

## Identification of lactic acid bacteria and quality parameter of Tempeh obtained from red kidney beans (*Phaseolus vulgaris*) and cowpeas (*Vigna unguiculata*)

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**ABSTRACT.** Tempeh fermentation generally uses soybeans as a substrate. However, the availability of soybeans in Indonesia, especially in the province of East Nusa Tenggara, is inadequate, so alternative fermentation substrates are important to consider. Red beans and cowpeas grow abundantly in East Nusa Tenggara. Therefore, researchers are interested in conducting research using local beans as a substrate for tempeh fermentation. This study aims to utilize red beans and cowpeas as substrates for tempeh fermentation with proximate analysis and isolate and identify lactic acid bacteria from bean soaking water, considering the important role of LAB in LAB the tempeh fermentation process. Red beans and cowpeas were employed, MRSA and MRSB media, test media, commercial tempeh starter, and LAB. Before the study started, traditional tempeh was prepared using a commercial starter (*Rhizopus* sp.). Red peanuts and cowpeas tempeh were firstly tested using the AOAC method, secondly isolated using the spread plate method, then purified and lastly characterized and molecularly identified using PCR method and DNA sequence analysis to obtain a phylogeny tree. Based on the proximate analysis results for water content, cowpea seeds increased during soaking to fermentation, which was 6.81% to 17.41%. For the dry matter of the two samples, red beans, 7.16% to 22.22%, experienced a decrease in cowpea from 93.19% to 82.59%. Kidney beans 92.84% to 77.78%. The ash content of cowpea is 4.66% to 4.16%. red beans 4.44% to 2.41%, cowpea crude fiber 6.04% to 4.67%, red beans 5.32% to 3.75%. cowpea carbohydrates 57.049% to 40.389%, red beans 54.364% 38.474%. The protein content of cowpeas was 30.870% to 36.210% in tempeh, red beans 33.173% to 36.062% in tempeh. Cyanide levels in cowpeas were 0.043% to 0.010%, red beans were 0.054% to 0.011% in tempeh. The lactic acid bacteria that were successfully isolated and identified were bacteria with isolate code KM2 related to *Enterococcus faecium* and KN2 isolate *Lactiplantibacillus plantarum*, lactic acid bacteria function to lower the pH in the soaking water and prepare a suitable pH for the growth of *Rhizopus* mold.

**Keywords:** crude fat content; molecular identification; phylogenetic tree; proximate analysis; Tempeh fermentation

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## INTRODUCTION

Tempeh is a good source of nutrition because it contains protein, carbohydrates, essential fatty acids, vitamins, and minerals and is a source of vegetable protein. Tempeh contains various nutrients needed by the body, such as protein, fat, carbohydrates, and minerals (Winarno *et al.*, 2021). Several studies have shown that tempeh is easier to digest than soybeans. Fermented tempeh is a product that has a better shape, texture and taste and is more easily absorbed and utilized by the body (Galand *et al.*, 2021). The moulds that grow on soybeans hydrolyze complex compounds into simple compounds that humans easily digest.

Tempeh which the Indonesian people commonly known, uses soybean (*Glycine max*) as raw material. Tempeh contains unique biochemical properties, including particular amino acids (isoleucine, leucine, lysine, tryptophan and methionine). Tempeh is becoming more widely consumed on a global scale. Tempeh can also be made from various legumes (Radita *et al.*, 2017). Soy tempeh is fermented in a controlled manner using a mould *Rhizopus oligosporus*. Apart from the mould *R. oligosporus*, other microorganisms are also known to participate in the tempeh fermentation process (Radita *et al.*, 2017). Tempeh fermentation consists of two stages, including lactic acid fermentation, which occurs naturally during the soaking process by lactic acid microbiota and *Rhizopus* fungal fermentation (Radita *et al.*, 2017).

Ethanol, hydroperoxide, bacteriocin, and lactic acid are the active metabolites generated by lactic acid bacteria. The metabolites formed are agents capable of killing microorganisms. Bacteriocin is used as an antibacterial. Bacteriocins, it was revealed, are critical in conquering infections caused by germs. Additionally, the lactic acid produced by LAB has the potential to reduce the pH of the surrounding environment. Low pH levels can help prevent spoilage bacterium contamination and eliminate harmful germs, particularly those found in the body (Sine & Soetarto, 2018).

This study aims to identify molecularly lactic acid bacteria from red bean tempeh and cowpea and determine the quality of tempe cowpea through a complete proximate analysis. The quality of fermented tempeh is a determining factor in whether fermented tempeh is feasible or not for consumption. Nutritional quality is an essential aspect of food ingredients; various food processing techniques result in changes in the content of an ingredient. Fermentation is one way to process food using microbes. Fermentation is a process of energy production in cells under anaerobic conditions. In fermentation, there are physical and chemical changes in the fermentation substrate. *Rhizopus* mould, which plays an essential role in tempe fermentation, can produce protease enzymes, especially *Rhizopus oligosporus*. The reshuffle of protein complex compounds into simpler compounds is essential in tempe fermentation. It is one of the main factors determining tempe quality, namely as a source of vegetable protein. has a high digestibility value (Setiawati *et al.*, 2017). The protein content expressed as total nitrogen content did not change during fermentation. Changes occurred in the levels of soluble protein and free amino acid levels.

