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Fungi, bacteria, and chemical components in the fermentation process of various maturity levels of drumstick trees (*Moringa oleifera* Lam.) in making tempeh, a fermented soybean food

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Abstract: This research focuses on the role of fungi and bacterial inhibition on the chemical quality of tempeh made from Horseradish (drumstick trees) tree seeds (*Moringa oleifera* Lam.) at various levels of maturity. The tempeh produced using these seeds harvested at different maturity stages as treatments before fermentation, namely: B0 = soybeans (control), B1 = mature/old seeds, B2 = medium seeds, and B3 = young seeds, with four replications, composing of totally sixteen test units. There were several stages carried out before the fermentation, namely: soaking, boiling, drying, inoculating, wrapping and then fermenting. In the preparation of yeast (starter culture), a method of Nurrohmah (2013) was applied and modified, while in the preparation of *Moringa oleifera*, the method of Jubaidah et al. (2016) was adopted and modified. The results showed that Rhizopus sp. could ferment *Moringa* seeds at all maturity stages. The higher the maturity level of the Moringa seeds, the faster Rhizopus sp. grew and developed, thereby better in inhibiting the growth of bacteria, particularly coliform bacteria. Regarding chemical quality parameters, tempeh made from medium and mature Moringa seeds contained better water (B3 contained 75.73% water), protein (B1 contained 17.21% protein), and fiber (B3 contained 9.39% fiber). Meanwhile, soybeans (B0) contained the highest ash (1.38%), fat (6.41%), and carbohydrate (17.60%) compared to all maturity levels of Moringa seeds.

Keywords: ash content; coliform bacteria; fermentation; fibre content; soybeans.
Abbreviations: ATP_adenosine triphosphate; HCI_hydrochloric acid; H_2SO_4_sulfuric acid; NaOH_sodium hydroxide; N_2_nitrogen gas; NH3_ammonia.

Introduction

Tempeh is a popular fermented food product in Indonesia and has been consumed for ages largely in Asian countries (Aaslyng and Højer, 2021; Elhalis, et al., 2023), well known as a nutritious plant-based protein source (Romulo and Surya, 2021; Subali et al., 2023; Rizzo, 2024; Teoh, et al., 2024). Tempeh is produced by fermenting soybeans for a specific period using the fungus *Rhizopus oligosporus* (Chen et al., 2020; Yarlina et al., 2023). Typically, tempeh has a white colour, resulting from the growth of fungal mycelia binding soybean seeds into a compact composition (Duniaji et al., 2019; Adanlawo et al., 2023). The nutritional content of tempeh meets body's nutritional needs. Raw tempeh contains 41% more protein than to raw beef, where a 100 grams of raw beef has 14.4g of protein while tempeh has 20.3g of protein (https://www/soupersage.com/compare-nutrition/tempeh-vs-raw-beef).

In recent years, interest in tempeh as a nutritious and functional food ingredient has increased, particularly in line with the growing awareness of the importance of plant-based protein in a healthy diet. It is estimated that tempeh production in Indonesia exceeds 100.000 tons, with a wide variety of textures and variations, from different raw materials, substitutes, flavour enhancers, aroma, and texture.

Unfortunately, in Indonesia, the increasing demand for tempeh is not aligned with soybean production. According to Central Bureau Statistics Indonesia data for 2023, soybean production until December reached 550.000 tons, whereas the demand was 2.7 million tons. This gap is due to limitation in the available lands for soybean cultivation, being only 285.000 hectares, whereas 2.5 million hectares are needed to meet demand. This situation calls for a serious response by utilizing alternative raw materials with nutritional values comparable to soybeans.

One promising raw material for tempeh production is Moringa seeds (*Moringa oleifera* L.), known for their high nutritional content and significant health potential. *Moringa oleifera,* or the Moringa tree, has long been valued for its rich nutritional

content, including protein, fibre, and essential vitamins and minerals. However, the use of Moringa seeds as a raw material for tempeh has not been explored as much as soybeans. Studies on the potential of Moringa seeds for tempeh production indicate that these seeds have beneficial nutritional properties, but challenges in the fermentation process should be solved to maximize their benefits.

Scientifically, many parts of the Moringa plant can be used as raw materials in chemical, health, and food industries (Cao, et al., 2023; Pareek, et al., 2023; Pello and Ricky, 2023). Proximate analysis has shown that *Moringa oleifera* leaves are rich in protein (22.99–29.36%), low in fat (4.03–9.51%), contain fibre (6.00–9.60%), and ash (8.05–10.38%). Fresh leaves of the plant contain vitamin C (187.96–278.50 mg/100 g), calcium (1.322–2.645%), phosphorus (0.152–0.304 g/100 g), and potassium (1.317–2.025 g/100 g) (Salma, 2020).

The growth of Moringa plants is influenced by genetic factors, cultural techniques, and growing conditions (climate and soil). Moringa can grow in various types of soil which does not require intensive care, due to its drought-resistance, making it easy to cultivate (Mashamaite et al., 2024; Kashyap et al., 2022). The maturity level of Moringa seed affects its water content. At optimal maturity, Moringa seed has high water content (46%), while ripe seed has 37.3%, and overripe seed has 7.9% (Sumarjan and Sumarjan, 2017).

The moringa has been found to have a fat content of 36.12%, protein content of 32.66%, carbohydrates of 14.43%, ash content of 4.79% and fibre content of 68.5% (Ramteke et al., 2024). However, it was found that so far, especially in Indonesia, Moringa seeds have only been used as vegetables' raw materials (Rahmatu et al., 2023).

In fact, the use of Moringa seeds as a soybean substitute, in addition to serving as a raw material for tempeh, can also inhibit bacterial growth (Aoki et al., 2023). The combination of Moringa seeds and Rhizopus sp. both have antimicrobial properties, particularly against *Escherichia coli* (*E. coli*), a common foodborne pathogen. This fungus produces various compounds such as rhizoxin, rhizonin, and rhizopuspeptidase, which have been shown to inhibit *E. coli* growth. Rhizopus sp. can inhibit the growth of *E. coli* through several inhibition mechanisms, including disruption of the cell wall. The fungus produces enzymes that break down the *E. coli* cell wall, ultimately leading to cell lysis and death. *Rhizopus* sp. can also inhibit protein synthesis by producing compounds that prevent protein synthesis in *E. coli*, stopping the bacteria from multiplying.

