is presented in Figure 1. It demonstrated a higher bacterial population on Cassava Flour (CF) and lower growth on Cassava-Potato Flour (CPF).

Also, the growth on the bread showed a higher bacterial population on Wheat-Flour Bread (WFB) and lower on Cassava-Soya Bean Flour Bread, CSFB.

Fungal growth on bread on day 3

Fungal growth on day 3 showed that the Wheat-Cassava Flour Bread (WCFB) was higher, followed by Wheat-Flour Bread (WFB). There was no fungal growth on the Cassava-Potato Flour Bread (CSPB), as shown in Figure 2.

Baking loss of finished product

The baking loss was evaluated by analyzing moisture loss. Moisture loss of the samples is presented in Figure 3. The result shows that moisture loss in Wheat-Flour Bread (WFB) was the highest, while Cassava-Potato Flour Bread (CPFB) had the lowest.

Sensory evaluation of bread

The results of the sensory evaluation of bread are presented in Table 2. The individual flour bread, i.e., Wheat-Flour Bread (WFB) and Cassava Flour Bread (CFB), showed that WFB had the highest score in color (5), flavor (5), taste (5), texture (5), and acceptability (5). Meanwhile, the composite flour bread showed that Wheat-Cassava Flour Bread (WCFB) had the highest score in color (4), flavor (4), taste (4), texture (4), and acceptability (5) compared to the Cassava-Potato Flour Bread (CPFB) (4,3,4,4 and 4) and Cassava-Soybean Flour Bread (CSFB) (4,4,4,4 and 3).

Table 1. Functional properties of various flours

Sample	SWC (%)	WHC (g H_2O/g flour)	OAC (g oil/g flour)
WF	11.00 ± 1^b	1.74 ± 0.24^a	1.80 ± 0.15^a
CF	6.33 ± 0.58^a	2.10 ± 0.08^b	2.41 ± 0.07^b
WCF	10.00 ± 1^b	1.83 ± 0.05^a	2.03 ± 0.07^a
CPF	12.33 ± 1.15^b	2.16 ± 0.07^b	2.21 ± 0.81^b
CSF	6.67 ± 0.57^a	1.98 ± 0.05^a	2.15 ± 0.10^b

Note: n= 3; values in mean ±STD, means with different superscripts varies significantly at $p<0.05$ down the column; WF: 100% wheat flour; CF: 100% cassava four; WCF: 50% wheat flour: 50% cassava flour; CPF: 50% cassava flour: 50% sweet potatoes; CSF: 50% cassava flour: 50% soybean flour

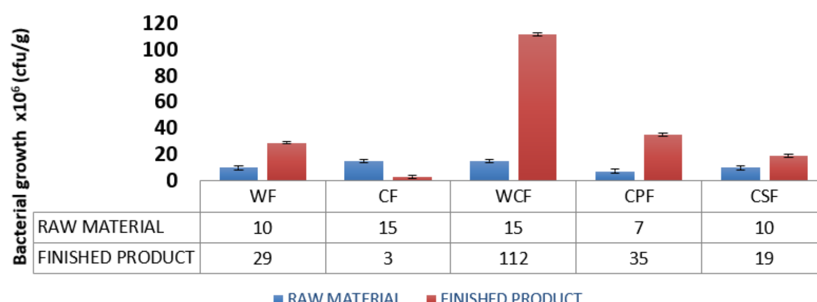

Figure 1. Bacterial density in flour and bread

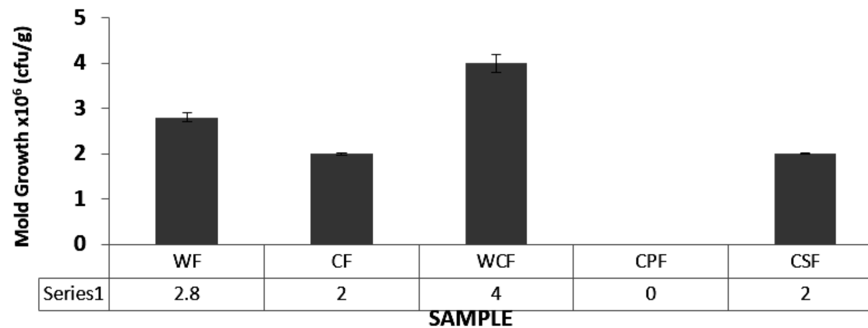

Figure 2. Fungal growth on bread using various flours

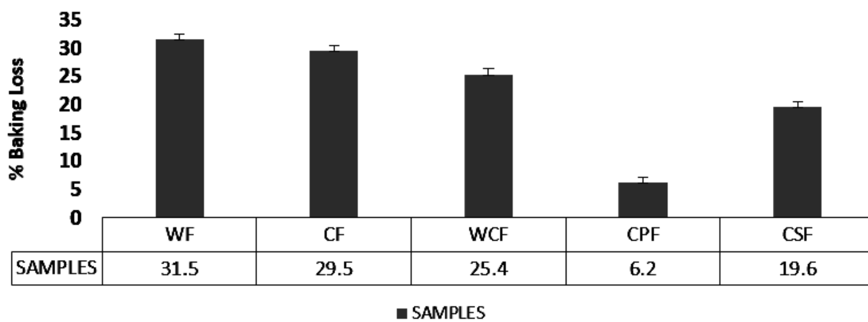

Figure 3. Baking loss in bread using various flours

Table 2. Sensory evaluation of bread using various flours

Sample	Color	Flavor	Taste	Texture	Acceptability
WFB	5	5	5	5	5
CFB	4	4	4	4	3
WCFB	4	4	4	4	5
CPFB	4	3	4	4	4
CSFB	4	4	3	4	3

Note: no: 50; 5: excellent, 4: very good, 3: good, 2: fair, 1: poor; WF: 100% wheat flour; CF: 100% cassava four; WCF: 50% wheat flour: 50% cassava flour; CPF: 50% cassava flour: 50% sweet potatoes; CSF: 50% cassava flour: 50% soybean flour

Discussion

The higher swelling capacity in WF than the CF is likely due to the gluten in wheat flour that forms continuous fine strands when immersed in water. The low swelling capacity of CF can be attributed to the large particle size or decreased interaction of the granules (Camel et al. 2019). However, swelling capacity improved when cassava flour was mixed with soybeans and sweet potatoes flours.

Water absorption capacity is defined as the ability of the flour or starch to hold or retain water against gravity that comprises bound water, hydrodynamic water, capillary water, and physically entrapped water (Andres et al. 2006). The increased water absorption of cassava flour and its addition reflects that a large amount of water is needed to prepare doughs with cassava flour. The loss interaction between amylose and amylopectin in the original starch granules and the weak binding forces cause the cassava flour to absorb more water (Ajatta et al. 2016). This property is essential for rapid dough formation while processing bakery products such as bread.

Oil absorption capacity is a process that involves the physical entrapment of oil by food products or materials when mixed with oil. It indicates the rate of protein or protein attachment to fat in food fortifications and is useful since fat acts as a flavor retainer and enhances the taste of food. The results showed composite flour CPF and CSF resisted oil absorption better than WF. A study by Althea-ann and Shuryo (1983) showed that the higher the amount of heat treatment to a protein, the more hydrophobic the protein, resulting from a higher number of hydrophobic groups exposed through the unfolding of the protein molecules.

The baking loss was analyzed in terms of the moisture loss of the bread, and the moisture content of the control bread was higher than the bread using composite flour. The loss of moisture content decreases with an increased proportion of composite flour during the baking process, and therefore, baking loss also decreases on composite flour bread.

All samples showed microbial growth. The composite of wheat-cassava flour bread has higher microbial density than other samples. The microbial density gradually increased during the three days of storage, probably due to the fat hydrolysis. The result of this study agrees with a previous study by Sewald and DeVries (2003) that hydrolysis of glycerides caused increased microbial growth on stored bread. The low growth observed in the Cassava-Potato Flour Bread (CPFB) after three days of storage than

the other composite flour due to low-fat content. It implied Cassava-Potato Flour Bread (CPFB) could be stored longer with good quality because its long shelf life is associated with low microbial growth.

The organoleptic test and sensory evaluation showed that wheat flour and composite flour bread were more satisfying. Sensory evaluation revealed that the composite bread had lower scores than the standard bread regarding color, flavor, taste, texture, and acceptability. However, there was no significant difference between wheat flour and the three composite flour bread regarding color, flavor, taste, texture, and acceptability.

In conclusion, based on the functional properties of the bread using composite flour, it can be concluded that mixed flour of legume/tuber or composite flours could be used in bread formulations. The organoleptic evaluation also showed that the experimental bread using various composite flour was acceptable to the panelists.

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Effect of basil (*Ocimum* sp.) essential oil addition in chitosan edible film on the quality of red snapper (*Lutjanus campechanus*) in cold storage

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Abstract. *Setyaningsih R, Pangastuti A, Farahdilla N. 2023. Effect of basil (*Ocimum *sp.) essential oil addition in chitosan edible film on the quality of red snapper (*Lutjanus campechanus*) in cold storage. Asian J Trop Biotechnol 21: 18-25*. One of the marine fish favored by the community is the red snapper (*Lutjanus campechanus* Poey, 1860). However, the quality of fresh fish will rapidly deteriorate during the auctioning, sorting, and marketing processes. Basil essential oil contains antimicrobial compounds that may improve the efficacy of edible film in preserving fish quality. This study aims to determine the effectiveness of using edible chitosan film with glycerol plasticizer and basil essential oil to increase the shelf life of red snapper and inhibit the growth of microorganisms. This study used two treatments consisting of basil essential oil (0%, 1.5%, 3%, and 4.5%), with sample testing on days 0, 3, and 6, and storing the samples at 4°C. The results showed that fish flesh's pH and water content revealed a statistically significant (P= 0.05) difference between treatments. However, Total Volatile Base (TVB) values for optimum treatment of 3% and 4.5% did not significantly differ. The Total Plate Count (TPC) showed that red snapper coated with an edible film treatment of 3% was the most optimal for inhibiting microbial growth. The panelists also preferred red snapper fillets with an edible film containing 3% basil essential oil because it increases the fillets' shelf life compared to other treatments.

Keywords: Basil essential oil, edible film, *Lutjanus campechanus,* red snapper, shelf life

INTRODUCTION

Red snapper (*Lutjanus campechanus* Poey, 1860) is a high-economic-value marine fish popular for public consumption and export commodities. Red snapper is highly nutritious and can improve health (Ihsan et al. 2019). Increasing market demand for fish, especially red snapper, needs to be followed by maintaining the quality of the fish. During the auctioning, sorting, and marketing process, the quality of fresh fish will quickly decline; one of the efforts to prevent the decline in fish quality is cold storage. Fish storage at cold temperatures can slow the growth of bacteria and affect the parameters of fish freshness. Unfortunately, preserving fresh fish in supermarkets and traditional markets is usually unhygienic, namely, only using ice placed in boxes or tables to attract consumers. This certainly allows contamination by microorganisms and accelerates fish spoilage (Syafitri et al. 2016).

Fish stored at cold temperatures have a longer shelf life than those stored at room temperature or without preservatives (Kresnasari 2021). However, to improve fish quality maintenance further, it is necessary to use effective packaging and preservative technologies. Therefore, as a substitute for plastic packaging, the edible film that can coat food products is better at maintaining humidity and oxygen levels and is antimicrobial. The edible film is also safe for consumption to reduce environmental pollution. In addition, natural preservatives can be added to increase the antimicrobials by edible packaging (Afrianti 2010; Qoeroti et al. 2021). One of the plants that can be used as an edible natural preservative is basil (*Ocimum* sp.).

Edible films are generally made of hydrophilic substances that have good mechanical and barrier properties to resist the transfer of gases, aroma compounds, and fats. Substances that are generally used as basic ingredients for edible films are proteins and polysaccharides. Polysaccharides have hydrocolloid properties which means they have low permeability to water vapor (Pavlath and Orts 2009). One of the natural ingredients that contain polysaccharides is chitosan. In making edible films, materials are needed to reduce fragility, increase flexibility and film resistance. These materials are commonly called plasticizers. One of the ingredients that can be used as a plasticizer is glycerol. The addition of glycerol greatly determines the quality of the film, especially if it is used for low temperature storage. Glycerol has a good correlation when combined with starch and can improve the physical and chemical properties of tensile strength, elongation, and transparency of edible films (Alfatahillah et al. 2021).

Basil leaves contain flavonoids, alkaloids, polyphenols, tannins, saponins, steroids, and essential oils, which can eradicate fungi and germs and inhibit the growth of food pathogens (Souhoka et al. 2019). Therefore, Basil leaves can be used as an active ingredient in edible films as a coating for the red snapper to extend its shelf life. In addition, edible films of basil essential oil are known to have antimicrobial activity. Therefore, the addition of basil essential oil is considered to have the potential as an

antimicrobial agent and can maintain the quality of fish flesh (Singh et al. 2020). Moreover, optimum microbial growth in tilapia fillets can be inhibited by packaging using an edible film of basil with a concentration of 1.5%; this packaging can increase the shelf life of fish until the ninth day (Agustin et al. 2020).

Concerning these problems, adding basil essential oil to chitosan edible film with glycerol plasticizer can potentially be used as packaging for red snapper, which is stored at cold temperatures before being sold. This study aims to determine the effect of the edible film added with basil essential oil on the quality of red snapper in cold storage. This treatment is expected to maintain the freshness of the fish in terms of its physical (appearance, texture of flesh, surface mucus, and odor), chemical (fish pH, water content, and total volatile base), microbiological, and sensory properties.

MATERIALS AND METHODS

Place and time of the study

The research was conducted from August to December 2021 at the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, and the Integrated Laboratory of Universitas Sebelas Maret, Surakarta, Indonesia.

Materials and tools

This study used red snapper filets measuring 5 cm x 5 cm (purchased from Balekambang Fish Market, Surakarta), basil leaves (purchased from Hardjodaksino Market, Surakarta), food-grade chitosan (CV. Chimultiguna), water, distilled water, ice cubes, fish-balls (Teman Laut brand), perchloric acid 6%, phenolphthalein indicator, NaOH 20%, H_3BO_3 3%, hydrochloric acid (HCl), Butterfield's phosphate-buffered solution, Plate Count Agar (PCA), thioglycolate agar, organoleptic assessment blanks, glacial acetic acid 1%, glycerol, vacuum, and 2-methyl-3-furaanthiol.

The tools used in this study were an analytical balance, thermometer, pH meter, mortar and pestle, oven, desiccator, Conway dish, container, Erlenmeyer, petri dish, incubator, magnetic stirrer, hot plate, storage area, tape, stir bar, glass beaker, banana leaf, scissors, micrometer, ruler, and stationery.

Experiment design

This study used a Completely Randomized Design (CRD) with four treatments and three replications for each treatment. In addition, this study used two experimental factors, namely edible film packaging made from chitosan with glycerol plasticizer and basil essential oil at a concentration of 0%, 1.5%, 3%, and 4.5%, and the sampling time was on the 0th, 3rd, and 6th day; data obtained from three replications. The fish was wrapped in edible film and stored at 4°C, then subjected to physical, chemical, and microbiological tests on the 0th, 3rd, and 6th days. Furthermore, a completely randomized design was used based on the number of experimental factors.

Working method

*Manufacture of basil essential oil (*Ocimum *sp.)*

Basil leaves were dried using the dry wind method at room temperature for seven days. Next, steam distillation apparatus was set up. First, 10 liters of distilled water were put into the distillation tank. Next, dried basil leaves were put into the distillation tank and processed for 5 hours. Finally, the resulting essential oil is transferred to a sterile container and ready to use (Agustin et al. 2020).

Manufacture of the edible films

As much as 3 grams of chitosan was slowly dissolved with 100 mL of 1% glacial acetic acid. Next, glycerol was added to as much as 0.4 ml/gram of chitosan and basil essential oil with a concentration of 0%; 1.5%; 3%; and 4.5%. The homogeneous edible film solution lay on the banana leaf sheets measuring a 15 x 15 cm^2 with a 5-7 mL volume and let dry up at room temperature for 24 hours. (i) Thickness test, checking the thickness of the edible film was done using a tool, the Brookfield CT 3-4500, and the results were compared with Japanese Industrial Standards (JIS). (ii) Tensile strength, the Tensile Strength (TS) at the fracture point and the elongation percentage were measured using the Universal Testing Instrument (UTI). The water concentration in the edible film is first balanced with the moisture content of the environment, which has a relative humidity of 70% at 25°C. Sample weighing is done every 2 hours. When the weight of the edible film sheet is constant, the moisture content in the sample is in balance with the environment. Furthermore, the tensile strength of the edible film was analyzed using a Brookfield CT 3-4500. (iii) Water vapor permeability test, the edible film was cut into 2 cm by 2 cm squares. A container containing 15 mL of sterile aquadest was then prepared. The edible film was then dried at 180°C for 3 hours use oven. Finally, the dried edible film was added to the prepared container and stored at 25°C (Huri and Nisa 2014). (iv) Elongation, the elongation percentage was calculated by comparing the edible film length after breaking it with the edible film length before being pulled by the Brookfield CT 3-4500 tool (Huri and Nisa 2014).