Cowpea (*Vigna unguiculata* (L.) Walp) and Red Bean (*Phaseolus vulgaris* L.). Widely found in East Nusa Tenggara, especially on the island of Timor, these two nuts are pretty abundant. Every 100 g of old cowpea seeds contains 18-25% protein, 1.4 g of fat, 50-60% carbohydrates, 6.8 g of fibre, and 5 g of ash (Stancheva *et al.*, 2016; Jayathilake *et al.*, 2018). This type of bean is drought tolerant, so it is generally planted on dry land in the dry season or on paddy fields after rice. In 100 g, red beans contain 22.7% protein, 3.5% mineral, 1% fat and 57.7% carbohydrates, out of which total carbohydrates have 38.6 % starch and 18.8% dietary fibre, 60% insoluble and 40% soluble. Its protein has the highest lysine content of about 5% (Shehzad *et al.*, 2015). Nuts generally contain protein, fat, carbohydrates, crude fibre, and ash content and contain trypsin inhibitor compounds and cyanide. Bavia *et al.* (2012) stated that during fermentation, *R. oligosporus* was also able to reduce trypsin inhibitor activity in peanuts. Therefore, it can be said that *R. oligosporus* was the best mould used in fermenting tempeh with peanuts as raw material.

In addition to having nutritional content, nuts generally contain cyanide (HCN) compounds. Cyanogenic glycosides are potentially toxic because they can decompose and release hydrogen cyanide (HCN). HCN with a CN- molecular structure can be released when a material is crushed, chewed, sliced, or damaged. When the digestive tract very quickly absorbs ingested HCN into the bloodstream (Bolarinwa *et al.*, 2016). HCN is a toxic compound that is naturally present in nuts. If consumed directly, it will cause harm to those who consume it (Wahono, 2016). HCN in food raw materials itself is a type of cyanide that is easily soluble in water, so if it is given treatment in the form of washing, soaking and boiling, it will reduce the HCN level.

## MATERIALS AND METHODS

The study took place during the period August to October 2020. The research included the manufacture of cowpea and red bean tempeh, proximate analysis (AOAC, 2005) and analysis of cyanide levels (AOAC, 2005) in the sample. Isolation and molecular identification by PCR method.

**Tempeh making process.** Preparation of Inoculum. The inoculum used for making cowpea and red bean tempeh was usar or pure culture of *Rhizopus oligosporus* (Raprima brand), cowpea, and red bean prepared as a fermentation substrate 500 g, placed on woven bamboo. Then soaked for 24 h, then peeled the skin of the beans, then boiled for 1 h. About 500 g of cowpeas and boiled red beans inoculated with 1% (w/w) (5 g) of usar or pure culture of *Rhizopus oligosporus*, levelled with stirring. Furthermore, cowpeas and red beans have been inoculated, each treatment wrapped in perforated

plastic. All packets of tempeh will incubate for 24-48 hours at room temperature (Alvina & Hamdani, 2019).

**Determination of water content (AOAC, 2005).** Weigh the sample powder or material mashed as much as 1-2 grams into a clean and dry weighing bottle to know its weight. Dry in the oven at a temperature of 100°C-105°C for a specific time, depending on the type of material. The greater the water content in a food, the longer the heating time required. Reheat in the oven for 30 minutes, relax in a desiccator and weigh; This treatment was repeated until a constant weight was reached (the difference in weighing was less than 0.2 grams, respectively). Weight reduction is the amount of water in the material.

$$\% \text{ ka} = \frac{(c+s)' - (c+s)''}{(c+s)' - c} \times 100\%$$

Notes:

(c + s)': cup weight and initial sample

(c + s)'': cup weight and final sample (AOAC, 2005).

**Determination of ash content by dry method (AOAC, 2005).** Glow a porcelain crucible with a lid in a muffle furnace. Cool in the oven, then place in the desiccator until cold. Only then weighed. Weigh the sample in a porcelain crucible of known weight (approximately 2 grams), then heat it on an electric stove so that the material becomes charcoal. Then incandescent in a muffle at 600°C for 6 h until it becomes whitish ash, allow the muffle to show room temperature, then open the lid. Then the crucible is put in a desiccator until it cools down, and then it is weighed (AOAC, 2005).

$$\% \text{ wb (wet basis)} = \frac{\text{ash weight (grams)}}{\text{sample weight (grams)}} \times 100\%$$

$$\% \text{ db (dry basis)} = \frac{\% \text{wb}}{1 - \text{water content}} \times 100\%$$

**Determination of carbohydrate content by the carbohydrate method by difference (Winarno, 2002).** Carbohydrate content by difference is used for food composition tables or proximate analysis. Usually, carbohydrate analysis (KH) is not carried out; total carbohydrates by difference are obtained by subtracting the number 100 with the presentation of other components such as water, ash, fat, and protein. The following formula calculates it:

$$\text{Total carbohydrates} = 100 - (\% \text{ water} + \% \text{ ash} + \% \text{ protein} + \% \text{ fat}) \text{ (Winarno, 2002).}$$

The KH level calculated as above (not analyzed separately) is called 'carbohydrate by difference'. of course, the level of accuracy of the data is not as high as when compared to a complete analysis of all major components. However, in certain cases the 'carbohydrate by difference' data is sufficient and acceptable.