Rhizopus sp., a type of fungus, plays an important role in tempeh production, a soybean fermentation product. This fungus breaks down proteins and carbohydrates in soybeans and other seeds, making them easier to digest and improving their nutritional value. The fungus grows by forming thick white mycelium and releases various enzymes that accelerate reactions during fermentation. The enzymes involved include protease, lipase, and amylase, which help break down proteins, fats, and carbohydrates in seeds like soybeans, enhancing their flavour and increasing their protein content and bioavailability (amount and absorption rate).

Studies on *Rhizopus* mould in tempeh have shown its benefits in reducing the occurrence of diarrhoea caused by *Escherichia coli* and *Salmonella typhi* compared to *Vibrio cholera* (Khikmah and Haloho, 2021).

Furthermore, antibacterial tests showed that two isolates of *Rhizopus* sp. could inhibit *Vibrio cholera*, demonstrating moderate inhibitory capacity. The use of *Rhizopus* sp., such as *R. oligosporus*, is widespread as a fungal inoculum in tempeh fermentation, which is highly advantageous (Martín-Miguélez, 2024; Romulo and Surya, 2021). The fermentation process of soybean does not only contribute to the antioxidative impacts but also emerge the antibacterial activity (Kusumah, et al., 2018).

Nutritional quality of tempeh can be achieved using *Rhizopus* species, while according to Ardiani et al. (2024), changes in texture, colour, and aroma can occur when using *Rhizopus stolonifer* and *R. oryzae* as inoculants.

Tempeh fermentation process involves the activity of microorganisms, particularly fungi and bacteria. *Rhizopus oligosporus* and *Rhizopus oryzae* are the two main species commonly used in tempeh fermentation, playing roles in protein conversion to more digestible forms, and influencing the flavour, texture, and organoleptic properties of the final product. Lactic acid bacteria can also contribute to the development of unique flavours and aromas in tempeh. Fungal moulds belong to the eukaryotic organisms group, forming fine white threads for spore growth (hyphae), which are visible in fermented tempeh produced by the action of *Rhizopus oligosporus*.

According to Gautier et al. (2022), the chemical components of Moringa seeds themselves, such as proteins, fats, carbohydrates, and phytochemicals, influence the fermentation outcome. Besides, they further added that bioactive compounds produced during fermentation, such as essential amino acids, unsaturated fatty acids, and antioxidants, can enhance the nutritional value of Moringa seeds tempeh and provide additional health benefits. However, factors such as the pre-fermentation processing level of Moringa seeds, the duration and conditions of fermentation, and the interactions between fungi and bacteria significantly affect the chemical composition and functional properties of the resulting tempeh. Based on the explanation above, this research was conducted to explore the interaction between fungi, bacteria, and chemical components during the fermentation of Moringa seeds tempeh from various maturity levels. This study aims to better understand how these variables contribute to the quality and nutritional value of the resulting tempeh, and to provide information that can be used to optimize Moringa-based tempeh production in the food industry.

Results and discussion

Water content

As can be seen in Figure 1, the highest average value of water content is 75.73% in young Moringa seeds (B3) while the lowest value of 60.83% was found in the control treatment soybeans (B0).

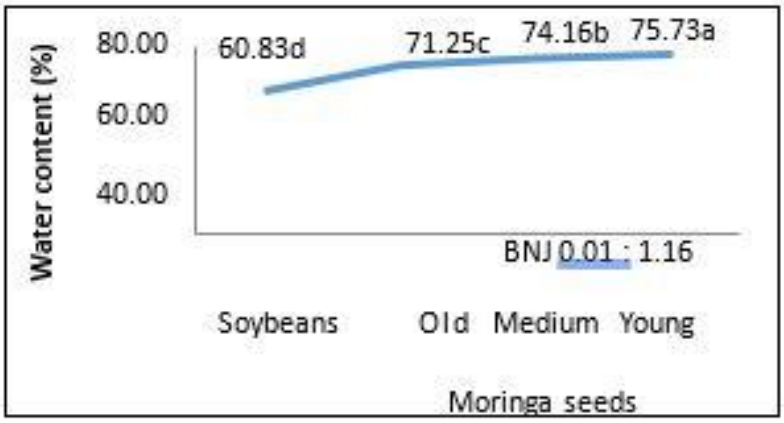

Figure 1. The average of water content (%).

The study of Gupta et al. (2022) proved that the moisture content of Moringa seeds declined and decreased by the time they were getting more mature. Water content also affects the freshness and shelf life of food, as well as the life of microorganisms. Hallsworth (2021) reported that viability and long-term integrity of microbial cells are maintained by water, which acts as a preservative.

Treatment with different maturity levels of Moringa seeds affects the amount of water content in tempeh. Usually, the suitable water activity to grow is at 0.90 to 0.99 at an aw value of 0.75 – 0.99 (Benkerroum, 2020). growth is influenced by the water content of soybeans before fermentation. There is a change in water content, when fermentation takes place. Suhartanti et al. (2019) revealed that for several soybeans' varieties, the longer the fermentation time produced the higher water content of tempeh. This is caused by the metabolic activity of fungi during fermentation which releases heat making the fermentation temperature increase (Damanik et al., 2018).

When tempeh is fermented, microbes break down carbohydrates and then produce water, energy (ATP), and carbon dioxide. Soybeans experience hydration during soaking and boiling. Because water will enter the cell walls of the soybeans causing their weight to increase. According to the Indonesian National Standard (SNI) 3144-201, the maximum water content of tempeh is 65% (Novelina et al., 2023), hence water content of Moringa seeds tempeh in this study does not comply with the standard since the old, medium, and young Moringa seeds tempeh contain the water which surpassed that maximum percentage.

Ash content

Ash content is a parameter of the nutritional value of product material produced by the components of organic substances contained in the product" (Arandini, 2022).

Figure 2 shows the highest percentage content average of ash is 1.38% found in the control treatment (B0) which used soybeans, while the lowest value is 0.29% found in the treatment of young Moringa seed (B3). This indicates that ash content of Moringa seed is lower compared to soybeans. Different maturity levels of Moringa seeds affect ash content in which the older the Moringa seed used, the more increased the content of the ash. A research study of Santoso et al. (2019) which utilized old Moringa seeds (already brown) found that these seeds had higher ash content, i.e., 3.3 – 6.5%. Adegbe et al. (2016) also reported that the ash content in Moringa seeds oil was 5%. Soaking and cooking in the process of making tempeh from Moringa seeds causes a loss of solids in the seeds which results a decrease in ash content (Damanik et al., 2018).