Physical properties test of red snapper fish

The external appearance of the fish was observed and assessed based on the assessment blank provided. The physical assessment consisted of four parameters: appearance, flesh texture, surface mucus, and odor. The texture of fresh fish flesh is elastic and compact. The texture of the fish is compared to fish balls and fresh fish. The smell of fresh fish is very distinctive according to its type and is similar to that of seaweed. The odor assessment parameters are based on ISO 8586:2012 with four odor descriptions, such as a comparison with vinegar to indicate a sour odor or comparison with expired nuggets; smelling rancid indicates a rancid odor; comparison with fresh fish indicates the smell of seaweed, while comparison with 2-methyl-3-furaanthiol indicates a mushroom odor (SNI 2346-2015).

Chemical properties test of red snapper fish

pH. Fish flesh samples weighed as much as 10 grams. Next, the sample was dissolved in 20 mL of sterile distilled water. The dissolved sample was then homogenized using a mortar for 1 minute. Finally, the pH of fish flesh is measured using a pH meter (Bawinto et al. 2015).

Water content. A porcelain cup was dry-sterilized using an oven at 105°-110°C for 1 hour. When finished, the cup was cooled in a desiccator for 30 minutes. The weight of the cup is weighed as weight (A). A total of 2 grams of sample was transferred to a cup and then weighed (B). Next, the sample cup was dried in an oven at 105°-110°C for 24 hours. When finished, the sample cup was cooled in a desiccator for 30 minutes and then re-weighed as weight (C). The water content formula calculates the sample's water content (Bawinto et al. 2015).

$$\text{Water content} = \frac{(B-C)}{(B-A)} \text{ X } 100\%$$

Total Volatile Base (TVB). The initial procedure was carried out by weighing 10 grams of sample, which had been added to 90 mL of 6% perchloric acid and homogenized for 2 minutes. The homogenization results were then filtered to obtain a clear, colored filtrate. Next, 50 mL of the filtrate was placed in a distillation tube and drip-dried with the phenolphthalein indicator. Then, as much as 10 mL of 20% NaOH was added to the solution until the color changed. Erlenmeyer was prepared and filled with 100 mL of 3% H_3BO_3 and 5 drops of Tashiro indicator. Then steam distillation was carried out for 7 minutes until 200 mL of distillate was obtained. Finally, the distillate was titrated using a 0.0197 N HCl solution.

Microbiological test with Total Plate Count (TPC) (SNI 2332-3: 2015)

Sample preparation. The fish flesh weighed 2 grams and was transferred to a sterile container. Next, 27 mL of Butterfield's Phosphate Buffered solution was added to the sterile container containing fish flesh and homogenized for 2 minutes. Next, as much as 1 mL of the homogenate was transferred to a test tube, and 9 mL of Butterfield's Phosphate Buffered solution was added (as a 10^{-2} dilution). The dilution step was repeated until the dilution was $10^{-5;}$ for each dilution, the sample was homogenized by rotating it 25 times with rotary evaporator.

Total Plate Count (TPC) test. A total of 1 mL of homogenate from each dilution was transferred to sterile petri dishes in duplicate. Next, as much as 12-15 mL of PCA agar was added to the petri dish, which already contained the homogenate. It was then homogenized and incubated at 37°C for 24 hours. If the sample contains bacteria, the bacteria will grow on PCA media after incubation, forming round, milky white colonies. All colonies formed were read and recorded. Microorganism incubation results were calculated using the Total Plate Count (TPC) method and compared to the quality standards listed in SNI 2729: 2013, with a maximum fish flesh of 5.0 x 10^5 colonies/g.

Sensory test (SNI 2346-2015)

Hedonic test. The hedonic test assessed several samples with a score of 1-9 according to the level of preference for the blank provided. The higher the score, the more the panelists like the assessed object. The assessment of very strong dislike have 1 point , and the assessment of very strong liking at 9 point.

Scoring. Samples were assessed for appearance, smell, and texture with a score of 1-9 on the blank provided. The higher the score, the higher the specifications of the flesh according to common fresh fish. Score 9 is given for its bright, intact appearance, strong aroma suitable for fresh fish, and very compact texture. Scores of 7 are given for intact but less bright appearance, strong odor, and compact texture. A value of 5 is given for a dull and incomplete appearance, starting to smell sour, and a less compact texture.

Data analysis

Analysis of the data results using one-way ANOVA (One-Way ANOVA) with a 95% confidence level and continued with the Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Edible film properties

Thickness is one of the parameters for assessing the quality of edible films. In this study, basil essential oil was added at 0%, 1.5%, 3%, and 4.5% concentrations. The thickness of the edible film obtained will affect other parameters such as tensile strength, water vapor transmission rate, and Elongation, whose quality will be compared with the Japan Industrial Standard (JIS). The thickness of all films ranged from 0.05 to 0.21 mm. Based on JIS, the films produced meet the standards because they have a thickness of no more than 0.25 mm (Nurindra et al. 2015).

The thickness of a film is affected by the area of the print and the volume of solution used. For example, a print area of 15 cm x 15 cm with a volume of 5-7 mL of edible film solution produces a thickness that meets JIS standards. Appropriate film thickness can reduce the risk of breaking when used as packaging. Film thickness is an important parameter because the thicker the film, the stiffer and physically harder the film will be, and the more quickly the film will break (Alfatahillah et al. 2021).

Tensile Strength (TS) is one of the feasibility parameters of edible films for food packaging. The results of the tensile strength test on edible films with the addition of basil essential oil at different concentrations are directly proportional to the thickness of the edible film. The thicker the edible film, the higher the tensile strength value produced. The higher tensile strength value becomes one of the quality parameters of edible films used in food packaging (Nguyen et al. 2020). The tensile strength value of the resulting edible film ranges from 3.65-8.52, and there are significant differences in each treatment of adding basil essential oil to make edible films (Table 1). This value meets the minimum tensile strength standard based

on JIS of 0.392266 MPa. Therefore, the edible film with the addition of basil essential oil meets the standards and has good quality by observing the results of the film tensile strength test (Nurindra et al. 2015).

The third parameter of edible film quality is water vapor permeability, also known as water vapor transmission or WVP. The film's resistance to inhibiting the transmission of food moisture in and out is one of the reasons this film can inhibit bacterial decay and prevent bacterial contamination. The results of the WVP edible film test that will be used as packaging for fresh red snapper fillets are directly proportional to the film's increase in thickness and tensile strength (Table 1). The Water Vapor Transmission rate (WVP) in the edible film produced in this study follows previous studies results. The thicker the edible film, the more WVP there will be in proportion to the amount of chitosan and other additives (Pramono et al. 2018). The denser the film, the easier it is for WVP to be inhibited so that the interaction between food and moisture from outside the package can be inhibited to maintain food quality (Agustin et al. 2020).

Determination of the best edible film used as packaging can be observed from the lowest WVP value (Katili et al. 2013). The range of WVP edible film values is 0.2579-0.37535 g/hour m2, which complies with JIS standards. According to the JIS standard, the maximum WVP value of the film is 0.4167 g/hour m2 (Setijawati 2017). The edible film that meets the JIS standard is good for packaging, especially food packaging, because it can optimally inhibit water vapor transmission.

The elongation value of the edible film, or the percent elongation of the film, is one of the parameters of film quality in food packaging. Film flexibility is important when a film is used as food packaging. Films that break easily are unsuitable for packaging food because they cannot completely cover the surface of the food and allow bacterial contamination. Edible film elongation in this study was inversely proportional to the film's thickness, WVP, and tensile strength. The higher the concentration of essential oil added, the more the elongation value of the film should decrease. The elongation value decreases due to decreased bond distance between the molecules. However, the test using the Brookfield CT 3-4500 showed an increase in Elongation directly proportional to adding essential oils to manufacture edible films. Nevertheless, the film elongation in this study ranges from 15.43% to 17.57%. The elongation value is low because it is less than 50% (Suryaningrum et al. 2005).

Physical properties of red snapper fillets

Physical assessment of red snapper fillets was measured from the external appearance of the fish body, the brightness of the eyes, the elasticity of the fish flesh, and the condition of the fish flesh. Fish that are still fresh have different bodies, flesh, and other organ colors from fish that have started to decompose due to biochemical reactions during the capture and marketing process, causing physical changes in the fish. Fish start to rot, has a faded flesh color, and secrete mucus due to biochemical reactions of enzymes and microbial decomposition. The fresh fish eyes are bright, while those fish starting to rot are cloudy and coated with mucus. The flesh of fresh fish is chewy and still watery because its protein is good enough to bind to water. Damaged fish flesh is characterized by a loss of elasticity (Waluyo and Kusuma 2017). Fresh fish has chewy and wet flesh because it has not lost much fluid from the flesh, and there is no dull body surface mucus, which makes fish unattractive (Nurilmala et al. 2018).

Ten panelists assessed red snapper fillets, resulting in a different assessment each day of testing (Table 2). Overall, the parameters for assessing mucus, flesh, smell, and texture of red snapper fillets packaged using edible film show that the value of fish packaged with the edible film but without the addition of basil essential oil (0% concentration) is the lowest compared to the addition of basil essential oil. Furthermore, adding basil essential oil at a 4.5% concentration on day 6 obtained the highest score for the mucus parameter, with a score of 7.63. Adding basil essential oil at 3% concentration obtained the highest rating from panelists until day six compared to 0%, 1.5%, and 4.5% concentrations.

On the sixth day, it was observed that the panelists preferred the physical appearance of the fish based on the parameters of mucus, flesh, smell, and texture of the fillets packaged using edible film with the addition of basil essential oil at a 3% concentration. Edible coating as packaging influences the appearance of fish flesh. Packaging fish fillets using edible coatings with high concentrations of essential oils makes the appearance of the flesh duller compared to low concentrations, which are more stable in maintaining the brightness of the flesh (Azzahra et al. 2013).

The normal pH value of fresh fish is 7, or to be precise, 6.9-7.2, with a decrease ranging from pH 2-6.5 that occurs during the rigor mortis phase. Rotten fish has an alkaline or high pH between 8 and 10 due to protein breakdown into alkaline compounds in the fish's body. The decrease in pH occurs due to the formation of lactic acid from anaerobic glycolysis results after the fish dies or does not receive oxygen (Waluyo and Kusuma 2017; Yuniarti et al. 2021). The higher the concentration of essential oils added to the edible coating, the more stable the pH of the fish fillets. At the highest concentration, the pH of fish fillets experienced a lower increase compared to fish packed with edible coatings with lower concentrations of essential oils. This is because essential oils contain antimicrobial compounds, which cause protein decomposition in fish fillets to occur more slowly (Azzahra et al. 2013).

The pH of red snapper fillets that experienced the most significant decrease was found in fillets treated without edible film packaging and with edible films but without the addition of basil essential oil. Red snapper fillets can be consumed until the 6th day in 1.5%-4.5% edible film packaging. Compared to concentrations of 1.5%, 3%, and 4.5%, treatment at 0% inhibits pH changes, causing acid accumulation in red snapper fillets to increase significantly and the pH value to decrease (Figure 1).

Table 1. Physical properties of chitosan edible film with the addition of basil essential oil

Component	Edible film's physical properties				Japanese Industrial Standard (JIS)
	Basil ess. oil 0%	Basil ess. oil 1.5%	Basil ess. oil 3%	Basil ess. oil 4.5%	
Tensile strength (MPa)	3.65a ± 0.12	5.04b ± 0.06	7.09c ± 0.04	8.52d ± 0.12	TS > 0.392266
WVP (g/hour m^2)	0.27a ± 0.02	0.28a ± 0.06	0.32a,b ± 0.04	0.37b ± 0.24	WVP < 0.4167 g/hour m2
Elongation (%)	15.43a ± 0.13	15.78a,b ± 0.15	16.07b ± 0.09	17.57c ± 0.15	10% < E < 50%

Note: ess: Essential; a,b,c: Similar letter notation in one line indicates no significant difference for each test parameter at Duncan's test level of 5%

Table 3. Total Plate Count (TPC) value of red snapper fillets packaged using chitosan edible film with the addition of basil essential oil

Storage time	Total Plate Count value (x105)				
	Control	Basil ess. oil 0%	Basil ess. oil 1.5%	Basil ess. oil 3%	Basil ess. oil 4.5%
0th Day	2.00^{ay} ± 0.00	$1.93^{ax,y}$ ± 0.06	1.93^{ax} ± 0.06	1.93^{ax}± 0.06	1.9^{ax} ± 0.10
3rd Day	3.20^{by} ± 0.00	$2.80^{bx,y}$± 0.00	2.63^{bx}± 0.06	2.57^{bx}± 0.06	2.27^{bx} ± 0.12
6th Day	5.63^{cy}± 3.78	$3.30^{cx,y}$± 0.00	3.03^{cx} ± 0.06	2.80^{cx}± 0.10	2.53^{cx}± 0.06

Note: ess: Essential; a,b,c: Similar letter notation in one column shows no significant difference for storage time at 5% Duncan test level; x,y: Notation of similar letters in one row indicates no significant difference for the treatment of different concentrations at Duncan's test level of 5%

Table 2. Physical value of red snapper fillet packaged in chitosan edible film with basil essential oil

Parameter	Treatment	0th day	3rd day	6th day
Outer mucus	Control	7.47	7.53	7.03
	0%	8.30	7.57	7.03
	1.5%	8.37	7.60	7.30
	3%	8.37	7.80	7.40
	4.5%	7.47	7.77	7.63
Flesh	Control	7.03	6.70	6.40
	0%	7.60	6.80	5.80
	1.5%	7.57	6.57	6.07
	3%	7.50	7.40	6.57
	4.5%	6.73	7.10	6.40
Odor	Control	7.53	6.73	5.17
	0%	7.60	6.67	3.77
	1.5%	7.43	7.07	5.10
	3%	7.70	7.43	5.53
	4.5%	7.47	7.30	5.47
Texture	Control	6.87	7.57	5.73
	0%	7.17	7.70	4.77
	1.5%	6.83	7.20	4.23
	3%	7.37	7.67	6.17
	4.5%	7.30	7.03	5.60

Note: Score 8.1-9: Clear mucus layer, transparent, shiny, bright, brilliant cutlet, the flesh tissue is very strong, the smell is fresh, and the texture is dense, compact, very elastic; Score 7.1-8: Clear mucus layer, transparent, quite bright, the cutlet is brilliant, strong flesh tissue, the smell of fresh fish, solid texture, compact, very elastic; Score 6.1-7: The mucus layer is starting to get a little cloudy, the cutlet is a little less bright, strong flesh tissue, the less specific smell of fresh fish, dense texture, compact, very elastic; Score 5.1-6: The mucus layer is getting cloudy, the cutlet is less bright, slightly less strong flesh tissue, neutral odor, slightly soft texture, a little less elastic; Score 3.1-5: The mucus has thickened and begun to change color, and the cutlet has faded, there is less strong flesh tissue, a slightly sour odor, a soft texture, and less elasticity

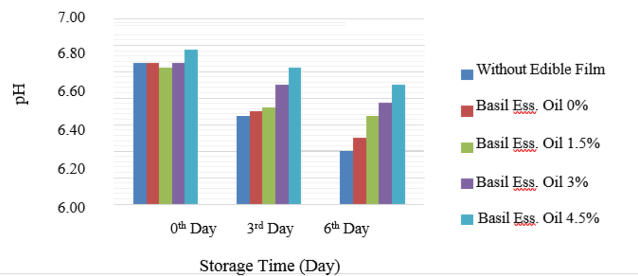

Figure 1. pH value of red snapper fillets packaged using edible chitosan film with the addition of basil essential oil

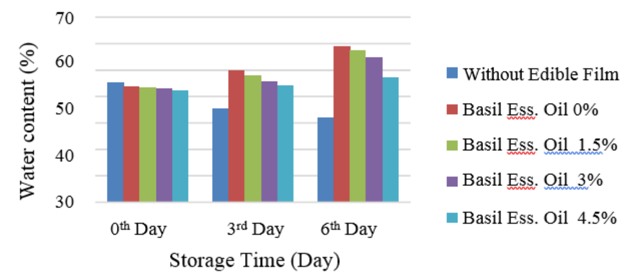

Figure 2. The water content of red snapper fillets packaged using edible chitosan film with the addition of basil essential oil

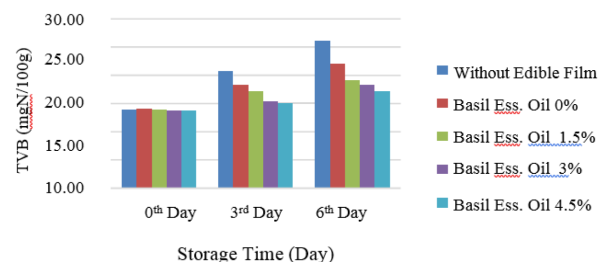

Figure 3. Total Volatile Base (TVB) of red snapper fillets coated with edible chitosan film with basil essential oil added

The water content is the second chemical parameter that determines the quality of fish fillets. The longer the fish fillets are stored in edible film packaging, the water content value will increase. That can happen because of chitosan's ability to inhibit water's evaporation in fish flesh. These results follow previous studies: fresh anchovies' water content with edible film increased within three days of storage (Mardyaningsih et al. 2014). However, fillets with edible film increased significantly in water content without adding basil essential oil, and at a concentration of 4.5%, the increase in water content occurred more slowly (Figure 2). The slow increase in water content in fillets with high concentrations of basil essential oil edible film indicates that the higher the concentration of essential oil added, the better the ability of the edible film to inhibit the rate of transmission of water vapor, which is one of the causes of rapid fish spoilage.