**Determination of crude protein (AOAC, 2005).** Weigh the sample by approximately 0.3 g, then add approximately 1.5 grams of selenium mixture catalyst. Put the weighed sample into a Kjeldahl flask, then add 20 ml of concentrated H<sub>2</sub>SO<sub>4</sub>. Digest until the colour of the solution becomes yellowish-green – transparent, then refrigerate for about 15 minutes. Add 300 ml of distilled water, then cool again. Add 100 ml of 40% NaOH (technical), then do the distillation. Collect the distillation results with 10 ml of 0.1 N H<sub>2</sub>SO<sub>4</sub> to which three drops of mixed indicator, namely Methylene Blue and Methylene Red, have been added. Titrate with 0.1 N NaOH until the colour changes from purple to blue-green. Determine the blank determination: pipette 10 ml H<sub>2</sub>SO<sub>4</sub> 0.1 N and add two drops of PP indicator, titrate with 0.1 N NaOH (6.25 is the protein-nitrogen conversion factor in the protein calculation formula).

$$\% \text{ Crude Protein} = \frac{\text{ml blank} - \text{ml sample} \times \text{N NaOH} \times 6,25}{\text{Sample weight (mg)}} \times 100\% \text{ (AOAC, 2005)}$$

**Determination of crude fat content (AOAC, 2005).** Take a fat flask whose size corresponds to the Soxhlet extraction tool, dry it in an oven, cool it in a desiccator and weigh it. Weigh 5 g of cowpea and red bean samples (before treatment, soaked, boiled and tempeh beans). In flour directly in a lead sieve, according to size, then cover with fat-free cotton wool. Place the sample's filter paper in the Soxhlet extraction apparatus, then place the condenser above it and the fat flask under it. Pour the petroleum ether solvent into the fat flask sufficiently, according to the size of the soxhlet used. Reflux for a minimum of 5 hours until the solvent that drops back into the fat flask is clear. Distil the solvent in the fat flask, and collect the solvent.

Furthermore, the fat flask containing the extracted fat was heated in an oven at 105°C. After drying to a constant weight and cooled in a desiccator, weigh the pumpkin and its fat. Fat weight can be calculated:

$$\% \text{ Fat} = \frac{\text{Fat weight (g)}}{\text{Sample weight}} \times 100 \text{ (AOAC, 2005)}$$

**Determination of coarse fibre content (AOAC, 2005).** The sample is weighed as much as approximately 1 gram (x) and put into the Heather extract tool. Add 50 ml H<sub>2</sub>SO<sub>4</sub> 0.3N, boil for 30 minutes. Add 25 ml of 1.5 N NaOH, and boil for 30 minutes. Prepare filter paper that has been heated in an oven at 105°C for 1 hour and then weigh (a). Filter the liquid using filter paper using a Buchner funnel. Filtering is done with a suction flask connected to a vacuum pump. Wash successively using 50 ml of hot water, 50 ml of 0.3 N H<sub>2</sub>SO<sub>4</sub>, 50 ml of hot water and 25 ml of acetone. Put the filter paper and its contents into a porcelain cup. Dry in an oven at 105 °C for 1 hour. Remove, calm in a desiccator and weigh (Y). Put the cup back into the furnace (400 - 6000C). Remove, cool and weigh (Z). The formula can calculate the weight of crude fibre:

$$\% \text{ Coarse Fiber} = \frac{Y-Z-a}{x} \times 100 \text{ (AOAC, 2005)}$$

**Quantitative HCN Determination (AOAC, 2005).** Weigh 10-20 g of the finely ground sample (20 mesh), add 100 ml of distilled water in a Kjeldahl flask and macerate for 2 hours. Then add another 100g of distilled water and steam distillation. The distillate is accommodated in an Erlenmeyer that has been filled with 20 ml 0,02 N AgNO<sub>3</sub> and 1 ml HNO<sub>1</sub>. After the distillate reached 150 ml, the distillate was stopped. The distillate was then filtered with Krus Gooch. The precipitate that may exist is washed off with water. The excess AgNO<sub>1</sub> in the distillate was titrated with K-thiocyanate using a ferric indicator.

**Isolation of LAB from the red bean and cowpea soaking water.** A total of 1 ml of the sample was aseptically added to 9 mL of sterile peptone water (0.1%, w/v) and homogenized. Then, a multilevel dilution was carried out until the seventh dilution. 0.1 mL samples were taken from the last three dilution series, and cultured on MRS Agar supplemented with 1% CaCO<sub>3</sub> using the spread plate method. They were incubated for 48 hours in an incubator at 37°C (Widodo, 2017).

**BAL purification.** Colonies that grew with a clear zone around them were purified using streaking on 1% CaCO<sub>3</sub> supplemented MRSA media with the quadrant method and incubated for 24 hours in an incubator at 37°C in order to obtain a single colony for further subculture as a single pure isolate (Widodo, 2017).