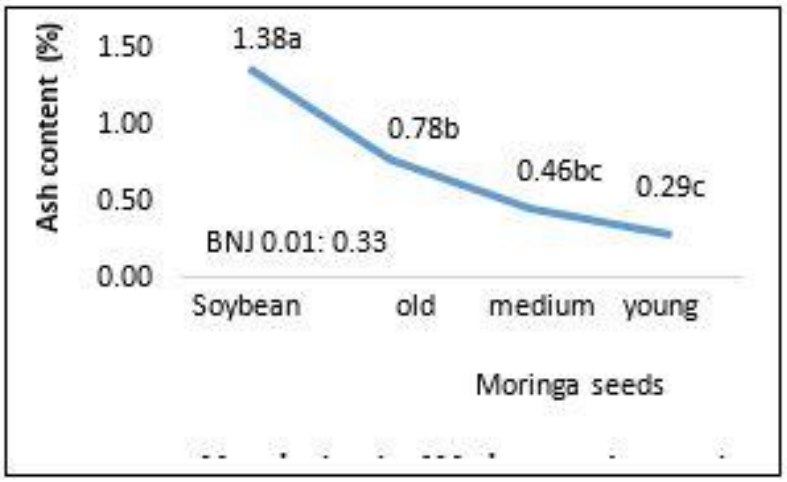

Figure 2. The average of ash content (%).

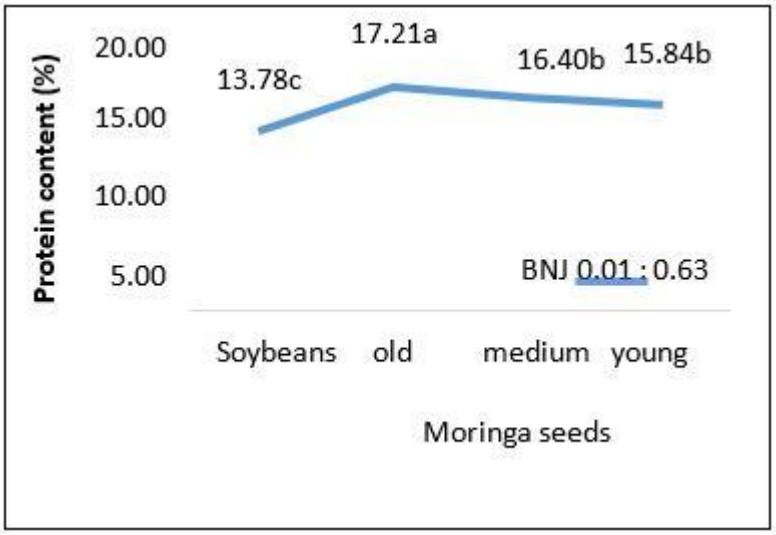

Figure 3. The average of protein content (%).

Organic matter and water are the main constituents of food (around 96%) and the rest are mineral elements known as ash content or organic substances (Saadilah et al., 2018). In a food product, ash content is commonly related to the amount of minerals contained (Khasaah et al., 2025). According to Mudambi and Radjagopal (1980) and Fitria et al. (2013), the ash content will decrease if the percentage of water content increases, also increasing the base weight of tempeh, but generally the ash content does not change when storing tempeh. Under the same conditions, the younger the Moringa seeds used, the more water they will produce due to microbial activity, so that the ash content of the Moringa seeds becomes lower.

Protein content

Figure 3. presents that the highest average of protein content is 17.21% in old Moringa seeds (B1), while the lowest one is 13.78% in the control treatment that used soybeans (B0). The maturity levels of Moringa seeds affected their protein content, by which the older the Moringa seeds, the higher the protein content of Moringa seeds tempeh.

This was in line with the study of Novenra (2019) who found that the fermented old *jengkol* or 'dog-fruit' seeds (pithecellobium jiringan (Jack) prain) contained higher protein compared to the young one, and this fermented result can also be used as raw material for fermentation using tempeh yeast. Yeast is known to synthesize proteins derived from its substrate. The synthesis of essential amino acids improves the composition of amino acids to become more balanced (Sakinah et al., 2019). During fermentation, proteins are degraded by fungi to produce dipeptides and then produce NH3 or N2 compounds which will become steam. The more protein being degraded, the lower the protein levels (Cempaka et al., 2018). The protein value that can be measured is also influenced by the water content of the food (Falinrungi et al., 2019).

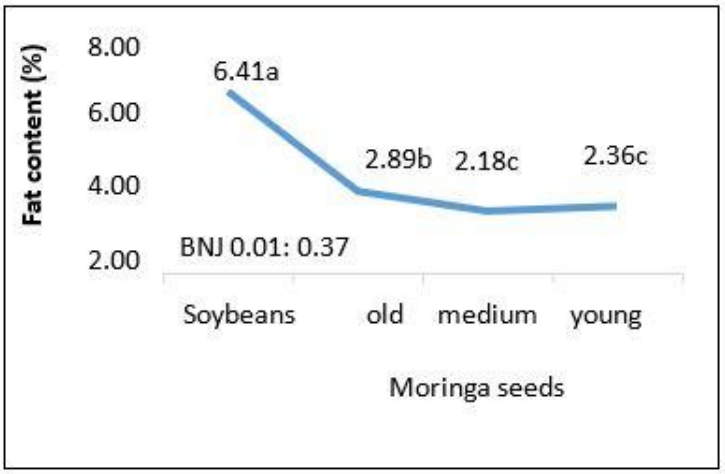

Figure 4. The average of fat content (%).

Fat content

The average percentage of the fat content is displayed on Figure 4. It revealed that the highest fat content is 6.41% in the control treatment using soybeans (B0), while the lowest one is 2.18% found in medium Moringa seeds (B2). Different maturity levels of Moringa seeds affected the fat content of Moringa seeds tempeh. Haron and Roab (2014) reported that raw soybeans contained higher fat, that was 10.60% and decreased to 8.39% after being fermented. All fat contents of tempeh resulted from all treatments tended to be smaller. This happened because during the fermentation, the lipase

enzyme in mushrooms will hydrolyze triglycerides to produce free fatty acids. Then, the resulting fat can be used as an energy source for the fungus to grow, causing the fat content to decrease (Damanik et al., 2018; Rizal et al., 2022). Moringa seed tempeh has the lowest fat content compared to the fat content of young Moringa seed tempeh.
The fat analysis towards young Moringa seed tempeh had weaknesses, in which the sample used could not be ground properly and uniformly. When a Moringa pod was cut and sliced, sometimes there were more seeds in one cut than in others. Auliana (2017) explained that the analysis results will not match what is desired and it might become valid. Fat content found in all treatments of Moringa seeds was lower compared to Indonesian National Standard 3144-2015 of soybeans tempeh that requires a minimum of 10%. However, even though the fat content was low, it was still considered to be of good quality especially for people who are on diet or are obese (Sari et al., 2016).