Another chemical parameter used as a reference for fish quality is the Total Volatile Base (TVB) level. Storage of fish immediately after being caught is known to optimally inhibit chemical reactions in dead fish flesh (Nurilmala et al. 2018). Red snapper fillets without edible film packaging experienced a higher TVB increase than fish fillets with edible film with added basil essential oil. The higher the concentration of essential oils, the more the increase in TVB of fish flesh was inhibited (Figure 3). The addition of basil essential oil has a significant effect proportional to the concentration. In this study essential oils with the highest concentration of 4.5% were more effective in maintaining the TVB value of fish flesh than at 0%, 1.5%, and 3% concentrations.

The parameter TVB values for fresh values are as follows: TVB values for very fresh fish are 10 mg N/100 g; Fresh fish has a TVB value of 10-20 mg/100 g. Fish with a TVB value of 20-30 mg N/100 g can be consumed, but the higher than those cannot be consumed. Fish without coatings and with the addition of low concentrations of essential oils can be consumed until the 6th day, while fish with higher coatings can be consumed until the 9th day (Tambunan and Chamidah 2021).

Microbiological properties of red snapper fillets

The third determination of fish freshness after physical and chemical tests of red snapper fillets are microbiological testing. The preservative used in this study was edible film packaging with basil essential oil, known to have antimicrobial properties. That formulation is expected to optimize the function of the edible film as a component to prevent more microbial contamination and inhibit bacterial growth. However, it is known that the control treatment is not maximally effective in inhibiting microbial growth, even with edible film packaging added with basil essential oil, which is only considered capable of inhibiting microbial growth. Moreover, the ANOVA statistical test showed a $P = 0.292$ or $P > 0.05$ value. Controlled treatment and packaging of red snapper fillets with edible film significantly impacted the 6-day shelf life. At the same time, the difference in concentration did not make a significant difference in the inhibition of bacterial growth (Table 3).

After the fish dies, it will undergo autolysis because enzymes in the body break down non-protein nitrogen (NPN) so that nitrogen levels in the form of amino acids, amines, and glucose in the fish's body increase and provide an optimum environment for bacterial growth. As a result, bacteria can produce fish decay marker compounds in trimethylamine, ammonia, amines, fatty acids, aldehydes, indol, sulfides, and mercaptans (Nurilmala et al. 2018).

According to Ababutain (2019), basil essential oil has antimicrobial activity against *Candida albicans, Escherichia coli,* and *Staphylococcus aureus* molds. *Ocimum basillicum* L. can be an inhibitor against *E. coli* and *S. aureus* bacteria which are resistant to erythromycin antibiotics. On the other hand, Agustin et al. (2020) stated that the higher the concentration of basil essential oil added to manufacture edible films, the more directly proportional it is to the inhibition of bacteria, which continues to increase. At a concentration of 1.5%, the antimicrobial activity of basil essential oil was in the moderate category. In addition, basil essential oil contains several terpene or antimicrobial compounds that can inhibit microbial growth in tilapia fillets.

Essential oils contain antibacterial compounds. Therefore, bacterial growth will be more inhibited if fish fillets coated with the edible film are stored at refrigerator or freezer temperatures. The results of the total bacteria test showed that the treatment of coating fish fillets with edible film with the addition of essential oils at chiller temperatures was close to the quality standards of SNI 7388:2009 (Mulyadi et al. 2016). Control treatment fish fillets stored for 6 days showed microbial growth of 5.63 colonies which exceeded the SNI quality standard with a maximum of 5 colonies at 10^5 dilution.

Sensory properties of red snapper fillets

Moreover, determining fishery product quality uses the human senses (organoleptic) as an assessment parameter. The fish flesh used as the test sample is in the form of fillets. One way to find out the freshness of fish flesh is by observing by slicing the fish from the tail. While the fish flesh from the tail is difficult to slice, indicating the fish belongs to the fresh fish category. Then, if the fish's flesh is bent and can immediately return to its original shape, it indicates that the fish is still fresh (Nurilmala et al. 2018). A decline in fish flesh quality occurs due to autolysis by the cathepsin enzyme, which decomposes protein into simpler compounds and is followed by a decrease in the fish flesh pH (Nurilmala et al. 2018).

Hedonic test

The affective test includes the hedonic test, which aims to measure the panelist's level of preference for the product. For example, the results of the assessment by ten panelists on red snapper fillets stored at cold temperatures using edible film with the addition of basil essential oil showed that on the 6th day, they had started to show signs of decay; thus, they received a score of 2-5 (Table 4).

Table 4. Hedonic values of red snapper fillet packaged with chitosan edible film and basil essential oil

Parameter	Treatment	0th Day	3rd Day	6th day
Control		6	6	4
	0%	6	6	5
Appearance	1.5%	7	6	5
	3%	5	5	5
	4.5%	6	6	5
Control		5	5	4
	0%	6	6	2
Aroma / odor	1.5%	6	6	4
	3%	6	6	5
	4.5%	5	5	4
Control		6	6	4
	0%	6	6	4
Texture	1.5%	6	6	5
	3%	6	6	5
	4.5%	6	6	5

Note: Value 7.1-9: Bright intact appearance, very strong aroma (typical of fresh fish), and very compact texture; 7-5.1: Less bright intact appearance, strong aroma (typical of fresh fish), and compact texture; 1-5: Appearance is dull and incomplete, starting to smell sour, and the texture is less compact

Table 5. Scoring values of red snapper fillet packaged with chitosan edible film and basil essential oil

Parameter	Treatment	0th Day	3rd Day	6th day
Control		7	7	6
	0%	7	7	7
Appearance	1.5%	8	7	7
	3%	7	7	6
	4.5%	6	7	5
Control		7	6	5
	0%	6	7	6
Aroma / odor	1.5%	7	7	7
	3%	7	6	6
	4.5%	7	6	5
Control		7	7	6
	0%	7	7	6
Texture	1.5%	8	7	7
	3%	7	7	7
	4.5%	7	7	6

Note: Score: 8.1-9: Most favored, 7.1-8: Like it a lot, 6.1-7: Just like, 5.1-6: Quite like it, 4.1-5: Neutral

Scoring test

The scoring test is carried out to determine the level of quality on a value scale of 1-9. The results of the panelists' assessment of red snapper fillets stored at cold temperatures using edible film with basil essential oil tended to favor still the fish's appearance, smell, and texture (Table 5).

Based on the panelists' organoleptic tests, both the hedonic tests and the scoring showed that the panelists preferred the appearance of fish with edible film packaging of basil essential oil at 1.5% and 3% concentrations. In addition, panelists liked the smell of fish fillets with an edible film of basil essential oil at 3% and the texture at 1.5% to 4.5% concentrations. On the sixth day, 0% gave a poor rating for the odor and texture parameters compared to 3% and 4.5% concentrations. This is possible because the basil essential oil covered the distinctive fresh smell of basil. In comparison, the texture changes are difficult to inhibit due to the metabolism's rapidity.

Moreover, organoleptic changes in fish can be caused by microbial activity and chemical reactions in the fish's flesh. Protein and fish fat degradation reactions occur due to proteolytic enzymes that degrade protein and lipase enzymes for degrade fat. The quality of fish flesh changes occurs during the pre-rigor phase, marked by the release of mucus from the fish skin surface. During the next phase, rigor mortis, the fish body will become stiff due to contraction and relaxation between actin and myosin to produce actomyosin (Nurilmala et al. 2018).

Changes in fish appearance, apart from being caused by chemical reactions in the flesh, are also caused by additives during the storage period, or in this case, by the edible film used. The higher the concentration of basil essential oil added to the edible film, the duller the resulting edible film will be, so while it is used to package fish, it will affect its appearance. The higher the concentration of essential oils added, the stronger the smell of basil will be to prevent the odor of rotting fish flesh. This is comparable to the results of the organoleptic test (Tables 4 and 5), in which panelists prefer the smell of fish fillets with edible film packaging with the addition of a high concentration of basil essential oil (3%). The basil essential oil odor can neutralize the bad smell of fish flesh compared to the 1.5%, 3%, and 4.5% concentrations.

This study concludes that edible film produced from chitosan with basil essential oil and used as a packaging material meets Japanese Industrial Standards (JIS) standards for thickness, tensile strength, water vapor transmission rate, and Elongation so that it can be used as packaging for red snapper fillets. Meanwhile, edible films with basil essential oil at a concentration of 3% were more acceptable to panelists and inhibited more changes in fillet quality (TVB, moisture content, and growth of microorganisms). Furthermore, adding basil essential oil makes the film brownish-yellow in color. It provides a distinctive fresh smell of basil, which can reduce the rotten or fishy smell of red snapper fillets that have been stored for several days.

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Effects of the candlenut seed oil supplementation on the fatty acids profile of Swiss Webster mice

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Abstract. *Wonggo D, Agustin YC, Wongso S, Putra SED. 2023. Effects of the candlenut seed oil supplementation on the fatty acids profile of Swiss Webster mice. Asian J Nat Prod Biochem 21: 26-33.* Candlenut (*Aleurites moluccana* (L.) Wild) is a widely distributed plant in Asia and Australia. Previous studies show the benefits of consuming foods containing Unsaturated Fatty Acids (UFA) that can reduce saturated fatty acids in the body. However, to date, there has been on the effect of candlenut seed oil on Fatty Acid (FA) profile, so this study intended to determine the highest candlenut seed oil supplemented to feed/food ingredients and its effect on unsaturated and Saturated Fatty Acids (SFA) profile in the blood and adipose tissue. Thirty-two Swiss Webster mice were divided into 2 groups, Group I (control group, n=16) was given normal (regular) feed, and Group II (test group, n=16) was assigned 16% candlenut seed oil-supplemented feed. The experiment was carried out for 13 weeks. At the end of the study (week 13th), the mice were dissected, and the fatty acids profile from the adipose was analyzed using a GC-FID instrument. Supplementation of 16% candlenut seed oil in feed increases total fat content to 180%, decreases Saturated Fatty Acids (SFA) to 17,71%, and increases unsaturated fatty acids (UFA) to 82,29%. Mice treated with 16% candlenut seed oil in the feed for 13 weeks showed a significant reduction in SFA level (38,975% ± 11,178 vs. 22,148% ± 2,853, p<0,05), accompanied by a significant increase in UFA (60,633% ± 10,924 vs. 77,693% ± 2,685, p<0,05). In addition, a significant increase in linolenic acid (1,1% ± 0,572 vs. 7,925% ± 1,305, p<0,05) and the UFA with more than one double bond (Linoleic acid and Linolenic acid) (26,65% ± 8,783 vs. 42,245% ± 2,322, p<0,05). Regular consumption of candlenut seed oil could increase UFA levels and decrease SFA levels. Therefore, candlenut seed oil might be the potential as a food additive to maintain health.

Keywords: Adipose, *Aleurites moluccana*, GC-FID, saturated fatty acid, unsaturated fatty acid

Abbreviations: ALA: Linolenic Acid; FA: Fatty Acid; GC-FID: Gas Chromatography-Flame Ionization Detector; LA: Linoleic Acid; MUFA: Mono Unsaturated Fatty Acid; PUFA: Poly Unsaturated Fatty Acid; SFA: Saturated Fatty Acid; UFA: Unsaturated Fatty Acid

INTRODUCTION

Candlenut (*Aleurites moluccana* (L.) Wild), a plant widely distributed in Asia and Australia, belongs to the Euphorbiaceae family. Candlenut is also known as kemiri (Indonesia and kukui (Hawaii). Several plant species in the *Aleurites* include *A. montana, A. trisperma, A. cordata, A. fordii,* and *A. moluccana*. Candlenut is used as a spice, biofuel, and traditional medicine. Candlenut seeds have the highest economic value compared to the leaves and stem. Candlenut seeds contain a high level of oil (60%), dominated by unsaturated fatty acids, such as Linolenic Acid (23,01%), Linoleic Acid (38,25%), and Oleic Acid (29,05%) (Tambun et al. 2020). Candlenut seed oil contains up to 66-91% of unsaturated fatty acids with as low as 7-10% saturated fatty acids (Shaah et al. 2021). Opportunities for exploring the potential of candlenut are widely open due to limited studies on this plant; therefore, it is necessary to study the use of candlenut seed oil as a feed supplement and its effect on fatty acid profiles and its toxicity in rats.

The high oil content in candlenut seed can be used as an unsaturated fatty acid source similar to peanut, sesame, and sunflower oil (Yanti et al. 2021). Candlenut seed oil has not been used as vegetable oil, known as "healthy oil." These essential unsaturated fatty acids cannot be synthesized in the human body (Watanabe and Tatsuno 2020), so these fatty acids could be obtained from candlenut seeds.

Fatty acids could be as saturated and unsaturated fatty acids based on the presence or absence of double bonds between carbon atoms. Unsaturated fatty acids are fatty acids that have one or more double bonds in their structure, and saturated fatty acid is a fatty acids without double bonds (Adeva-Andany et al. 2019). Moreover, unsaturated fatty acids, which only have one double bond, are known as Mono Unsaturated Fatty Acids (MUFA). Unsaturated fatty acids with more than one double bond are called Poly Unsaturated Fatty Acids (PUFA).

Unsaturated fatty acids, such as Omega-3 (Linolenic Acid), Omega-6 (Linoleic Acid), and Omega-9 (Oleic Acid), have health benefits, including cardiovascular disease, cancer prevention, lower risk of diabetes, and anti-inflammation agent (Medeiros-De-Moraes et al. 2018;

Moloudizargari et al. 2018; Priatni et al. 2018; Shahidi and Ambigaipalan 2018; Gammone et al. 2019). Contrarily, saturated fatty acids, such as palmitic acid, stearic acid, myristic acid, and lauric acid, were linked to high cholesterol levels and cardiovascular disease. The altered lifestyle, mainly dietary modification, could effectively prevent cardiovascular disease as the leading cause of death worldwide. The study aims to replace a diet with high saturated fat with an unsaturated one using candlenut seed oil.

On the other hand, candlenut seeds contain phorbol ester and saponin, the two most plenteous substances found in candlenut seeds that are responsible for their toxicity (Corcoran et al. 2020; Lawani and Winter 2022). Phorbol ester is a toxic diterpene generally found in Euphorbiaceae and Thymelaceae. Candlenut seed is usually consumed as an herbal medicine for weight loss, could cause vomiting, diarrhea, gastrointestinal nuisance, cardiac arrest, and also death (González-Stuart and Rivera 2019; Corcoran et al. 2020; Rosa et al. 2022; Lawani and Winter 2022); due to its adverse effects. Maximum intake at ≥2 g/kg BW did not cause any mortality (de Castilho et al. 2021). Candlenut has also been banned for consumption as a weight-loss supplement in Brazil, Chile, and Argentine. In-vivo study indicates clinical signs of toxicity, including ataxia, anesthesia, and no response to audio and visual stimuli on rat models after oral administration of candlenut extract at a concentration of 2 g/kg body weight (de Castilho et al. 2021). The toxic effect after consuming other Euphorbiaceae plants, such as *Jatropha curcas* (Sawadogo et al. 2018), *Euphorbia bivonae* (Athmouni et al. 2019), and *Alchornea cordifolia* (Ansah et al. 2011) have also been reported.