**Characterization of lactic acid bacterial isolates.** The characterization of isolates included morphology, physiology, and biochemistry. Morphological characterization consisted of Gram staining, endospore staining, and motility tests, while physiological and biochemical characterization consisted of catalase, temperature, and salt tests (Widodo, 2017).

**DNA amplification of 16S rRNA encoding with PCR (Polymerase Chain Reaction).** The DNA sample amplification reaction was carried out in a 0.2 ml PCR tube. In each PCR test tube, the following were added: 0.25 µl of RBC Taq (5 unit/ml), five µl of 10 x buffer Taq (containing Mg<sup>2+</sup>), four µl of dNTP 2.5mM, as much as 1.25 µl (20 pmol) dan 1.25 µl (20 pmol) each of universal primer

63F (5' -CAGGCCTAACACATGCAAGTC-3') dan universal primer 1387R (5' -GGGCGWGTGTACAAGGC -3'), 2.5 µl (100 ng) of genome extract and ddH<sub>2</sub>O until the volume becomes 50 µl. PCR amplification was carried out using a PCR PTC 100 (MJ Research, Inc) at the initial denaturation temperature of 95°C for 5 minutes, followed by 30 cycles of primer attachment at 94°C for 30 seconds and extension at 50°C for 1 minute, 72°C for 2 minutes, and the final step of 72°C for 5 minutes. The PCR product was taken and stored at 4°C for further examination using 1% agarose electrophoresis in TAE 1x, 100 V for 30 minutes (Widodo, 2017).

**DNA sequence analysis of 16S rRNA encoders.** DNA sequencing encoding 16S rRNA was carried out by 1st Base through PT. Indonesian Science Genetics. The analysis of the sequencing results was carried out by BLASTing the nucleotide sequences from the 16S rRNA sequencing results with the database available on the website [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov). Multiple alignments were performed using the Clustal W program. Furthermore, the visualization of kinship using a combination phylogenetic tree Program TREEVIEW X with a neighbour-joining plot (Thompson *et al.*, 1995).

## RESULTS AND DISCUSSION

**Proximate analysis.** Proximate Analysis Results (pre-treatment, treatment and fermentation results).

**Table 1.** Complete proximate analysis.

Sample	Water content (%)	Dry materials (%)	Ash (%)	Crude Fat (%)	Coarse Fiber (%)	Carbohydrate (%)	Protein (%)
Cowpeas	6.81	93.19	4.66	0.611	6.04	57.049	30.870
Soaked Cowpeas	7.32	92.68	3.96	1.016	6.25	53.446	34.258
Boiled Cowpeas	4.84	95.16	3.62	1.630	1.79	57.525	32.385
Cowpeas Tempe	17.41	82.59	4.16	1.831	4.67	40.389	36.210
Red Beans	7.16	92.84	4.44	0.860	5.32	54.367	33.173
Soaked Red Beans	8.81	91.19	4.24	0.819	5.45	50.621	35.510
Boiled Red Beans	4.78	95.22	2.82	1.309	2.85	56.255	34.836
Red Beans Tempe	22.22	77.78	2.41	0.834	3.75	38.474	36.062

Proximate analysis showed that the results varied in water content, ash content, crude fat, crude fibre, carbohydrates and protein in each treatment. Cowpea (*V. unguiculata* (L.) Walp) had the highest protein content after being fermented into tempeh than cowpea before treatment. There was an increase at the time of soaking, namely 30.870% to 34.258%; after being boiled, the protein content in cowpeas decreased to 32.385%, and increased again when fermented to 36.210%, the total increase in protein in control up to tempeh was an increase in protein by 5.34%. Controlled red bean protein content was 33.173%, soaked at 35.510%, decreased when boiled at 34.836% and increased again when fermented into tempeh, namely 36.062%; the total protein increase in red beans to tempeh was protein, which increased by 2.89%. Protein levels decrease during boiling because food processing at high temperatures will cause protein denaturation so that coagulation occurs and decreases its solubility (Diniyah, 2015). During tempeh fermentation, the protein content of the mould that grows on the tempeh produces enzymes that break down complex compounds. *Rhizopus oligosporus* produces protease enzymes. The reshuffling of protein complex compounds into simpler compounds, namely amino acids, is essential in tempeh fermentation and is one of the main factors determining the quality of tempeh, namely as a source of vegetable protein that has a high digestibility value because it is easier to be absorbed and utilized by the body directly. (Manini *et al.*, 2016; Sine & Soetarto, 2018). During the fermentation process, several proteins are used by the mould *Rhizopus oligosporus* as a nitrogen source for its growth (Sayudi *et al.*, 2015). In the manufacture of tempeh, the part that is wasted is the skin; the skin is not the part where the protein is stored but the polysaccharide, which is not soluble in water. The boiling process mainly causes protein loss in the manufacture of tempeh, and the tempeh moulds ferment using nitrogen from the protein substrate for

the life of the mould (Winarno *et al.*, 2021). Protein levels in red beans have changed as well as cowpeas. Legumes contain relatively protein; in the tempeh fermentation process, the protein content will change due to the treatment carried out during the fermentation process.