Carbohydrate content

As can be seen on Figure 5, the highest average of carbohydrate is 17.60% in the control treatment which applied soybeans (B0), whereas the lowest one is 5.78% found in the treatment of young Moringa seed tempeh (B3).
Maturity levels of Moringa seeds affected carbohydrate content of Moringa seed tempeh, in which the older the Moringa seeds produced higher carbohydrate content. Özcan (2020) reported that fat content was getting increased along with the maturity of its seed. It was explained that carbohydrate breakdown occurred by the activity of microorganisms during fermentation. Therefore, it reduced carbohydrates because starch was hydrolyzed by the α-amylase enzyme to produce simple sugars. This simple sugar was then utilized by microorganisms as an energy source (Simwaka et al., 2017; Sakinah, 2019).

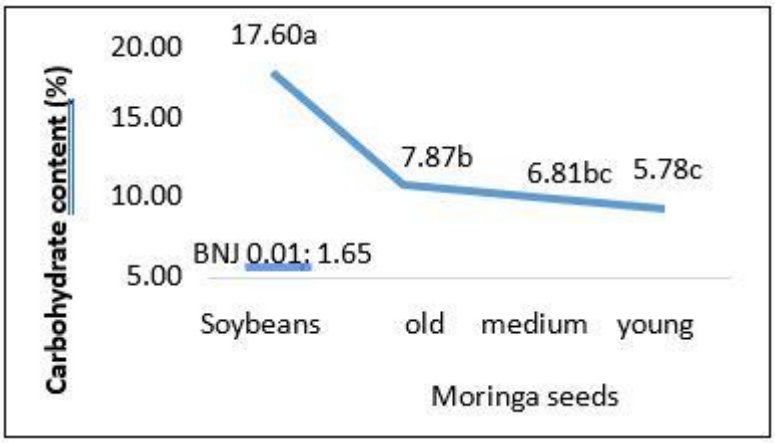

Figure 5. The average of carbohydrate content (%).

Fiber content
Figure 6 presents the average percentage of fiber content, in which the highest (9.39%) was found in the treatment of young Moringa seed (B3) while the lowest (3.01%) found in the control treatment of soybeans (B0).
Maturity levels of Moringa seeds affected the fiber content of Moringa seeds tempeh, in which the older Moringa seed used had lower fiber content. The study of Thakur et al. (2022) proved that the carbohydrate contents decreased in the soybean after the process of fermentation. They reported that raw soybean reached 21.31% carbohydrate content then it decreased to 14.90% after being fermented. This happened because *Rhizopus sp* can break down crude fiber content (Van den Hil et al., 2010; Endrawati and Kusumaningtyas, 2017). Fiber helps the digestive process in the intestine (Van den Hil et al, 2010; Endrawati and Kusumaningtyas, 2017). Having enough fiber can reduce cholesterol levels, hypertension, diabetes, and also providing antioxidants and lowering cancer (Fasulo, 2025).

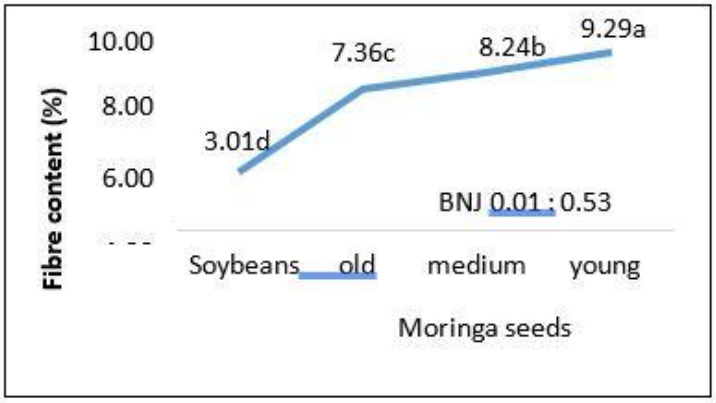

Figure 6. The average of fibre content (%).

Microscopic characteristics of in tempeh

Figure 7 shows that the sporangium structure in the *Rhizopus* sp. already formed at 72 hours. The sporangium structure is clearly seen in the treatment of old Moringa seed (B1) and young Moringa seed (B2). Meanwhile, in the treatment of young Moringa seed (B3), sporangium has formed even though it is not yet completely filled.

Microscopically, Rhizopus sp. has its colony characteristics, namely greyish white on the mycelium while blackish grey on the spores. It has smooth hyphae, short sporangiophores, and a round shape (globossa) on the sporangium. Thus, this is similar to the characteristics of *Rhizopus oligosporus*, but further research needs to be done (Virgianti, 2015; Hartanti et al., 2015). Hernawati and Vita (2019) stated that *R. oryzae* and *R. oligisporus* are fungi that are often used in the fermentation process of tempeh. *R. oligosporus* inoculum is good for making tempeh because it can produce antibiotics, biosynthesis of B vitamins which are beneficial for the body.

Coliform bacteria suspected test

Based on the analysis results in laboratory shown on Figure 8, it is known that all treatments of B1, B2, and B3 negatively contained coliform bacteria. This was based on the fact that within 24 hours, there were no gas bubbles in the Durham tube, indicating that there was no bacteria presence.

If there is a positive reaction, a certain amount of gas will be formed in the Durham tube because carbon gas will give pressure to the part of the Durham tube in the closed reaction (Putri and Kurnia, 2018).

Some amount of bioactive have the characteristic of anti-bacteria produced by *Rhizopus* sp. as the result of being isolated from commercial tempeh. The mould is antagonistic to the enteric bacterial pathogens such as *Salmonella typhimurium*, *E. coli*, and *Shigella flexneri* (Virgianti, 2015).