Considering the potential use of candlenut seed oil for health and the high number of toxicity reports, it is crucial to determine the safe dose of candlenut seed oil supplemented in food. Furthermore, this study aimed to determine the highest content of candlenut seed oil in feed/food and its effect on saturated and unsaturated fatty acids profile in blood and adipose after consumption of candlenut seed oil.

MATERIALS AND METHODS

Animal model

Healthy Swiss Webster mice (*Mus musculus*) aged 3-4 months were distributed into polypropylene cages (20x30 cm). The animals were placed in a room at 27°C with free access to food and water (ad libitum). This research was approved by the Institutional Ethical Committee University of Surabaya, Indonesia, No. 192/KE/VIII/2021.

Proximate analysis

The proximate analysis comprises protein, water, ash, fat, carbohydrate, and crude fiber. Protein analysis: 0,5 g of each sample was digested with 1 mL H2SO4 and 1 g $CuSO_4$ at 300-400°C for 2 hours. The mixture was then cooled at room temperature and titrated with 0,1N NaOH. Analysis of moisture content: 2 g of sample was heated in the oven for 5 hours at 105°C. Analysis of ash content: 2 g of sample was heated in the furnace at 550°C for 4 hours. Fat content was performed by hydrolyzing the sample: 1,5 g of sample was added with 250 mL 3M HCL for 1 hour, then neutralized using aquadest. The dried sample was mixed with 60 mL hexane in the Soxhlet for 95 minutes, then dried in the oven at 105°C. Carbohydrate content was calculated using the formula: 100% - (water content + protein content + total fat content + ash content). Analysis of crude fiber content: 5 g of the sample was mixed with 200 mL of HCl 3% and refluxed for 3 hours. The mixture was cooled to room temperature, neutralized with NaOH 30%, then added with three drops of acetic acid 3% and 500 mL water. Ten mL of the solution was added with 25 mL Luffschroll solution and 15 mL water, then boiled for 3 minutes and cooled for 10 minutes. And then, the mixture was added with 15 mL KI 20% and 25 mL H2SO4 25% and titrated with 0,1N Na2S2O3.

Toxicity test of candlenut seed oil

Twelve mice were divided into two groups, Group I (Control Group, n=6) was given regular feed without candlenut seed oil addition. Group II (Test Group, n=6) was given candlenut seed oil with an increasing concentration of 2% per week in the feed, namely 0% of candlenut seed oil in the 1st week and 16% at the week 9th, At the end of the ninth week, 12 mice (6 from each group) were dissected, and blood samples were collected from the heart and inserted into the tubes containing EDTA. The fatty acids profile was then analyzed using Gas Chromatography-Flame Ionization Detector (GC-FID). The highest concentration of candlenut seed oil added, which did not cause toxicity in the animal model, was then used to determine the effect of candlenut seed oil addition on adipose tissue.

Fatty acids profile of candlenut seed oil effect on adipose tissue

Thirty-two Swiss Webster mice were divided into 2 groups, Group I (control group, n=16) was given normal (regular) feed, and Group II (test group, n=16) was assigned 16% candlenut seed oil-supplemented feed. The experiment was carried out for 13 weeks. At the end of the study (week 13th), the mice were dissected, and the fatty acids profile from the adipose was analyzed using a GC-FID instrument.

Each tube containing 0,1 g of blood/adipose sample was mixed with 1 mL KOH 1N in methanol and then put in the 85°C water bath for 15 minutes. The mixture was then cooled at room temperature before adding 1 mL of Boron Trifluoride (BF3), sprayed with N_2 gas, and put in the 90°C water bath for 30 min. It is followed by adding 1 mL n-hexane and 6 mL saturated NaCl. The mixture was centrifugated at 1,500 rpm for 10 min. The supernatant was collected and added with 1 g of anhydrous Na_2SO_4. The supernatant was taken out using a syringe, transferred to a new vial, and analyzed further using a GC-FID instrument. The standard solution for reference or blank in the GC-FID instrument containing 100 μL Tridecanoic Acid 10,000

ppm and 100 µL Tricosanoic Acid 1,000 ppm was treated the same as the sample.

Statistical analysis

Data were analyzed using the Statistical Product and Service Solution (SPSS program). All data were expressed as mean ± Standard Deviation (SD), and statistical differences between means were determined by independent T-test. P<0,05 was regarded as statistically significant for all statistical analyses.

RESULTS AND DISCUSSION

Proximate analysis for feed

The results of the proximate are shown in Table 1. Carbohydrates, protein, and water content are the top 3 components of the feed. The significant constituent change in feed supplemented with candlenut seed oil is the fat content. It increased 3x compared to the normal feed, while water, ash, protein, crude fiber, and carbohydrate content were insignificant changes.

Fatty acids profile of the feed

The saturated fatty acids content in the oil-supplemented feed decreased from 32.35% to 17.71%, while the unsaturated fatty acids increased from 14.64% to 82.29%. On the oil-supplemented feed, saturated fatty acids, i.e., lauric acid, myristic acid, and palmitic acid, were lower than normal. Linolenic acid was much higher in oil-supplemented feed (Table 2).

Fatty acids profile of blood

The fatty acids profile of Swiss Webster mice fed with candlenut seed oil-supplemented feed, which increased gradually to 16% in week 9^{th,} showed no significant results on unsaturated fatty acids between the control (normal) group and oil-supplemented group (Figure 1).

At the same time, no toxicity or death was observed in this experiment. This finding showed that 16% candlenut seed oil showed no toxicity leading to death. The addition of the oil concentration was stopped at a concentration of 16% because this concentration was considered too high for the usual additional oil in a food ingredient.

Fatty acids profile of adipose tissue

Mice treated with 16% candlenut seed oil supplementation in feed for 13 weeks had higher weight gain than the control group (39.437g ± 3.054 vs. 35.062g ± 1.692, p<0.05) (Figure 2).

Furthermore, the total saturated fatty acids in the oil-supplemented group significantly decreased (38.975% ± 11.178 vs. 22.148% ± 2.853, p<0.05), while the total unsaturated fatty acids increased significantly (60.633% ± 10.924 vs. 77.693% ± 2.685, p<0.05) (Figure 3).

Based on the number of double bonds of unsaturated fatty acids showed that the unsaturated fatty acids with more than one double bond (Linoleic acid and Linolenic acid) had a significant increase compared to the control (26.65% ± 8.783 vs. 42.245% ± 2.322, p<0.05), and the unsaturated fatty acids with only one double bond (Palmitoleic acid and Oleic acid) also increased, but insignificant (33.985% ± 6.965 vs. 35.448% ± 0,6, p<0.05) (Figure 4).

The results showed that the saturated fatty acids, i.e., myristic acid, palmitic acid, and stearic acid in the oil-supplemented group were lower than the control (2.643% ± 2.412 vs. 1.095% ± 0.265, p<0.05), (26.925% ± 4.241 vs. 17.3% ± 1.995, p<0.05), and (9.443% ± 4.734 vs. 3.778% ± 0.747, p<0.05), respectively, and a combination of myristic acid and palmitic acid that known as harmful fatty acids, decreased significantly (29.535% ± 6.585 vs. 18.37% ± 2.18, p<0.05) (Figure 5).

Furthermore, it showed that only the level of linolenic acid increased significantly (1.1% ± 0.572 vs. 7.925% ± 1.305, p<0.05). In contrast, oleic acid and linoleic acid increased, but insignificant (31.575% ± 6.186 vs. 33.35% ± 0.889, p<0,05) dan (25.55% ± 8.281 vs. 34.3% ± 1.715, p<0.05), respectively. Palmitoleic acid decreased (2.4% ± 1.023 vs. 2.098% ± 0.873, p<0.05) (Figure 6).

Table 1. The results of proximate analysis of normal feed and oil-supplemented feed

Matrix	Proximate analysis results		% Change from normal feed
	Normal feed	16% Oil-supplemented feed	
Water	11.5	9.02	-21.2
Ash	5.82	5.52	-5.1
Fat	5.63	15.8	180.4
Protein	16	14.9	6.9
Crude Fiber	6.52	5.55	14.8
Carbohydrate	54.6	49.2	9.8

Table 2. Fatty acids content of normal feed and supplemented feed

Fatty acids		Concentration (%)	
		Normal feed	16% Oil-supplemented Feed
Saturated	Lauric acid	3.29	0.44
	Myristic acid	1.41	0.34
	Palmitic acid	24.2	13.8
	Stearic acid	2.73	2.87
	Others	0.7	0.25
	Total	32.35	17.71
Unsaturated	Palmitoleic acid	0.19	0.08
	Oleic acid	29	26.4
	Linoleic acid	35.5	36.9
	Linolenic acid	2.1	18.4
	Others	0.75	0.46
	Total	67.65	82.29

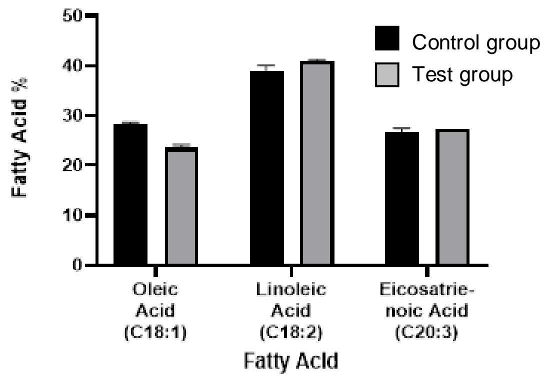

Figure 1. Profile of unsaturated fatty acids in the blood of mice after 9 weeks consuming feed supplemented with increasing gradually of candlenut seed oil to 16%

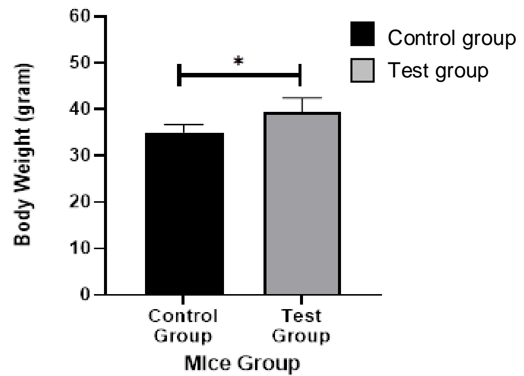

Figure 2. Body weight of mice body treated with normal feed and oil-supplemented feed. *Indicate significant differences between the control and the test groups with p<0.05

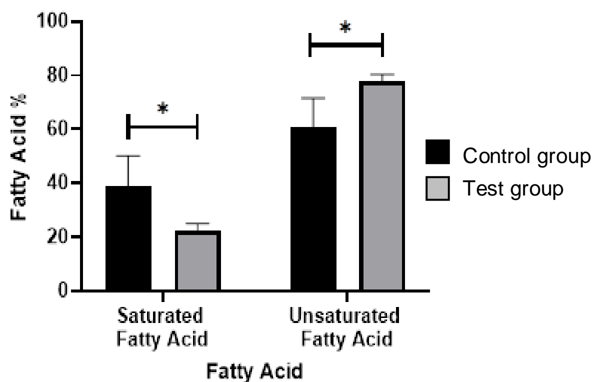

Figure 3. Saturated and unsaturated fatty acids in adipose tissue in mice treated with normal and oil-supplemented feed. *Indicate significance between the control and the test groups with p<0.05

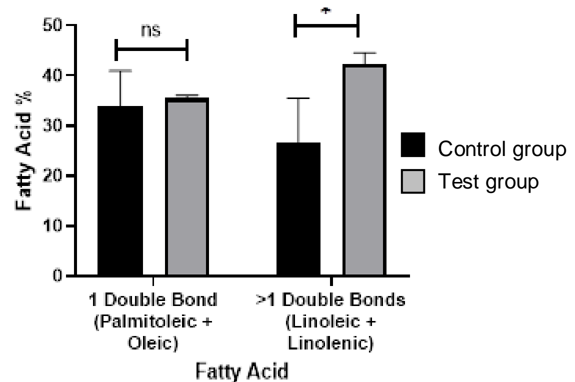

Figure 4. Percentage of 1 and >1 double bond groups on unsaturated fatty acid of mice of the control group and test group. Ns indicates no significant differences between the control group and the test group. *Indicate significant differences between the control and the test groups with p<0.05

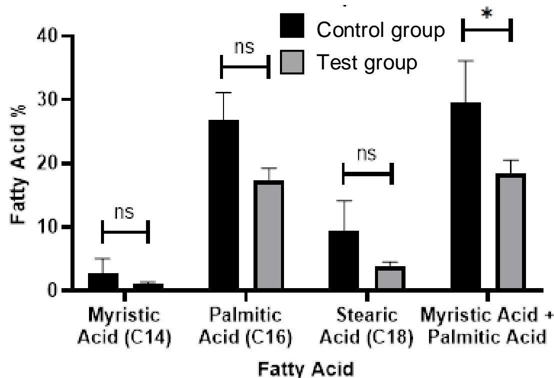

Figure 5. Percentage of Saturated Fatty Acids in adipose tissue of mice of the control and test groups. Ns indicates no significant differences between the control group and the test group. *Indicate significant differences between the control and the test groups with p<0.05

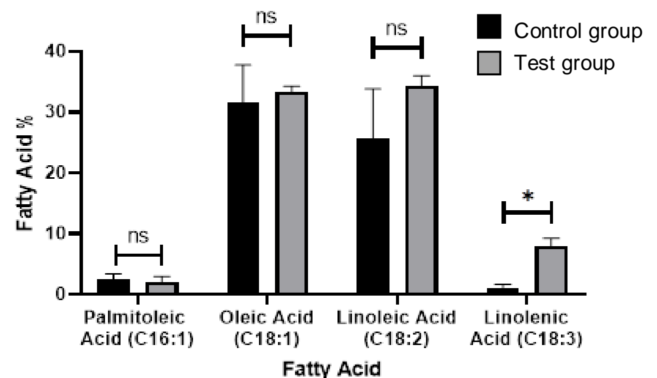

Figure 6. Percentage of unsaturated fatty acid in adipose tissue of mice of the control and test groups. Ns indicates no significant differences between the control group and the test group. *Indicate significant differences between the control and the test groups with p<0.05

Discussion

Supplementation of 16% candlenut oil in feed reduces saturated fatty acids and increases unsaturated fatty acids significantly. Furthermore, an increase was observed in linolenic acid and unsaturated fatty acids with more than one double bond. Decreased saturated fatty acid content (38,975% to 22,148%) and increased unsaturated fatty acids (60,633% to 77,693%) indicate the potential of candlenut seed oil as a food ingredient with beneficial health effects.

Increased levels of total fatty acids in feed supplemented with candlenut seed oil are due to the high oil content in candlenut seed (Cabral et al. 2016; da Silva et al. 2020). In this study, total fatty acids increased by 180% in the oil-supplemented feed compared to normal (regular) feed. The mice fed the high-fat diet (oil-supplemented feed) showed a significant increase in body weight compared to the control group (Figure 2). A study by Baumgardner et al. (2008) showed that adding corn oil increased weight gain in mice because of its high calories. Most of the fatty acid content in candlenut seed oil is unsaturated fatty acids with various health benefits. However, it is still necessary to determine the maximum limit of candlenut seed oil consumption so there is no adverse effect.

The unsaturated fatty acids profile in the blood was used to determine the highest level of candlenut seed oil supplementation in the feed that does not cause any adverse effect. (Figure 1). The fatty acids profile in adipose tissue was also determined because adipose tissue is the primary storage that/accumulates fatty acids. Blood fatty acids represent transient levels due to the breakdown of fatty acid stores within adipose tissue (Hodson et al. 2008). Fatty acids consumed are transported into the bloodstream as parts of more complicated lipid structures like triacylglycerols and phospholipids (Calder 2015). The fatty acids are then taken to adipose tissue to be re-esterified and stored as an excessive energy reservoir (Kuriyama et al. 2005; Suganami et al. 2012). The absorbed fatty acids accumulate and multiply in the body, especially in adipose tissue (Figueiredo et al. 2017).