The tempeh fermentation process involves three supporting factors, namely raw materials or substrates that support tempeh moulds and environmental factors that affect mould growth (temperature, pH, and water content). Oxygen is needed for mould growth. Therefore plastic bags used as wrappers are given small holes with a distance between one hole and another about 2 cm. Excess water vapour will inhibit the growth of mould. Based on the results of measurements of water content in this study, it was shown that the water content of cowpea and red bean tempeh was higher than that of cowpea and red bean seeds (table 2), water content increased, soaking was 6.81% to 7.32%, boiling experienced 4.84% and 17.41 % fermentation. Red beans with water content in the control 7.16%, soaking 8.81%, boiling decreased to 4.78% and increased when fermented to 22.22%, in cowpea the water content increased from control to fermentation by 10.6%, red beans water content increased by 15.06% of the control until it becomes tempeh, this is because during the fermentation there is a release of moisture by the mould as a result of the decomposition of complex compounds that are blocked by the plastic packaging. As the fermentation time increases, the macromolecular reshuffle becomes more intensive so that the water content of tempeh increases. According to Sine and Soetarto (2018), during tempeh fermentation, water is produced due to the breakdown of carbohydrates by microorganisms; water is one of the products of aerobic fermentation. During tempeh fermentation, microorganisms digest the substrate and produce water, carbon dioxide, and energy. In addition, the water content of cowpea seeds before fermentation occurs also affects the growth of moulds. Water also acts as a reactant. In this case, the mould only uses the free water on the substrate.

The results of the dry matter measurement of the two samples decreased, namely the control of cowpea at 93.19%, soaking to 92.68%, boiling increased to 95.16%, while during fermentation decreased to 82.59% in cowpea tempeh, the total change from control to tempeh was 10.6%. Red beans soaked 92.84% to 91.19%, boiling increased to 95.22%, while fermentation decreased to 77.78% in red bean tempeh, the total change from control to tempeh was 15.06%.

The calculation of total carbohydrates in cowpea control were 57.049%, soaked 53.446 %, boiled 57.525%, after fermented into tempeh 40,389%, the total decrease in carbohydrates control cowpeas to tempeh was 16.66%. The control of red bean total carbohydrates was 54.367%, soaking 50.621%, boiling 56.255%, and 38.474% in red bean tempeh and the total decrease in carbohydrates in red bean control tempeh was 15.89%. The carbohydrate content in both samples of peanuts before treatment was greater than that of tempeh; this was due to the breakdown of carbohydrates into simpler monomers. In this case, the amylase enzyme produced by moulds plays a role in converting carbohydrates into monosaccharides. In addition, moulds also use carbohydrates as a source of energy for *R. oligosporus* that produces carbohydrates enzymes (polygalacturonase, endocellulase, xylanase, arabinanase and to a lesser extent  $\alpha$ -D-galactosidase,  $\beta$ -B-galactosidase,  $\beta$ -D-xylosidase,  $\alpha$ -L-arabinofuranosidase and  $\alpha$ -D-glucosidase), lipase, protease dan fitas (Rizal *et al.*, 2020).

In this study, the ash content in cowpea and red bean tempeh decreased compared to the ash content in the seeds of the two beans, namely in control cowpea 4.66%, when soaked decreased to 3.96%, boiled to 3.62% and tempeh 4.16%. The ash content of cowpea decreased by 0.5% from control to tempeh. The analysis results on red bean control were 4.44%, soaking 4.24%, boiling 2.82% and 2.41% in red bean tempeh, and the total decrease in ash content in red bean to tempeh was 2.03%. This can be caused by hydrolysis during soaking; Agume *et al.* (2017) stated that the minerals contained in cowpea seeds are dissolved in water during soaking. Besides, boiling also affects the decrease in mineral content. The amount of ash content is related to the minerals in a material (Astawan *et al.*, 2013). Suppose the ash content in the material decreases. In that case, the mineral content of the material will also decrease so that the high ash content in cowpea and red bean seeds

shows that the mineral content in cowpea seeds is higher than the mineral content in the tempeh of the two beans.

The results of the crude fat test on cowpea control were 0.611%, 1.016% soaking, and 1.630% boiling; after being fermented into tempeh, crude fat increased to 1.831%. The total increase in crude fat content of cowpeas from control to tempeh was 1.22%. In the red bean control, the crude fat test results were 0.860 %, soaked 0.819 %, boiled 1.309 %, and crude fat decreased to 0.834% in red bean tempeh, and the total decrease was 0.026%. According to Settaluri *et al.* (2012), nuts' fat content is 1–2%. Linoleic acid and linolenic acid are the most important essential fatty acids needed by the body to grow and maintain physiological functions (Settaluri *et al.*, 2012). During the tempeh fermentation process, there is a tendency to increase the degree of unsaturation of fat. Thus, the number of polyunsaturated fatty acids increases. In the process, palmitic acid and linoleic acid decreased slightly, while the increase occurred in oleic and linolenic fatty acids (linolenic acid was not found in soybeans). Unsaturated fatty acids have a lowering effect on serum cholesterol content so that they can neutralize the adverse effects of sterols in the body (Jesch & Carr, 2017).