Bacterial gram staining

Figure 9, shows that bacteria classified as positive gram. It is based on the results of Gram staining, which revealed bacteria that appeared purple in color. Positive bacterial gram has cell walls composed of a thick layer of peptidoglikan so that when crystal violet dropped on them, these bacterial cell walls will absorb the purple color and do not fade when alcohol is given (Suarjana et al., 2017). The red color in cell walls will be shown by negative bacterial gram such as *E. Coli* (Rahayu and Muhammad, 2017). Moringa seeds can reduce the amounts of coliform bacteria by up to 99.9% (Al-Jadabi et al., 2023). Moringa seeds have inhibitory power against the growth of *Enterobacter aerogenes*, *Staphyloccusaureus*, *Shigella* spp., *E. Coli*, and *Salmonella bacteria* (Saudale and Early, 2018). Besides, *Moringa oleifera* were proven to have antibacterial action against harmful bacteria including *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* (El-Sherby et al., 2024).

Wigunarti et al. (2019) evidenced that brown, old, dry Moringa seeds were able to inhibit bacteria *E. Coli* and *Staphylococcus aureus*. This is because Moringa seeds contain pterygospermin, moringine, 4 α L- amnosyloxy benzyl isothiocynate, ben oil, as well as flocculants in the cotyledons of Moringa seeds. These substances have antimicrobial properties, which can kill and inhibit the growth and activity of several positive and negative gram bacteria (Sundale & Early, 2018).

Materials and methods

Equipment used

A hot plate, aluminum foil, mortar, pestle, and plastic sieve. Tools for moisture and ash content analysis include an analytical balance with 0.1 mg accuracy, a temperature-controlled oven, porcelain crucibles, a muffle furnace, and a desiccator. Tools for fat and fibre analysis include filter paper, cotton, Soxhlet apparatus, a boiling flask (200 ml), Erlenmeyer flasks, a rotary vacuum evaporator, a vacuum pump, and a Buchner funnel. Tools for protein analysis include a UV-Vis spectrophotometer, stirrer, glass funnel, and test tubes. Microbiological analysis tools include an inoculation loop, Petri dishes, micropipettes, pipette tips, various glassware, graduated cylinders, volumetric flasks, dropper pipettes, Durham tubes, an autoclave, and volumetric pipettes.

Plant materials

Fresh seeds of *Moringa oleifera* (horseradish) tree were harvested from different levels of maturity, with the following standards: Mature Moringa seeds have pale green skin, large seeds, and having triangular shape with papery wings (Figure 10). Medium ripe seeds are green with large, white seeds (Figure 11). Young seeds are dark green, and the seeds are small and white (Figure 12). Soybeans were obtained from a traditional market. Additional materials used for the preparation of tempeh, as well as chemical and microbiological analyses, included: yeast (starter culture), glutinous rice, distilled water, and banana leaves. The chemicals used include nutrient (NA) of sea-wed jelly powder, distilled water, safranin, logol, H_2SO_4 (1.25%), HCl (0.02 N), NaOH (1 M), NaOH (3.25%), and ethanol.

The experiment

This study was an experiment involving one element: the variation of Moringa seed maturity level (B0 = soybeans [control], B1 = mature/old seeds, B2 = medium seeds, B3 = young seeds). Each treatment was repeated four times, resulting in 16 experimental units. The experimental design used was a Completely Randomized Design (CRD). Observed parameters include moisture content, ash content, fat, protein, carbohydrates, crude fibre, the microscopic characteristics of tempeh fungi, and suspected coliform bacterial testing.

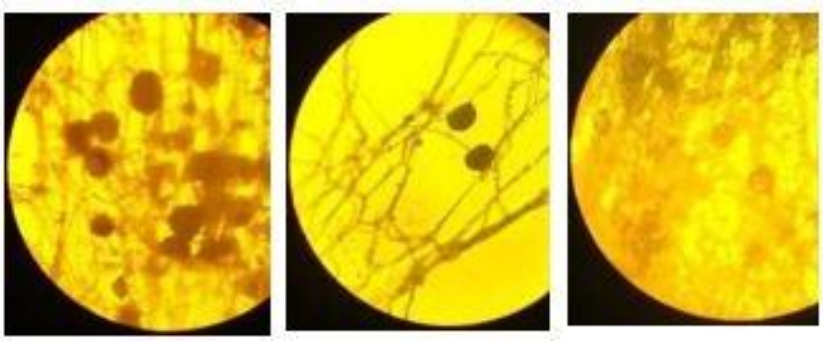

Figure 7. Sporangium Rhizopus sp. After 72 hours in old Moringa seed tempeh (B1), medium Moringa seed tempeh (B2), young Moringa seed tempeh (B3). Magnification 10 x10.

Preparation of yeast (starter culture)

The tempeh starter culture preparation was based on the method of Nurrohmah (2013) by modifying the work steps. In Nurrohmah's method, the rice was ground at the beginning, whereas in this research, the rice was ground at the final stage. The first step was to prepare glutinous rice, which was then screened to select good quality grains and remove impurities. The rice was washed under running water and soaked for one hour to soften. After soaking, the rice was drained using a plastic sieve to separate the water, waste, and rice. The drained rice was then steamed to achieve a soft texture and drained. The next step involved preparing a banana leaf-lined tray to hold the steamed rice, which was left to air dry for 24 hours. Once the rice was dry, it was sprinkled with the starter culture and mixed well using a wooden spoon. It was then wrapped in aluminum foil and inoculated with fungi for 24 hours. After that, the rice was roasted in an aluminum container on a hot plate until it was brown and dry. Finally, it was cooled, ground into a fine powder using a mortar and pestle, and sieved through a 40-mesh sieve to obtain powdered starter culture.

Preparation of moringa seed tempeh

The preparation of Moringa seed tempeh adopted the modified method of Jubaidah et al. (2016), particularly in terms of soaking duration, which only took one hour in this research.

The first step was to sort Moringa seeds based on their maturity levels (old, medium, and young). The seeds were then peeled, washed, and soaked for one hour. After that, the seeds were boiled at medium heat (old seeds for 1 hour 30 minutes, medium seeds for 1 hour, and young seeds for 50 minutes). The seeds were drained and air-dried for one hour, after which 35 grams of each type of Moringa seed was weighed. The next step was to inoculate the seeds with 0.8 grams of starter culture, equivalent to 2% of the total seed weight, and mix well. The seeds were then wrapped in banana leaves. The final step was the fermentation process, which lasted three days (72 hours) at room temperature (25-30°C).