Supplementation of 16% candlenut seed oil in the feed increased unsaturated fatty acids in blood and adipose tissue. In this study, mice fed with test feed (oil-supplemented feed) for 13 weeks showed an increase of UFA up to 17% compared to the control group. Huber et al. (2007) showed that mice fed with a high unsaturated fatty acid diet for six weeks with an average daily feed consumption of 7,6 g/day were able to increase levels of unsaturated fatty acids in the adipose tissue by 5 to 14% compared to mice with normal feed (Huber et al. 2007). Another study by Shin and Ajuwon (2018) showed that mice fed with a high unsaturated fatty acid diet for 12 weeks showed an increase in the average daily consumption of feed accompanied by increasing unsaturated fatty acids by 2-7% than mice with normal feed (Shin and Ajuwon 2018). The fatty acids in the feed are deposited in the body, especially in adipose tissue, for energy storage (Kuriyama et al. 2005; Suganami et al. 2012).

Animal models supplemented with 16% candlenut seed oil in their diet for 13 weeks also significantly decreased saturated fatty acids and increased unsaturated fatty acids (Figure 3). A study by Pedrosa et al. (2002) that used candlenut leaf extract showed only a decrease in LDL levels without interfering with their HDL content. This difference in results may be due to differences in lipoprotein cholesterol distribution (Krause and Hartman 1984). These results indicate that candlenut oil can potentially prevent cardiovascular disease caused by the blood accumulation of Low-Density Lipoprotein (LDL) cholesterol. The increase in LDL levels will lead to the formation of plaque or atherosclerosis and subsequent stroke and abrupt heart attack (Luo et al. 2022).

Extraction of candlenut seed produces 42-62% oil, with total unsaturated fatty acids of as much as 66-91% and a low level of saturated fatty acids (7-10%) (Shaah et al. 2021). Consuming a high level of unsaturated fatty acids was closely related to cardiovascular risk prevention, cancer, diabetes, obesity, and autoimmune deterrence (Medeiros-De-Moraes et al. 2018; Moloudizargari et al. 2018; Shahidi and Ambigaipalan 2018; Gammone et al. 2019). Unsaturated fat influences the genes that control fat metabolism and inflammation, so the risk of cardiovascular disease decreases (Larsen et al. 2021). One example is the downregulation of FGF18 expression, a Fibroblast Growth Factor (FGF) family involved in apoptosis and cell survival. Furthermore, the upregulation of SEPTIN 14, a protein implicated in cell proliferation, was also observed. The expression change in FGF18 and SEPTIN14 may lead to the prevention of atherosclerosis and reduced Vascular Smooth Muscle Cell (VSMC) proliferation level (Larsen et al. 2021).

Unsaturated fatty acids are fatty acids that have double bonds in their structure. There are two types of unsaturated fatty acids, unsaturated fatty acids with only one double bond (Mono Unsaturated Fatty Acid/MUFA) and unsaturated fatty acids with more than one double bond (Poly Unsaturated Fatty Acid/PUFA). Both have an equal effect on preventing cardiovascular disease (Miller et al. 2016). However, Lada and Rudel (2003) showed that MUFA was better at lowering LDL than PUFA because MUFA could lower HDL levels without lowering LDL levels, while PUFA can reduce LDL and HDL levels. Figure 4 shows that only PUFA levels increased significantly (26.65% ± 8.783 vs. 42.245% ± 2.322, $p<0,05$) in the oil-supplemented group. Previous studies showed that PUFA could reduce triglyceride levels and improve endothelial function, better at preventing heart disease than MUFA (Miller et al. 2016). However, Sheashea et al. (2021) showed that MUFA was more consistent in preventing heart disease. In addition, PUFA and MUFA did not have a different effect on the rate of fat oxidation (Jones et al. 2008; Casas-Agustench et al. 2009).

This study revealed that all detected saturated fatty acids, i.e., myristic acid, palmitic acid, and stearic acid, were decreased (Figure 5). It is consistent with the study by Mensink (2016) that three unsaturated fatty acids are the most abundant in humans and play a significant role in the body. Saturated Fatty Acid (SFA) is a type of fatty acid

with no double bonds in its chain (Adeva-Andany et al. 2019) and is also called "bad fat." Interestingly, whereas stearic acid is classified as a saturated fatty acid, it hasn't played a significant role in lipid metabolism; thus, it showed no effect on cardiovascular risk and had a neuroprotective effect against cerebral ischemia (Chen et al. 2020). The study showed that saturated fatty acids, namely myristic acid and palmitic acid, were decreased in the mice treated with oil-supplemented feed. These saturated fatty acids are associated with significant health problems, such as cholesterol and heart disease (Fatima et al. 2019; Saraswathi et al. 2022). Palmitic acid is known for its role as an intracellular signaling molecule and is closely related to metabolic syndrome, cancer, a neurodegenerative disorder, and cardiovascular disease (Fatima et al. 2019). Myristic acid, although accumulated in small amounts, has a significant role in cholesterol-upregulating action (hypercholesterolemia) and insulin resistance causing diabetes (Saraswathi et al. 2022).

Four unsaturated fatty acids were detected in this study, i.e., Palmitoleic Acid, Oleic Acid, Linoleic Acid, and Linolenic Acid (Figure 6). The four unsaturated fatty acids are also the most abundant unsaturated fatty acids found in adipose tissue (Ratcliffe et al. 2020). The unsaturated fatty acids belonging to MUFA are Palmitoleic Acid and Oleic Acid, while those belonging to PUFA are Linoleic Acid and Linolenic Acid. Palmitoleic acid, or (9Z)-hexadec-9-enoic acid, is Omega-7 monounsaturated fatty acid that contributes to anti-obesity, diabetes, and cardiovascular risk (Hu et al. 2019). The other unsaturated fatty acid in high levels was oleic acid or Omega-9. It is used as an anti-inflammation, anti-cancer, and emulsifier or solubilizing agent in the industry (Farag and Gad 2022). Omega 9 is commonly used for lowering LDL (bad cholesterol) and increasing HDL (good cholesterol) (Alagawany et al. 2022). Omega-7 and Omega-9 in food can reduce cholesterol levels due to an immunosuppressive effect, lowering triglycerides and reducing lymphocyte proliferation by Concanavalin A (ConA) (Yaqoob et al. 1994).

Omega-6 (Linoleic Acid) (LA) was the highest unsaturated fatty acid found in this study (Figure 6). LA is abundant in the body and in our food (Marangoni et al. 2020). LA is metabolized into other PUFAs with more carbon chains and is associated with immune and inflammatory responses (Patterson et al. 2012). Linolenic acid or Omega-3 Alpha-Linolenic Acid (ALA) is commonly found in flaxseed, chia seed, walnut, and fish oil (Shahidi and Ambigaipalan 2018). ALA act as Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) precursor. Increasing ALA levels in the body are linked to cardiovascular health benefits (Watanabe and Tatsuno 2020; Weinberg et al. 2021), lowering cholesterol level (Rouhanipour et al. 2022), cancer prevention (Wei et al. 2022), and anti-inflammation (Ishihara et al. 2019). LA and ALA combination also served as a proven anti-inflammation agent, even though the complex mechanism is not fully understood yet (Innes and Calder 2018).

Candlenut has been known to contain phorbol ester and saponin, found in abundant amounts in candlenut seed, and is believed to be responsible for its toxicity (Rosa et al. 2022; Lawani and Winter 2022). However, adding 16% candlenut seed oil to the mice feed does not cause death in the animal model, and this shows that candlenut is not detrimental up to 16%. Further research was needed to determine the long-term effects, metabolism, and mechanisms of its toxicity to ensure the safety of candlenut seed oil consumption. Vomiting, diarrhea, gastrointestinal nuisance, cardiac arrest, and death are reported after consuming candlenuts as an herbal weight-loss supplement (Lawani and Winter 2022; Rosa et al. 2022). Due to the adverse effects, the candlenut supplement has been banned for consumption in Brazil, Chile, and Argentine. In addition, an in-vivo study indicates clinical signs of toxicity, including ataxia, anesthesia, and no response to audio and visual stimuli on rat models after oral administration of candlenut extract at a concentration of 2 mg/kg body weight (de Castilho et al. 2021). Other Euphorbiaceae plants, such as *J. curcas* (Sawadogo et al. 2018), *E. bivonae* (Athmouni et al. 2019), and *A. cordifolia* (Ansah et al. 2011), also reported having toxic effects.

Supplementation of 16% candlenut seed oil in the feed reduces saturated fatty acids and increases unsaturated fatty acids significantly (38.975% to 22.148% and 60.633% to 77.693%, respectively). Furthermore, an increase was observed in linolenic acid and unsaturated fatty acids with more than one double bond. However, no significant changes were observed in the other unsaturated fatty acids. Decreased levels of saturated fatty acids and increased levels of unsaturated fatty acids indicate the potential of candlenut to be used as a food ingredient with health benefits.

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Cholesterol-lowering activity by lactic acid bacteria isolated from yogurt from Boyolali, Indonesia

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Abstract. *Nurcahyani I, Susilowati A, Pangastuti A. 2023. Cholesterol-lowering activity by lactic acid bacteria isolated from yogurt from Boyolali, Indonesia. Asian J Nat Prod Biochem 21: 34-45.* Hypercholesterolemia is considered a risk factor for cardiovascular disease, as it is characterized by increased cholesterol levels in the bloodstream that exceed the established normal range. At present, cardiovascular disease stands as the foremost cause of mortality worldwide, with a global death toll of 17.7 million. The employment of Lactic Acid Bacteria as a probiotic has the potential to reduce cholesterol levels via the enzymatic of Bile Salt Hydrolase (BSH), which facilitates the deconjugation of bile salts, as well as its capacity to assimilate cholesterol directly. The consumption of fermented dairy products that have undergone appropriate bacterial fermentation has been found to potentially contribute to a reduction in blood cholesterol. The region of Boyolali in Indonesia is known for its yogurt production; regularly consuming probiotic products has been suggested as a dietary approach to promoting long-term hypocholesterolemia effects. The objective of this research was to isolate and evaluate the efficacy of Lactic Acid Bacteria (LAB) in reducing cholesterol levels, and to identify the specific isolates derived from yogurt originating from Boyolali. The activity of the BSH enzyme was evaluated through qualitative testing by observing precipitation zones on the growth media. A quantitative assessment was also conducted using the UV-vis spectrophotometer method at λ570 nm. LAB isolates' activity in bile salt deconjugation and cholesterol assimilation was also measured using the UV-vis spectrophotometer method at λ660 nm and λ550 nm, respectively. The bacterial identification process was conducted through the examination of both macroscopic and microscopic morphology. The study employed a One-way Analysis of Variance (ANOVA) to compare the release of amino acids (specifically glycine and taurine), the release of free cholic acid, and the disparity in cholesterol levels between media that were inoculated with LAB and those that were not. The BSH enzyme activity was observed to be 1.11-3.82 U/mL for sodium glycocholate substrate and 0.85-3.13 U/mL for sodium taurocholic substrate. The recorded levels of bile salt activity were 0.52-1.26 µmol/mL for the sodium glycocholate substrate and 0.43-0.9 µmol/mL for the sodium taurocholic substrate. The percentage of assimilated cholesterol ranges from 38.69% to 71.98%.

Keywords: Bile salt hydrolase, lactic acid bacteria, lowering cholesterol

INTRODUCTION

According to the World Heart Federation (2003), Death from Cardiovascular Disease (CVD) jumped globallly from 12.1 million in 1990 to 20.5 million in 2021. Cardiovascular disease was the leading cause of death worldwidw in 2021. Hypercholesterolemia is considered a risk factor for cardiovascular disease. This condition is characterized by increased bloodstream cholesterol levels exceeding the normal range (Freed 1994). Individuals diagnosed with hypercholesterolemia are at a significantly elevated risk of experiencing a heart attack, with a threefold increase in risk compared to those with a typical blood lipid profile. Numerous methods have been employed to decrease cholesterol levels, including pharmacological agents to manage hypercholesterolemia. However, this intervention is associated with high costs and adverse effects, as Kumar et al. (2012) reported.

Recently, numerous investigations have been carried out on the functionality of Lactic Acid Bacteria (LAB), commonly known as probiotic bacteria, that can potentially lower cholesterol levels. Studies conducted by Zeng et al. (2010) and Ishimwe et al. (2015) have demonstrated the efficacy of diets incorporating LAB strains in reducing overall cholesterol levels and decreasing lipoprotein cholesterol concentration at lower densities. Prior studies have indicated that certain LAB strains can lower cholesterol levels. Specifically, *Lactobacillus casei* (Klaver and Van Der Meer 1993), *Lactobacillus plantarum, Lactobacillus paracasei* (Belviso et al. 2009), *Lactobacillus lactic* (Kimoto et al. 2002), and *Enterococcus faecium* (Hlivak et al. 2005; Ayyash et al. 2018) have been identified as such strains. The consumption of dairy products that have undergone fermentation by appropriate bacterial strains has been shown to reduce blood cholesterol. However, it is worth noting that the bacterial strains present in yogurt products are typically not indigenous to the human gastrointestinal tract. According to Lertcanawanichakul et al. (2015), regular intake of probiotic products may serve as a dietary intervention for inducing sustained hypercholesterolemic effects.

The process of cholesterol reduction by lactic acid bacteria is attributed to the enzymatic activity of Bile Salt Hydrolase (BSH), which deconjugates bile salts and

facilitates cholesterol assimilation (Burhan et al. 2017). The BSH enzyme is responsible for the hydrolysis of conjugated bile salts, which involves the amide bond cleavage in the conjugated bile salts. The liberation of free cholic acid occurs through the activity of the BSH enzyme, leading to the release of amino acid residues and deconjugated bile salts (Kumar et al. 2012). According to De Smet et al. (1994), unbound cholic acid and no cost attached to it exhibit lower solubility and reduced efficacy in lipid absorption within the intestinal tract. Consequently, it is eliminated from the body through fecal excretion. According to Kumar et al. (2012), cholesterol reduction can be achieved by deconjugating bile salts by LAB. According to Begley et al. (2006), the bile salts lost through fecal excretion increase the demand for cholesterol to facilitate the enteropathic synthesis of bile salts. Consequently, this process leads to a reduction in cholesterol within the body.

MATERIALS AND METHODS

Materials

Yogurt samples were obtained from Boyolali District, Central Java Province, Indonesia.

Procedure

Yogurt samples

The sample liquid was obtained from yogurt products from home production in Dusun 3, Kiringan, Boyolali District, Boyolali Regency, Central Java, with a YoGood trademark. In addition, for the yogurt milk starter, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were used.

Isolation of LAB from Boyolali Yogurt

Distilled water was used to dilute Boyolali yogurt at dilution levels ranging from 10^{-1} to 10^{-5}. In each dilution series, 100 µL of the sample liquid was applied onto the MRS (De Man, Rogosa, and Sharpe) agar surface, fortified with 0.5% calcium carbonate ($CaCO_3$). The agar plates were then incubated at 37°C for 1-2 x 24 hours. The colonies that exhibited distinct, clear zones on MRS agar were carefully selected and re-inoculated using the streak method to form quadrant streaks on MRS Agar. These cultures were then incubated under identical conditions. This process was repeated until uniform colonies were obtained. The resulting isolates were subjected to catalase testing, and those with negative catalase activity were identified as LAB isolates. The selected LAB isolates were subjected to inoculation on MRS Agar slant and subsequently preserved at 4°C as a stock culture. Furthermore, the culture was transferred to MRS Broth medium for subsequent examinations. The formation of a clear zone on MRS Agar media containing 0.5% CaCO3 is attributed to the interaction between bacterial acids and calcium in the media. Bacteria capable of producing clear zones are likely to be Lactic Acid Bacteria (LAB) due to their ability to produce lactic acid.

Qualitative measurement of BSH (Bile Salt Hydrolase) activity

Bile salt hydrolase activity was measured using the methodology described by Dashkevicz and Feighner (1989) and Ahn et al. (2003). MRS A medium was aseptically prepared using the following components: MRS B at a concentration of 525 g/L, agar at 7.5 g/L, and 0.3% bile salt and $CaCl_2$ at 0.375 g/L. The resulting medium was rendered sterile. Subsequently, the aseptic disc paper was immersed in LAB isolate that had undergone an 18-hour culture period, following which the disc paper was deposited onto MRS agar. The media containing LAB isolates underwent incubation for 72 hours at a temperature of 37°C. The BSH activity is distinguished by the emergence of a zone of bile salt precipitation encircling the colony on $CaCl_2$-containing agar media. This phenomenon arises from the interaction between cholic acid and $CaCl_2$, forming precipitated salt. The manifestation of a distinct area devoid of bacterial growth in the vicinity of the colony is indicative of the hydrolytic activity facilitated by the BSH enzyme.