The results of the crude fibre test on cowpea control were 6.04%, soaked 6.25%, decreased when boiled 1.79%, after being fermented into tempeh, the crude fibre increased to 4.67%. The total decrease in crude fibre from control to tempeh was 1.37%. The test results on the red bean crude fibre control were 5.32%, soaking 5.45%, boiling decreased to 2.85%, and crude fibre increased again to 3.75% in red bean tempeh. The total change in crude fibre from the control to tempeh was 1.57%.

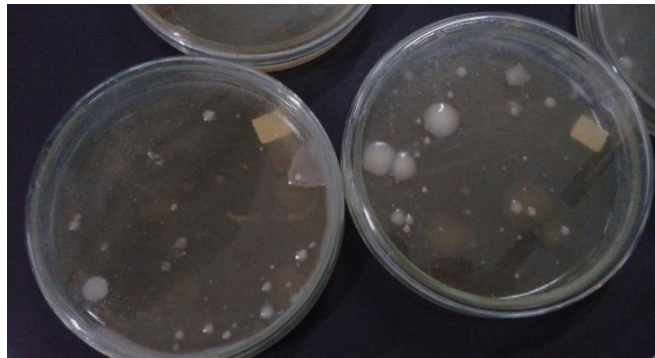
**Cyanide content.** Cyanide (HCN) test results in cowpeas and red beans (pre-treatment, treatment and fermentation results).

Table 2. Cyanide Content in cowpeas and red beans.

Sample	Treatment	%HCN (average)
Cowpeas	Control (nuts before treatment)	0.043
	Soaking	0.040
	Boiling	0.012
	Tempeh	0.010
Red Beans	Control (nuts before treatment)	0.054
	Soaking	0.047
	Boiling	0.012
	Tempeh	0.011

The test results of cyanide levels in cowpeas before treatment, soaking and boiling, and those that have been fermented showed a decrease in each treatment, a reasonably high decrease occurred during boiling. In table 2, cyanide levels in cowpea control were 0.043%, soaking was 0.040%, boiled 0.012 %, cowpeas tempeh 0.010 %, but the decrease in cyanide levels from cowpeas to tempeh was cyanide content decreased by 0.033%. Control of red bean 0.054%, soaking 0.047 %, boiling 0.012 %, red bean tempeh 0.011 %, the total decrease in cyanide levels in control of red bean to tempeh was 0.043 %. Traditionally, the reduction or elimination of cyanide in agricultural commodities has been widely carried out by using tools and materials available in household capacity. It can be done by peeling the skin of cyanide foods before processing (Ramli *et al.*, 2021), followed by soaking before cooking or fermenting for several days. This treatment destroyed linamarin, and the cyanide content was wasted so that the content was only about 10-40 mg/kg. In addition, hydrogen cyanide will be easily lost by boiling, as long as it is not tightly closed. Through heating, enzymes that play a role in the breakdown of linamarin become inactive so that Hcn is not formed (Ramli *et al.*, 2021). The decreasing value of cyanide levels can be influenced by the ability of moulds to degrade anti-nutritional substances (Nkhata *et al.*, 2018). The mould *Rhizopus oligosporus* is a fermentation agent capable of degrading anti-nutritional substances in soybeans. One of them is a compound of phytic acid and phosphoric acid. In this case, Fermentation can reduce interfering compounds in a food material to improve the final product (Nkhata *et al.*, 2018).

**Isolation of Lactic Acid Bacteria from dripping water of Red Beans (*Phaseolus vulgaris* L) and Cowpeas (*Vigna unguiculata* (L.) Walp.).** The presence of contaminant bacteria in the inoculum will reduce tempeh quality because it also grows and can even cause disease. A soaking process precedes the making of tempeh. In addition to softening soybean seeds and increasing their weight, baking is also able to produce vitamins and prevent the development of pathogenic bacteria. *Lactobacillus plantarum* is a Lactic Acid Bacteria; soaking water containing LAB can reduce the growth of pathogenic bacteria. LAB was isolated from soaking water after the beans were soaked for 24 h; a sign of LAB growth in the soaking water was the decreased pH value so that the soaking water conditions became acidic. Grown on MRS media. This medium is a selective medium for the growth of lactic acid bacteria. First, dilution is carried out to reduce the number of microbial populations contained in the media. Observations on colony morphology included shape, edge, elevation and colony colour. Observation of cell morphology included Gram staining test, cell shape and motility test, and physiological properties test, namely catalase test. The isolates selected in this study were KN2 (cowpeas soaking water) and KM2 (red bean soaking water).