Microscopic characterization of tempeh fungi

The microscopic morphology of tempeh fungi was observed under a microscope with 100x magnification. Hyphae and spores were placed on a glass slide using an inoculation needle to avoid clumping. Then, the preparation was stained with methylene blue solution and covered with a cover slip. Observations were made on morphological structures such as rhizoids, stolons, reproductive structures (shape and colour of spores), and sporangiospores (spore-producing stalks), as cited in Rosidah (2023).

Coliform bacteria suspected test

Tempeh samples were diluted through five dilution series, namely 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. First, 5 g of Moringa tempeh was grind and put into the first test tube to be homogenized (dilution 10^{-1}).

Dilution 10^{-2} was done by taking 1 ml from the first test tube of 10^{-1} to be put into the second test tube containing 9 ml distilled water to be homogenized. Dilution 10^{-3} was done by taking 1 ml from the second test tube of 10^{-2} to be put into the third test tube containing 9 ml distilled water to be homogenized. Dilution 10^{-4} was done by taking 1 ml from the third test tube of 10^{-3} to be put into the fourth test tube containing 9 ml distilled water to be homogenized. Dilution 10^{-5} was done by taking 1 ml from the fourth test tube of 10^{-4} to be put into the fifth test tube containing 9 ml distilled water to be homogenized (Kartika et al., 2014).

The dilution process was proceeded to more 4 series, i.e., 10^{-6}, 10^{-7}, 10^{-8}, dan 10^{-9}. The dilution of 10^{-6} was done by taking 1 ml from the fifth test tube of 10^{-5} to be put into 10^{-6} test tube containing 9 ml of Lactose Broth. The dilution of 10^{-7} was done by taking 1 ml from test tube of 10^{-6} to be put into 10^{-7} test tube containing 9 ml of Lactose Broth with an inverted Durham tube. The dilution of 10^{-8} was done by taking 1 ml from test tube of 10^{-7} to be put into 10^{-8} test tube containing 9 ml of Lactose Broth with an inverted Durham tube. And the last, the dilution of 10^{-9} was done by taking 1 ml from test tube of 10^{-8} to be put into 10^{-9} test tube containing 9 ml of Lactose Broth with an inverted Durham tube. All these tubes were incubated at 37°C for 24 hours. After that, the number of tubes that formed gas was recorded (Kartika et al., 2014).

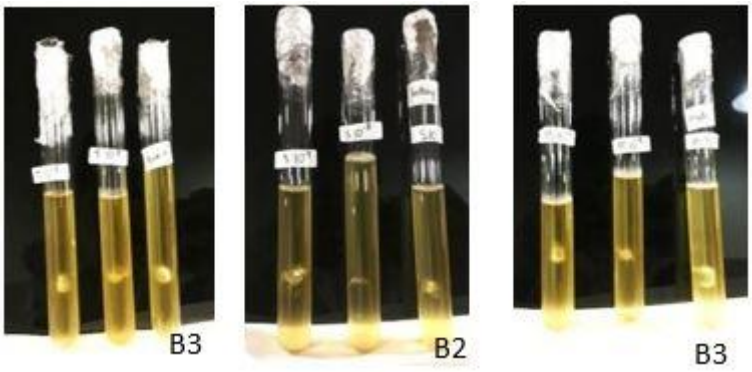

Figure 8. Negative *coliform* bacteria in old Moringa seed tempeh (B1), medium Moringa seed tempeh (B2), young Moringa seed tempeh (B3).

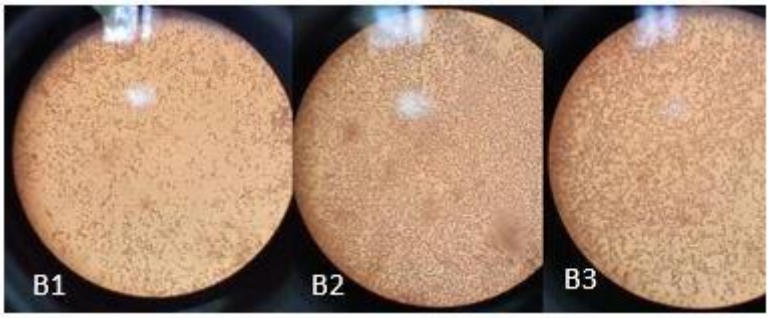

Figure 9. Bacterial gram staining to old Moringa seed tempeh (B1), medium Moringa seed tempeh (B2), and young Moringa seed tempeh (B3).

Selective mediums were used to isolate bacteria to find out their characteristics. Each sample in 10^{-7}, 10^{-8}, and 10^{-9} tube was taken 1 ml to be inoculated on nutrient of seaweed-jelly and then followed by streaking on the seaweed-jelly MacConkey. The sample was smeared using a loop the smear was carried out using the quadrant streak method B and incubated at 37^{o}C for 24 hours with the position of upside-down cup. If the result was positive the colour would be greyish or colourless with diameter between 1 – 2 mm (Wasita and I Made, 2016).

Bacteria gram staining

Gram staining was conducted on inoculated sample in MacConkey sea-weed jelly media with the purpose to identify gram and form of grown bacteria. Using a sterile tube, a few colony was taken, and then a thin layer was made over the sterile glass object that had previously been dripped with distilled water, and then waited until it dried. Fixation was carried out by touching the bottom surface of the glass object three times in a row to the surface of the Bunsan flame, so that bacterial smears were not washed away when the staining was carried out. Next, a crystal violet solution was given and waited around 20 – 60 seconds and then washed with running water in direction almost parallel to the water flow to reduce bacterial loss due to washing. Then, a solution of Lugol iodine was given and left for one minute. This was useful as a mordant to increase the affinity of the cells for the staining process, after that it was washed in running water. The next step was decolorizing by 95% alcohol and should not be too much before being washed in running water. After that, safranin was given for 45 seconds, was washed in running water and dried. Under a microscope, it was observed and resulted in pink rods (negative gram rods) (Wasita and I Made, 2016).

Conclusion

The experiment showed that *Rhizopus* sp. Fungus can ferment at all levels of maturity of moringa seeds. The older the Moringa seeds caused faster *Rhizopus* sp. development and better inhibition of the bacterial growth. This inhibition was also supported by Moringa seeds which also contained iodate as a bacterial inhibitor.