Quantitative measurement of BSH (Bile Salt Hydrolase) activity

The BSH activity quantification was conducted through the assessment of free amino acids resulting from the conjugation of bile salts by LAB isolates, following the methodology described by Tanaka et al. (2000). After a 20-hour incubation period at 37°C, LAB cultures were subjected to cell harvesting via centrifugation at 9,700 xg for 15 minutes. The resulting cells were washed twice with sodium phosphate buffer (100 mM, pH 6.8), supplemented with dithiothreitol (10 mM), and resuspended in the same buffer. Subsequently, the cell's Optical Density (OD) was determined through 600 nm absorption.

The sonication process was employed to eliminate intracellular enzymes from the cell suspension. It was achieved by subjecting the suspension to ultrasonic waves for 60 seconds and cooling on ice for two cycles. The resulting mixture was centrifuged at a force of 9,700 x g for 15 minutes. A reaction mixture was assembled, comprising 1.8 mL of sodium phosphate buffer (100 mM, pH 6.0), 100 µL of conjugated bile salt (200 mM), 100 µL of dithiothreitol (10 mM), and 100 µL of cell-free extract. The mixture underwent incubation at a temperature of 37ºC for 30 minutes. Subsequently, the control sample was subjected to a mixture of 200 µL and trichloroacetic acid (200 µL) to halt the reaction. Both sample types underwent centrifugation at a velocity of 9,700 x g for 15 minutes.

A 200 µL sample of supernatant was mixed with an equal volume of distilled water (200 µL) and subsequently combined with a ninhydrin reagent solution consisting of 1% ninhydrin solution (0.5 mL), 30% glycerol (1.2 mL) and 500 mM sodium citrate buffer (pH 5.5; 0.2 mL) to a total volume of 1.9 mL. Subsequently, the amalgam was subjected to vortexing and boiling for 14 minutes. Next, the solution was allowed to cool down, and its absorbance was gauged at a wavelength of 570 nm, with glycine and taurine employed as standards. A single unit of BSH activity (U/mL) is precisely characterized as the quantity of

enzyme that releases one micromole (1 µL) of amino acids from the substrate within a minute.

Measurement of deconjugated sodium glycocholate and sodium tauroccolate

A sterile MRS broth of 10 mL was enriched with sodium glycocholate (6 mM) and sodium taurocholate (6 mM). Subsequently, MRS B was inoculated with a 1% LAB isolate and incubated at 37°C for 20 hours. The liberation of unbound cholic acid is the foundation for the quantitative assessment of bile salt deconjugation. The culture that underwent incubation was subjected to pH adjustment by adding 1N NaOH, followed by centrifugation at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was collected and subjected to further pH adjustment by adding 10N HCL until a pH of 1.0 was achieved.

Subsequently, 1 milliliter (1 mL) of the supernatant was transferred and introduced into a solution containing 2 mL of ethyl acetate. Subsequently, the mixture was subjected to vortexing for 60 seconds and allowed to rest until the separation phase. Following this, a volume of 2 mL from the ethyl acetate layer was extracted and subsequently transferred to a glass tube, where it was subjected to evaporation at a temperature of 60ºC. The resulting residue was solubilized in a solution of 0.01N NaOH (1 mL) and subjected to vortex agitation. A solution containing 1% furfuraldehyde (1 mL) and H_2SO_4 (16N, 1 mL) was introduced into the mixture, which was subsequently subjected to vortexing for 1 minute. The mixture was then heated at 65°C in a water bath for 10 minutes. Following the cooling process, the mixture underwent the addition of glacial acetic acid and was subjected to vortexing for 1 minute. The solution's absorbance was assessed at a wavelength of 660 nm, and the quantity of cholic acid discharged was determined by employing a standard cholic acid gradient concentration curve, as per Shehata et al. (2019).

Cholesterol assimilation measurement

PEG 600 cholesterol was introduced into the MRS broth medium, resulting in a final concentration of 100 µg/mL. One percent LAB inoculum was introduced into MRS broth-Cholesterol-PEG 600 after incubating at 37°C for 24 hours. The mixture was then incubated for an additional 24 hours at 37°C. Next, to conduct cholesterol analysis, the LAB suspension underwent centrifugation at 4,000 rotations per minute for 10 minutes at 4°C. Then, 1 mL of the resulting supernatant was extracted for further examination.

Furthermore, 1 mL of the sample was treated with 33% potassium hydroxide (KOH) and 1 mL of absolute ethanol. Subsequently, the solution underwent vortexing for 1 minute. It was incubated at 37°C for 15 minutes, then left at ambient temperature. Subsequently, a mixture comprising 2 mL of deionized water and 2 mL of hexane was introduced into the solution, followed by vortexing for 1 minute while allowing for the separation phase at ambient temperature. Subsequently, 1 mL of the hexane layer was carefully dispensed into a glass tube and subjected to solvent evaporation using a water bath operating at 65°C. Following the formation of the residue, a volume of 2 mL containing 50 mg per deciliter of o-phthalaldehyde reagent prepared in acetic acid was introduced to the sample and subsequently homogenized. After mixing, 0.5 mL of sulfuric acid (H_2SO_4) was introduced to each tube, and the resulting solution was vortexed for 1 minute. Subsequently, the solution was incubated for 20 minutes at ambient temperature. The outcome entails the quantification of the solution's absorbance at a wavelength of 550 nm through the employment of a UV spectrophotometer. The cholesterol assimilation value was determined using the standard cholesterol curve and measuring the sample's absorbance value against varying cholesterol concentrations (ranging from 0 to 0.1 mg/mL) in MRS broth. The cholesterol assimilation value was calculated by subtracting the cholesterol value before incubation from the value of the LAB isolate sample after 24 hours of incubation, measured in units of µg/mL. In addition, the proportion of assimilated cholesterol calculations involved dividing the value of cholesterol assimilation by the value of cholesterol at 0 hours.

Cholesterol assimilation by LAB is determined using the following formula: (i) Cholesterol assimilation (µg/mL) = [cholesterol (µg/mL)]0h − [cholesterol (µg/mL)]24h. (ii) Cholesterol assimilation by each LAB isolate is also calculated in percentage: assimilated cholesterol (%) = [cholesterol assimilation (µg/mL)/ cholesterol (µg/mL)0h] × 100%.

Characterization of LAB isolates

The LAB isolates were characterized through morphological observations, which involved examining the growth of colonies on culture media. The characterization process included observations of the shape of the colonies when viewed from above, the surface of the colonies when viewed from above , the edges of the colonies when viewed from the side, and the color of the colonies (Dwidjoseputro 2005). The selected isolates underwent testing for catalase activity and gram staining on specific colonies. The catalase test involves the introduction of a 3% hydrogen peroxide solution to the bacterial inoculum on a glass surface. Bubbles indicate a positive catalase reaction, while the absence indicates a negative one. The bacterial isolates that were catalase negative were observed to lack the ability to produce the catalase enzyme, which is responsible for the decomposition of hydrogen peroxide into water and oxygen. In addition, using indicators such as the clear zone on $CaCO_3$-containing media and the catalase test facilitates the identification of LAB isolates.

Data analysis

The enzyme activity test results were analyzed using both quantitative and qualitative methods. The deconjugation and assimilation processes of cholesterol were quantitatively described during the study. The study conducted the quantitative analysis using one-way ANOVA to compare the release of amino acids, free cholic acid, and cholesterol in LAB-inoculated media.

RESULTS AND DISCUSSION

Characterization of lactic acid bacteria

This study collected LAB (Lactic Acid Bacteria) isolates from yogurt products produced in Dusun 3, Kiringan, Boyolali District, Central Java, Indonesia, under the brand name YoGood. The nomenclature of the isolate is derived from the commercial product appellation, succeeded by the isolate numeral corresponding to each bacterium that was effectively extracted. Thirteen isolates were obtained from yogurt isolation, which exhibited growth on MRS agar media supplemented with 0.5% $CaCO_3$. The formation of a clear zone surrounding the colony can be attributed to the alkaline properties of $CaCO_3$, which effectively counteracts the acid production by lactic acid bacteria. Clear zones in the isolate signify its classification as a lactic acid bacteria group member. It has been observed that lactic acid can hydrolyze CaCO3, forming a distinct zone on MRS agar media. This phenomenon has been documented in studies conducted by Sun et al. (2014) and Pisol et al. (2015).

Table 1 displays the outcomes of the morphological observations. The colonies generally exhibited a circular shape with flat edges, convex angles of elevation, smooth surfaces, and a milky white hue. The observations above align with the morphological attributes of the colonies that are presumed to be lactic acid bacteria. Specifically, the colonies exhibit a circular shape with flat edges and a white to yellowish-white hue (Kurnia et al. 2020). According to Candra et al. (2007), the isolates were identified as a single species (strain) based on their colony morphology as observed macroscopically.

Subsequently, the selected isolates underwent biochemical tests, specifically the catalase test. The catalase test is a diagnostic assay utilized to ascertain the presence or absence of catalase enzyme activity in a given bacterium. The test is conducted by adding 1-2 droplets of hydrogen peroxide to the bacterial colonies. The presence of bubbles characterizes Catalase positive bacteria upon observation, whereas the absence of bubbles identifies catalase-negative bacteria. According to Delvia et al. (2015), a positive catalase test signifies the ability of bacteria to break down harmful hydrogen peroxide molecules into water and oxygen. The findings of the catalase assay revealed that 13 isolates exhibited a negative catalase reaction, thereby suggesting their classification within the lactic acid bacteria cohort. The features above align with LAB's typical attributes: gram-positive, non-spore-forming, and lacking in catalase enzyme production (catalase negative). Most of the isolates exhibit facultative anaerobic characteristics and can generate lactic acid as the primary product of fermentation, as reported by Widodo (2003).

After going through the catalase test, 13 bacterial isolates were tested further in the form of a qualitative BSH (Bile Salt Hydrolase) enzyme test. By observing the formation of a precipitation zone in MRS A growth medium containing CaCl 0.37 g/L and 0.3% oxgall, 9 bacterial isolates were selected that could produce the BSH enzyme qualitatively. The nine isolates that passed the test were subjected to microscopic morphological observations. Gram staining was carried out as a colorant for the character of the isolates based on the structure of the bacterial cell wall. The observations of the ninth isolate had Gram-positive characters, which were observed under a microscope with 40-100x magnification, as shown in Table 2.

Gram staining determines whether bacteria are Gram-positive or gram-negative. Gram-positive bacteria have a purple tint on their cells because the bacterial cell wall has thicker peptidoglycan properties than gram-negative bacteria. The crystal violet color can be bound and maintained by thick peptidoglycan. Gram-negative bacteria, on the other hand, have a red color on their cells, indicating that they cannot bind crystal violet color and are solely stained by safranin (Yulvizar 2013). The findings revealed that nine bacterial isolates were Gram-positive bacteria with stem cell shape/bacillus. Lactic acid bacteria are known to b e Gram-positive bacteria. Bacteria isolated from Sumbawa mare milk are Gram-positive bacteria with variations in the shape of long and short bacilli (Sujaya et al. 2008). So, based on the data, it is clear that the nine bacterial isolates identified from yogurt from Boyolali are lactic acid bacteria.

Table 1. Morphological characteristics of bacterial colonies from Boyolali Yogurt, Boyolali, Indonesia

Isolate Name	Macroscopic Observations				Catalase Test
	Shape	Elevation	Border	Surface	
YG-1	Bulbous	Convex	Flat	Smooth	-
YG-2	Bulbous	Convex	Flat	Smooth	-
YG-3	Bulbous	Convex	Flat	Smooth	-
YG-4	Bulbous	Convex	Flat	Smooth	-
YG-5	Bulbous	Convex	Flat	Smooth	-
YG-6	Bulbous	Convex	Flat	Smooth	-
YG-7	Bulbous	Convex	Flat	Smooth	-
YG-8	Bulbous	Convex	Flat	Smooth	-
YG-9	Bulbous	Convex	Flat	Smooth	-
YG-10	Bulbous	Convex	Flat	Smooth	-
YG-11	Bulbous	Convex	Flat	Smooth	-
YG-12	Bulbous	Convex	Flat	Smooth	-
YG-13	Bulbous	Convex	Flat	Smooth	-

Table 2. Microscopic morphological characteristics of bacterial colonies from Boyolali Yogurt, Boyolali, Indonesia (via Gram staining)

Isolate name	Microscopic Observation	
	Shape	Gram
YG-2	Rod	+
YG-3	Rod	+
YG-4	Rod	+
YG-5	Rod	+
YG-8	Rod	+
YG-9	Rod	+
YG-10	Rod	+
YG-12	Rod	+
YG-13	Rod	+

Bile salt hydrolase enzyme activity

Nine isolates were successfully selected: YG-2, YG-3, YG-4, YG-5, YG-8, YG-9, YG-10, YG-12, and YG-13. The isolates selected had good growth, and a qualitative BSH (Bile Salt Hydrolase) enzyme assay was performed to assess the presence or absence of enzyme activity produced by the bacterial isolates. Nine LAB isolates on MRS agar media containing 0.3% oxgall demonstrated BSH enzyme activity. Bacterial isolates that manufacture BSH enzymes have a cloudy white precipitation zone and/or a clear zone around them (Dong et al. 2012) or cloudy white granular colony formation (Xiong et al. 2016). Figure 1 depicts the qualitative results of the BSH enzyme test.

Table 3 displays the obtained outcomes. All nine isolates can produce the BSH enzyme. The deposition of bile acids in the form of free bile acids/cholic acid observed in the precipitation zone surrounding the isolate on MRS agar growth media supplemented with oxgall is attributed to the deconjugation of bile acids by BSH enzymes, as reported by Sirilun et al. (2010). The study by Julendra et al. (2017) revealed that the MRS media with 0.3% oxgall content was utilized as a substrate for the BSH enzyme in the qualitative test. Additionally, the inclusion of CaCl2 in the media enhanced the activity of the BSH enzyme and facilitated the precipitation of the enzyme deconjugation outcomes. The manifestation of a transparent or haloed zone surrounding bacterial isolates indicates BSH enzyme activity resulting from the precipitation of unbound bile salts. The reason for this is that isolates exhibiting positive BSH possess the capacity to sustain their proficiency in the process of hydrolyzing conjugated bile salt compounds. According to Dong et al. (2012), positive strains for BSH exhibit greater efficacy in the hydrolysis of glycolic-conjugated bile salts than taurocholate-conjugated bile salts.

The nine isolates exhibiting qualitative enzyme activity will undergo quantitative testing to ascertain the enzyme activity levels in units per milliliter (U/mL). The findings indicated that the LAB isolates exhibited varying levels of BSH enzyme production ($P<0.05$). The results indicate that the enzyme can hydrolyze sodium glycocholate and sodium taurocholate substrates, as presented in Table 4. The activity of the BSH enzyme was observed on two different substrates, namely glycocholate and taurocholic. The resulting BSH activity on the glycocholate substrate ranged from 1.11 to 3.82 (U/mL), while on the taurocholic substrate, it ranged from 0.87 to 3.13 (U/mL). The YG-2 isolate exhibited the greatest enzyme activity, with a recorded value of 3.82 (U/mL) for the sodium glycocholate substrate and 3.13 (U/mL) for the taurocholic substrate. The YG-4 isolate exhibited the least BSH activity on the sodium glycocholate substrate, measuring at 1.11 (U/mL), while the YG-10 isolate exhibited the least BSH activity on the sodium taurocholic substrate, measuring 0.85 (U/mL).