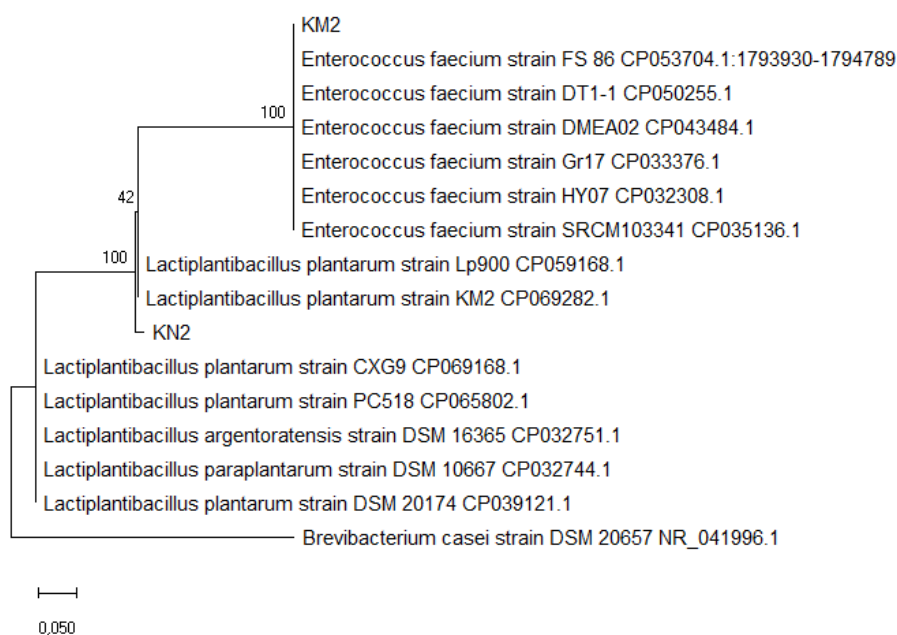


**Fig 1.** Bacterial isolation result of isolation from bean soaking water.

Fig. 1. shows a milky white bacterial colony, round in shape, has smooth margin and a convex elevation. The staining results were gram-positive, spore negative, catalase-negative, and non-motile. It is suspected that the bacteria that have been isolated are lactic acid bacteria. Lactic acid bacteria are gram-positive bacteria, coccus, catalase-negative and spore harmful and are non-motile bacteria (Bergey *et al.*, 2013; Yudianti *et al.*, 2020). Lactic acid bacteria play a vital role in the tempeh fermentation process. The acidification process that occurs due to the activity of lactic acid bacteria in the soaking process causes good conditions for the growth of *Rhizopus* mould and can also inhibit the growth of pathogenic microbes (Radita *et al.*, 2017).

**Lactic Acid Bacteria Identification from Soaked Red Bean (*Phaseolus vulgaris*) and Cowpea (*Vigna unguiculata*).** Following isolation, molecular identification was performed using the PCR technique; the isolates used were KN2 and KM2 to determine the LAB strains isolated. DNA sequences were then matched to lactic acid bacteria from various genera in the Lactobacillaceae Genbank family using the PCR findings. The alignment of 16S rRNA DNA sequences revealed that the LAB isolates KN2 and KM2 were Lactobacillaceae family members. Multiple alignments were performed using the Clustal W program. Furthermore, the kinship visualization was performed using a phylogenetic tree combination of the TREEVIEW X program with the Neighbor-Joining plot.





**Fig 2.** Phylogenetic tree of lactic acid bacteria from 2 water samples of cowpeas and red bean tempeh soaked based on 16S rRNA gene sequence analysis using the Neighbor Joining method.

Bacteria with isolate code KM2 are related to *Enterococcus faecium* strain FS 86 CP053704.1:1793930-1794789; *Enterococcus faecium* is an ordinary member of the human gut microflora (Fiore *et al.*, 2019), which is often used as a probiotic. Nevertheless, certain enterococci strains cause urinary tract infections, bacteremia, endocarditis, and other infectious illnesses (Fiore *et al.*, 2019). Bacteriophages can treat infections caused by this antibiotic-resistant strain of *E. faecium* (Shlezinger *et al.*, 2019). *E. faecium* is one microbe classified into LAB that can produce bacteriocins. *E. faecium* is a gram-positive cocci bacterium with a diameter of 0.5–1  $\mu$ m that colonizes in chains, pairs, or solitarily. These bacteria are facultative anaerobes, meaning they can survive and multiply without oxygen. In optimum or acidic conditions, it can thrive at temperatures ranging from 10 to 45 degrees Celsius and in isotonic or hypertonic settings (Fiore *et al.*, 2019). Catabolize various energy sources, including carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and other-keto acids. These bacteria are facultative anaerobes and can live and reproduce with or without oxygen. *E. faecium* is a microorganism that can survive in very extreme environments, including very alkaline pH and high salt concentrations (Ramsey *et al.*, 2014). *E. faecium* is considered a superbug. Because these bacteria can travel to many organs of the body, including the digestive tract and skin, and survive for a long time on inanimate objects, their multi-drug resistant characteristics make them malignant pathogens (Heidari *et al.*, 2015). The application of *E. faecium* in biotechnology produces antibacterial peptides called bacteriocins. Bacteriocins have a bactericidal and bacteriostatic effect against spoilage or spoilage bacteria. These microbes can be used to process foodstuffs such as cheese, milk, meat and vegetables. *E. faecium* can also be used as a probiotic for harmful bacteria in the digestive tract (Hanchi *et al.*, 2018).