The maturity levels of Moringa seeds had a significant effect on the chemical properties of Moringa seeds tempeh. The water and fibre content in Moringa seed tempeh decreased as the Moringa seeds were getting older. Likewise, the level of ash, protein, and carbohydrates also increased as the Moringa seed used were getting older. The highest fat content was found in old Moringa seed tempeh and the lowest one was in medium Moringa seed tempeh.

Authors' contributions:

RDR: conceived and designed the research; TAI and AM: collected and analysed the data; MNS: performed the analysis results and supplied the supporting theories/references; AN: wrote the research article/paper

References

Adegbe AA, Larayetan RA, Omojuwa TJ (2016) Proximate analysis, physicochemical properties and chemical constituents characterization of *Moringa oleifera* (Moringaceae) seed oil using GC-MS analysis. American Journal of Chemistry. 6(2): 23-28.

Al-Jadabi N, Laaouan M, El Hajjaji S, Mabrouju J, Benbouzid M, Dhiba D (2023) The dual performance of *Moringa oleifera* seed-as eco-friendly natural coagulant and as an antimicrobial for wastewater treatment: A review. Sustainability. 15(5): 4280.

Aoki H, Nakatsuka-Mori T, Ueno Y, Nabeshima Y (2023) Analysis of functional ingredients of tempe-like fermented *Moringa oleifera* seeds (*Moringa tempe*) prepared with Rhizopus species. Journal of Bioscience and Bioengineering. 135:4.

Arandini NPCA, Singapurwa NMAS, Semariyani AAM (2022) Preservation of tofu with liquid smoke from coconut shell. SEAS (Sustainable Environment Agricultural Science). 6(2): 102 – 111.

Ardiani I, A'yun Q, Nazua KS (2024) Variation in Rhizopus species that play a role in making tempeh in the Bekasi area. BIOSEL (Biology Science and Education). 13(1): 10-18.

Auliana R (2017) Pembuatan tempe bekatul dan kandungan gizinya. HEJ (Home Economics Journal). 1(2): 47-58.

Benkerroum N (2020) Aflatoxins: Producing-molds, structure, health issues and incidence in Southeast Asian and Sub-Sharan African Countries. Int J Environ Res Public Health. 17(4): 1215.

Cao J, Shi T, Wang H, Zhu F, Wang J, Wang Y, Cao F, Su E (2023) *Moringa oleifera* leaf protein: Extraction, characteristics and applications. Journal of Food Composition and Analysis. 119: 105234.

Cempaka L, Eliza N, Ardiansyah A, Handoko DD, Astuti RM (2018) Proximate composition, total phenolic content, and sensory analysis of rice bran tempeh. Makara Journal of Science. 22(2): 89-94.

Damanik RNS, Pratiwi DYW, Widyastuti N, Rustanti N (2018) Nutritional composition changes during tempeh gembus processing. In IOP Conference Series: Earth and Environmental Science. 116(1): 12 – 26.

El-Sherby GM, Alluqmani AJ, Elsehemy IA, Kalaba, MH (2024) Antibacterial, antioxidant, cytotoxicity, and phytochemical screening of moringa oleifera leaves. Scientific Reports. 14:1.

Falinrungi A, Rostiati R, Gatot SH (2019) Karakteristik mutu fisikokimia dan organoleptik greenies cake daun kelor (Moringa oleifera L.). e-J. Agrotekbis. 7 (4):11-18.

Fasulo MMS (2025) How fibre supports overall health and lowers risk of colorectal cancer. In: https://www.uclahealth.org, February 27, 2025.

Gautier A, Duarte CM, Sousa I (2022) *Moringa oleifera* seeds: Characterization and potential uses as food. Foods. 11:162.

Gupta N, Chinnappa M, Singh PM, Kumar R, Sagar V (2022) Determination of the physio-biochemical changes occurring during seed development, maturation, and desiccation tolerance in *Moringa oleifera* Lam. South African Journal of Botany. 144: 430-436.

Hallsworth E (2022) What is preservative of microbes. Microbial Biotechnology. 15(1): 191-214.

Hartanti AT, Gayuh R, Iman H (2015) *Rhizopus* species from fresh tempeh collected from several regions in Indonesia. Hayati Journal of Biosciences. 22: 136-142.

Haron H, Raob, N (2014) Changes in macronutrient, total phenolic and anti- nutrient. Nutr Food Sci. 4: 265.

Hernawati D, Vita M (2019) Variasi inokulum rhizopus sp. pada pembuatan tempe berbahan dasar kedelai dan bungkil kacang tanah. Bioma: Jurnal Biologi Makassar. 4(1): 58 – 67.

Jubaidah S, Nurhasnawati H, Wijaya H (2016) Penetapan kadar protein tempe jagung (*Zea mays* l.) dengan kombinasi kedelai (glycine max (l.) merill) secara spektrofotometri sinar tampak. J. Ilm. Manuntung. 2: 111–119.

Kashyap P, Kumar S, Riar CS, Jindal N, Baniwal P, Guiné RPF, Correia PMR, Mehra R, Kumar H (2022) Recent advances in drumstick (*Moringa oleifera*) leaves bioactive compounds: Composition, health benefits, bio-accessibility, and dietary applications. Antioxidants (Basel). 11(2):402.

Khasanah S, Nugroho M, Astuti FY (2025) Proximate analysis of donuts substituted with corn cob flour (*Zea mays*). G-Tech: Jurnal Teknologi Terapan. 9(1): 313-319.

Kusumah D, Kabuyama Y, Maeda I (2018) Promotion of fungal growth, antibacterial and antioxidative activities in tempe produced with soybeans thermally treated using steam pressure. Food Science and Technology Research. 24(3): 395-402.

Martín-Miguélez JM, Bross J, Prado D, MerinoE, Moré RP, Otero J, Aduriz AL, Delgado J (2024) Review: *Rhizopus* sp. beyond tempeh. An occidental approach to mold-based fermentations. International Journal of Gastronomy and Food Science. 39: 101090.

Mehra R, Kumar, H (2024) Recent advances in drumstick (*Moringa oleifera*) leaves bioactive compounds: Composition, health benefits, bio-accessibility, and dietary applications. Antioxidants (Basel). 11(2): 402.

Liu K, Mahmood K (2015) Nutrient composition and protein extractability of oat forage harvested at different maturity stages as compared to grain. Journal of Agricultural Science. 7(12): 288 – 296.

Mashamaite CV, Ramatsitsi MN, Manyevere A (2024) *Moringa oleifera* Lam: A versatile climate-smart plant for nutritional security and therapeutic usage in semi-arid regions. Journal of Agriculture and Food Research. 16, 101217: 1-12.