Overall, the findings of this investigation suggest that the total activity of BSH enzymes on the glycocholate substrate was greater than that observed on the sodium taurocholate substrates (as presented in Table 4). This observation implies that the BSH enzymes exhibit more hydrolysis towards bile salts conjugated with glycine. Variations in enzyme activity can be attributed to the substrate specificity of the BSH enzyme, as noted by De Smet et al. (1994). The enzymatic substrate can be influenced by the existence of amino acid segments, such as glycine and taurine, as conjugates, or steroid side chains that serve as substrates for the BSH enzyme, as reported by Gilliland et al. (1985). According to Begley et al. (2005), bacterial strains that exhibit positivity for the BSH enzyme display distinct variations in their affinity and activity towards conjugated substrates. The differential affinity exhibited by certain bacterial strains towards specific substrates, particularly the hydrolysis of BSH, has been identified as a crucial factor for their survival in vivo (Prete et al. 2020). The study's findings suggest that the metabolism of glycine-conjugated bile acids is preferred by several bacterial strains over taurine.

The greater efficacy of BSH enzymes on glycocholate substrates in comparison to sodium taurocholate substrates can be attributed to the heightened toxicity of glycine-conjugated bile salts towards bacterial cells under low pH conditions in the intestinal tract, as opposed to taurine-conjugated bile salts, which exhibit lower toxicity. According to Miremadi et al. (2014) and Anwar and Sameer (2018), the ratio of taurine-conjugated bile salts to glycine-conjugated bile salts in the human digestive tract is smaller at 1:3. This results in higher efficiency of the BSH enzyme in the hydrolysis of glycolic bile acids during bile acid metabolism in vivo.

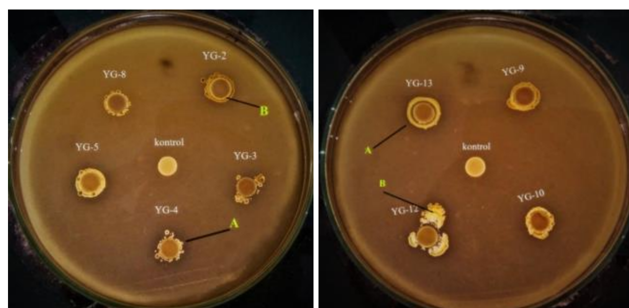

Figure 1. Qualitative BSH enzyme activity test results on MRS A media containing 0.3% oxgall. Note: A: Clear zone, B: Precipitation zone, Control: Without administration of LAB isolate

Table 3. Qualitative BSH enzyme activity by LAB isolates on MRS A media containing 0.3% Oxgall

Isolate name	BSH enzyme activity
YG-2	+
YG-3	+
YG-4	+
YG-5	+
YG-8	+
YG-9	+
YG-10	+
YG-12	+
YG-13	+

Note: Has BSH enzyme activity (+), does not have BSH enzyme activity (-)

Table 4. Quantitative BSH enzyme activity by LAB isolates on sodium glycocholate and sodium taurocholic substrates

Isolate name	BSH Enzyme Activity (U/mL) [1]			
	Sodium Glycocolate [2]		Sodium Taurocholic [2]	
	Sample	Control [3]	Sample	Control [3]
YG-2	3.82 ± 0.0478 [i]	0.32 ± 0.0095 [a]	3.13 ± 0.2003 [h]	0.16 ± 0.0000 [a]
YG-3	2.39 ± 0.2705 [g]	0.29 ± 0.0005 [a]	1.68 ± 0.1143 [e]	0.15 ± 0.0008 [a]
YG-4	1.11 ± 0.0269 [b, c]	0.37 ± 0.0005 [a]	1.55 ± 0.1362 [d, e]	0.16 ± 0.0064 [a]
YG-5	1.41 ± 0.1069 [c, d, e]	0.36 ± 0.0008 [a]	2.00 ± 0.2031 [f]	0.18 ± 0.0046 [a]
YG-8	1.51 ± 0.1518 [d, e]	0.33 ± 0.0009 [a]	2.47 ± 0.2206 [g]	0.18 ± 0.0002 [a]
YG-9	2.50 ± 0.2589 [g]	0.38 ± 0.0011[a]	1.23 ± 0.0710 [c, d]	0.19 ± 0.0000 [a]
YG-10	2.56 ± 0.0499 [g]	0.36 ± 0.0086 [a]	0.85 ± 0.0007 [b]	0.19 ± 0.0000 [a]
YG-12	1.57 ± 0.1158 [d, e]	0.31 ± 0.0004 [a]	0.91 ± 0.0031 [b]	0.17 ± 0.0002 [a]
YG-13	3.03 ± 0.2234 [h]	0.28 ± 0.0002 [a]	0.87 ± 0.0594 [b]	0.16 ± 0.0004 [a]

Note: 1. One unit of BSH activity (U/mL) is measured based on the amount of enzyme that releases 1 µmol of amino acids from the substrate per minute. 2. Enzyme substrate used. 3. Control containing reaction mixture (cell-free extract, conjugated bile salt)+ Trichloroacetic acid to halt reaction. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a-i indicates the results in the column with different lowercase letters indicate a significant difference ($P \leq 0.05$)

The BSH enzyme activity generated by LAB obtained from yogurt originating from Boyolali was observed to be greater in amount compared to the BSH enzyme activity reported in prior investigations. Kumar et al. (2012) conducted a study wherein a Lactobacillus strain sourced from fecal matter was found to produce BSH enzyme activity on sodium glycocholate substrate. The enzyme activity was measured to be 0.517 U/mL by *L. plantarum* Lp21. On the other hand, *L. plantarum* Lp91 produced the highest BSH enzyme activity of 0.299 U/mL on sodium taurocholic substrate. Liong and Shah's (2005) investigation revealed that L. acidophilus exhibited a greater overall BSH enzyme activity than *L. casei* when tested on sodium glycocholate and taurocholic substrates. The enzymatic activity of BSH on substrates of sodium glycocholate was observed to range between 0.45-1.81 U/mL, while on substrates of sodium taurocholic, the activity ranged between 1.06-1.47 U/mL. In addition to the findings of Thakkar et al. (2016), it has been observed that LAB derived from multiple fermented food sources can generate BSH enzymes within the range of 0.048 to 0.097 µmol/mL per minute. The study by Bhat and Bajaj (2020) revealed that the LAB isolates M5 and M9, obtained from breast milk, exhibited enzyme activity capable of releasing 0.057 and 0.052 µmol/mL glycine per minute, respectively.

The activity variance of the resultant BSH enzymes is subject to the influence of the environmental conditions in which Lactic Acid Bacteria (LAB) thrive. Tanaka et al. (2000) and Begley et al. (2006) have reported that LAB isolates derived from the digestive tract and mammalian feces can produce BSH enzymes in an environment abundant in conjugated and deconjugated bile acids. Strains or species derived from environments lacking bile salts, such as dairy products and fermented vegetables, cannot synthesize BSH enzymes. As per prior literature, it was asserted that the function of BSH enzymes was exclusively associated with commensal bacteria present in the gastrointestinal tract. The study reported that all Lactobacillus strains obtained from the intestine could hydrolyze glycine and taurine-conjugated bile acids (Reyes et al. 2014).

Numerous studies have demonstrated that BSH enzyme activity can be produced by isolates derived from environments beyond the digestive tract. The study conducted by Prete et al. (2020) demonstrated through chromatographic analysis that *L. plantarum*, a food-borne strain, can modify the bile acid profile by producing unconjugated bile acids. The observed disparity in the reaction between the control and sample groups suggests that all strains have the potential to deconjugate bile acids, despite not being derived from the intestinal environment.

The capacity of *L. plantarum* to synthesize BSH enzymes and perform bile acid deconjugation can be regarded as an adaptive mechanism to its surroundings. The lactic acid bacterium, *L. plantarum*, can inhabit diverse ecological niches, such as the fermentation of milk, meat, and different kinds of vegetables, as well as the human gastrointestinal tract (De Vries et al. 2006). According to Klereebezem et al. (2003), *L. plantarum* has a genome size of 3.3Mb, one of the largest among lactobacilli. This characteristic allows the bacterium to adapt to diverse environmental niches. The genome of *L. plantarum* WCFSI, as reported by Molenaar et al. in 2005, is anticipated to contain four BSH-associated genes, denoted as bsh1 to bsh4, which are dispersed throughout the genome of the Lb strain. The study exclusively examined the bsh1 gene, which is accountable for the metabolism of bile acids in *L. plantarum*.

Out of the 13 isolates that were obtained from yogurt in the present investigation, it was observed that only 9 isolates exhibited the capacity to generate BSH enzyme activity on both sodium glycocholate and sodium taurocholate substrates. This finding demonstrates that the capacity to produce BSH enzymes was not universal among LAB isolates obtained from yogurt. The BSH enzyme activity level in YG-2 isolates was higher than that of several LAB strains previously studied in humans and the intestine. The production of enzyme activity by the nine isolates can be considered an adaptive mechanism employed by bacterial isolates to ensure their survival in the intestinal milieu. Prete et al. (2020) reported that BSH activity in *L. plantarum* isolated from food-borne sources was found to be equivalent to that of human isolates.

Bacterial isolates may possess one or multiple bsh-coding genes that exhibit activity within the intestinal milieu, despite the isolate's non-gastrointestinal origin. This phenomenon represents an adaptive mechanism of the isolate to the environment, facilitating the metabolism of bile acids. As per the findings of Reyes et al. (2014), it was observed that the bsh coding sequence was present across all significant phyla, and the enzymatic activity of the produced enzymes varied across different environments.

Christiaens et al. (1992) conducted a study to assess the BSH activity and growth of *L. plantarum* 80 obtained from forage grass, with regards to the existence of conjugated bile acids in vitro. The present investigation has demonstrated that the bsh LP80 gene exhibits homology with the DNA of intestinal lactobacilli, thereby leading to the expression of the BSH enzyme. The potential correlation between the silage ecosystem and the digestive ecosystem can be elucidated by the existence of the BSH enzyme in Lactobacillus strains that have been extracted from fodder grasses, as reported by Tannock (1990) and Van Renterghem et al. (1991).

The liquid containing bile salts is a surface-active compound that can permeate and interact with the lipophilic cytoplasmic membrane of bacteria, resulting in alterations and impairment of the bacterial membrane architecture. The surface active compounds can activate lipolytic enzymes that can interact with fatty acids present in the cell membrane, ultimately influencing the permeability of the cell and leading to cellular damage, as Astuti and Rahmawati (2010) stated. The crucial criterion to identify LAB isolates as potential probiotic candidates is their capacity to endure the gastrointestinal tract's harsh conditions. The resistance of LAB to oxgall, or bile salts, is closely linked to the activity of the BSH enzyme. This enzyme is responsible for the hydrolysis of conjugated bile salts into a deconjugated form, thereby mitigating the toxic effects of bile acids on bacterial cells (Zulfidin et al. 2018).

Lactic Acid Bacteria (LAB) possessing the capability to synthesize the BSH enzyme exhibit a favorable trait of thriving and establishing themselves in the distal portion of the small intestine, which is the site of enterohepatic circulation. De Smet et al. (1995) reported in an in vitro investigation that the activity of BSH serves as a resistance mechanism against intracellular acidification caused by conjugated bile salts. It is in line with the research by Bustos et al. (2012), who showed strains that were able to hydrolyze bile salts or had BSH enzymes (+) were more resistant to exposure to some conjugated bile salts and strains that did not have BSH enzymes (-) showed a significant percentage of cell death. From the process of hydrolysis of bile salts by BSH enzymes, conjugated bile salts will dissociate so that these substances can enter cells through active transport or passive diffusion, so that when inside the cells, conjugated bile salts will be converted into a weaker form for bacterial cells, namely deconjugated bile salts. The result of hydrolysis in the form of free amino acids released from the bile salt deconjugation process can be used as a carbon, nitrogen, or energy source by bacteria. According to Begley et al. (2006), the metabolic breakdown of glycine results in the production of ammonia and carbon dioxide, whereas the metabolic breakdown of taurine yields ammonia, carbon dioxide, and sulfate.

Apart from the function of BSH, whereby LAB can indirectly lower cholesterol levels, the enzymatic deconjugation of bile salts in the gastrointestinal tract can enhance the elimination of bile salts. According to Noh et al. (1997), the C-24 N-acyl amide bond connecting the bile acids and amino acids in conjugated bile salts can be cleaved by the BSH enzyme, leading to the formation of deconjugated bile salts. The deconjugation of bile salts reduces solubility and hinders absorption within the intestinal lumen, in contrast to their conjugated counterparts. Due to this enzymatic process, free bile acids are liberated and subsequently eliminated through fecal excretion (De Smet et al. 1994; De Rodas et al. 1996). Cholesterol, the precursor of bile salts, can substitute for the lost molecules of bile salts during the excretion process, thereby reducing the cholesterol level in the serum.

Several studies have demonstrated that LAB isolates possessing the capacity to synthesize BSH enzymes exhibit the potential to decrease Low-Density Lipoprotein (LDL) levels in the bloodstream. According to Ooi et al. (2010) and Malpeli et al. (2015), ingesting yogurt containing *L. reutei* CRL 1098 for four weeks has significantly reduced total cholesterol and LDL-c. Additionally, a six-week trial involving capsules containing BSH-active strains of *L. acidophilus* and inulin has significantly reduced LDL and total cholesterol, significantly. The BSH enzyme facilitates the formation of deconjugated bile salts, specifically free cholic acid, which are subsequently eliminated via the fecal route. This process reduces the amount of bile acid reabsorbed into the liver from the digestive tract, increasing the demand for cholesterol. Therefore, to maintain homeostasis of bile acids, the body will sequester surplus cholesterol within the system, thereby reducing cholesterol levels in the bloodstream. In the given circumstances, there will be a surge in the requirement for endogenous cholesterol synthesis, leading to the activation of LDL-α receptors in the liver. It, in turn, will result in an upsurge in the uptake of LDL-C by the liver, ultimately leading to a decrease in LDL-C concentrations (Nur et al. 2021; Bhat and Bajaj 2019).

Bile salt deconjugation activity

The findings indicated that the nine LAB isolates could deconjugate both types of bile salts utilized as substrates, as presented in Table 5. The LAB isolates exhibited deconjugation capabilities within the range of 0.52-1.26 (μmol/mL) on the sodium glycocholate substrate, while on the taurocholic substrate, their ability to deconjugate bile salts ranged from 0.43-0.9 (μmol/mL). The YG-2 isolate demonstrated the most significant deconjugation activity on the glycocholate substrate, yielding a value of 1.26 μmol/mL. Similarly, the YG-2 isolate exhibited the highest activity level on the taurocholic substrate, with a 0.9 μmol/mL value. The deconjugation potential of nine isolates was determined by measuring the liberation of free cholic acid. A positive correlation was observed between the amount of cholic acid released and the level of deconjugation activity.

Table 5. Deconjugation activity of sodium glycocolate substrate and sodium taurocholic substrate by LAB isolates

Isolate name	Deconjugation activity (µmol /mL) [1]	
	Sodium Glycocolate [2]	Sodium Taurocholic [2]
YG-2	1.26 ± 0.0026 p	0.90 ± 0.0015 n
YG-3	1.25 ± 0.0027 p	0.48 ± 0.0006 d
YG-4	0.93 ± 0.0040 o	0.80 ± 0.0010 l
YG-5	0.81 ± 0.0007 l	0.47 ± 0.0031 d
YG-8	0.65 ± 0.0006 j	0.67 ± 0.0018 k
YG-9	0.55 ± 0.0004 g	0.43 ± 0.0008 c
YG-10	0.52 ± 0.0013 e	0.48 ± 0.0012 d
YG-12	0.82 ± 0.0010 m	0.54 ± 0.0018 f
YG-13	0.61 ± 0.0014 h	0.64 ± 0.0007 i
Control [3]	0.20 ± 0.0031 a	0.12 ± 0.0014 b

Note: 1. Bile salt deconjugation activity is measured based on the amount of cholic acid released 1 µmol of amino acids from the substrate per milliliter (mL). 2. Substrate used. 3. Control, containing media + conjugated bile salt, without, LAB isolate. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a-p indicates the results in the column with different lowercase letters indicating a significant difference ($P \leq 0.05$)

Generally, the outcomes of the bile salt deconjugation examination revealed that the LAB isolates exhibited a greater capacity to liberate free cholic acid on glycocholate substrates than taurocholate substrates ($P \leq 0.05$). It suggests that the variance in substrates can influence the activity of bile salt deconjugation, as shown in Table 5. The results indicate a significant difference in the bile salt deconjugation activity between the two substrates, as evidenced by using distinct lowercase letters to denote each result in the column. The subsets in Duncan's table are represented by distinct alphabet letters for each column. The ANOVA test revealed a notable dissimilarity, indicating that sodium glycocholate substrates' deconjugation was more efficient than sodium taurocholic. A higher quantity of liberated cholic acid evidenced it.