Bacteriocins produced by LAB *E. faecium* can be used as a preservative (bio preservative), especially in processing livestock products such as milk and meat. The effect of preservation with bio preservatives is caused by one of the metabolites produced by these bacteria, namely bacteriocin. Bacteriocinogenic from LAB can prolong the life of tempeh (Wikandari *et al.*, 2021); this is because the nature of bacteriocin can inhibit pathogenic bacteria and contaminants that are not expected to grow in tempeh. In the soaking water of tempeh (soaking water), the dominant microbe usually comes from the order Lactobacillales (Radita *et al.*, 2017).

Isolates with isolate code KN2 are related to *Lactiplantibacillus plantarum*, a gram-positive lactic acid bacteria. *Lpb. plantarum* exhibits ecological and metabolic adaptability and can inhabit a

wide range of ecological niches, including fermented foods, meats, plants, and the digestive tract of mammals (Filannino *et al.*, 2014). *Lpb. Plantarum* is commonly found in fermented foods, strains *Lpb. plantarum* has been considered a promising probiotic candidate for the food and human medicine industry as a bio-preservative and alternative bio-therapy (Gonzalez *et al.*, 2021). *Lpb. Plantarum* also has potential antimicrobial activity; this is indicated by species *Lpb. plantarum* has a broad spectrum of antibacterial activity against many food spoilage microbes (such as bacteria, yeasts and fungi) and various enteropathogenic bacteria, inhibiting Gram-positive and harmful bacteria (Dinev *et al.*, 2018). In addition, *Lpb. Plantarum* can fight pathogenic microbes in food; based on this ability *Lpb. plantarum* is used as a starter culture for various fermented food products (Arena *et al.*, 2016). Bacteria from the *Lpb. plantarum* species are typically gram-positive lactic acid bacteria species, non-sporulating (non-spore-forming), rod-shaped, occurring both singly or grouped in short chains *Lpb. plantarum* exhibits ecological and metabolic adaptability and can inhabit a range of ecological niches, including fermented foods, meats, plants, and the mammalian gastrointestinal tract (Filannino *et al.*, 2014). This species is thought to adapt to stressors better than other members of the *Lactobacillus* genus, and researchers believe it is the sequence of the *Lpb. plantarum* genome that allows this microbe to be so flexible; however, it does prefer an anaerobic environment to replicate in. It is good at utilising and breaking down a range of carbon sources wherever they are available. This versatility means that the species can be found and isolated from various sources, including saliva, the human intestine, dairy products, plant material, and silage. Strains from this species are also used to ferment different foods such as sauerkraut, pickles, and sourdough bread (Yudianti *et al.*, 2020). The ability of *Lpb. plantarum* to inhibit the growth or adhesion of pathogenic microbes has been well-studied in vitro. However, because assessing similar characteristics in vivo is more complicated, such evidence is substantially more limited; strains of this species possess a wide range of probiotic characteristics and have shown promise in numerous experimental disease models and human trials (Fidanza *et al.*, 2021). LAB can grow spontaneously in fermentation at 35-37°C (Manini *et al.*, 2016); LAB plays a vital role in many fermented foods worldwide. BAL in fermented foods is important for health (Caggianiello *et al.*, 2016). One of the most important microorganisms that mediate acidification is the lactic acid bacteria (LAB) group. They produce lactic acid as their main fermentation product from the culture medium (Bintsis, 2018). In tempeh fermentation, acidic conditions can be made by adding organic acids such as lactic acid (Huang *et al.*, 2018). However, tempeh fermentation in Indonesia does not use organic acids, but acidic conditions occur naturally by various types of bacteria. This can cause the quality of tempeh to be inconsistent, and sometimes, it can cause a bitter taste of tempeh (Radita *et al.*, 2017).

Microbial diversity in tempeh can depend on environmental conditions, place of fermentation, type of sample, water used, and aseptic conditions applied. In this Soaking water of red beans and cowpeas, only two types of LAB were obtained due to limited equipment and environmental conditions in Kefamenanu, East Nusa Tenggara Province, Indonesia.

## CONCLUSION

This study showed a change in pre-treatment; in cowpeas, the moisture content increased from control to fermentation by 10.6%. Ash content decreased by 0.5%, fat content increased by 1.22%, crude fibre decreased by 1.37%, dry matter decreased by 10.6%, carbohydrates decreased by 16.66%, protein increased by 5.34%, cyanide content decreased by 0.033%. Meanwhile, red bean water content increased by 15.06%, ash content decreased by 2.03%, fat content decreased by 0.026%, crude fibre decreased by 1.57%, dry matter decreased by 15.06%, and carbohydrates decreased by 15.89%, protein increased by 2.89%. Cyanide levels in red beans decreased by 0.043%. Changes occur in each treatment, especially in the boiling process. Quantitatively, there is a decline in most nutrients, but qualitatively it becomes more easily digested by the body. The lactic acid bacteria that were successfully isolated and identified are bacteria with the code KM2 isolate related to *Enterococcus faecium* and KN2 isolate *Lactiplantibacillus plantarum*. These two microbes are

lactic acid bacteria from the phylum Firmicutes; these two bacteria are suspected of having probiotic potential. The lower pH value in the soaking water is due to LAB's activity, a bacteria that provides significant resistance during the tempeh fermentation process.

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