Novelina, Wati L, Hari PD, A'yun Q (2023) The effect of the addition of red kidney bean skin powder (*Phaseolus vulgaris* L.) on the physical, chemical, and organoleptic characteristics of red kidney bean tempeh. AJARCDE: Asian Journal of Applied Research for Community Development and Empowerment. 7(3): 1-7.

Novenra N (2019) Pengaruh tingkat kematangan biji dan jumlah inokulum terhadap kadar protein tempe berbahan dasar jengkol (*Pithecellobium jiringa* (jack) prain.) dan uji organoleptiknya. Doctoral Dissertation, Universitas Negeri Padang, Indonesia.
Özcan MM (2020) Moringa spp: Composition and bioactive properties. South African Journal of Botany. 29: 25 – 31.
Pareek A, Pant M, Gupta MM, Kashnia P, Ratan Y, Jain V, Pareek A, Chuturgoon AA (2023) *Moringa oleifera*: An updated comprehensive review of its pharmacological activities, ethnomedicinal, phytopharmaceutical formulation, clinical, phytochemical, and toxicological aspects. Int J Mol Sci. 24(3): 2098.
Pello FM, Ricky DR (2023) The extraction and nano particles production of Moringa leaf bioactive compounds and their identification. JNS: Journal of Nutrition Science. 4(1): 26-36.
Rahayu SA, Muhammad HG (2017) Uji cemaran air minum masyarakat sekitar margahayu raya bandung dengan identifikasi bakteri escherichia coli. Indonesian Journal of Pharmaceutical Science and Technology. 4 (2), 89 – 97.
Rahmatu R, Sangaji MN, Adehani, Rahmi, Alam N (2023) Inhibitory power of Moringa (*Moringa oleifera*) seed extract from various levels of maturity on Eschericia coli bacteria. In the 2nd International Interdisciplinary Conference on Environmental Sciences and Sustainable Developments 2022 Environment and Sustainable Development (IICESSD-ESD 2022). Atlantis Press.
Ramteke TR, Kuril L, Suryawanshi OP (2024) Quality evaluation of moringa (*Moringa oleifera*) seed and defatted cake. International Journal of Advanced Biochemistry Research. 8(6): 226-231.
Rizal S, Kustyawati ME, Suharyono AS, Suyarto VA (2022) Chanes of nutritional composition of tempeh during fermentation with the addition of saccharomyces cerevisiae. Bio Diversity. 23(3): 1553 – 1559.
Romulo A, Surya R (2021) Tempe: A traditional fermented food of Indonesia and its health benefits. International Journal of Gastronomy and Food Science. 26: 100413.
Rosidah R, Azizah AS, Megawati HP (2023) Fungal morphological analysis on leaf packaged tempeh and plastic packaged tempeh. Jurnal Pengabdian Masyarakat Biologi dan Sains. 2(1): 48 – 57.
Salma S (2020) Nutritional and functional properties of *Moringa oleifera*. Department of Biochemistry and Molecular Biology, Bangladesh Agriculture University, Bangladesh. www.journals.elsevier.com/metabolism-open.
Saadilah M. Rostiati R, Syahraeni K (2018) Karakteristik kimia tepung biji kelor (*Moringa oleifera* lam.) dari berbagai ketinggian tempat tumbuh. Agrotekbis. 6 (1): 100 – 108.
Sakinah N, Endang P, Nurheni SP (2019) Kandungan gizi dan mutu protein tepung biji kelor terfermentasi. Jurnal Teknologi dan Industri Pangan. 30 (2): 15 – 24.
Santoso BB, Parwata IGM, Jayaputra (2019) Keragaan hasil analisis proksimat biji kelor (M*oringa oleifera* lam.) pada berbagai teknik pengeringan biji. Jurnal Ilmiah Rekayasa Pertanian dan Biosistem. 7(2): 221-229.
Sari KP, Jamaluddin, Sukainah A (2016) Fortifikasi Tempe Berbahan Dasar Kedelai dan Biji Nangka. Jurnal Pendidikan Teknologi Pertanian. 2: 16-26.
Saudale F, Early B (2018) Aktivitas antibakteri ekstrak polar dan non polar biji kelor (*Moringa oleifera*) asal pulau Timor NTT. Jurnal Sains dan Teknologi. *7*(1): 564 – 572.
Simwaka JE, Chamba MVM, Huiming Z, Masamba KG, Luo Y (2017) Effect of fermentation on physicochemical and anti-nutrional factors of complementary foods from millet, sorghum, pumpkin, and amaranth seed flours. Int Food Res J. 24:1869-1879.
Suarjana IGKINKB, Hapsari M, Ketut T (2017) Modul isolasi dan identifikasi bakteri. Fakultas Kedokteran Hewan, Universitas Udayana, Indonesia.
Suhartanti PD, Handajani S, Nandariyah (2019) Physical characteristics of the seeds of soybean (*Glycine max*) varieties and the effect of fermentation time on the chemical characteristics of tempeh. Cell Biology & Development. 3(1): 13-18.
Sumarjan B, Sumarjan (2017) Viabilitas biji kelor (*Moringa oleifera* lam) dan pertumbuhan bibit pada berbagai tingkat kematangan buah. Crop Agro. 10(1):7789-7797.
Thakur P, Kumar K, Ahmed N, Yadav AN, Kumar S, Rizvil QUEH, Chauhan D, Jan S (2022) Impact of diverse processing treatments on nutritional and anti-nutritional characteristics of soybean (*Glycine max* L.). Journal of Applied Biology & Biotechnology. 10(3): 97 – 205.
Virgianti DP (2015) Uji antagonis jamur tempe (rhizopus sp) terhadap bakteri patogen enterik. Biosfera. 32 (3): 88 – 95.
Wadji SA, Sri K, Susanti, PH (2017) Uji aktivitas antibakteri campuran ekstrak biji kelor (*Moringa oleifera*) dan daun kersen (muntingia calabura) terhadap pseudomonas aeruginosa dan Bacillus subtilis. J. Trop. Biodiv. Biotech. 2: 10-15.
Wasita IKS, I Made AH (2016) Identifikasi bakteri escherichia coli serotipe 0157 dengan media sorbitol Mac Conkey Agar (SMAC) pada jamu beras kencur dari pedagang jam gendong di Kota Denpasar. E-Jurnal Medika. 5 (11): 13-18.