The bile salt deconjugation test and the BSH enzyme activity test indicate greater activity on the glycocholate substrate than the sodium taurocholate substrate in this study. The observed variation in activity suggests that the BSH enzyme exhibits substrate recognition capabilities towards bile acids, specifically towards the cholicate steroid core and amino acid groups (glycine or taurine). The identification of substrates is primarily based on the amino acid groups they contain. According to Taranto and de Valdez (1999), most BSH enzymes exhibit greater efficacy in the hydrolysis of bile salts conjugated with glycine instead of taurine. Furthermore, examining the configuration of the BSH enzyme across diverse species has revealed the presence of specific residues within the enzyme's active site that serve as a crucial element in the binding of the substrate. The recognition of substrates by the active site and the provision of information regarding selective substrates in BSH enzymes have been reported in previous studies (Rossocha et al. 2005).

According to a study conducted by Liong and Shah (2005), it was found that most BSH enzymes exhibit higher efficacy in the hydrolysis of glycine-conjugated bile salts as compared to taurine-conjugated bile salts. The study observed that eleven strains of *L. casei* and *L. acidophilus* released a greater amount of cholic acid in deconjugating bile salts devoid of deconjugated sodium glycocholate, in contrast to sodium taurocholate. De Smet et al. (1995) observed that the BSH enzyme exhibits a higher specificity for the hydrolysis of Glycodeoxycholic Acid (GDCA) as compared to Taurodeoxycholic Acid (TDCA). The research conducted by Moser and Savage (2001) demonstrates that the steroid structure of bile salts plays a crucial role in determining the specific substrate specificity of BSH. Conversely, the analysis of conjugated bile acid hydrolases by *Clostridium perfringens* reveals a lack of specificity for particular substrates, which can be attributed to the absence of active site residues on the enzyme that is necessary for binding to the conjugated bile acid substrate (Rossocha et al. 2005). In essence, the BSH enzyme possesses specific active site residues that enable it to bind to the appropriate conjugated bile acid substrate.

It is a widely acknowledged fact that many probiotic strains possess multiple homologous BSH genes. Certain BSH genes found in bacteria can confer advantages, such as optimizing bacterial defense mechanisms in response to fluctuating environmental circumstances. The activity of enzymes towards substrates is subject to variation, further modulated by the BSH gene. The BSH gene exhibits specificity towards distinct bile salt compositions and protects varying durations of exposure to bile salts (Begley et al. 2006). The findings indicate that the isolates exhibited a greater capacity for deconjugating the two bile salts than in earlier investigations. Specifically, LAB isolates obtained from breast milk demonstrated the ability to deconjugate taurocholic bile salts within the range of 0.06-0.25 µmol cholic acid per mL, with the most notable performance being exhibited by *Pediococcus pentocaceus* 1-A38 (Nuraida et al. 2011). According to Pranata et al. (2016), *L. plantarum* 1.R.1.3.2 and *Lactobacillus acidophilus*, which were isolated from LAB, exhibited the ability to deconjugate taurocholic bile salts at a concentration of 0.58 µmol/mL. Similarly, Pato (2003) reported that six LAB isolates obtained from curd could release cholic acid at concentrations ranging from 0.21 µmol/mL to 0.45 µmol.

Probiotic bacteria that exhibit Bile Salt Hydrolase (BSH) activity can induce a hypocholesterolemic response by catalyzing the deconjugation of bile salts. The deconjugation of bile salts has been observed to decrease serum cholesterol levels by stimulating the production of bile acids to compensate for the loss of cholesterol in fecal matter. Additionally, this reaction has been found to diminish the solubility and absorption of cholesterol in the intestinal lumen, as reported by Lye et al. (2010). The solubility of cholesterol is greatly influenced by its interaction with bile salts, given its insolubility in aqueous solutions. According to Wang (2003), the efficacy of bile salts in emulsifying decreases when they are deconjugated. As a result, cholesterol becomes less soluble, which can hinder the formation of micelles in the intestinal tract.

During the deconjugation of bile salts, the resultant cholic acid reduces pH levels (<5.0) within the fermentation medium. This acidic bacterial environment can interfere with the formation of stable cholesterol micelles in the body. Consequently, cholesterol may

precipitate with free bile salts resulting from deconjugation (Klaver and Van der Meer 1993). The phenomenon of cholesterol deposition and subsequent cholic acid formation has been observed to impede cholesterol absorption within the gastrointestinal system potentially. The deposition of cholesterol alongside free bile acids represents a direct mechanism for reducing cholesterol levels, in addition to the assimilation of cholesterol by Lactic Acid Bacteria (LAB). The phenomenon of cholesterol deposition necessitates the presence of a BSH enzyme that exhibits optimal activity within a pH range of 5-6. Under pH less than 6 conditions, the deconjugated bile salts tend to coalesce with cholesterol, leading to precipitation. However, an elevation in the pH level of the medium to 7 can facilitate the dissolution of cholesterol back into the medium (Tahri et al. 1996).

Cholesterol assimilation activity

The selected lactic acid bacteria exhibit the trait of being capable of cholesterol assimilation. The direct assimilation of cholesterol within the intestine can diminish the absorption of cholesterol ingested from the digestive tract into the bloodstream. Therefore, using probiotics that possess hypocholesterolemic or cholesterol-lowering properties presents a viable option for averting the onset of cardiovascular disease.

The study revealed that nine distinct strains of Lactic Acid Bacteria (LAB) obtained from yogurt in Boyolali exhibited the capacity to lower cholesterol levels in the media. It was evidenced by the significant assimilation of cholesterol, with values ranging from 39.59 to 111.92 µg/mL, as presented in Table 6. The assimilated cholesterol value is a metric derived from the disparity between the quantity of cholesterol recorded during the initial incubation and the quantity recorded following a 24-hour incubation period. LAB YG-2 Isolate exhibited the greatest assimilated cholesterol value of 111.92 µg/mL, while YG-4 isolate demonstrated the lowest value of 39.59 µg/mL. A negative correlation exists between the disparity in cholesterol quantity in the media and the extent of cholesterol level reduction observed in the stated media.

Moreover, calculating the percentage of assimilated cholesterol involves dividing the difference into cholesterol levels in the media, previously determined, by the cholesterol level at the initial time. Table 6 and Figure 2 indicates that LAB isolates exhibit varying degrees of cholesterol assimilation, ranging from 38% to 71%. The YG-13 isolate yielded the highest percentage, measuring 71.98%, whereas the YG-4 isolate produced the lowest percentage, measuring 38.69%. The study's findings indicate that YG-13 exhibited the highest assimilation percentage value of 71.98% among the LAB isolates tested. However, it is noteworthy that the highest assimilated cholesterol value was observed in the YG-2 LAB isolate, which recorded a value of 111.92 µg/mL. It is important to note that the observed value difference could be attributed to variations in the total cholesterol level at 0 hours across the tested LAB isolates during measurement. Therefore, it is recommended to employ percentage calculations to compare the results accurately.

Table 6. Assimilated cholesterol value and percent assimilation of cholesterol by bacterial isolates

Isolate name	Assimilated cholesterol [1] (µg/mL)	% of Assimilated cholesterol [2]
YG-2	111.92 ± 0.3712 ^{f}	68.82 ± 0.3138 $^{e, f}$
YG-3	60.93 ± 1.3867 ^{c}	54.59 ± 0.4368 ^{c}
YG-4	39.59 ± 0.0818 ^{b}	38.69 ± 0.0692 ^{b}
YG-5	103.24 ± 1.1330 ^{e,f}	62.59 ± 0.8818 $^{d, e}$
YG-8	94.40 ± 0.0750 ^{e}	56.07 ± 0.0477 $^{c, d}$
YG-9	77.28 ± 0.1531 ^{d}	48.83 ± 0.1108 ^{c}
YG-10	73.24 ± 10.373 ^{d}	53.88 ± 7.6343 ^{c}
YG-12	77.02 ± 0.2591 ^{d}	52.37 ± 0.1608 ^{c}
YG-13	72.20 ± 0.2095 ^{d}	71.98 ± 0.2091 ^{f}
Control [3]	4.84± 2.0721 ^{a}	3.98 ± 1.6124 ^{a}

Note: 1. Assimilated cholesterol is measured based on the difference in the amount of cholesterol released in µg per milliliter (mL) at 0-hour and 24 hours. 2. The percentage of cholesterol is measured by dividing the difference between the amount of assimilated cholesterol and the value of cholesterol at the 0-hour. 3. Control, containing media + cholesterol, without LAB isolate inoculation. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a- f indicates the results in the column with different lowercase letters indicating a significant difference (P ≤ 0.05)

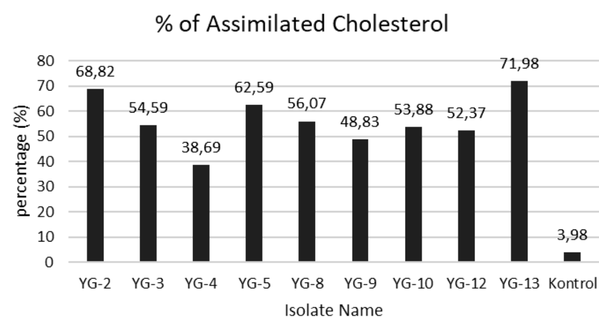

Figure 2. Assimilated cholesterol percentage

Statistically significant differences were observed between the control, cholesterol assimilation results, and cholesterol percentage (P<0.05). The graph illustrates that isolate YG-13 exhibited the highest percentage of assimilated cholesterol. The observed disparity in percentage between the isolate samples and the control can be attributed to the absence of LAB isolates in the control treatment, which was conducted using growth media that contained cholesterol. As a result, the control treatment did not exhibit any cholesterol assimilation activity. In the control group where LAB was not introduced, the total cholesterol levels remained elevated at the initial time point and after 24 hours of incubation. Consequently, the difference in total cholesterol levels was minimal when assimilated cholesterol was calculated, resulting in a low percentage. In the treatment group where LAB was administered, it was observed that the initial high levels of cholesterol decreased significantly after 24 hours. Furthermore, there was a substantial difference in the total cholesterol levels following the assimilation activity. This

finding demonstrates that incorporating LAB isolates can elicit a cholesterol-lowering effect in the medium.

The present investigation revealed a higher cholesterol level assimilation activity exhibited by the LAB isolates under examination than that demonstrated by LAB isolates obtained from prior research sources. According to a study by Nuraida et al. in 2011, LAB isolates obtained from breast milk exhibited cholesterol assimilation activity ranging from 0.86-14.97 µg/mL. The highest assimilation activity was observed in *Pediococcus pentosaceus* 1-A38. Burhan et al. (2017) conducted a study wherein they examined 8 LAB strains that were isolated from *dangke* (a traditional food from Enrekang, Sulawesi) and beef. The study yielded *L. fermentum* B111K and *L. plantarum* IIA-1A5 strains, which exhibited the potential to reduce cholesterol in vitro. The cholesterol assimilation rates of *L. fermentum* B111K and *L. plantarum* IIA-1A5 were 4.10% and 8.10%, respectively.

The variance in the cholesterol assimilation capacity of the bacteria examined in this investigation, as compared to prior studies, was impacted by the existence or nonexistence of bile salts in the testing milieu (Lye et al. 2010) and disparities in the cholesterol origins employed in the trial (Kusumawati 2002). Notably, cholesterol exhibits a considerably low solubility in water, thereby rendering it arduous to dissolve in water-based MRS B media. The limited solubility of cholesterol in aqueous solutions significantly impacts the bacterial assimilation of cholesterol. Lye et al. (2010) assert that the presence of bile salts impacts the capacity to eliminate cholesterol. Most tested strains exhibit the capacity to decrease or eradicate elevated cholesterol levels in test media containing bile salts, as opposed to control media lacking bile salts. Bile salts, classified as biological surfactants, can reduce surface tension, thereby enhancing the cellular attachment of cholesterol. These bile salts have a superior capacity to dissolve cholesterol than their solubility in aqueous solutions.

The present investigation yielded a comparatively elevated level of cholesterol assimilation activity in comparison to prior research endeavors. This outcome was attributed to the inclusion of 0.3% oxgall and pure cholesterol in the culture medium, whereas earlier studies solely employed pure cholesterol without supplementing bile salts. Consistent with the findings of Lye et al.'s (2010) investigation, it was observed that adding oxgall to the media resulted in a decrease in cholesterol concentrations. This effect was attributed to the activity of a *Lactobacilli* strain that was incubated at 37°C for 20 hours. Specifically, the strain *L. bulgaricus* FTDC 1311 exhibited the greatest cholesterol assimilation activity. The observed assimilated cholesterol activity in media containing cholesterol and the specified substance was 62.42 µg/mL. In contrast, media containing solely cholesterol exhibited an assimilated cholesterol activity of 27.89 µg/mL.

The variations observed in the cholesterol assimilation capacity among the bacterial strains examined were associated with the distinct characteristics of each strain, specifically the composition and physicochemical attributes of the peptidoglycan present in their respective cell walls. This finding was reported by Kimoto et al. (2002). Within the cholesterol assimilation mechanism, a portion of the cholesterol will undergo direct uptake or absorption, subsequently integrating with the bacterial cellular membrane. The study conducted by Lye et al. (2010) demonstrated the attachment of cholesterol by *L. bulgaricus* FTDC 1311, as evidenced by the use of Scanning Electron Microscopy (SEM). The direct binding of cholesterol to the surface of *lactobacilli* cells during fermentation was observed, resulting in a textured cell surface compared to the bacterial cell surface in the control group. These findings suggest probiotic conditions may induce *L. bulgaricus* FTDC 1311 cholesterol attachment. Kimoto et al. (2002) conducted research that revealed that the distribution of fatty acids in bacterial cells varied between media containing cholesterol and media lacking cholesterol. The interaction between cholesterol and the bacterial cell membrane can alter the fatty acid composition of the bacterial cell. This phenomenon has been widely discussed in various media outlets. According to Noh et al. (1997), Lactobacilli bacteria that can assimilate cholesterol exhibit greater resistance to interference from sonication. Therefore, it can be attributed to the fact that cholesterol absorption during the assimilation process can alter the composition of the bacteria's cell wall and cell membrane.

Lim et al. (2017) conducted a study wherein the qualitative analysis of pellet cells of LAB 4 and LAB 12 isolates was performed using fluorescence microscopy with the Neil Red staining method. The study results indicated that both LAB cells could assimilate cholesterol, as evidenced by the appearance of a higher intensity of red fluorescence on the surface of the bacterial cell from the media containing cholesterol compared to the control, wherein LAB isolates were incubated without cholesterol. The process of cholesterol assimilation by probiotic bacterial strains in the digestive tract has reduced cholesterol absorption by enterocytes. This reduction in cholesterol absorption has been linked to a decreased risk of developing coronary heart disease, as reported by Aquino et al. (2017). The inhibition of micelle formation in the intestine can occur due to cholesterol binding to the cell surface. The disruption of micelle formation results in reduced cholesterol entering the systemic circulation. Furthermore, decreasing cholesterol levels (Bhat and Bajaj. 2019).

The capacity to reduce cholesterol levels is a significant probiotic characteristic, as elevated serum cholesterol levels are recognized as a primary contributor to the heightened likelihood of developing metabolic disorders, such as cardiovascular disease, obesity, and coronary heart disease (Park et al. 2018). According to Wang et al. (2018), using probiotics is a non-pharmacological strategy that can mitigate the risk factors associated with cardiovascular disease by decreasing elevated levels of serum cholesterol within the human body. The consumption of probiotics derived from fermented products containing specific bacterial strains has been shown to elicit a reduction in levels of cholesterol concentration. According to Pan et al. (2011), it is necessary to regularly consume probiotic products to achieve long-term hypocholesterolemic effects,

as the strains responsible for these effects are not naturally present in the human digestive tract.

In conclusion, nine isolates obtained from yogurt originating from Boyolali exhibited in vitro cholesterol-lowering activity by producing BSH enzymes, deconjugating bile salts, and assimilating cholesterol. Therefore, of the nine LAB isolates, the YG-2 isolate exhibited the most significant BSH enzyme activity and bile salt deconjugation, measuring 3.82 U/mL and 1.26 (μmol/mL) for sodium glycocholate. The YG-13 isolates exhibited the highest cholesterol assimilation capacity, specifically 71.98%. The correlation between the activity of BSH enzymes and bile salt deconjugation was demonstrated by the higher activity observed against glycocholate substrates compared to sodium taurocholate.

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